A Translational Approach to Investigate Mechanisms Underlying Intergenerational Inheritance of Depression

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Abstract

Background: Stress during early life such as exposure to prenatal and postnatal depression or receiving reduced levels of parental care can produce long-lasting behavioural effects. Such long-term disruptions in stress-related behaviours have been seen in both human and rodent studies in offspring exposed to a variety early-life stressors such as maternal depression. Importantly, offspring exposed to early life stress have increased susceptibility to maternal depression themselves suggesting a mechanism by which stress could be intergenerationally inherited through maternal stress.

Aims: The overall aim of this study was to explore the major possible mechanisms underlying how maternal stress and reduced care is able to increase the risk of developing stress-related behavioral disorders in the offspring. This included: 1) programming neuroendocrine and immune changes; 2) epigenetic alterations driving changes in regulation of stress-related genes; 3) alterations in endocrine and immune factors in milk.

Methods: This study investigated a rat model of intergenerational inheritance of maternal depressed maternal care stress and two human studies of maternal depression. Endocrine and immune factors were measured using ELISA and epigenetic changes in a rat model and 2 human cohorts using bisulphite pyrosequecning. RNASeq was used to investigate genomewide RNA, transcriptome, changes across generations.

Results: A literature review found supporting evidence for the hypotheses and revealed key targets for endocrine, immune and epigenetic regulation that became a focus of the following studies. Analyses in *Study 1* showed alterations between the generations in endocrine factors important for stress and maternal care with further changes in specific immune factors and epigenetic alterations at key genes involved in stress regulation. Tanscriptome analyses in *Study 2* revealed changes in genes important for calcium binding signaling and levels of shared gene expression between generations. Epigenetic investigations in *Study 3* found associations with maternal depression and stress with at at homologous gene regions tested in *Study 1* suggesting a level of conservation in

mechanisms important for stress regulation in maternal depression. Within *Study 4* milk from stressed dams there were reduced levels of endocrine stress factors important in regulation of stress and maternal behavior.

Conclusions: This thesis found important evidence to support the involvement of immune and endocrine factors in the transmission of maternal stress together with epigenetic changes at key genes and transcriptome alterations.

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List of abbreviations

ACTH	Adrenocorticotrophic Hormone
AVP	Arginine Vasopressin
α-1AGP	Alpha-1-Acid Glycoprotein
α-MSH	Alpha-melanocyte-stimulating hormone
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
CRH	Corticosteroid releasing hormone
CSS	Chronic Social Stress
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FSH	Follicle- stimulating hormone
GH	Growth hormone
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GR (NR3C1 gene)	Glucocorticoid Receptor
HPA	Hypothalamic-pituitary-adrenal axis
ICAM-1	Intercellular adhesion molecule-1
IL-1β	Interleukin 1 beta
IL-6	Interleukin-6
IL-18	Interleukin-18
IFNγ	Interferon gamma
LH	Luteinizing hormone
PCR	Polymerase chain reaction
PVN	Paraventricular nucleus
PTSD	Post-traumatic stress disorder
TIMP1	metallopeptidase inhibitor 1/ a tissue inhibitor of metalloproteinases
TSH	Thyroid-stimulating hormone
TNFα	Tumour Necrosis Factor alpha

VEGF	Vascular endothelial growth factor
WCHADS	Wirral Child Health and Development Study
WHO	World Health Organization

1 Chapter 1: Literature review

1.1 Introduction

Perinatal maternal depression is associated with an increased risk for stress-related behavioral and emotional problems in the offspring (Murgatroyd and Spengler, 2011a). Prenatal and postnatal stress and maternal depression may impact offspring through possibly different pathways. For example, during pregnancy maternal stress may influence intrauterine environment and so impact alterations in neurodevelopment of the growing foetus. Postnatal maternal depression could influence offspring through variations in parental care that could be influenced by stress and in turn may induce stress-related behaviours in the offspring. One might therefore consider that prenatal and postnatal maternal depressive symptoms might independently influence development of stress-regulatory pathways in offspring. A further concept is that offspring exposed to perinatal stress and maternal depression have increased risks for developing maternal depression themselves. It is therefore possible that such maternal depression could be inherited through multiple generations. Such generational inheritance can be referred to as intergenerational. It is important to clarify this terminology. In inter/trans-generational inheritance it is speculated that an environmental factor induces a epigenetic change: the term *intergenerational* would refer the adult female animal (F0) and the first (F1) and second (F2) generation of offspring being effects because the adult, the foetus, and the primordial germ cells in the foetus would be directly exposed to that particular environmental factors. *Transgenerational* would refer to subsequent generations (i.e. F3 and F4 etc.) also being affected in the absence of exposure to the particular environmental factor (Skvortsova et al., 2018). Such epigenetic factors could be important in driving inheritance of stress-related behaviours across generations, possibly in combination with perinatal postnatal behaviour or prenatal uterine environment.

Dysregulation of the stress-regulatory Hypothalamic-Pituitary Adrenal (HPA) axis is a major component of depression. A possibility is that the dysregulation of this in prenatal and postnatal depression could program alterations in the function of the HPA axis in offspring. Further possibilities are altered regulation of key hormones important in controlling maternal behavior or other neuropeptides important in depression. If these might be alternatively regulated in the mother then prenatal effects might be seen through the placenta or postnatal changes through inducing depressed maternal care. We should also consider the immune system and the role that inflammation plays in depression and the stress-response. Immune changes are well described effects of early stress (Murgatroyd, 2018)

The possibility is that there may be large numbers of genes impacted by maternal depression and early life stress. For example a recent large genome-wide expression study has found that some psychiatric disorders, including depression, share global gene expression patterns (Gandal et al., 2018). One question is whether such changes might be by the next generation.

Finally, there is possible role of lactation. If maternal depression might influence factors in the milk that might in turn influence stress-related behavior of the offspring. Milk contains numerous important endocrine and immune factors that might influence the suckling offspring.

Studying a rodent model of transgenerational stress and maternal depression allows to investigate these components and test these hypotheses. It is also important to investigate whether such hypotheses might also translate to human studies.

This review chapter investigates these different hypotheses and collects information form relevant studies that will help to inform the subsequent chapters that explore each of these possible mechanisms.

1.2 Aims

The aim of this part of this literature review is to collect ideas and mechanisms important in the intergenerational inheritance of depression. Studies from a rat model have shown that maternal behavior is a key driver of this; however, it is unclear at the molecular level as to how this is driven. Using rat studies and human findings, the chapter explores the possible role of specific stress, endocrine and immune factors, together with epigenetic changes, transcriptome alterations and changes in milk factors.

As well as exploring any mechanisms, this chapter also looks to identify key factors that can be tested in a rat model and the possible translational value to human studies. This thesis is novel in that it looks to explore several key hypotheses by which stress may be transgenerationally inherited, using both rodent models and human cohorts.

1.3 Objectives

1. Investigate and identify specific stress-related factors and hormones involved in the possible intergenerational transmission of maternal behavior and depression.
2. Investigate the possible role of immune factors in the intergenerational transmission of maternal behavior and depression.
3. Identify possible epigenetically regulated candidate genes that might be involved in the intergenerational transmission of maternal behavior and depression.
4. Explore the possible role of stress in the regulation of milk factors and identify key hormones that might be involved in the intergenerational transmission of maternal behavior and depression.

1.4 The regulation of stress and depression

1.4.1 Depression

Depression is a well-defined mental health condition; the Diagnostic and Statistical Manual of Mental Disorders—Fourth Edition (DSM-IV) is used in the USA, while the ICD-10 (International Statistical Classification of Diseases) is used worldwide. DSM-IV defines a depressive episode as low mood or anhedonia (loss of interest in previously enjoyable activities) for a minimum of two weeks. Major depression disorder has a significant impact on society, as it is the most prevalent mental health condition, and is the biggest medical burden to middle-aged members of the public. About 80% of patients with a depressive episode will have a recurrence at some point in their life. In developed countries, it is the largest cause of disability; approximately 1 in 10 people suffer from depression each year, and approximately 2 in 10 will develop depression within their lifetime (Juruena, 2014). As depression has such a significant impact on public health, diagnosis and treatment is effective. Current treatment of depression is not effective, with 30-50% of patients not responding to antidepressant therapy (Juruena, 2014).

It is well known that stressful life events predispose to the development of depression, particularly early life stress in the form of childhood adversity is known to significantly increase the risk of depression and anxiety in adult life; about 25-33% of children exposed to maltreatment develop major depression in adulthood (Raine, 2002; Heim and Binder, 2012; Baes et al., 2014; Juruena, 2014). There is a wide range of early-life stressors and early traumatic experiences that affect society such as parental loss, childhood illness, family violence, and neglect i.e. deprivation of food, clothing, shelter, and love.

It is thought that genetic differences, gender, personality and family history, combine with environmental factors, as early-life stressors, as mechanisms that underlie the risk for the development of major depression (Heim and Binder, 2012).

1.4.2 Maternal Depression

Postnatal depression afflicts from 10% to 20% of mothers following birth. The risk factors linked to development of postnatal depression include prior history of depressive disorder and/or premenstrual dysphoric disorder, depression during pregnancy, inadequate social support, and negative life events. Unlike the more common "baby blues," reported1 to effect more than 50% of new mothers, postnatal depression can engender long-term risks to children including cognitive and behavioural problems (Biaggi et al., 2016).

Mood and anxiety disorders in the prenatal and postnatal period affect a large number of childbearing women, and carry a significant risk to the well-being of mother and child. A review by Choi (Choi and Sikkema, 2016) found evidence to suggest that mood and anxiety disorders in the prenatal period, are a significant predictor of negative obstetric and infant/child outcomes. Postnatal mood and anxiety disorders were found to disrupt mother-child bonding, and cause problems with child growth and long-term development (Choi and Sikkema, 2016)

In the prenatal period, the foetal brain is developing and growing with significant plasticity. Hence, having a better understanding of prenatal stress is important in order to understand how exposure to stress during this key period affects development of mental health in the offspring (Cruceanu et al., 2017). In the postnatal period, mother-offspring interactions also affect the development of the plastic brain; tactile and olfactory stimuli have particularly been implicated as important to the developing brain. For instance, preterm human infants demonstrate enhanced growth and neurodevelopment when stimulated by touch, and maternal tactile stimulation can attenuate infant stress responses. The activation of neural pathways can be stimulated by maternal odours the offspring associates with its early rearing environment. This response to odours at the neural level is enhanced when combined with tactile stimulation in the form of licking (Kingston and Tough, 2014).

It is important to note that although they are usually studied separately, there is high correlation between prenatal and postnatal stress. Therefore, a mother with a history of

mental illness or abuse during childhood exposes the foetus to prenatal stress, and is more likely to expose the baby to postnatal stress due to increased risk of dysfunctional mother-infant bonding, or child abuse etc. As a result, it can be difficult to study prenatal and postnatal stress independently, as they go hand in hand (Cruceanu et al., 2017). However, it is important to distinguish the effects of prenatal compared with postnatal stress on offspring to allow testing the relative effect of maternal mental health compared to various postnatal factors, which may help identify which periods and factors have the most important effect on child development. It is also important to know which period is more crucial i.e. prenatally or postnatally, due to allows for possible treatments (Kingston and Tough, 2014). (Brummelte and Galea, 2016) (**Figure 1.1**) shows a schematic of the effect of the prenatal depression and postnatal care on infant development.

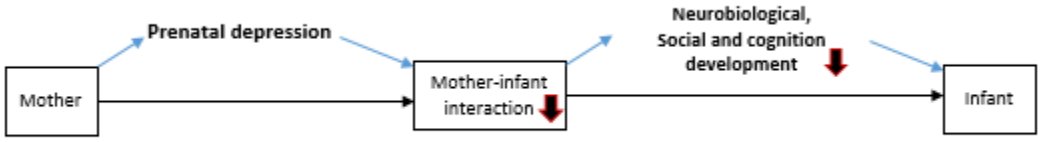

Figure 1.1. Association between prenatal depression, mother-infant interaction and child cognition. *Prenatal depression, mother-infant interaction could contribute to impaired neurobiological development of child.*

1.4.3 The endocrine regulation of stress

Our neuroendocrine system is key to the regulation of the stress response. A stress response is the way in which an organism responds to sudden changes in their environment, either physical or emotional that may disrupt their homeostasis (Babenko et al., 2015). This response is important to allow the organism to maintain homeostasis and to deal with changes in environments whether they be actual, anticipated, or even perceived. Stress either perceived through visual, audial, olfactory, and sensory or cognitively can activate various parts of the brain that connect to the hypothalamus and stimulate the HPA axis by causing the release of corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) from the paraventricular nucleus (PVN) of the hypothalamus. CRH and AVP stimulate the release of adrenocorticotrophic hormone

(ACTH) from the anterior pituitary gland. This in turn stimulates the secretion of glucocorticoids which are the main stress steroids, from the adrenal cortex. In humans, the major stress glucocorticoid is cortisol, while rodents release a related steroid, corticosterone. Negative feedback occurs via cortisol or corticosterone binding to a receptor in cell called the glucocorticoid receptor (GR) which is able to bind at GR binding sites in genes allowing to regulate these neuropeptides and allow levels to return back to baseline once homeostasis is restored (Nephew et al., 2017) (**Figure 1.2**). The HPA axis is therefore key to the regulation of the stress response.

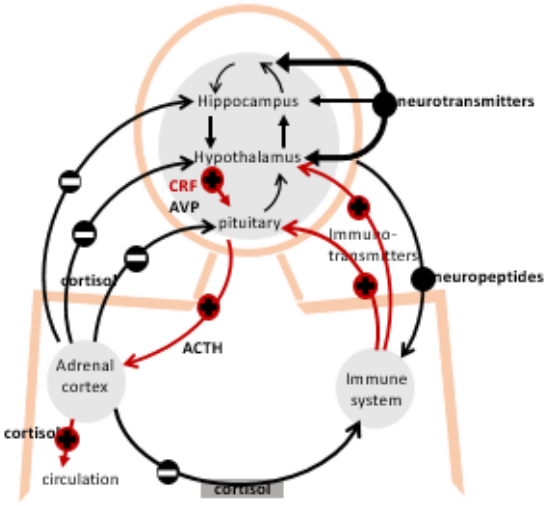

***Figure 1.2. Schematic of the Hypothalamic Pituitary Axis (HPA**). The HPA is activated and releases CRH and AVP from the hypothalamus. This induces the release of ACTH from the pituitary that stimulates the release of cortisol from the adrenal cortex into the circulation into blood. Cortisol induces many of the responses associated with stress. Cortisol also dampens activity of the immune system. Cortisol negatively feedback inhibits HPA axis activity at the pituitary, hypothalamus and hippocampus.*

A stress response generally promotes survival as it forces an organism to rapidly adapt to a sudden change in an environment. Stress may be acute or chronic. Acute stress is characterized by a danger that suddenly occurs and may activates what is known as a fight-or-flight response. For example, an organism needs to quickly decide to run, fight or hide in the presence of a predator. This is usually regulated through adrenaline release from the adrenal glands. Chronic stress is characterized by the persistent presence of sources of stress that an organism might encounter every day, such as for example the constant presence of a nearby predator. This type of stress may involve long-term

stimulation of the fight-or-flight response and involves the neuroendocrine pathway known as the hypothalamic-pituitary-adrenal (HPA) axis. However, when a stress response is too strong or continually activated, or exaggerated in relation to the perceived stress, disease or death of an organism may result (Murgatroyd and Spengler, 2011a).

Humans respond to stress through the same basic endocrine mechanisms described above, similar to all other organisms. However, in humans, stress is a very complex phenomenon, influenced and resulting from numerous environmental changes that may vary in the level of their actual physical or emotional threats.

1.4.4 The role of early-life stress and the HPA axis in depression

Some stressors at specific periods in an organism's life, especially during early life and development can cause long-lasting alterations to the neuroendocrine system. For example, early life stressors can cause changes to physiology and behaviour that are long lasting together with long-lasting alterations of the HPA axis. Rodent studies in the literature, show changes to the HPA axis such as increased glucocorticoid response to subsequent stressors, increased plasma levels of ACTH, reduced GR expression in the hippocampus, as well as changes in CRH and AVP in the hypothalamus (Murgatroyd and Spengler, 2011a).

There is much evidence reporting that early life stress can induce long-term dysfunction of the HPA, which carries on in to adult life. This dysfunction increases susceptibility to depression; hyperactivity of the HPA axis in major depression is one of the most well defined findings in psychiatry (Juruena, 2014). HPA hyperactivity is characterized by chronically increased secretion of CRH, decreased negative feedback, and increased circulating ACTH and cortisol. A significant proportion of patients with major depression have increased concentrations of cortisol, an inappropriately high cortisol response to ACTH, as well as pituitary and adrenal enlargement (Murgatroyd and Spengler, 2011a). Although the mechanism underlying the HPA dysfunction is unclear, one proposed mechanism is that circulating glucocorticoids lead to an impairment of feedback

inhibition. The observed changes to the HPA axis usually improve after the depressive episode has resolved (Holsboer, 2000).

1.4.5 The role of the neuroendocrine system in regulating maternal behaviour and alterations following early-life stress

Maternal care involves the consistent and coordinated expression of a variety of behaviours over a period of time. Adverse changes in maternal care can have profound impacts on neurodevelopment and behaviour of offspring. This complex behavioural pattern depends on a number of integrated neuroendocrine mechanisms involving, amongst others, the actions of the stress HPA hormones (CRH), (AVP), oxytocin, and prolactin (Murgatroyd and Nephew, 2013). Some of these factors important for maternal behavior, such as oxytocin and prolactin are also regulated following exposure to a stressor, as part of the HPA axis (Slattery and Neumann, 2008).

1.5. Intergenerational inheritance of stress and depression

1.5.1 Impact of maternal depression on stress-related behaviour of offspring

Alterations in social behaviour are well known in offspring (in both human and rodent studies) exposed to early life stressors such as reduced maternal care or maternal depression (Murgatroyd et al., 2015a). Another aspect is that earlier exposure to early-life stress can increase the risk for those individuals to develop postnatal depression (Heim and Binder, 2012; Heim and Nemeroff, 2001).

There is much evidence for the observation that exposure to maternal depression can predispose to depression in later life. One study found that in those who experienced multiple adversities during childhood, the risk of developing depression increased up to 4-fold (Felitti et al., 1998). In addition results from another study showed a correlation between early life stress and the development of mental health conditions in adult life; the more severe the childhood adversity, the more likely to develop depression in later life (Edwards et al., 2003). Numerous national studies such as the National Comorbidity

Survey (Molnar et al., 2001) , the Ontario Health Survey (MacMillan et al., 2001) and a New Zealand community survey (Mullen et al., 1996) all reported similar results, where early life adversity increases risk of depression. Even twin studies have demonstrated concordant findings of early life stress having a role, beyond genetic factors (Kendler et al., 2000; Heim and Binder, 2012).

Studies have shown long lasting effects of early life stress on the brain and its stress regulatory HPA system, may lead to the development of a vulnerable phenotype with increased sensitivity to stress and risk for a range of behavioural stress related disorders. Support for this hypothesis comes from a numerous studies in animal models that provide the direct and causal evidence that early adverse experience, such as maternal separation or variations in low or high levels of maternal care, lead to changes in HPA regulation a connected network of brain regions (Sanchez et al., 2001; Talge et al., 2007; Lupien et al., 2009). These effects appear to be present across species and in different models of adversity involving, stress in early life.

A review by Kingston and Tough (Kingston and Tough, 2014) on human studies showed in that there was a small to moderate effect of prenatal distress on behaviour, and social development in school-age children. In addition, almost 70% of the studies demonstrated a significant association with these traits and postnatal depression.

Research using various animal models and studies in human populations have shown that prenatal and postnatal stress can induce a long-lasting effect stress-related behaviour in the offspring (Murgatroyd and Spengler, 2011a).

The early maternal environment of an offspring can have long lasting effects. In rodents, the behavior of mother rats (dams) in the early postnatal life can may affect how the babies (pups) respond to stress, and subsequently how they mother their own offspring. This can relate to circumstantial outcomes as a result of particular actions by the mothers, like interaction through touch, for example arch-back nursing, licking and grooming (Meaney, 2010). This has been shown, in seminal studies, to induce increased levels of Glucocorticoid Recepter (GR) and increased HPA axis feedback (leading to more controlled regulation), and lower levels of depression and stress-related behaviors as well

as reduced levels of fear. This licking and grooming have been shown to induce epigenetic regulation of the GR gene promoter in the hippocampus of the pups (Weaver et al., 2006). Further studies have shown that such postnatal behaviours like increased care by unstressed mothers can reduce the effects of stress and promote hippocampal neurogenesis, as well as influencing and how the offspring themselves behaved as parents (Del Cerro et al., 2010). This can be considered adaption, how generations adapt to changing environments.

In 2012 a study on the Wirral Child Health and Development Study (WCHADS), was the first to translate the rodent licking and grooming to human investigations. (Sharp et al., 2012) showed that touch, in the form of stroking by the mother, could reduce the impact of prenatal stress. If one considered that stroking could be a human variation of licking and grooming in rats and mice then this would support the rodent work in its ability to reduce the effects of prenatal stress on depression in the young.

1.5.2 Role of maternal depression in leading to changes in care behavior that in turn influence offspring behaviour, i.e. intergenerational inheritance

As discussed, there are numerous studies that show that maternal stress and depression can induce an increased risk for the offspring to also developing stress-related disorders (Lyons-Ruth et al., 1997; National Research Council, 2009).

Although the increased risk of major depression in offspring of depressed parents is well defined, there is very limited information on the risk to the third generation and to what extent this might even extend beyond to further generations.

1.5.3 Role of maternal stress and depression in the regulation of the HPA axis in offspring

Changes to serum cortisol status in patients with maternal depression have been well described in the literature; chronic maternal depressive state (greater than one month)

tends to cause hypocortisolaemia, while short-term immediate postnatal low mood tends to cause hypercortisolaemia. Cortisol status in children has also been reported to change with maternal depressive states. On exposure to reduced parental care, nursery-reared Rhesus monkeys exhibited lower basal cortisol levels compared to controls raised by their mother. In human offspring, exposure to the early life stress of postnatal depression also causes low basal cortisol levels, when compared with controls exposed to only moderate early life stress (Nephew et al., 2017).

One probable mechanism is that the as time passes during chronic stress, the stress-induced hypercortisolaemia observed initially, may transform into hypocortisolaemia as a protective development; it is thought that the hypocortisolaemia may develop in order to protect the brain and physiological processes from long term exposure to excess serum cortisol (Nephew et al., 2017).

1.5.4 Transgenerational inheritance of depression.

1.5.4.1 Rodent studies and the intergenerational CSS maternal depression rat study

In a CSS rat model F0 mothers were subject to a resident male intruder during lactational periods. Maternal care and maternal aggression were the measured in the dams on early (day 2), mid (day 9), and late (day 16) stages of lactation to assess the effects of early-life CSS at different time points during lactation (Carini and Nephew, 2013; Nephew and Murgatroyd, 2013). After a 60-min pup removal, maternal care testing was performed, which included reintroduction of all pups into the home cage and video recording of the dam for 30 min. Total maternal care included the combined durations of pup grooming and nursing.

This showed that there were significant differences in total maternal care in the F0 mothers at day 9 only. In the F1 dams there was reduction in only day 2 and in the F2 dams there were significant reductions in maternal care on all three days of lactation. This shows that there is an increased reduction in maternal care across the generations. In locomotor activity and allogrooming there were increases from F0 to F2 dams suggesting

again increases in depression and anxiety-related behaviours across the generations (**Figure 1.3**). This therefore demonstrates that some aspects of maternal care is transgenerationally inherited. However, these behaviours are not as strong as those effects seen in the F0 lactating dams (Carini and Nephew, 2013; Nephew and Bridges, 2011; Murgatroyd et al., 2015b) or the lactating F1 Dam offspring (Carini and Nephew, 2013; Nephew and Murgatroyd, 2013; Murgatroyd et al., 2015b).

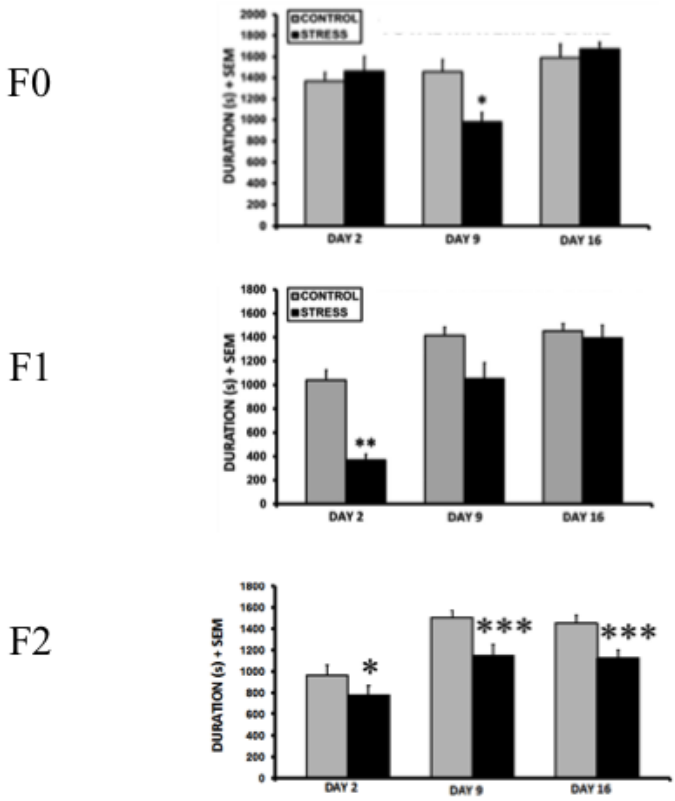

Figure 1.3. Maternal care behavior in F0, F1 and F2 Dams following exposure of the F0 dam to CSS. *Duration of total maternal care (pup grooming, nursing, nesting) on days 2, 9, and 16 of lactation during a 30 min maternal care observation in control and early life CSS (stress) dams in F0, F1 and F2 dams. *, ** and *** Indicate a significant effect of treatment (t-test, p <⍰ 0.05, 0.01 and 0.005 respectively) (Nephew et al. 2018).*

This data from the transgenerational CSS study firstly show that the CSS maternal stressor causes reductions in maternal care and increases stress behaviour. Secondly, that the female offspring of the F0 dams subjected to reduced maternal care, when mothers themselves, give lower levels of maternal care and show increased stress. Furthermore, their offspring, i.e. grandchildren of the F0 dams also show reduced maternal care.

Thirdly, the reductions in maternal care become more exaggerated through the generations, i.e. the effect of the stressor actually increases through the generations.

The mechanisms by which the transgenerational inheritance of CSS has yet to be investigated or determined. It is unclear what endocrine and immune markers may be regulated through the generations in response to the CSS in the F0 dams and whether the decreased maternal behaviour accompanied by changes in plasma corticosterone, prolactin, oxytocin, bdnf or immune markers in a transgenerational pattern of inheritance. It is also unknown as to whether any epigenetic changes of key genes in the brain, such as those involved in HPA regulation, are maintained, i.e. inherited, across the 3 generations that may underlie alterations in the stress behavior (**Table 1.1)**.

Table 1.1. Inheritance of maternal care behavior resulting from stress in F0 to F1 and F2

Mechanisms	**F0**	**F1**	**F2**
Maternal care	d2- , d9 ↓ , d16-	D2 ↓ , d9-, d12-	D2 ↓ ,d9 ↓ , d16↓
Maternal restless	d2- , d9 ↑ , d16-	d2 ↑ , d9-, d12-	d2 ↑ , d9 , d16 ↑
Milk intake	d2 - ,d9 ↓ , d16↓	d2 ↓ , d9-, d12-	d2- , d9-, d12-
HPA regulation, corticosterone, ACTH	?	?	?
Maternal-related hormone	?	?	?
Growth hormones and reproductive hormones	?	?	?
Immune factors	?	?	?
Behaviour-related neuropeptides	?	?	?
Epigenetics regulation	?	?	?
Endocrine and immune factors in milk	?	?	?

This mechanism implies that HPA axis function is dependent on the length of exposure to stress and the type of stressor. The former in this case refers to acute or chronic stress, and the latter refers to depressed maternal care. This is further supported by rodent studies demonstrating attenuated corticosterone levels in response to early-life exposure to maternal separation (Faure et al., 2006; McEwen et al., 2016). A potentially related finding is the lower milk corticosterone levels of F1 CSS dam mothers, i.e. F2 dams received lower levels as pups. Interestingly, juvenile offspring of dams given corticosterone in the drinking water also display decreased basal corticosterone concentrations (McCormick et al., 2000), similar to the effect observed in the F2 offspring in the present study, where their F1 mothers exhibited elevated levels. Several other studies in rodents have shown that ingestion of glucocorticoids via mother's milk has beneficial programming effects in offspring that persist into adulthood, including better spatial memory (Casolini et al., 1997) and reduced anxiety and altered HPA axis response to stress (Catalani et al., 2000). This suggests that the reduced corticosteroid in the F1 CSS milk could mediate the depressed maternal care, increased restlessness and hypocorticosolemia of the F2 dams.

Exposure of the foetus to maternal glucocorticoids may be important in foetal neurodevelopment (Cottrell and Seckl, 2009); cortisol is key to normal development of the brain, and maternal and infant cortisol levels are well correlated. However, exposure to greater amounts of cortisol has long-term effects on neuroendocrine structure, function and on behavior. One animal study treated pregnant rats with glucocorticoids and found that neurogenesis was significantly inhibited in the subsequent offspring, and neuronal structure and synapse formation were altered (Noorlander et al., 2014). A human study observed greater amygdala volumes (a brain region known to play a key role in the processing of emotions) when maternal cortisol was high in early pregnancy, which was associated with increased evidence of affective symptoms in 7-year-old girls (Newman et al., 2016).

1.5.4.2 Human studies of transgenerational inheritance of depression

In a study of 800 depressed and never depressed women together with data from their children and grandmothers, a positive correlation was found between the mother's depression and the grandmother's depression, as well as depression in the children (aged 15) and grandmaternal depression (Hammen et al., 2004). In another study on 251 women, it was seen found that those individuals with higher risks of major depression were the subjects with biological parents and grandparents generations affected by major depression. Interestingly, grandparental major depression on its own, did not increase the risk in grandchildren and leaving one to conclude that the grandparental depression impacts the risk that the parental depression subsequently has on the grandchildren (Weissman et al., 2016). (Pettit et al., 2008) also studied three generations to test how grandparental and parental major depression could influence grandchild behavior. This revealed that parental major depression increased the risk of grandchild anxious/depressed scores. However, grandparental major depression did not predict higher grandchild anxious/depressed scores although it did interact with parental major depression to predict this. Therefore, grandchildren with parents and grandparents affected by major depression had the highest risk anxiety/depression score. The results of these three studies therefore suggest the presence of the intergenerational transmission of depression.

1.5.4.3 Transgenerational epigenetic inheritance

Transgenerational epigenetic inheritance describes the process by which alterations in disease, behaviour or phenotypes are inherited through epigenetic changes that are separate from the DNA sequence (Murgatroyd and Spengler, 2011b). For example, epigenetic inheritance could allow the inheritance of information through parental-offspring interactions that could be affected by stress or depression. Such maternal stress such a prenatal or postnatal depression is known to be able to induce changes in HPA axis regulation in offspring through via transgenerational epigenetic inheritance to one

generation (Roth and David Sweatt, 2011). However, research is beginning to show that changes to the HPA axis can actually extend to multiple generations (Murgatroyd, 2018).

Though transgenerational epigenetic inheritance is a well-known subject and well-studied in animal models, there is relatively little human research following maternal depression through multiple generations with only a few observational studies. Most of the studies look at the effect of prenatal stress and depression on the offspring's HPA activity (Glover et al., 2010). Part of the reason that it is hard to research is that there are a number of limitations to such studies. Firstly, there is variation between how different mothers respond to a variety of stressors and in their levels of perceived stress which tends to correlate not so well with HPA activity (Glover et al., 2010). Many studies tend to be relatively small with multiple measurements that reduce statistical power. However, taken together, maternal prenatal and postnatal stress does tend to correlate with HPA activity in offspring, which seem to be inherited by epigenetic mechanisms (Meaney et al., 2007; Murgatroyd and Spengler, 2011b; Beijers et al., 2014). However, there is relatively little evidence looking at the second generation and beyond. One of the most well know cohorts and seminal studies was in pregnant women in the Dutch Winter Famine of 1944/1945 who were exposed to extremely low levels of food reaching 500 kcal per day. Those in late pregnancy gave birth to babies of reduced birth weight and that those female offspring in turn also gave birth to smaller babies (Painter et al., 2008). This supports that reduced birth weight from prenatal exposure to famine was inherited, and supports the hypothesis of developmental origins of health and disease (DOHaD), that exposure to specific environmental influences during development may have significant consequences on an individual's later health. Another study of women who were given the synthetic oestrogen diethylstilboestrol, had significantly increased reproductive abnormalities and cancers in their children and increased risk of hypospadias in the third generation, in boys via the maternal line (Kalfa et al., 2011).

1.5.5 Molecular mechanisms by which the HPA axis can be programmed by early life stress

As described above prenatal or postnatal stress or early life stress can lead to changes in HPA axis activity in offspring and this can occur through epigenetic changes. The concept is that epigenetic changes occurring in stress-related genes important in HPA axis function could alter expression of those genes such that different levels of key components of the HPA axis are altered (Murgatroyd and Spengler, 2011b).

1.5.5.1 Concept of gene expression

Gene expression is where information in a gene (i.e. a section of DNA) is converted to a functional protein, by first copying the gene into ribonucleic acid (RNA). It can be split into two parts-transcription and translation. Transcription is the process where DNA is copied into RNA, while translation is the process where RNA is used as a template to create a protein.

The enzyme RNA polymerase is used for transcription, which takes place in the nucleus. There are three steps to transcription: initiation, elongation and termination. Initiation occurs by the RNA pol molecule binding to the 5'end of the gene at the promoter region, with the help of transcription factors that must bind to the promoter before the RNA polymerase can bind. RNA polymerase then "reads" the DNA and adds nucleotides to the 3' end of the RNA, so that the original DNA strand is copied into an RNA molecule (**Figure 1.4**).

After transcription, the RNA is transported to the cytoplasm from the nucleus were it undergoes translation to become its protein equivalent. Here ribosomes essentially convert each three-nucleotide sequence of mRNA into its corresponding amino acid, with the help of tRNA. The ribosome is also responsible for linking each amino acid by covalent bonds to form a polypeptide protein chain.

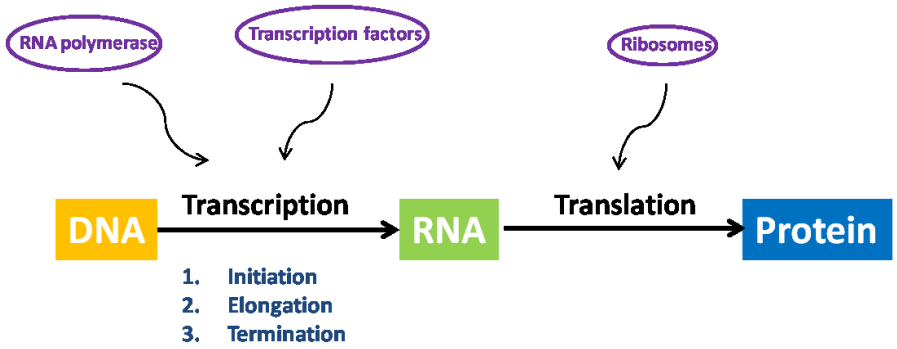

Figure 1.4. Showing concept of gene expression. *There are the steps of transcription, while the important molecules that facilitate each process are in purple.*

1.5.5.2 Epigenetics

The DNA sequence of an organism is identical in all its cells, however epigenetic mechanisms can have an effect on which parts of the sequence are expressed by adjusting the accessibility of the DNA to the gene transcription machinery. In effect, the genome can be thought of as containing two levels of information; the inherited DNA sequence from the organisms parents, which is mainly the same in all cells and tissues, as well as the varied expression achieved by epigenetic mechanism, which are cell and tissue specific (Murgatroyd and Spengler, 2011b).

Epigenetics is the way in which environmental factors can alter genetic expression by switching genes "on" and "off", without altering the original DNA sequence (Heim and Binder, 2012). For instance, epigenetic changes can influence the activity of neurons in a developing brain, which can translate to marked alterations in an organism's behavior. These environmental factors have their influence early in life during development and differentiation. Epigenetic mechanisms include chromatin modification, microRNA expression and DNA methylation. The latter, DNA methylation, is one of the best-understood epigenetic mechanisms and it involves adding methyl groups to the DNA sequence in this way regulating whether the gene is available for transcription; promoters of genes with methyl groups attached are inaccessible to the cell's transcription machinery and are therefore silenced. Hence we can say the gene is "switched off" (Alyamani and Murgatroyd, 2018). The process of DNA methylation involves the addition

of a methyl group at the cytosine side chain in cytosine-guanine dinucleotides (CpG), by the family of enzymes called DNA methyltransferases (Murgatroyd and Spengler, 2011b). The insertion of methyl groups changes the appearance and structure of DNA, which may either: 1, directly block DNA binding of transcription factors or 2, attract factors that preferentially bind to methylated or unmethylated DNA to interfere with transcription factor accessibility. Clusters of CpG (called CpG islands) are found throughout the genome, however many are found in the 5' promoter region. Hence, methylation here has a significant impact on gene expression, as it blocks the promoter region and inhibits expression (Alyamani and Murgatroyd, 2018) (**Figure 1.5**).

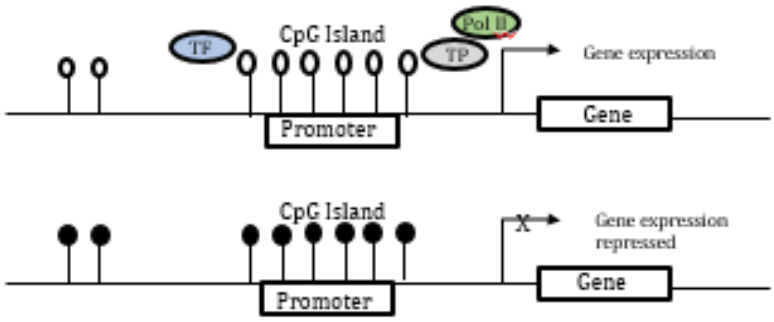

Figure 1.5. How DNA methylation can alter gene expression. *DNA Methylation can affect gene expression by interfering with binding of transcription factors (TF) and RNA polymerase II (Pol II).This schematic shows a gene with an unmethylated CpG island, and thus active, promoter region. As the CpG island is not methylated, TFs are able to bind and facilitate attachment of (Pol II for transcription. The gene below has been 'switched off' where the promoter region is methylated, and this represses gene expression as TFs are unable to bind and leading to repressed gene expression.*

1.8.3 Epigenetic programming by early life stress

It is hypothesised that maternal stress and the mother's care determine offspring behavioural traits and their stress responses through epigenetic mechanisms (Murgatroyd and Spengler, 2011b). For example experiments in rodents show that experiences during sensitive periods of development influence DNA methylation patterns of several genes and that these patterns represent stable epigenetic modifications that

alter gene transcription throughout the lifespan and promote specific behavioural outcomes (Murgatroyd et al., 2009).

Potential associations between epigenetic regulation of HPA axis genes and clinical outcomes remain relatively under studied, with DNA methylation of NR3C1 and FKBP5 being most studied in direct relation to risk for human stress-related disease. (**Tables 1.2** and **1.3**) (**Figure 1.6**). These genes are particularly important as they are key factors in the regulation of the activity of cortisol.

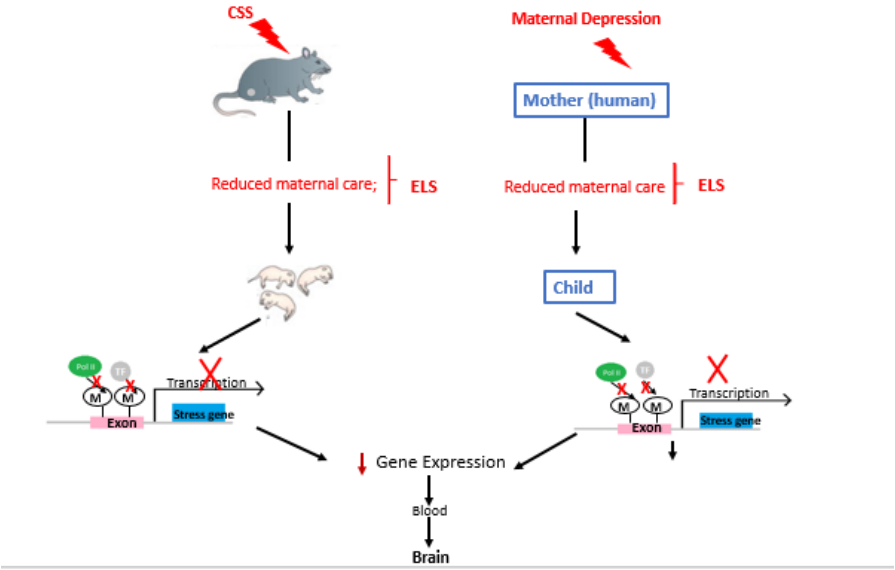

Figure 1.6. Schematic demonstration of the hypothesis of epigenetic regulation by stress-regulatory genes by early life stress in rats and humans. *Chronic social stress (CSS) on the dam results in altered maternal behaviour which causes early life stress (ELS) to the pups. This in turn results in DNA methylation that reduces expression of a candidate gene in a rat.* ***Right.*** *Maternal depression in humans causes ELS to the offspring, which results in decreased expression of a gene via the same epigenetic mechanism as the rat.*

1.5.5.3 Epigenetic regulation of the *NR3C1* gene by early-life stress

The NR3C1 gene codes for the Glucocorticoid receptor (GR). This gene is regulated by a very complicated promoter structure with at least eleven untranslated alternative first exons regulated by a complex promoter structure with one promoter for each of the alternative first exons. It is thought that these alternative first exon transcripts are important for the regulation of GR levels in within different cell or tissue or environmental conditions by differentially controlling translation efficiency and RNA stability (Bockmühl et al., 2015). Although GR is expressed in all cells ubiquitously, its levels are tightly

controlled according to tissue, or even cell, type. Therefore, within the brain, levels are higher in areas involved in the stress response such as in the hypothalamus and hippocampus (Karanth et al., 1997; Uchida et al., 2008; Booij et al., 2013).

Many studies in rats and mice have investigated the role of early life stress on long-term GR regulation. One of the seminal studies from the group of Meaney (as previously mentioned) (Meaney, 2010) have shown that rat pups that received higher levels of maternal care from mothers, also showed increased levels of GR in the hippocampus. In 2004 Weaver *et al.* together with Meaney demonstrated that in rat pups of high maternal care mothers *Nr3c1* methylation was decreased in hippocampal samples within the GR gene's alternative first exon 1-7 promoter, originally identified as being regulated in the hippocampus (Weaver *et al*. 2004). These differences in methylation rates lasted into the offspring's adulthood. Many further studies have been conducted. (Daniels et al., 2009) produced conflicting results, investigating the impact of separating rat pups from their mothers between postnatal day 2 and 14 and finding no methylation changes. However, numerous other studies have indeed supported Meaney's findings finding increased methylation at *Nr3c1* exon 1-7 promoter following various early life stressors, as detailed in (**Table 1.2).**

Table 1.2. Rodent studies investigating the epigenetic regulation of Nr3c1 following Prenatal, postnatal, early-life and adult stressors

Reference	Animal	Form of stress	Model	Tissues	Methylation
(Weaver et al., 2007)	Rat	Increased Early maternal care	High licking and grooming	Hipp	1-7 ↓
(Niknazar et al., 2017)	Rat	Preconception paternal stress	Males stressed prior to mating	Hipp	1-7 ↓
(Mifsud et al., 2017)	Rat	Adult acute stress	Forced swim test stress	Hipp	1-7↓
(Brubaker et al., 2015)	Mus	ELS	Maternal separation	Hipp	1-7 N/D
(Daniels et al., 2009)	Rats	ELS	Maternal separation	Hipp	1-7 N/D
(Kember et al., 2012)	Mus	ELS	Maternal separation	Hipp	1-7 ↑
(Henningsen et al., 2012)	Rat	Early maternal care	High licking and grooming	Hipp	1-7 N/D
(Schuessler et al., 2005)	Mus	Early Maternal care	High licking and grooming	Hipp	1-7 ↑
(Kosten et al., 2014)	Rat	Early maternal care	Reduced care by altering litters	Hipp	1-7 ↑

(Kundakovic et al., 2013)	Mus	Early life stress	Maternal separation	Hipp	1-7 ↑
(Mueller and Bale, 2008)	Mus	prenatal stress	Chronic stress to pregnant dams	Brain	1-7↑
(Witzmann et al., 2012)	Rat	Adult stress	Chronic stress	Hipp, PVN	1-7 ↑
(Tran et al., 2013)	Rat	Adult stress	Repeated water avoidance stress	Amy	1-7 ↑

Hipp; hippocampus, Mus; mouse, ELS; early life stress, N/D; not different, PVN; paraventricular nucleus, Amy; amygdala.

The results of the above animal studies have led to human studies also investigating *NR3C1* methylation (Turecki and Meaney, 2016) (**Table 1.3)**. The rat *Nr3c1* 1-7 promoter, is the homologue of exon 1-F in human *NR3C1* gene. The first study to look at GR in the brain was conducted by (McGowan et al., 2008) looked at two groups of suicide victims- one with a history of childhood abuse, and another without. In brains from these subjects they found increased DNA methylation at the promoter region of the *NR3C1* gene homologus to the rat studies (Weaver et al. 2004) supporting the animal studies that alterations to methylation of the *Nr3c1* gene in the hippocampus occur in response to early life environment (**Figure 1.7**).

Table 1.3. Human studies investigating the epigenetic regulation of NR3C1 following, postnatal, early-life and adulthood stressors. *studies investigating prenatal stress

Reference	Stress	Study, Age collected	Tissues	Methylation
(Perroud et al., 2011)	Sexual abuse	30-40yrs	Blood	1-F ↑
(Tyrka et al., 2012)	Early life trauma	27.3yrs	Blood	1-F ↑
(Schuessler et al., 2005)	Childhood abuse and bulimia	25yrs	Blood	1-F N/D
(Yehuda et al., 2013)	Early life trauma	Pre-treatment, post-treatment, 3 months follow up.	Blood	1-F ↑
(Melas et al., 2013)	Childhood adversity (depressed)	55yrs	Blood	1-F ↑
(Perroud et al., 2013)	Childhood trauma, bipolar	44.6yrs	Blood	1-F ↑
	Child physical abuse	25yrs	Blood	↓
(Van Der Knaap et al., 2014)	Early-life trauma	16.3yrs	Blood	1-D ↓, 1-F ↑
(Van Der Knaap et al., 2014)	Child abuse and neglect	5-14yrs	Saliva	1-F ↑
(Martín-Blanco et al., 2014)	childhood abuse	29.4yrs	Blood	1-F ↑

(Romens et al., 2015)	Child abuse and neglect	12yrs	Blood	1-F ↑
(McGowan et al., 2008)	Child abuse, suicide	34yrs	Hipp	1-F ↑
(Labonté et al., 2012)	Child abuse, suicide	37yrs	Hipp	1-B ↑, 1-C ↑, 1-H ↓
(Oberlander et al., 2008)	Maternal depression	At birth	Cord blood	1-F ↑
(Radtke et al., 2011)	Maternal stress (violence)	14.1yrs	Blood	1-F ↑ *
(Mulligan et al., 2012)	Maternal stress	At birth, War stress	Cord blood	1-F ↑ *
(Hompes et al., 2013)	Maternal anxiety	At birth,	Cord blood	1-F ↑ *
(Conradt et al., 2013)	Maternal depression	At birth	Placenta	1-F ↑ *
(Essex et al., 2013)	Childhood stress	15.1yrs	Buccal	1-F ↑
(Perros et al., 2014)	Maternal stress	Mother 45yrs, children 17yrs, exposure to Tutsi genocide	Blood	1-F ↑
(Yehuda et al., 2013)	Preconception stress, PTSD	50yrs	Blood	1-F ↑
(Murgatroyd et al., 2015a)	Maternal depression	30yrs	Saliva	1-F ↑ *

Hipp; hippocampus, PTSD; Post-traumatic stress disorder.

Oberlander et al. in the first study to explore *NR3C1* methylation in blood cells from humans in relation to stress, specifically maternal depression, found increased levels of DNA methylation in the Exon 1-F promoter in cord blood samples taken from mothers who showed increased levels of anxiety and depression during pregnancy at the 2nd and 3rd trimesters (Oberlander et al., 2008). Perroud and colleagues (Perroud et al., 2011) again focusing on exon 1-F they found that in people with MDD those individuals exposed to childhood physical abuse and neglect showed significantly increased exon 1F methylation compared to those people with MDD but who never exposed to childhood abuse. Furthermore, there was a significant and positive dose-response association between the number of type of childhood abuse and neglect and methylation. (Conradt et al., 2013) reported that newborn offspring exposed to maternal depression *in utero* had increased methylation within exon 1f as well as adverse neurobehavioural outcomes. (Radtke et al., 2011) examined DNA methylation using peripheral blood samples taken from children (n=24) aged up to 19 years old whose mothers had been exposed to violence before, during and after their pregnancy. Increased methylation rates in children

were significantly associated with maternal exposure to violence during their pregnancy. Methylation was seen in 7 of the 24 children, in 5 of the 10 CpG sites examined and at rates of up to 10%. Strikingly, there was no association between child *NR3C1* methylation and maternal exposure to violence either before or after pregnancy suggesting an importance of prenatal stress in inducing these methylation changes. Maternal *NR3C1* methylation was not significantly correlated with methylation levels in their children and was unaffected by exposure to violence. This study was the first in humans to show an apparently sustained dysregulation of the HPA axis associated with previous early life psychological stress.

In a study using buccal DNA from healthy individuals (n=92) (Edelman et al., 2012) were able to show that methylation at a single CpG site within the 1-F promoter correlated with cortisol response to stress. In 2010, Alt *et al*. (Alt et al., 2010) conducted a study exploring the possible association between methylation of *NR3C1* and depression. The authors assessed *NR3C1* methylation in post-mortem samples from depressed patients (n=6) in multiple limbic brain regions compared to controls (n=6). Hippocampal exon 1f transcripts were reduced in depressed patients. However, these data demonstrated very low overall levels of methylation in both depressed and control brains, whilst the *NR3C1* promoter for exon 1f was completely unmethylated in all of the samples taken. Thus, the mechanism for this down-regulation in depressed brains appeared to be entirely independent of methylation patterns. However, this study included only a very small number of samples. More recently Na *et al.* (Na et al., 2014) compared methylation levels of *NR3C1*'s promoter region in depressed patients (n=45) and controls (n=72). The authors found hypomethylation, rather than hypermethylation, at two CpG sites in patients. Neither the (Alt et al., 2010) nor the (Na et al., 2014) studies were able to provide definitive data regarding HPA axis functioning or childhood trauma. This would appear to be crucial, as illustrated by the following studies incorporating early adversity into models of depression, HPA axis dysfunction and methylation of *NR3C1* at different life stages.

(Perroud et al., 2011) investigated the correlation of the severity of childhood maltreatment with methylation rates in *NR3C1* for patients diagnosed with borderline

personality disorder (BPD) (n=101), depression (n=99) or depression with co-morbid post-traumatic stress disorder (n=15). They were able to show highly significant associations between methylation of *NR3C1* and the severity and number of sexual abuse episodes. In a study by (Melas et al., 2013) salivary DNA from depressed adults (n=92) were compared with controls (n=82) increased methylation at *NR3C1* significantly associated with early parental death.

Very few studies have identified candidates for human maternal behaviours equivalent to rat LG-ABN. However, in rats the effects of licking and grooming have been shown to be mimicked by stroking pups with a brush (Mulligan et al., 2012). A study by Sharp *et al.* (2012) (Sharp et al., 2012) demonstrated moderation of the effects of prenatal maternal depression upon emotional and physiological outcomes in human infants through mothers stroking their babies in their first weeks of life. A very recent follow-up study by (Murgatroyd et al., 2015a) has showed reduced *NR3C1* methylation associated with maternal stroking in these children, suggesting the possible role of epigenetic mechanisms in the long-term effects of early life stress and maternal care. Interestingly, the same study also found interactive effects between prenatal and postnatal maternal depression on methylation of *NR3C1*'s exon 1f. Infants of mothers with low prenatal depression showed increased methylation when exposed to increased postnatal depression - consistent with an interplay between prenatal and postnatal environments. Finally, it is important to remember that the reason that CpGs in this region of the NR3C1 gene were investigated in these human studies and found to relate to early-life stress, is through the important seminar work of the animal studies.

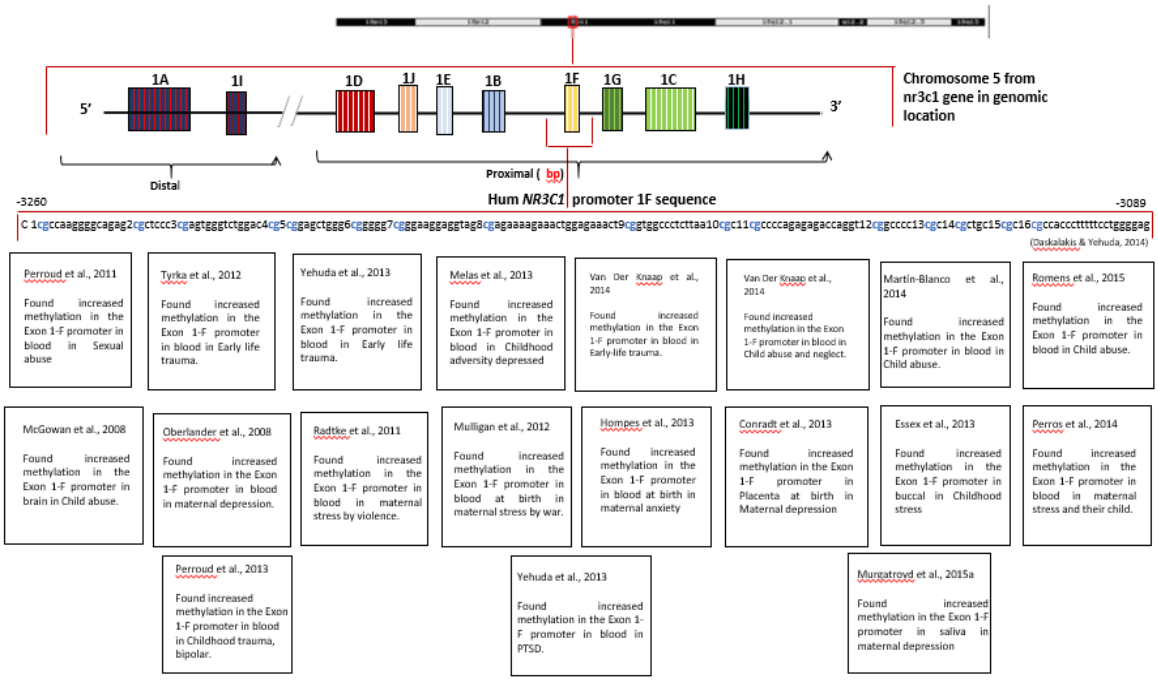

Figure 1.7. Human studies investigating the epigenetic regulation of NR3C1 1.7 promoter following various prenatal, postnatal, early-life and adulthood stressors

1.5.5.4 Epigenetic regulation of *FKBP5* by early-life stress

FK506 binding protein 51 is a co-chaperone to GR, which regulates its sensitivity. This is encoded for by the *FKBP5* gene. (Klengel et al., 2013) demonstrated an increase in DNA methylation of this gene in response to childhood trauma. These patients have a greater risk of developing adult stress-related psychiatric conditions, and it is thought that the demethylation of *FKBP5* leads to long-term dysregulation of the HPA axis,

Numerous studies in rodents, particularly mice, have shown changes in methylation at *fkbp5* following stress (**Table 1.4)**. For example mice injected with corticosterone for 3 months showed that decreased DNA methylation at CpGs within the promoter of *fkbp5* together with enhanced expression of *fkbp5* (Lee et al., 2010). Remarkably, DNA methylation increased again when the corticosterone injections were stopped after 3 months (Sabbagh et al., 2014).

Table 1.4. Rodent studies investigating the epigenetic regulation of the fkbp5 following various prenatal, postnatal, early-life and adulthood stressors

Reference	Animal	Form of stress	Model	Tissues	Methylation
(Lee et al., 2010)	Mus	Chronic Pregnant stress	Chronic exposer to CORT Via drinking water	Blood, Hipp	Intron 5 ↑
(Ewald et al., 2014)	Mus	chronic stress	5 doses of CORT of drinking water	Blood, Hipp	Intron 1 ↑ Intron 5 ↑
(Sabbagh et al., 2014)	Mus	Depression-like behaviours.	Forced stress and tail suspension	Blood	Intron 5 ↑
(Kitraki et al., 2015)	Rat	Prenatal exposure	perinatal BPA exposure	Hipp	Intron 5 ↑
(Green et al., 2016)	Rat	Adult rats	Chronic stress exposure	Hipp	Intron N/D
(St-Cyr et al., 2017)	Rat	Adult rats	Prenatal stress	Amy	Intron V ↑

Mus; mouse, Hipp; hippocampus, N/D; not different, Amy; amygdala.

In human studies, increased DNA methylation of the *FKBP5* gene has been associated with a stress exposure **(Table 1.5)**. Reduced expression of specific transcript variants in patients with MDD (Roy et al., 2017) while another study showed DNA methylation contributes to the upregulation of *FKBP5* transcription with age that effects stress resilience (Sabbagh et al., 2014). A study using MRI found a positive association with *FKBP5* promoter methylation and thickness of the transverse frontopolar gyrus in patients with major depression (Han et al., 2017). A landmark study has shown that childhood trauma can reduce DNA methylation specifically in the intron 7 of the *FKBP5* gene in subjects presenting with a specific SNP (Sabbagh et al., 2014), suggesting an interaction between genotype, DNA methylation, and the effects of environment on the *FKBP5* gene transcription.

Table 1.5. Human studies investigating the epigenetic regulation of the FKBP5 promoter following various prenatal, postnatal, early-life and adulthood stressors

Reference	Stress	Study, Age collected	Tissues	Methylation
(Kang et al., 2013)	PSTD	N=123, 116 control	Blood	Intron 7 ↑
(Klengel et al., 2013)	PSTD	Case-control:in vitro cell lone, N=76, 41yrs	Blood	Intron 7 ↑
(Menke et al., 2013)	MMD	N=68 ,18–65 yrs	Blood	Intron N/D
(Yehuda et al., 2013)	Early-life trauma	Pre-treatment, post-treatment, 3 months follow up.	Blood	Exon 7-8 ↑
(Höhne et al., 2014)	MMD	Case-control; pro-spective, N=116	Blood	Intron 7 ↑

(R Fries et al., 2016)	MMD	Case-control, N=68, 51yrs	Blood	Intron 7 ↑
(Tyrka et al., 2015)	childhood maltreatment	N= 69 preschool, 3-5yrs.	Blood	Intron 7 ↓
(Kertes et al., 2016)	chronic stress, war trauma or prenatal stress	24 mother, newborn 26yrs	Blood, placenta	Intron 5 ↑
(Yehuda et al., 2016)	PSTD	N=*76,* Holocaust survivors	Blood	Intron7 ↑
(Han et al., 2017)	MMD	N= 202, 20-69yrs	Blood MRI	Intron 7 ↓
(Mulder et al., 2017)	maternal stressors	298 14-months-old infants	Blood	Intron 5 ↑
(Parade et al., 2017)	childhood maltreatment	231 preschoolers	Saliva	Intron 7 ↑
(Roy et al., 2017)	MMD	N=48,	Blood	Intron 2 N/D

PTSD; Post-traumatic stress disorder, MMD; major depression, N/D; not different.

1.5.5.5 Epigenetic regulation of BDNF following early-life stress

BDNF is a small dimeric protein that is part of the neurotrophin family of proteins. It is particularly expressed in the cerebral cortex and hippocampus, but can be found throughout the brain. The main function of BDNF is to do with neurodevelopment and maintenance of the nervous system; it plays a role in the survival and proliferation of neurons, especially in learning and memory (Ismail et al., 2015). There is numerous evidence that BDNF expression is decreased by experiencing stress and that a lack of neurotrophic support causes depression.

Similarly to the *NR3C1* gene the human and rat *bdnf* genes have a complex gene structure, consisting of 11 exons (I–V, Vh, VI-VIII, VIIIh, IX), 9 of which (exon I–VII, IX) contain functional promoters. Epigenetic mechanisms have been shown to be very important for *BDNF* expression regulation (Zheleznyakova et al., 2016) Some rodent studies have shown that early-life stress can induce long-lasting changes in the methylation level of the *bdnf* promoter IV which and that this associates with lower *bdnf* expression level in the brain **(Table 1.6)**. Furthermore, the offspring of stressed females showed increased of the *bdnf* in the brain prefrontal cortex and hippocampus, (Roth et al., 2009; Roth and Sweatt, 2011).

Table 1.6. Rodent studies investigating the epigenetic regulation of the bdnf promoter following various prenatal, postnatal, early-life and adulthood stressors

Reference	Animal	Form of stress	Model	Tissues	Methylation
(Roth et al., 2009)	Rat	Infant rats	Abuse and neglect	Cortex	IV ↑
(Fuchikami et al., 2011)	Rat	PSTD	Contextual fear conditioning	Hipp	IV ↑
(Roth et al., 2011)	Rat	Adult rats PSTD	Stress manipulations (cat exposure.	Hipp	IV ↑
(Boersma et al., 2014)	Rat	Adult rat	Prenatal stress	Amy, Hipp	IV ↑
(Wei et al., 2016)	Mus	Resveratrol	Forced swim	Amy, Hipp	IV N/D
(Blaze et al., 2015)	Rat	Adult rats	Maltreatment Infant with a stressed caregiver	cortex	IV ↓, I ↓
(Mpofana et al., 2016)	Rat	ELS	Maternal separation	Blood	IV N/D
(Deng et al., 2017)	Rat	Adult rats ELS	chronic mild stress	Amy	IV ↑
(Makhathini et al., 2017)	Rat	Adult rats	Repetitive restrain stress	Hipp	IV N/D
(Sagarkar et al., 2017)	Rat	Anxiety-like behaviours	Minimal traumatic brain injury	Amy	IV ↑, I ↑, IX ↑
(Yamazaki et al., 2018)	Rat	ELS	Maternal fructose consumption	Hipp	IV ↑

PSTD; Post-traumatic stress disorder, Hipp; hippocampus, Mus; mouse, Amy; amygdala, ELS; early life stress, N/D; not different.

Another study by Boersma et al (Boersma et al., 2014) found that rats exposed to prenatal stress had increased *bdnf* methylation, and subsequently decreased *bdnf* mRNA expression, in the amygdala and hippocampus of their offspring. This suggests that stress can alter DNA methylation that relates to gene activity. In addition, this also supports that DNA methylation at *bdnf* can be inherited across generations.

There are many human studies support these findings of increased *bdnf* methylation described in rats following stress (**Table 1.7)**. Keller and colleagues were the first to investigate the *BDNF* DNA methylation changes in human brains. Investigating suicide victims in comparison with controls (Keller et al., 2010) and using bisulfite pyrosequencing and the authors analysed the methylation levels of CpG sites at *BDNF* promoter IV in the Wernicke area of the brain. They found significant increased methylation of the average levels of 4 CpG sites in suicide completers. Importantly, they also found an association between the average methylation level of these 4 CpG sites and the *BDNF* transcript IV levels supporting a role of methylation at these CpGs in regulating *BDNF* expression.

Table 1.7. Human studies investigating the epigenetic regulation of the BDNF promoter following various prenatal, postnatal, early-life and adulthood stressors

Reference	Stress	Study, Age collected	Tissues	Methylation
(Tadić et al., 2009)	MMD	N=161 Borderline, 156 healthy	Blood	IV ↓
(Keller et al., 2010)	Suicide, MMD	N = 120	Wernicke brain area	IV ↑
(Fuchikami et al., 2011)	MDD	N=20 Japanese, 18 healthy	Blood	IV ↑ I ↑
(Roth et al., 2011)	PSTD	-	Hipp	IV ↑
(D'addario et al., 2012)	childhood abuse	N=154	Blood	I ↑
(D'Addario et al., 2013)	MMD	N=85, with healthy	Blood	I ↑
(Kang et al., 2013)	suicidal attempts	N=108, age at onset	Blood	VI ↑
(Perroud et al., 2013)	childhood maltreatment	N= 115 suicidal	Blood	IV ↑ I ↑
(Thaler et al., 2014)	childhood abuse	N=64 women, 32 normal	Blood	VI ↑
(Braithwaite et al., 2015)	Maternal depressive	N=57 pregnant women, infant at 2 months of age.	Buccal swabs	IV ↑
(Chagnon et al., 2015)	Anxiety, depression	N=65 elderly women, no disorder	Saliva	VI ↑
(Kang et al., 2015b)	depression	732 , ≥ 65 yrs	Blood	VI ↑
(Moser et al., 2015)	Maternal separation. PTSD	N=46 women with violence and their young children	Saliva, (fMRI) brains.	IV ↑
(Stenz et al., 2015)	Prenatal stress	N=98 post-mortem by forensic physicians.	Cortex, Hipp, blood	IV ↑ I ↑
(Unternaehrer et al., 2015)	Low/high maternal care	A cross-sectional, 709, 24 yrs	Blood	VI ↑

MMD; major depression, PSTD; Post-traumatic stress disorder, Hipp; hippocampus.

Fuchikami et al. were the first to examine blood DNA methylation changes in *BDNF* in relation to depression. Again using bisulphite pyrosequecning to look at two CpG islands in promoter I and promoter IV of *BDNF* gene they found in major depressive disorder patients compared with healthy individuals, various significant differences with some CpG sites showing increased and decreased methylation (Fuchikami et al., 2011). Several other studies have also examined *BDNF* methylation in relation to depression. Chagnon et al., found a significantly higher methylation level in promoter VI in elderly women with anxiety and depression compared with controls (Chagnon et al., 2015). Most other studies have also focussed on methylation at *BDNF* promoter VI or only found changes there.

Kang et al found increased methylation at *BDNF* promoter VI with a history of suicidal attempts (Kang et al., 2013), increased promoter VI methylation levels in late-life depression (Kang et al., 2015a) and increased methylation individuals with breast cancer and depression (Kang et al., 2015b). Perround et al. found increased methylation at both promoter I and promoter IV in for bipolar depression patients (Perroud et al., 2013) while D'Addario and colleagues found increased DNA methylation at *BDNF* promoter I in bipolar disorder patients. Tadic and colleagues found lower levels of the analysed in the *BDNF* promoter IV in major depressive patients than controls but this was only 1 CpG out of 12 CpGs they analysed.

Looking at early life stress, Perround et al. (Perroud et al., 2013) in a study of bipolar depression that a larger number of childhood maltreatment was significantly associated with a higher methylation status of *BDNF* I and IV promoters. Thaler et al. found increased DNA methylation in the *BDNF* promoter IV **(Fig. 1.8)** in women with bulimic eating syndromes (Thaler et al., 2014) especially in those with a history of severe childhood abuse.

A study at the University of Geneva Hospital looked at 46 women who had post-traumatic stress disorder following domestic violence, and their infant children. These children were separated from their mothers and the subsequent behaviour of both parties was observed. In addition, DNA samples were taken from maternal saliva, and functional magnetic resonance imaging (fMRI) was carried out on maternal brains.

This revealed that maternal anxiety was significantly increased following separation from their child and that methylation of the *BDNF* gene was increased in the promoter region, causing *BDNF* expression to be decreased in the amygdala and hippocampus during separation, compared to during a non-stressful condition. In addition, there was significant correlation between the level of maternal anxiety, and the amount of *BDNF* methylation (Moser et al., 2015) An additional study of the influence of childhood maternal care on *BDNF* methylation level performed by Unternaehrer et al. (Unternaehrer et al., 2015) showed greater whole blood DNA methylation in the low versus high maternal care group in the *BDNF* exon VI.

These results all support the theory that depression relates to increased DNA methylation at *BDNF* and that stress and early life adversity may cause long term epigenetic changes to *BDNF* (Zheleznyakova et al., 2016) (**Figure 1.8**). However, it is unclear if *BDNF* methylation changes might show transgenerational inheritance between generations perhaps as a mechanism of transmission of anxiety between generations (Moser et al., 2015).

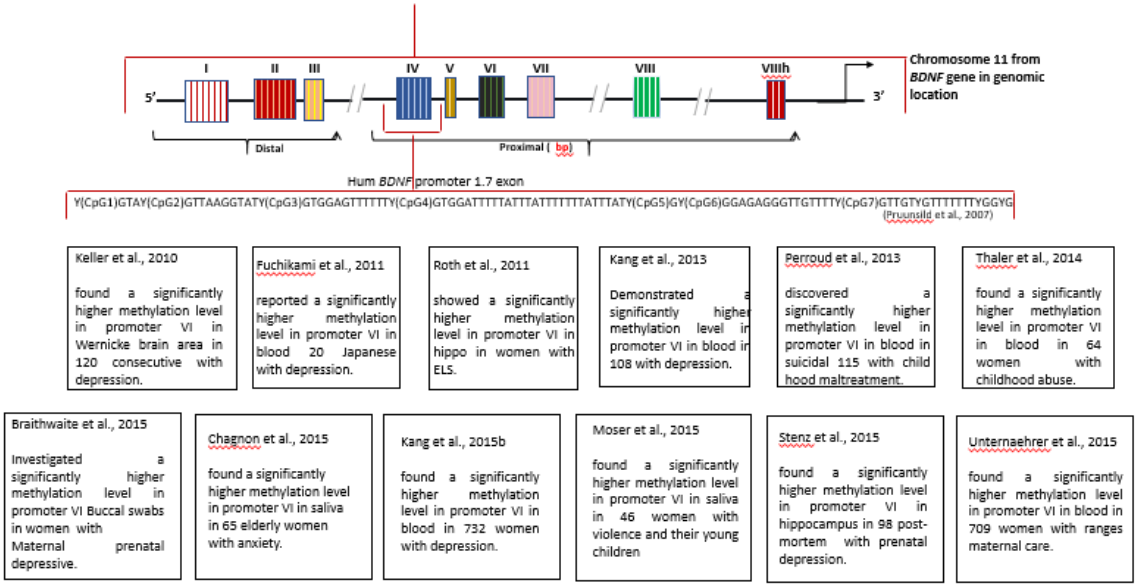

Figure 1.8. Human studies investigating the epigenetic regulation of BDNF 1.7 promoter following various prenatal, postnatal, early-life and adulthood stressors

1.5.6 Inflammatory factors in depression

There is much evidence in the literature that shows an increased activity of the innate immune system is patients with depression. This relates to an hypothesis of the social transduction theory of depression whereby exposure to social stress, particularly during early life, alters immune responses, which then can lead to risk for depression (Slavich and Irwin, 2014). Several studies have found higher levels of certain inflammatory cytokines in depressed people compared to non-depressed individuals including IL-1β,

IL-6, IFN-γ, TNF-α (Dowlati et al., 2010; Pike and Irwin, 2006). This increase in circulating cytokines has also been found in relation to increases in cytokine gene expression. For example, individuals with major depression had higher mRNA levels of TNF-α, IL-1β, IL-6 and INF-α (Cattaneo et al., 2013; Tsao et al., 2006).

Further studies have also linked increased levels of pro-inflammatory cytokines to increased severity of depression (Song and Wang, 2011), and poorer response to antidepressant therapies (Cattaneo et al., 2013). The links between increased cytokine levels and greater immune activation in patients with depression has also been demonstrated in the brains of depressed and suicide patients at post-mortem. For example, one study found increased levels of pro-inflammatory cytokines in depressed patients who had committed suicide, compared with controls (Shelton et al., 2011).

1.5.6.1 Early life stress and programming inflammation

Numerous studies show that exposure to prenatal and postnatal stress and depression can lead to long-term immune effects in the offspring, particularly increased inflammation, that can last into adulthood (Murgatroyd, 2018). One study showed immune changes in young adults who had previously been exposed to prenatal maternal stress (Entringer et al., 2015; Murgatroyd, 2018). A study by (Slopen et al., 2015) found increased levels of the pro-inflammatory marker C-reactive protein (CRP), in adults who had increased levels of childhood social adversity (Slopen et al., 2015). Similarly, Danese et al. found a higher level of CRP in adults age 32 that had experienced adverse childhood experiences (Danese et al., 2009). A study by O'Connor et al found reduced immune function in infants of mothers who had exhibited prenatal stress and anxiety (O'Connor et al., 2013), low-grade chronic inflammation associates with reduced immune competence. In a study by Murgatroyd increased levels of the pro-inflammatory cytokines CRP and IL-6 were found in adults who had experienced high levels of prenatal and early life stress (Murgatroyd, 2018)

1.5.7 Effect of stress on regulating factors in milk

A further mechanism by which a mother might transfer her stress to her offspring is through milk during lactation. A mother's stress might program stress-regulation in her offspring through lactation in several possible ways. For example, maternal stress might: 1) impair mother's lactation such as leading to reduced levels of milk production for the offspring in turn impacting postnatal development; 2) interfere with production of nutrients in the milk important for development; 3) alter levels of immune factors in the milk important for offspring immune development; 4) change quantities of hormones in the milk that the offspring uses in its development of its stress response circuits, known as lactocrine programming; 5) impact offspring behaviour such that the newborn is less able to suckle properly.

1.5.7.1 Physiology of lactation

The two main hormones involved in lactation are prolactin, which is secreted from the anterior pituitary, and oxytocin, which is secreted from the posterior pituitary in a pulsatile manner. Prolactin is released in response to stimulation of the nipple, and suckling of the infant, and is involved in milk synthesis. Oxytocin functions to regulate a neuroendocrine reflex called the milk ejection reflex in response to infant suckling. Oxytocin can also be released in response to various environmental factors such as hearing a baby cry or by preparing to breastfeed. Importantly, oxytocin is inhibited by stimuli such as stress, fear and anxiety (Sriraman, 2017; Leng et al., 2005).

1.5.7.2 Milk constituents

Breast milk is composed of numerous factors and cells such as T-cells, macrophages and stem cells (Ballard and Morrow, 2013). Factors include immune factors and endocrine hormones and steroids. These factors are thought to help regulate systems such as the endocrine and immune systems of the offspring, at least until they can function independently (Palmer, 2011). These factors exist in particularly high concentrations that

are greater than plasma concentrations, and they include hypothalamic releasing factors, pituitary hormones, thyroid hormones, growth factors and many others (Palmer, 2011; Polk, 1992) (**Table 1.8)**.

Table 1.8. Immune, endocrine and growth factors present in either rodent or human milk

Class of factors	Factors
Pituitary hormones	ACTH, Oxytocin, Prolactin, Growth hormone, Melatonin
Steroids	Corticosteroids, Oestrogen, Progesterone
Thyroid hormones	Thyroid stimulating factor, T3, T4
Hypothalamic releasing hormones	CRH, GHRH, GnRH, TRH
Metabolic hormones	Insulin, Leptin, Adiponectin, Ghrelin
Growth factors	EGF, PDGF, VEGF, NGF, IGF, erythropoietin, calcitonin, lactoferrin
Cytokines	IL-6, IL-7, IL-8, IL-10, FNy, TNFα, TGFβ
Imunoglobulins	IgM, IgG, IgA

Together with nutrients such as fats, proteins, carbohydrates, vitamins and minerals found in breast milk the immune and endocrine factors are crucial in neurodevelopment. The World Health Organisation (WHO) recommends that infants should be exclusively breastfed for the first six months of life (Martin et al., 2016b). Several studies have shown that breastfeeding is important for neurodevelopment and cognition in the infant for example (Grantham-McGregor et al., 1999).

1.5.7.3 Effect of environment on milk production and milk constituents

Multiple factors, such as stress, depression, fatigue, or long periods of separation between the mother and infant can affect the production of milk possibly through oxytocin mechanisms. Therapies that reduce stress have been shown to increase milk production. For example one study found that mothers exposed to music-based, visual and verbal interventions over 6 days significantly increased volume of milk production (using a milk pump) and milk fat concentration (Keith et al., 2012) (**Figure 1.9**).

Some research shows that depression may decrease the prevalence of breastfeeding. One study has shown that a negative correlation between depression and breastfeeding at 6 weeks after birth that no longer correlated at 12 weeks suggesting that depression in the early postnatal period might lower the prevalence of breastfeeding (Hatton et al., 2005). Animal studies also show that stress can inhibit lactation, depending on the type of stressful stimuli (Dewey, 2001). One mechanism is that maternal stress has been shown to reduction oxytocin levels (Lau, 2001; Dewey, 2001) that in turn reduces milk ejection reflex. It is also possible that prolactin, may also be affected by stress (Torner, 2016)

1.5.7.5 Effects of stress on cortisol/corticosterone levels in milk

In a cross-sectional study by Benjamin et al. (Murgatroyd et al., 2015b) on maternal and infant cortisol levels and breastfeeding status in 54 mothers and their infants (20 partially or fully breastfed and 34 formula fed) found significant correlation of cortisol at bedtime between breastfeeding mothers and infants. Interestingly, this correlation was not found with formula fed infants and mothers. The authors suggested mechanisms might be that environmental factors of skin-to-skin contact and maternal-fetal physical interaction are typically increased in breastfeeding dyads compared with formula fed dyads (Mörelius et al., 2015), and there is increased correlation of cortisol in the former group. They further suggest the possibility of a transfer of cortisol in human milk that may also contribute to the increased correlation observed (Neelon et al., 2015).

Multiple rodent studies have found that the increased ingestion of cortisol (e.g. via mother's milk or experimental supplementation) is beneficial to the offspring, as it improves spatial memory, reduces anxiety, and improves the function of the HPA axis (Casolini et al., 1997; Catalani et al., 2000). The opposite, however, has been described in human studies, where infants ingesting higher levels of cortisol are found to have greater anxiety and stress related behaviours (Grey et al., 2013).

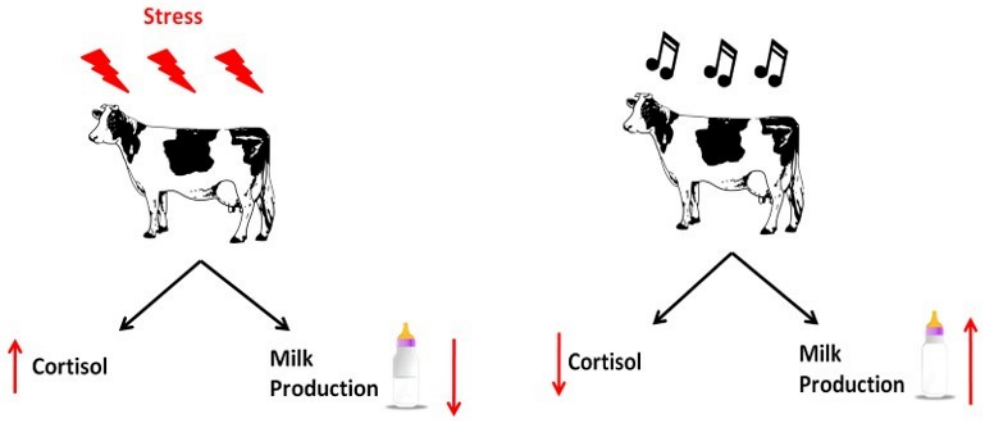

Figure 1.9. Schematic showing the effect of stress on milk corticosterone levels in cows*. Stress caused a reduction in milk cortisol and lower milk volume compared to cows that received music therapy and showed a reduction in cortisol and increase in milk production.*

1.5.7.6 Effect of stress on BDNF levels in milk

A study by Ismail et al looked at BDNF levels in infant serum and maternal breast milk in epileptic infants under 2 years of age, compared with healthy breastfed infants. They found significantly higher BDNF levels in the epileptic infants and in their mother's breast milk, compared to the serum of healthy control infants and their mother's milk (Ismail et al., 2015).

1.5.7.7 Transgenerational milk inheritance

"lactocrine programming" a term first used by Bagnell describes the process by which hormones in the mother's milk are able to influence behavioural, developmental and physiological processes within in the suckling offspring (Bagnell et al., 2009) Studies support the concept that the hormones within the mother's milk, when ingested, are able to bind to receptors within the sucking offspring activating hormonal signaling cascades in the offspring. It is therefore important to understand the effect of depression and stress on the levels of these hormones in breastmilk, as these may in turn impact stress-regulatory systems and behaviour in the offspring.

In the previously mentioned three generational chronic social stress (CSS) rat model, to study the effect of stress on the inheritance of maternal depression, anxiety and care

behaviours, an intergenerational reduction in maternal care was found. In the study Murgatroyd et al. reported reduced milk intake in the first generation (F1) on days 9 and 16, and reduced milk intake in the second generation (F1) on day 2 (Nephew et al., 2017) supporting that milk intake was reduced as a result of impaired maternal care observed in those generations. Interestingly, the female F1 dams also demonstrated reduced oxytocin receptor, vasopressin, and prolactin gene expression in the hypothalamus and amygdala.

1.6 Summary of possible mechanisms underlying transgenerational inheritance of stress and maternal depression

1.6.1 The role of maternal behaviour in programming transgenerational inheritance of infant behaviour

Non-genomic transmission of behavioural traits has been shown for maternal care in rats. Several studies have shown that maternal care of offspring was highly correlated to the behaviour exhibited by their own mothers in the first postnatal weeks (Francis et al., 1999; Pan et al., 2014; Champagne, 2011; Nephew and Murgatroyd, 2013; Nephew et al., 2017). Behaviour in turn could alter endocrine and immune regulators of stress in the offspring.

1.6.2 Transgenerational inheritance of epigenetic factors regulating maternal care

It is hypothesised that maternal stress and the mother's care determine offspring behavioural traits and their stress responses through epigenetic mechanisms (Murgatroyd and Spengler, 2011a). For example experiments in rodents show that experiences during sensitive periods of development influence DNA methylation patterns of several genes and that these patterns represent stable epigenetic modifications that alter gene transcription throughout the lifespan and promote specific behavioural outcomes (Murgatroyd et al., 2009).

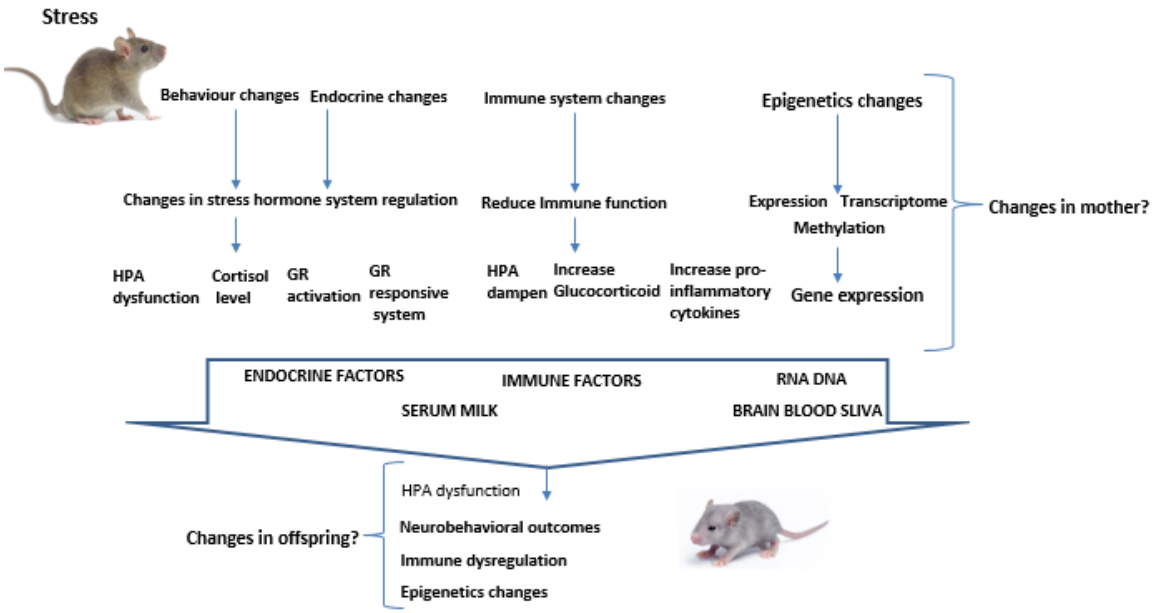

Figure 1.10. Summary of the mechanisms underlying transgenerational inheritance of behavior

1.6.3 Summary

This chapter provides evidence from previously published studies that: 1, stress may be transgenerationally inherited in both rodent models and human cohorts; 2, epigenetic marking of key genes are modulated by early life stress and further associate with later life depression; 3, endocrine factors are effects by early life exposure to stress; 4, the immune system is sensitive to early stress; 5, factors in milk can be regulated by stress in the mother and may influence stress regulation in the offspring.

1.6.4. Aims of all chapters

The aim of this thesis is to test for mechanisms involved in transgenerationally inherited stress using both rodent studies and human cohorts: Study 1 (Chapter 3) we will investigate and identify stress-related hormones and immune factors involved in the

possible intergenerational transmission of maternal behavior and depression using a rodent model. Study 2 (Chapter 4) we will perform a hypothesis-free genomewide screen using RNAseq to identify genes that are transgenerationally inherited following maternal stress and depression. Study 3 (Chapter 5) we will test epigenetic regulation of candidate genes in human studies of maternal stress and infants exposed to early stress. Study 4 (Chapter 6) we will explore the possible role of stress in the regulation of milk factors and identify key hormones that might be involved in the intergenerational transmission of maternal behavior and depression.

2 Chapter 2: METHODS AND MATERIALS

2.1 Introduction

This study uses a rodent model and two human studies to investigate, in a translational manner, the role of factors important in the intergenerational inheritance of stress through maternal depression (**Figure 2.1**). Stress-related, endocrine, immune and growth factors were measured in serum and milk using ELISA assays. Gene expression was measured in brains using real-time PCR and next generation sequencing, and DNA methylation studied using bisulphite sequencing.

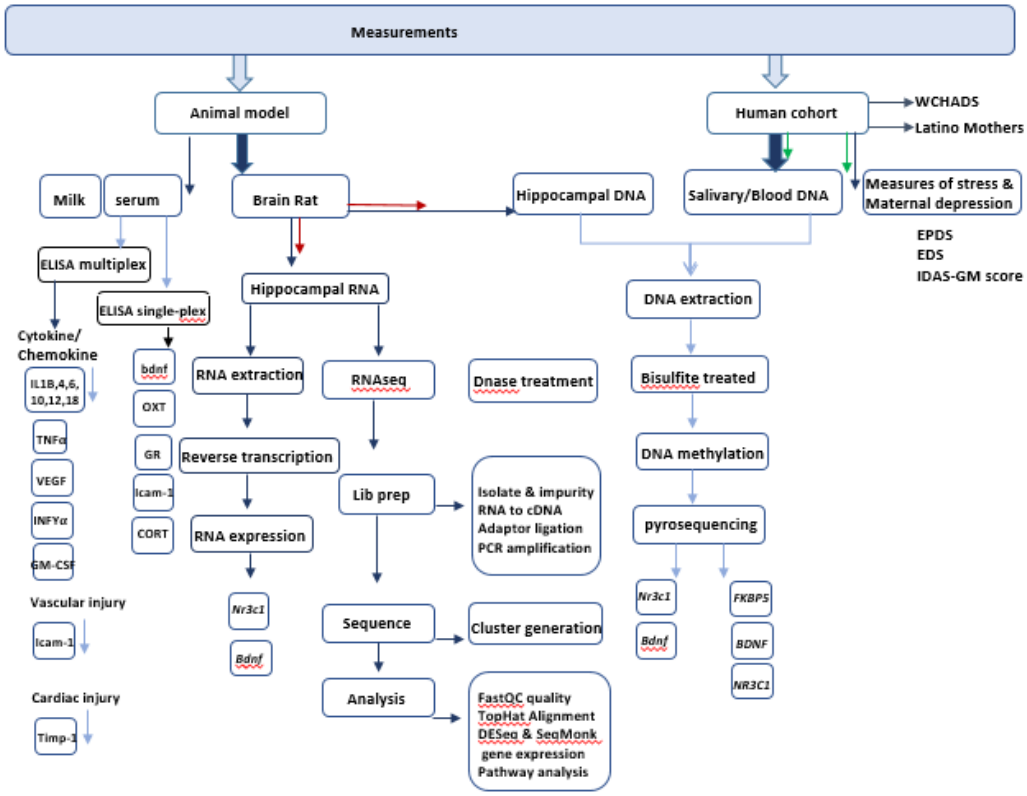

Figure 2.1. Timeline Flow chart of measurements

2.1 CSS Animal Model

The Sprague-Dawley rats were exposed to CSS and bred and behaviourally tested at Tufts University in collaboration with Dr Ben Nephew (Murgatroyd and Nephew, 2013). These animal experiments were performed in accordance with the guidelines of the Committee

of the Care and Use of Laboratory Animals Resources, National Research Council, and the research protocol was approved by the Tufts Institutional Animal Care and Use Committee.

The intergenerational maternal depression model involved subjecting F0 dams to a Chronic Social Stress (CSS) procedure from the 2nd to the 16th day of lactation. The CSS consisted of introducing a novel male intruder (this was smaller than the female so could not hurt the dam) while controls were left undisturbed, (Carini et al., 2013). Twelve dams of CSS and control were produced. The offspring of the F0 control and CSS dams are the F1 control and F1 CSS animals. On day 23 after weaning all the F1 offspring were housed in groups of four until 70 days old when two from each litter were mated with other Sprague-Dawley males. The F2 control and CSS animals were treated identically throughout the study. The final F2 sample sizes were n=14 and n=14 for the control and CSS groups respectively. Importantly, and there were no significant treatment differences in litter size or number or bodyweights at the F2 juvenile or adult stage, (all p's > 0.2) (Carini and Nephew, 2013). See (**Figure 2.2)** for an overview of the CSS model and the F0 to F2 generations. Brains, milk and serum were shipped to the MMU for analysis as part of this project. Brain punches from the hippocampus were taken.

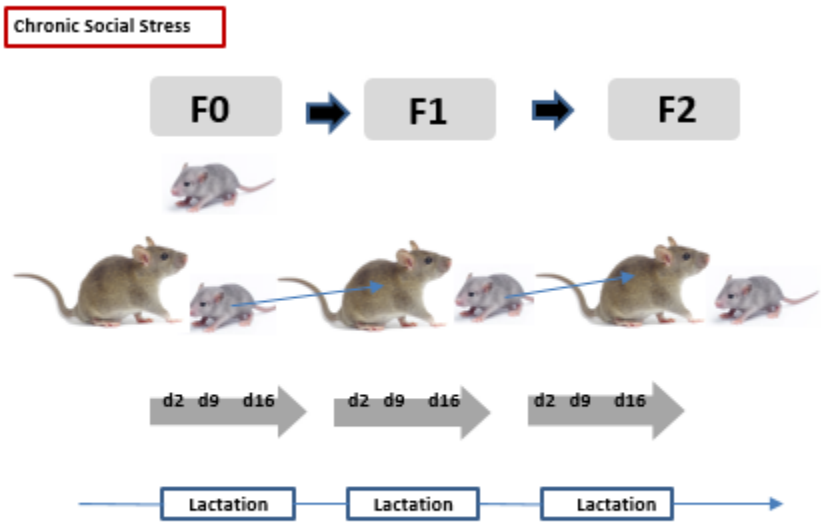

Figure 2.2. Schematic of the intergenerational model for chronic social stress. *F0 Dams are exposed to stress (by introduction of novel male intruder) during lactation in specific days (d) for maternal behaviour and endocrine measures. Her offspring (F1) are then allowed to grow and then tested and these offspring (F2) then tested for maternal behaviour and endocrine and immune measures.*

2.2 Human Study Populations

Two human cohorts were studied; the Wirral Child Health and Development Study (WCHADS), and the Latino Mothers Study. This was to allow testing DNA methylation with depression in 2 separate studies. It also allowed testing DNA methylation in children exposed to maternal depression in the WCHADS and the effects of stress (in the form of perceived discrimination) on DNA methylation in the Latino study.

2.2.1 WCHADS

The Wirral Child Health and Development Study (WCHADS) is a longitudinal study of prenatal and infancy origins of conduct disorders involving 1233 mothers with babies living in the Wirral area of the UK. A random subsample of 316 mothers were recruited as an intensive sample for which DNA was available from mothers and their offspring at multiple time points. The mothers at 20 and 32 weeks gestation were tested for prenatal depression and anxiety. The mothers and infants were then tested at 5, 9, and 29 weeks, and at 14 months for postnatal depression, depressive symptoms and anxiety. DNA was extracted from saliva obtained from the infants-children for 8 time points (within this study we used DNA collected at age 3). This DNA was used for methylation analyses.

2.2.2 Latino study

The Latino study is a longitudinal study of healthy pregnant Latina women (n = 150) living in North Carolina (NC) were enrolled in the study between May 2016 to March 2017. The mean age was 27.6 years old and exclusion criteria included those with severe depressive symptoms, a history of psychotic or bipolar disorder, or substance dependence to avoid

confounders and control for severe mood symptoms. Data collection was completed in English or Spanish, depending on participants preference, by a trained research assistant at the prenatal visit at 24-32-week gestation and a postnatal visit 4-6 weeks following birth. DNA was extracted from the mothers from blood samples by Hudson Santos from South Carolina and shipped to Manchester. This study was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill (#15-3027).

2.2.3 Measures of stress

Perceived Discrimination: The Everyday Discrimination Scale (EDS), a nine-item questionnaire, was used to measure routine, day-to-day experiences of discrimination at prenatal and postnatal. The stem question is: "In your day-to-day life, how often do any of the following things happen to you?" Sample items include: "You are treated with less courtesy than other people are," "People act as if they think you are dishonest" and, "You are called names or insulted." Participants then link main reason for these discrimination experiences, including gender, race, ancestry, religion. In addition, participants were asked whether they have felt any type of ethnicity-based discrimination during their lifetime. The EDS is a widely used measure of subjective experiences of discrimination (Williams et al., 1997), with validated Spanish translation (Campo-Arias et al., 2016); It correlates with measures of institutional racial discrimination and interpersonal prejudice (Krieger et al., 2005) and does not prime the subjects to think about race, which limits cues to prejudice prior to responding to the questions (Deitch et al., 2003).

2.3.4 Measure of maternal depression

Inventory of Depression and Anxiety Symptoms - General Depression Scale (IDAS-GD): This was used in the Latino study to comprehensively assess depressive symptoms (O'hara et al., 2012) to account for negative mood prenatally and postnatally. A higher IDAS-GD scores indicates more severe symptoms. For control non-depressed women the Typical

IDAS-GD score is around 32.4 and for high-risk women it is 37.4. Scores between 44.6 and 57.3 signify depression (Schiller et al., 2013; Medeiros et al., 2018; Fletcher et al., 2015)

Edinburgh Postnatal Depression Scale (EPDS): This is a 10-item self-report questionnaire that was developed to identify women who have postnatal depression and is an established screening tool for monitoring depressive symptoms in the postnatal period, as well as parental. It is usually used as a single score and has been used in longitudinal research studies, in many cross-sectional studies on maternal wellbeing, as well as in clinical practice. Up to 3 points are given for each question with 0 – 8 points signifying low probability of depression, 8 – 12 points suggestive of mild most likely just dealing with a new baby or the baby blues, 13 – 14 points suggest signs leading to possibility of Postnatal Depression, and 15 + points signifies a high probability of experiencing clinical depression. Importantly, this test is not diagnostic (Cox et al., 1987).

2.3.4 Analysis of serum proteins

ELISA was used to measure the concentration of specific factors in the serum using single-plex assays or customisable multiplex assays (MILLIPLEX MAP, Millipore) (**Table 2.1**). ELISA allows the detection of specific proteins by specific antibodies, as part of an immunoassay. Through a series of incubation and washing steps, these antibodies, which are linked, to an enzyme, will detect protein coating the bottom of a well on a microtiter plate. When exposed to a substrate, antibody-bound enzyme will cause a color change, indicating the presence of the protein-of-interest in the sample. A variation of this procedure is Luminex xMAP Technology where a microsphere (bead) array is used enabling multiple assays to be simultaneously performed from a single samples, i.e. multiplexing (Baker et al., 2012).

Three assay panels were used to measure of pituitary hormones (ACTH, bdnf, TSH and prolactin), neuropeptides (α-MSH, β-Endorphin, Neurotensin, Orexin A, Oxytocin, Substance P), and anti-inflammatory cytokines (GM-CSF, sIcam-1, IFNγ, IL-2, IL-4, IL-6, IL-10, TNFα and VEGF). Three assay panels were used to measure of cytokine (IL-1β, IL-4, IL-

6, IL-10, IL-14, TNFα, IFNγ, VEGF and GM-CSF), vascular injury, (Timp-1), and cardiac injury (Icam-1) were purchased from Millipore and R&D Systems (R&D Systems, UK) was conducted on a Luminex 200 Bio-Plex Platform. Immediately prior to the initiation of the study, the Bio-Plex platform underwent a complete on-site maintenance cycle. Samples were thawed directly on the day of analysis. Working wash solutions and protein standards were prepared within 1 hour of beginning the assay by reconstituting the standard in assay diluent and performing serial dilutions according to manufacturer specifications. A magnetic plate washer was utilized during the plate washing stages. Following processing, protein concentrations were calculated and analyzed with the xPONENT software (Luminex, v.3.1.871). The majority of samples were run in duplicate in an individual assay to eliminate interassay variation.

Table 2.1. List of endocrine and immune factors measured in rat plasma using (ELISA) single-plex and multiplex immunoassays

ELISA kit (Supplier)	Supplier
Oxytocin ELISA kit (ENZO)	Oxytocin
Corticosterone ELISA kit (ENZO)	Corticosterone
GXR ELISA kit Cat no: 80-0060 (ENZO)	GxR bdnf, OXT, GR, ICAM-1, CORT (single)
MILLIPLEX R MAP RAT Neuropeptides Magnetic Bead Panel (Millipore)	α-MSH, β-Endorphin, Neurotensin, Orexin A, Oxytocin, Substance P
MILLIPLEX R MAP RAT Pituitary hormones Magnetic Bead Panel (Millipore)	ACTH, bdnf, TSH and prolactin
MILLIPLEX R MAP RAT Anti-inflammatory cytokines Magnetic Bead Panel (Millipore)	GM-CSF, sICAM-1, IFNγ, IL-2, IL-4, IL-6, IL-10, TNFα and VEGF
MILLIPLEX R MAP RAT Cytokine/Chemokine) Magnetic Bead Panel (Millipore)	IL-1β, IL-4, IL-6, IL-10, TNFα, IFNY. and L1B,4,6,10,12,18 fromTNFα, INFYα, GM-CSF, VEGF.

MILLIPLEX R MAP RAT Cardiac Injury Magnetic Bead Panel 1 (Millipore)	Timp-1
MILLIPLEX R MAP RAT Vascular Injury Magnetic Bead Panel 2 (Millipore)	ICAM-1

3.2.3 Microdisection of hippocampus using brain punches

Frozen rat brains were sectioned using a LEICA cryostat at -18°. A scalpel was used to cut off the cerebellum and the rest of the brain sample was stuck to a chuck using Neg50 glue and placed in the pre-chilled LEICA cryostat to freeze.

The brain was sectioned cronally until the hippocampus was arrived at. A section was placed on a frosted slide and melted to the slide before staining for 2 minutes in cresyl violet fast (company). This was then washed in ethanol for 2 minutes and water for 1 minutes. The brain slice was visualized using a light microscope. Once the hippocampus is seen brain punches were taken across the hippocampus using a tissue borer (1 mm diameter) (**Figure 2.3**). Two punches were taken from both sides of the hippocampus. The punches were then placed in a pre-frozen Eppendorf tube and stored at -80° until extracted for DNA and RNA.

Figure 2.3. A cresyl violet stain of rat hippocampus and a frozen brain with a punch taken to show the diameter of the punch in relation to the hippocampus.

3.2.3 Simultaneous RNA and DNA extraction from brain punches

Brain punches were extracted for both RNA and DNA **(**see **Figure 2.3** for the scheme**)**. This allowed to reduce the number of animals needed and allowed to correlate RNA expression and DNA methylation within the individual animals. The frozen brain punches

were digested in 800 µl of Trisure (Bioline) using a vortexer and a 23-gauge needle. This was incubated for 5 minutes at RT followed by adding of 160 µl of chloroform. Samples were then shaked vigorously for 15sec and incubated for 5 minutes at RT. Samples were then centrifuged at 12,000xg for 15 minutes at 4°C. Three phases appeared: the upper colorless portion that contains RNA, the lower portion containing the solvent and the middle layer containing the DNA and protein. The upper phase (RNA) was transferred into clean into Eppendorf tubes and the RNA precipitated by adding 500 µl of ice-cold isopropanol followed by sample incubation for 10 minutes at room temperature. This was then centrifuged at 12,000xg for 10 minutes at 4°C. The supernatant was removed and RNA wash was performed by adding 1ml of 75% ethanol to the pellet and vortexed then centrifuged at 7500xg for 5 minutes at4°C. Finally, the pellet was air dried and dissolved in 25 µl DEPC-treated water.

The middle layer (DNA and protein) was taken and DNA extracted following a DNA extraction kit (Bioline). 180 µl of the lysis buffer GL was added, followed by 200 µl of G3. After incubation for 10 minutes at 70°C in a heat block (Labnet or Grants), 210 µl of 100% ethanol was used to wash the sample, and the resulting sample transferred to a spin column and centrifuged (SIGMA 3-16K) to allow DNA binding. Subsequent column washes were applied to remove unwanted proteins and salts. 30 µl of elution buffer G was added to the spin column to elute the DNA.

The DNA and RNA quality and quantity was measured by using the NanoDrop 1000 spectrophotometer (ThermoScientific). The RNA concentrations and purity were determined by assessing the ratio of absorbance at 260/280 nm and 260/230 nm. Ratios of 260/280 nm of ~1.8 and ~2.0 were accepted as “pure” for DNA and RNA, respectively, with 260/230 values in the range of around 2.0-2.2 and similar in all samples.

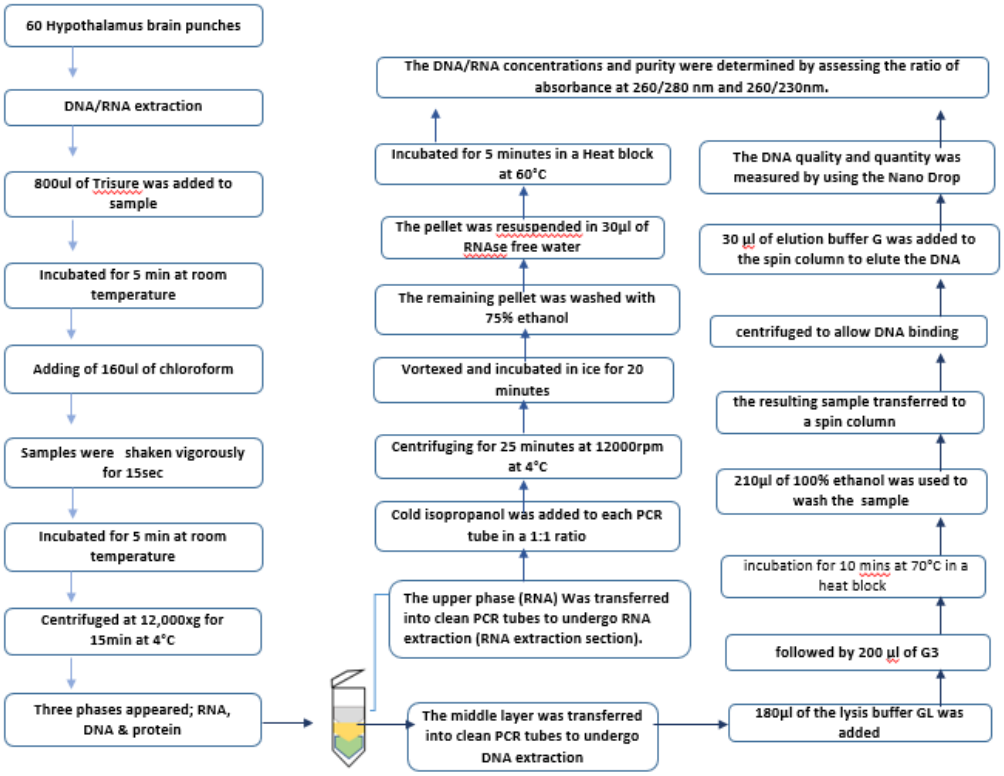

Figure 2.4. Flowchart of the Simultaneous RNA and DNA extraction from brain punches

3.2.3.2 Bisulfite conversion of DNA

200ng of extracted DNA, for each sample, was bisulfite treated and purified using the EpiTect Fast DNA Bisulfite Kit 200 (QIAGEN). See (**Figure 2.5)** for scheme. In summary, sodium bisulfite solution and sodium hydroxide (DNA protection buffer) were both mixed with the samples, before incubation in a thermocycler (SureCycler 8800; Agilent Technologies Inc., Santa Clara, USA) with the following settings: 95°C (5 minutes), 60°C (20 minutes), 95°C (5 minutes), 60°C (20 minutes) to ensure denaturation of the DNA and optimal deamination. The DNA was added to a spin column following and desulfonation buffer added followed by wash buffer and finally, elution buffer to dissolve the DNA from the spin column membrane and elute the bisulfite DNA.

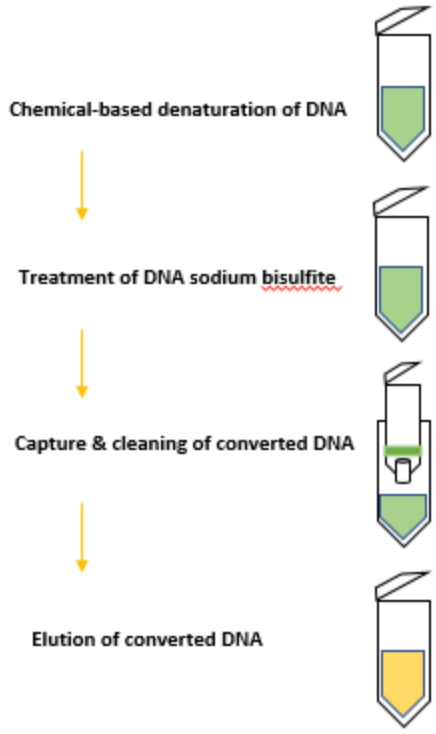

Figure 2. 5. Flowchart of the Bisulfite treated DNA

3.2.3.3 Bisulphite PCR

Bisulfite DNA was amplified by Polymerase chain reaction (PCR) with primers specific to bisulphite converted DNA and modified with a Biotin for the pyrosequencing method (see table 2.4). The total volume of reaction mixture was 20 µl: 10 µl myTaqHS, 1 µl Forward primer (10 µM), 1 µl Reverse primer (10 µM), 6 µl water MiliQ H2O. Reaction mixtures were made up as a master mix and plated out in a 96-well plate before additon of 2 µl of bisulfite DNA (which equals about 10 ng) DNA to each tube of the plate. The plate was placed in a thermocycler (PCR Agilent Technologies SureCycler 8800; Agilent) with the following program: 95°C (30 minutes), 58°C (30 minutes), 72°C (30 minutes) (45 cycles) that worked for both PCRs. A number of programs with different annealing temperatures from 55°C -60°C were tried, together with various primer concentrations and the products tested by gel electrophoresis, and the best program was recorded (**Table 2.2**).

Table 2.2. Primer sequences, locations and sequences of regions targeted by bisulphite pyrosequencing

Gene (location)	Species	Primers; Forward (F), Reverse (R), Sequencing (S), Biotinylation (B)	PCR cycle	Sequence analysed (CpGs numbered) 5'–3'
Nr3c1	Rat	F-TTGGTTTGGGAGGGAAAT; R-(B)AACTATCCCCTCCAAAACTCTAACTAC S-CCCTCCAAAACTCTAACTACC	94°C 30sm 56°C 30s 72°C 30s 50 cyc.	(CpG1)CR(CpG2)AAACTAAACR(CpG3)AAAACR(CpG4)AAAAAAAAATAAC
bdnf	Rat	F-AAAGGGGGTTTAGGGAGTTAT R-(B)ACTATCATATAATACCTCCTCTACC S-AGGTTTGGTTTTTGTG	94°C 30sm 56°C 30s 72°C 30s 50 cyc.	(CpG1)CR(CpG2)AAACTAAACR(CpG3)AAAACR(CpG4)AAAAAAAAATAAC
NR3C1 (hg19;chr5: 143,404,044-143,404,076)	Human	F-(B)AATTTTTTAGGAAAAAGGGTGG F-AACCCCTTTCCAAATAACACACTTC S-AACTCCCCAATAAATCTAAAAC	94°C 1m 60°C 1m 72°C 1m 50 cyc.	CR(CpG1)CR(CpG2)AAACTAAACR(CpG3)AAAACR(CpG4)AAAAAAAAATAAC
BDNF (hg19; chr11:27,701,578-27,701,672)	Human	F-GATTTTGGTAATTCGTGTATTAGAGTGTT R-(B)AATGGAGTTTTCGTTGATGGGGTGCA S-AGATTAAATGGAGTTTTCGTTGAT	94°C 1m 56°C 1m 72°C 1m 50 cyc.	Y(CpG1)GTAY(CpG2)GTTAAGGTATY(CpG3)GTGGAGTTTTTTY(CpG4)GTGGATTTTTATTTATTTTTTTATTTATY(CpG5)GY(CpG6)GGAGAGGGTTGTTTTY(CpG7)GTTGTYGTTTTTTTYGGYG
FKBP5 (hg19; Chr6: 35,558,48-35,558,567)	Human	F-GGATTTGTAGTTGGGATAATAATTTGG R-(B)TCTTACCTCCAACACTACTACTAAAA S-GGAGTTATAGTGTAGGTTT	94°C 1m 60°C 1m 72°C 1m 50 cyc.	TTTY(CpG1)GTGATTTTTGTGAAGGGTATAATTY(CpG2)GTTTAGTTTTGAAAAG

3.2.3.4 Bisulfite Pyrosequencing

Bisulfite pyrosequencing was done to measure the degree of DNA methylation at individual CpG sites. It was carried out on the PyroMark Q24 system (Qiagen, Hilden, Germany) using the PyroMark Q24 Advanced CpG reagents following the manufacturer's instructions. In rat samples, promoter IV of the *bdnf* gene and promoter 1.7 of the *Nr3c1* gene were analyzed. In human samples, *BDNF* promoter 1.4, *NR3C1* promoter 1.**F** and the *FKBP5* promoter were analysed.

For each 10 µl of PCR product, 1 µl of Streptavidin Sepharose High Performance beads (GE Healthcare), 40 µl of binding buffer, and 29 µl of ddH2O were added in a 24-well PCR plate. The plate was placed on a shaker for 10 minutes at 1400rpm at room temperature. The samples were then treated using the PyroMark Q24 workstation (Qiagen) as follows: 5 seconds in 70% ethanol, 5 seconds in denaturation buffer, and 10 seconds in wash

buffer. Following this, the samples were added to 25 µl of 0.3µM diluted sequencing primer in a Q24 sequencing plate, and heated for 2 minutes at 80°C before setting aside at room temparature. The PyoMark Q24 advanced CPG Reagents (Qiagen) were added to the sequencing cartridge, using the volumes determined by the PyroMark Q24 Advanced software. Both the cartridge and the Q24 sequencing plate were placed in the Q24 sequencer (Qiagen), and the methylation levels determined using the PyroMark Q24 advanced software. See (**Figure 2.6)** for a scheme.

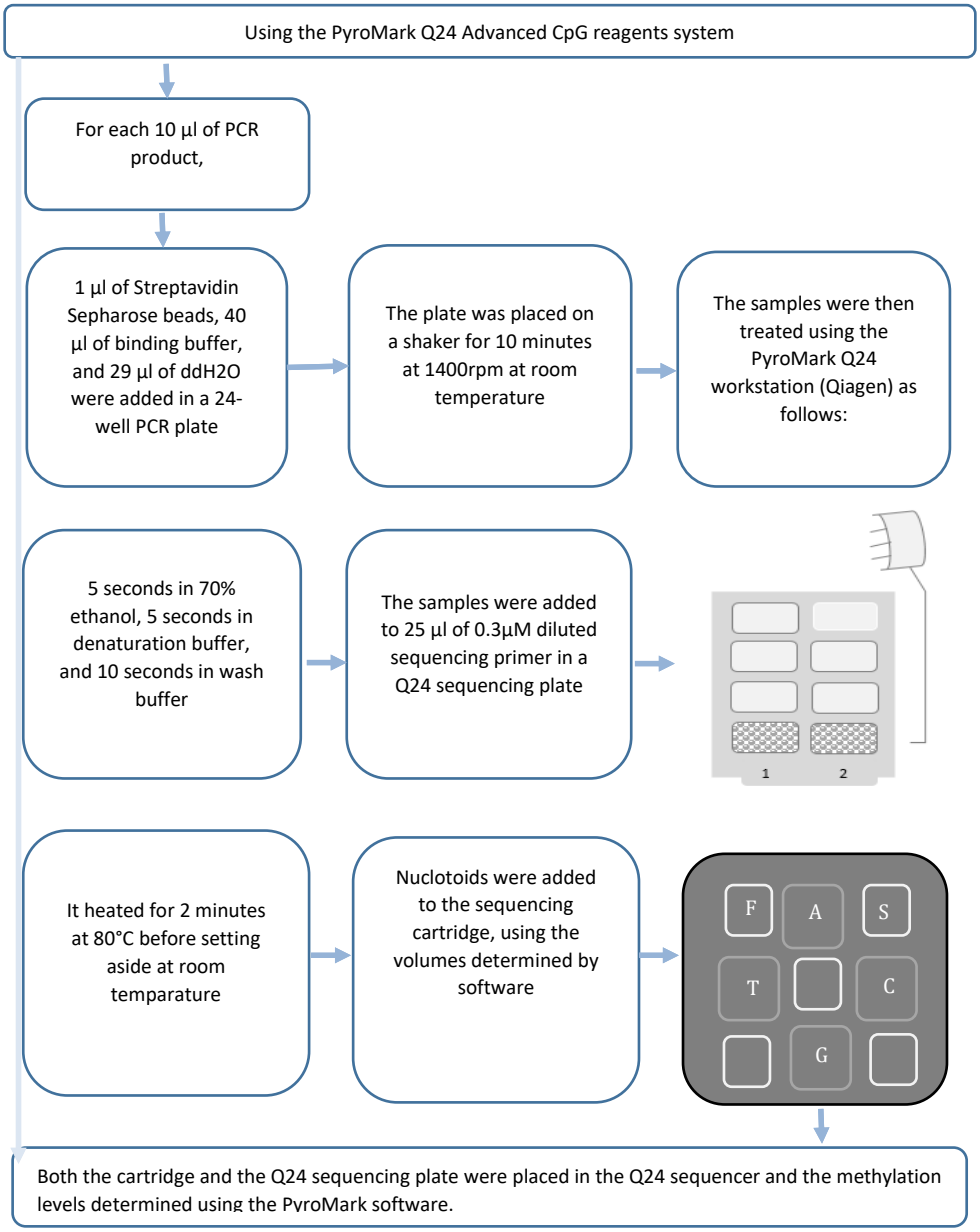

Figure 2.6. Flowchart of Bisulfite Pyrosequencing

3.2.4 RNA reverse transcription

The quality and quantity of the RNA was measured using the NanoDrop 2000 spectrophotometer (ThermoScientific). The RNA concentrations and purity were determined by assessing the ratio of absorbance at 260/280 nm and 260/230 nm.

The GoScript Reverse Transcription system was used to convert the RNA to cDNA by reverse transcription. Reaction mixtures (5 µl) were made as follows: total RNA (200ng), Hexamer primer (1 µl), and nuclease free water. The reaction mixtures were added to a new PCR plate and then incubated at 70°C for 5 minutes, before 10 seconds of centrifugation. Each 5 µl RNA reaction mix was added to 15 µl of reverse transcription reaction mix (20 µl). The total 20 µl was placed in a thermocycler (PCR Agilent Technologies SureCycler 8800) with the following settings: 95°C (5 minutes), 60°C (1 hour), 72°C (15 minutes).

3.2.4.2 Real-time polymerase chain reaction (RT-PCR)

The cDNA from the previous step, was then amplified using RT-PCR. Briefly, the following reaction mixture was made up in each well of a PCR plate for 60 samples (30 *bdnf*, 30 GR): 3 µl sample of cDNA, 15 µl SyBR (Bioline, UK), 1.5 µl of *bdnf*/GR Forward primer, 1.5 µl of *bdnf*/GR Reverse primer and 9 µl of nuclease free water. The PCR plate was placed in a real-time PCR system (Stratagene Mx3000P): 95°C (30 seconds), 60°C (30 seconds), 72°C (30 seconds) (40 cycles).

Expression levels for oxt, oxt receptor (oxtr), avp, avp V1a receptor, the long form of the prolactin receptor, GR (*Nr3c1*) and MR (*Nr3c2*) were normalized against three combined housekeeping genes, β-actin, hypoxanthine phosphoribosyltransferase (hprt) and glyceraldehyde-3-phosphate dehydrogenase (gapdh). Sequences of primers for real-time PCR are given in (**Table 2.3)**.

Table 2.3. Primer sequences for real-time PCR

Primers	Species	Sequence (5'-3')	PCR cycle
B-actin	Rat	-TTGCTGACAGGATGCAGAA; R-ACCAATCCACACAGAGTACTT	94°C 2m 94°C 5s 62°C 5 72°C 10s 45cyc
Hprt	Rat	F-CATCACCATCTTCCAGGAGC; R-TAAGCAGTTGGTGGTGCAGG	94°C 2m 94°C 5s 58°C 5 72°C 10s 45cyc
Gapdh	Rat	F-TGGTCAAGCAGTACAGCCCC; R-TACTGGCCACATCAACAGGA	94°C 2m 94°C 5s 60°C 5 72°C 10s 45cyc
Nr3c1	Rat	F-AGGGGAGGGGGAGCGTAATGG; R-CCTCTGCTGCTTGGAATCTGC	94°C 2m 94°C 5s 58°C 5 72°C 10s 45cyc
Bdnf	Rat	F-AGGGGAGGGGGAGCGTAATGG; R-CCTCTGCTGCTTGGAATCTGC	94°C 2m 94°C 5s 58°C 5 72°C 10s 45cyc

3.2.4.2.1 Gel electrophoresis

Gel electrophoresis (Bio_RAD) was used to confirm that the PCR products were adequately amplified, using 2% agarose gel and Midori Green.

3.2.4.2.1 Statistical analysis

The cytokine and hormone levels were analysed with either 2-tailed or 1-tailed t-tests (these were not corrected for multiple comparisons), following tests for distribution. The use of a 1-tailed t-tests for the ICAM-1 analysis in the F1 rats was justified in the case of a priori results indicating direction in previous studies of the F2 adults. Pearson correlations were used to test for associations with DNA methylation in the human studies, following tests for distribution. All graphical results are presented as

mean + SEM, and the level of statistical significance was $p \leq 0.05$. Excel was used for t-tests and graphs and SPSS for correlations.

3.2.3.4 RNASeq

RNASeq was performed according to methods provided as part of Chapter **4**. Briefly, RNA was used to make a cDNA library prep and sequenced using an Illumina NextSeq Next Generation Sequencer.

3.2.3.4 Analysis of rat milk

ELISA was performed on rat milk according to methods provided as part of Chapter **6**. Breifly, multiplex ELISA plates were used that were read using the Luminex Multiplex plate reader.

2.1 Materials

2.1.1 Equipment

Table 2.4. Equipment used for laboratory

Equipment	Supplier	Use
NanoDrop 2000 spectrophotometer	ThermoScientific	Quantification and purity of DNA and RNA samples
Bioanalyzer 2100	Agilent	Qualification of DNA and RNA samples
Nanod Qubit 3 Flurometer	Invitrogen	Quantification and purity of DNA and RNA samples
Thermocycler, SureCycler 8800;	Agilent Technologies Inc., Santa Clara, USA	PCR amplification, with Stepdown option
Stratagene Mx3000P	Stratagene	Real-time PCR for cDNA expression and Genotyping
Eppendrof PCR Mastercycler	Eppendorf	Bisulphite PCR amplification
PyroMark Q24 system	Qiagen, Hilden, Germany	Pyrosequencing for CpG methylation
Gel electrophoresis	BIORAD	Separation of DNA according to their size
NextSeq® Sequencer system	Illumina	Next-generation sequencer
Luminex XMAP TM Technology	Millipore	Multiplex ELISA analysis.

Bio Tek ® INSTRUMENTS elX405 #250371 (U.S.A)	Bio Tek	Magnetic plate washer for ELISA
Chemi/UV/Stain-tray & ChemiDoc UV (Bio-RAD)	BIORAD	Ultraviolet radiation touch imaging system
Centrifuge SIGMA 3-16K	Sigma	For DNA extraction
Centrifuge 5415	Eppendorf	A non-refrigerated bench-top centrifuge for bisulphite DNA conversion
Heater Techne Dri-Block DB-2D	Sigma	heater for denaturation of DNA prior to bisulphite pyrosequencing
Shaker	Thermo PeQlab	For bisulfite sequencing
Adjustable Pipette with Tips capable of delivering 25 µl to 1000 ul. Lot 16271071	Gilson- Thermo scientific	For all experimental analyses.
Multichannel Pipettes capable of delivering 5 µl to 50 µl or 25 µl to 200 µl.	BIORAD	For pipetting multiple DNA samples.
Laboratory vortexemixer	VortexGenie	mix small vials of liquid, attached to a rubber piece
Sonicator (Grant Ultrasonic cleaner # XUBA1)	Grant	Ultrasonic vibration so as to fragment
Titer plate shaker (IKA MTS 2/4 digital)	IKA	Assay and run plates, timed
Automatic plate washer for magnetic beads	Bio Tek	Wash of magnetic microspheres with automatic buffer switching
Microwave	Sigma	Melting of agarose
PyroMark Q24 Workstation Qiagen- Q24 biomark tray	Qiagen	Washing and denaturing of PCR products for pyrosequencing
Image/Gel doc system	BIORAD	Imaging and documentation of agarose gels
Cytostat	LEICA CM3050S	Brain sectioning and dissection

2.1.2 Molecular biology kits

Table 2.5. Kits used for laboratory

Kit	Supplier	Use
EpiTect Fast DNA Bisulfite Kit 200	QIAGEN	Conversion and *purification* of DNA

ISOLATE ll Genomic DNA kit	Bioline	Isolation of high-quality total cellular RNA
PyoMark Q24 advanced CPG Reagents	Qiagen	Advance quantitative CpG methylation to improve quantification of sequence
RNA 6000 Nano Reagents part 1 Kit-no 5067-1511 RNA 7000 Nano Chips	Agilent	RNA quality and quantity
Bisulphite Kit	Qiagen	Conversion of DNA for methylation analysis
GoScript Reverstranscripase Kit	Bioline	Reverse transcriptase for efficient synthesis of first-strand cDNA optimized in preparation for PCR amplification
Library Prep kit NEBNext Ultra RNA Library Prep Kit for Illumina E7530S BioLabs no.: 0111612	NEB	input for libraries to be sequence on the Illumina
RNA module NEBNext poly(A) mRNA Magnetic Isolation Module (BioLabs)	NEB	Isolation of poly(A)+ RNA. based on Oligo beads for binding of poly(A)+ RNA
NEBNext Multiplex Oligos for Illumina kit- Biolab E7335S No.: 0121610, (set 1, NEB E7335); (set 2, NEB E7500)	NEB	Library production base index primers with universal primer
RNATURBO DNA-FREE TM Kit-Invitrogen by Thermo Fisher Scientific Lot: 00505703	Thermo Fisher	Single- and double-stranded DNA
Luminex 100/200 Calibration Kit	Illumina	Calibration and verification
DNA extraction kit	Bioline	Rapid purification of high-quality DNA to be ready for PCR, sequencing
cDNA via the iScript cDNA Synthesis kit	BIORAD	First-strand cDNA synthesis kit for gene expression analysis using real-time PCR
High sensitivity DNA Reagents (Agilent Technologies) Lot No. 1749	Agilent	Separation, sizing and quantification

2.1.3 Glass and Plastics

Table 2.6. Glass and Plastics used for laboratory

Glass and Plastics	Supplier	Use
Conical flask	Pyrex	Agarose gel preparation

Reagent reservoirs	Starlabs	ELISA
Polypropylene microfuge tubes (0.5m., 1.5ml)	Starlabs	Most experiments
96 well PCR plates PCR plate consumables STARLAB lot.15189- caps strip MicroAmp GIAGENS lot. No.154038416 Mat.No. 1071591	UNSKIRTED	SKIRTED for the Eppendorf PCR UNSKIRTED for the Stratagene
Optical film- Lot no: 112571	BIORAD	For sealing 96 Well PCR plate seals
25G needle	Starlabs	DNA and RNA extraction
Q24 plate holder	Qiagen	For Pyrosequencing
Filter RNA and RNase-free tips. (10 µl, 200 µl, 1ml)	Starlabs	For all DNA work and PCRS
Non-Filter tips (10 µl , 200 µl, 1ml)	Starlabs	ELISA and non-contamination risk experiments
Brain micropunches	F.S.T	For brain microdissection
Magnetic rack 15 ml Tube- 2408 – DYNAL Invitrogen bead separations	Invitrogen	For RNA library prep
Parafilm 4 in. X125 FT.roll Pechirvey WI 54952	Heathrow Scientific	For loading agarose gels
Pyromark Sequencing cartridge	Qiagen	For Pyrosequencing

2.1.4 Chemicals

Table 2.7. Chemicals used for laboratory

Chemicals	Supplier	Use
Trisure 38033	Bioline	isolation of RNA, DNA
Chloroform	Sigma	substances dissolve
Isopropanol	Sigma	Dissolve, as cleaning fluid
Ethanol (70% ,75%,80%, 100%)	Sigma	denaturing and dissolving
RNAse free H_2O Mat. No. 1012888	QIAGEN	high purity
MyTaqHS Mix,2x Cat No.Bio-25046 Bioline	Bioline	Enzyme for PCR amplification
Agarose MELFORD MBI 200 Cas:9012-36-6 No.20509- TBEx1	MELFORD	diagnostic tool to visualize the fragments to move the DNA across
DNA stain madiore green advance 1ml Cat. No MG04	NippoN Genetics EuRopE GMBH	detecting nucleic acid in agarose gels
DNA loading buffer	Bioline	Mix with DNA samples for prepare DNA markers for loading on agarose
hyperladder 25bp. 50bp	Bioline	mass determination of DNA throughput agarose gels to determine the size of DNA fragments.

Bead (streptavidin sepharQse)	GE Healthcare	(protein) Streptavidin strong interact with biotin used to purify antigens
SYBR (Bioline, UK)	Bioline	binds to all dsDNA used in combination with a primer to detect PCR products in real-time.
Richard-Allan Scientific NeG 50 glue	Thermo Scientific	to prevent tissue separation from the slide
Sheath Fluid (XMAP®) #B64487(U.S.A)	Thermo Fisher Scientific	the delivery medium that carries the sample to the optics component of Luminex

2.1.5 Software

Table 2.8. Software used to process Experiments

Software	Supplier	Use
PyroMark Q24 Advanced software	Qiagen	used with the QIAGEN kits
PrimerDesign…for PCR product to seq	Qiagen	Check for validated designs on pyrosequencing Design primer/past seq/generate primer/
GraphPad	GraphPad Software Inc. (through MMU)	Table data for ELISA analysis
SPSS	SPSS Inc. (through MMU)	correlation, regression, tables, scatter plot
Exel	Microsoft (through MMU)	Makes figures
Quantify-one programme softwere- UV light box BIO-RAD – printer Sony 100V 1.5A 50/60H2	BioRad	For image calibration for quantify DNA/RNA
Bacespace	Illumina	allows to *use* the apps to *analyze* RNASeq analysis data
Babrahm bio. RNAseq analysis. Fast QC. Top HAT Alignment. Desseq2. HISAT2	Babrahm Bioinformatics	Analysing RNA sequence data in sequencing for analysis for (trimming, mapping and gene expression)
SeqMonk	BaseSpace	mapped next generation sequence data of genomic Gene expression -RNASeq
luminex 200 TM with XPONENT ® SOFTWARE	Millipore	assay workflow graphical user for Elisa data
link program for automatic plate washer for magnetic beads of Bio Tek ®	Bio Tek	For wash plate of ELISA

Agilent high sensitivity DNA Assay- 2001 expert - Bioanalyzer system	Agilent	RNA quality, total RNA with RNA Integrity Number (RIN) and graphs
DAVID protein pathway. analyses	DAVID bioinformatics	Gene ontology analyses and pathways
PANTHER (**protein** analysis through evolutionary relationships)	Thomas lab at the University of Southern California	Gene ontology analyses
Gene Ontology enrichment analysis and visualization tool	UniProtKB-GOA, European Bioinformatics Institute, Cambridge, UK	Gene ontology analyses

2.1.6 Solutions

Table 2.9. Solutions used for laboratory

Solutions	Recipe	Use
Pyrosequencing denaturation buffer	0.2 M NaOH	Pyrosequencing
Pyrosequencing wash buffer	PH 7.6 (10 Mm Tris-Acetate)	Pyrosequencing
10xTBE	Dissolve 108 g Tris and 55 g Boric acid in 900 ml distilled water, add 40 ml 0.5 M Na_2EDTA (pH 8.0) Adjust volume to 1 Liter.,	DNA and RNA gel electrophoresis
Sheath Fluid (XMAP®) #B64487(U.S.A)	(XMAP®)	Luminex

3 Study 1: Inheritance of endocrine, immune and epigenetic changes in a rat model showing inter-generational transmission of maternal depression

3.1 Introduction

Early adversities such as exposure to prenatal and postnatal stress such as maternal depression and reduced maternal care can produce long-lasting physiological and behavioural effects on offspring and future generations. In a rat model, exposure of dams to chronic social stress (CSS) during lactation reduces their levels of maternal care (Carini et al., 2013; Carini and Nephew, 2013; Murgatroyd et al., 2015b; Nephew and Bridges, 2011). Interestingly, stress-related behaviours are increased and levels of maternal care reduced in their F1 offspring (Carini and Nephew, 2013; Murgatroyd and Nephew, 2013; Murgatroyd et al., 2015b). Furthermore, the F2 generation show even further exaggerated increases in maternal stress and reductions in maternal care behaviour supporting an intergenerational transmission of increasing maternal depression.

Factors governing such transmission of maternal depression across the generations could include stress-regulatory factors important in the HPA axis, as discussed. Importantly in the CSS animal model the F1 animals were exposed to postnatal depressed maternal care while the F2 were exposed to both prenatal and postnatal maternal stress. Perhaps this might be reflected in an altered regulation of key factors between the generations. This could include ACTH and corticosterone that together with the glucocorticoid receptor are important in regulating the HPA axis (See Fig. 2.1) and prenatal and postnatal depression. Importantly, the HPA axis becomes differently regulated during pregnancy in the mother as the placenta, a transient endocrine gland, is formed; the placenta is also able to regulate the levels of corticosterone passing through to the developing offspring. Some studies have suggested interactions between HPA axis regulation across the prenatal and postnatal period (Murgatroyd and Spengler, 2011b; Laurent et al., 2013). Postnatally, the HPA axis exerts strong control over maternal behaviour through the regulation of stress-response.

It is possible that hormones important in the regulation of maternal behaviour could also be important. For example, oxytocin and prolactin are crucial in regulating maternal care, as well as milk production (Nephew and Murgatroyd, 2013). The dysregulation of oxytocin in maternal depression is well described and further studies have shown that altered maternal care and maternal depression can in turn influence infant's oxytocin together with behavioural changes (Priel et al., 2019).

Serum levels of bdnf have been strongly linked to maternal depression (Fung et al., 2015) and studies have found associations with the regulation of bdnf in adulthood following variations in maternal care received as an infant (see **Table 1.5**). A number of other neuropeptides linked to depression include Substance P (Schwarz and Ackenheil, 2002), B-endorphin (Hegadoren et al., 2009), alpha-MSH (Kokare et al., 2010) and Orexin (Shariq et al., 2018) are also important candidates that could exert changes in behaviour postnatally or influence stress prenatally. Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are crucial in regulating the ovarian cycle and pregnancy. Additionally, these hormones are associated with stress and regulation of mood with studies reporting altered levels of these among women with depression (Huerta et al., 1995). These hormones, important in the hypothalamic–pituitary–gonadal axis, are suppressed by the HPA axis and stress. Indeed low postnatal serum LH/FSH ratio has been shown to be a robust predictor of postnatal depression (Pillai et al., 2017). Therefore, measure of these factors could allow us to understand the role of stress in regulating depression in offspring.

Alterations in the regulation of the thyroid during pregnancy through thyroid stimulating hormone (TSH) abnormalities have been associated with adverse neuropsychological function and behaviour problems in infants (Andersen et al., 2017) as with Growth hormone (GH) (Devesa et al., 2011). These factors, important in metabolism are also important for neurodevelopment, particularly in cognition but also in behaviour. One possibility is that metabolic pathways might be impacted by maternal depression that consequently impact neurodevelopment of behaviour in the offspring. There are further

growth factors with additional links to immune modulation, such as VEGF and GM-CSF. It appears that early neurodevelopment and immune development, as well as development of the HPA axis, are tightly linked. So perhaps there are factors linking these processes that might become dysregulated through exposure to depression prenatal and postnatally. Another factors important in neurodevelopment is ICAM-1 that is also linked to inflammation. For example, one study has shown that increased concentration in this factor in children born preterm associated with increased risks of cognitive dysfuction (Leviton et al., 2018). This factor is also important in regulating the blood-brain barrier that could be important in allowing peripheral inflammatory factors to impact neuroinflammation.

Finally, there are immune factors to consider in the context of inflammation and its role in modulating the stress-response together with depression and its regulation during early prenatal and postnatal stress (Murgatroyd, 2018). Alterations in the levels and regulation of the interleukins IL-2, IL-4, IL-6, IL-8, IL-and 10 have all been clearly linked to risk for depression (Talarowska et al., 2016) as have interferons (Franscina Pinto and Andrade, 2016) and Tumour necrosis factors (TNFs) (Postal et al., 2016).

The involvement of epigenetic mechanisms in the regulation of maternal depression and its transmission through the generations would be a plausible hypothesis supporting an intergeneration epigenetic process. Numerous studies have investigated DNA methylation at key candidate genes involved in stress regulation through prenatal and postnatal stress. As the F1 and F2 animals were exposed to postnatal and prenatal plus postnatal maternal stress this might be reflected in altered epigenetic regulation of key genes between the generations, or perhaps epigenetic marks are directly inherited through the generations. Importantly, we also test the for sex-specific effects in endocrine and immune studies to determine if transgenerational effects might differentially targets female offspring.

This chapter looks to investigate the regulation of the above key factors linked to maternal behaviour, stress response, depression, neurodevelopment and inflammation

to investigate their possible roles in the generational transmission of maternal depression and stress-related changes.

3.2 Aims

The aim of this part of the project is to investigate whether specific stress, endocrine and immune factors, together with epigenetic changes are differently regulated in response to maternal depression and whether any possible changes might be inherited.

To do this, the CSS rat model of transgenerational changes in maternal care is studied using ELISA to investigate endocrine and immune markers, real-time PCR to test gene expression and bisulphite sequencing to measure DNA methylation.

3.3 Objectives

1. Measure endocrine markers of HPA regulation in generations of the CSS rat model.
2. Measure endocrine factors important in maternal care in generations of the CSS rat model.
3. Measure immune regulatory factors markers in generations of the CSS rat model.
4. Measure epigenetic regulation of specific stress-related genes in generations of the CSS rat model.

3.4 Results

Serum levels of hormones involved in the endocrine regulation of HPA axis (Corticosterone and ACTH) and the endocrine regulation of lactation and maternal care (Oxytocin and Prolactin) together with bdnf and TSH were firstly measured in the F2 animals to check for transgenerational changes.

LH, FSH, GH together with Orexin A were then also measured together with the above analytes in F1 and F0 animals to test whether they were inherited through the 3 generations. Finally, Immune measures were also made of key inflammatory markers in F2 and then F1 and F0 animals of key factors.

3.4.1 Endocrine regulation

Serum levels of endocrine factors important in HPA axis regulation, Prolactin, bdnf, TSH and related to stress, were measured in the F2 animals. Specifically, F2 Dams from F0 CSS and control were compared at day 23 of lactation.

3.4.1.1 HPA regulation in F2

Serum levels of Corticosterone and ACTH were measured in the F2 animals as a function of HPA axis regulation at 2 timepoints: as a pup and as a lactating dam. This revealed significantly lower levels in the CSS F2 Dams compared to the controls at day 23 of lactation and significantly lower levels in F2 pups (**Figure 3.1**).

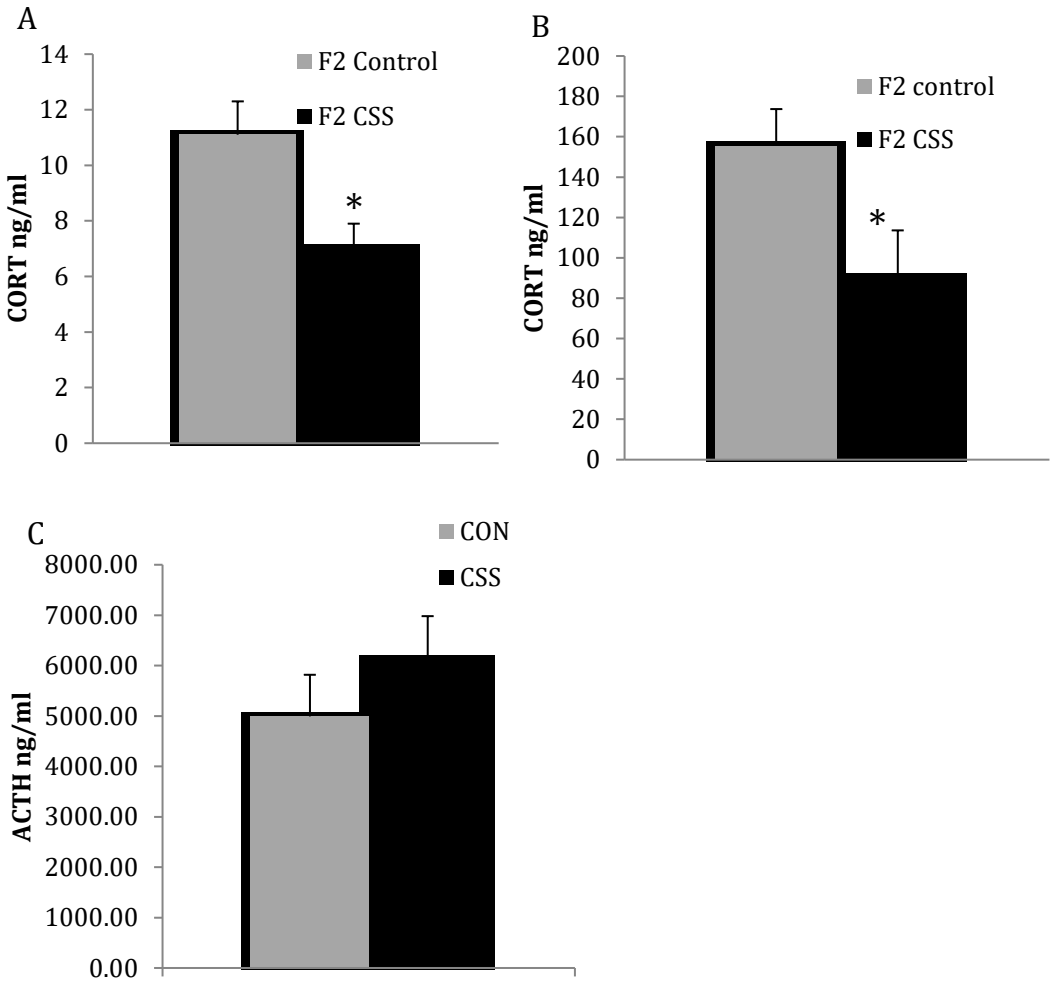

Figure 3.1. HPA axis function in F2 CSS and control dams. *Mean + SEM of serum levels of corticosterone (CORT) in F2 pups (d16) (A) and Dams (lactation d23) (B) and ACTH in Dams (lactation d23) (C) in CSS and control dams. * Indicates a significant effect of treatment (2-tailed t-test, $p < 0.05$). n= 11 control and 13 CSS Dams and 12 control and 12 CSS pups.*

3.4.1.2 Regulation of Maternal-related hormones in F2

Serum levels of Prolactin and Oxytocin were measured in the F2 Dams animals. This revealed no significant differences between CSS and controls at day 23 of lactation in Oxytocin (p= 0.54) and Prolactin (p= 0.69) (**Figure 3.2**).

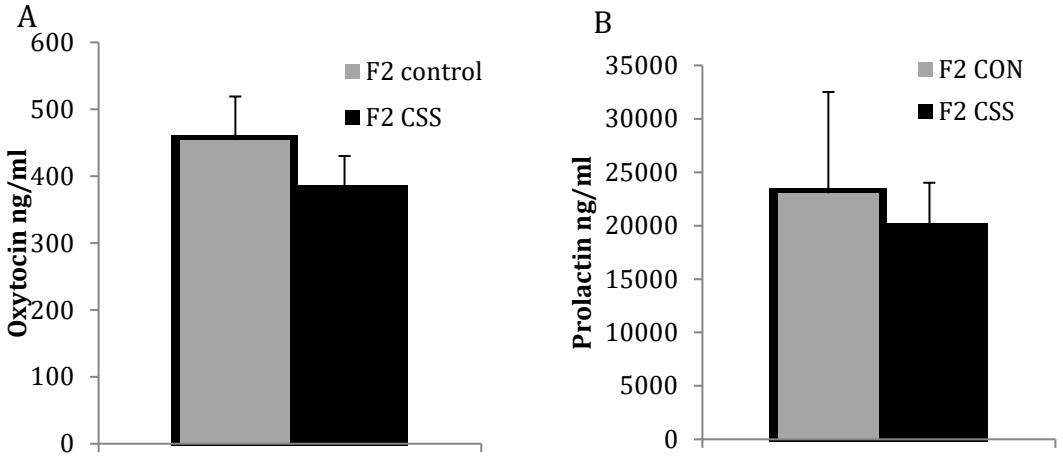

Figure 3.2. Oxytocin and Prolactin levels. *Mean + SEM of serum levels of Oxytocin (A) and Prolactin (B) in CSS and control Dams (lactation d23). * Indicates a significant effect of treatment (2-tailed t-test, $p < 0.05$). Oxytocin n= 11 control and 13 CSS Dams; Prolactin n=7 control and 8 CSS pups.*

3.4.1.3 bdnf and TSH regulation in F2

Serum levels of bdnf were measured in the F2 Dams together with TSH. This revealed no significant differences between CSS and controls at day 23 of lactation in bdnf (2 –tail t-test p=0.1) and TSH (2 –tail t-test p=0.35) (**Figure 3.3**).

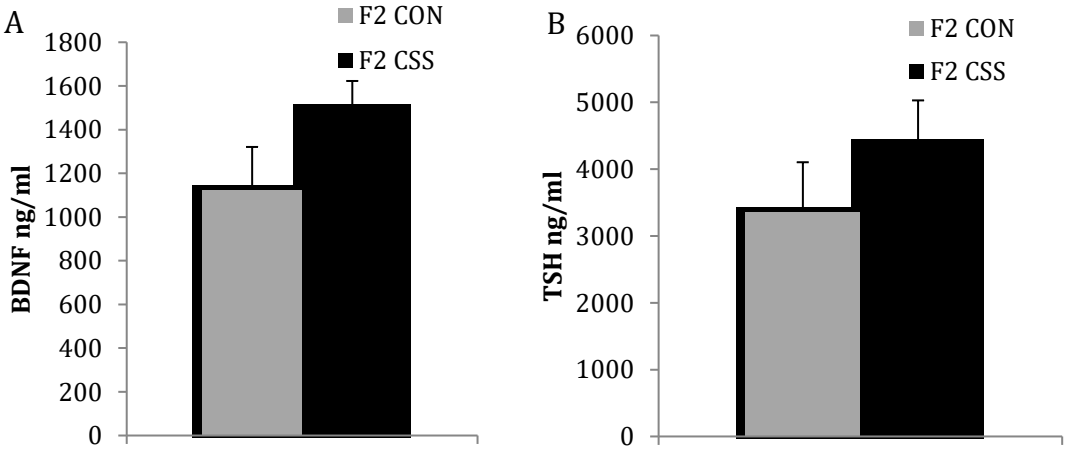

Figure 3.3. **bdnf** ***and TSH levels.***

Mean + SEM of serum levels of bdnf *(A) and* TSH *(B) in CSS and control Dams (lactation d23). * Indicates a significant effect of treatment (2-tailed t-test, $p < 0.05$). n= 11 control and 13 CSS Dams and 11 control and 13 CSS pups.*

3.4.1.4 HPA regulation in F1

Serum levels of ACTH were measured in the F1 animals as a function of HPA axis regulation as a lactating mothers. This revealed no significant differences between CSS and controls at day 23 of lactation (2–tail t-test p=0.35) or in the adults (male, p=0.93; female, p=0.45) (**Figure 3.4**).

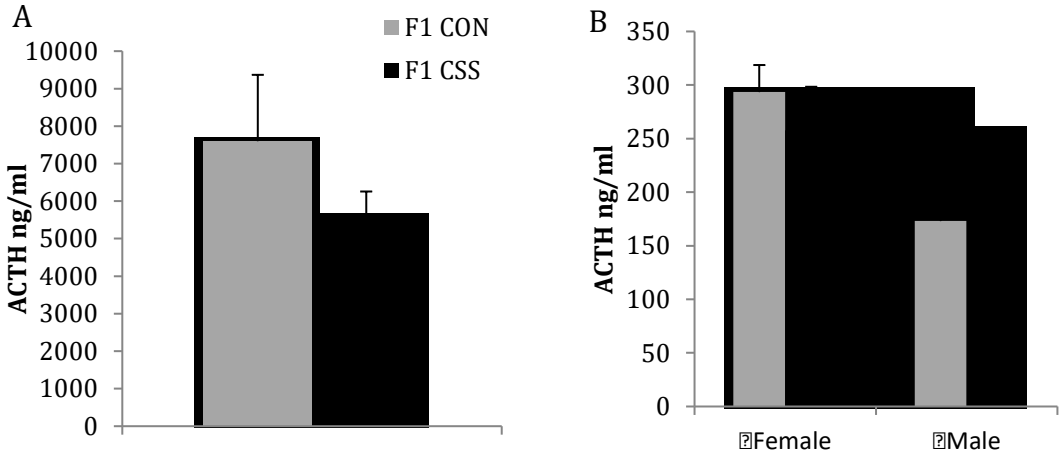

Figure 3.4. Corticosterone and ACTH levels in F1 Dams and adults. *Mean + SEM of serum levels of* ACTH *(A) in CSS and control mothers (lactation d23) and male and female adults (B). * Indicates a significant effect of treatment (2-tailed t-test, p < 0.05). n= 11 control and 13 CSS Dams and 13 male and 12 female control and 12 male and 11 female CSS pups.*

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3.4.1.5 Regulation of Maternal-related hormones in F1

Serum levels of Prolactin and Oxytocin were measured in the F1 Dams animals. Importantly, the Oxytocin assay was performed by a different method (Radio-Immuo-Assay) in the lab of Dr Ben Nephew as our oxytocin ELISA assay did not work in the dams. When calculating the results this revealed no significant differences between CSS and controls at day 23 of lactation in Oxytocin (p= 0.28) and Prolactin (p= 0.78). There were also no differences in CSS and control adults for Oxytocin (male p= 0.88, female p= 0.92), but significantly reduced Prolactin in females but not males (p= male 0.45, female 0.04) (**Figure 3.5**).

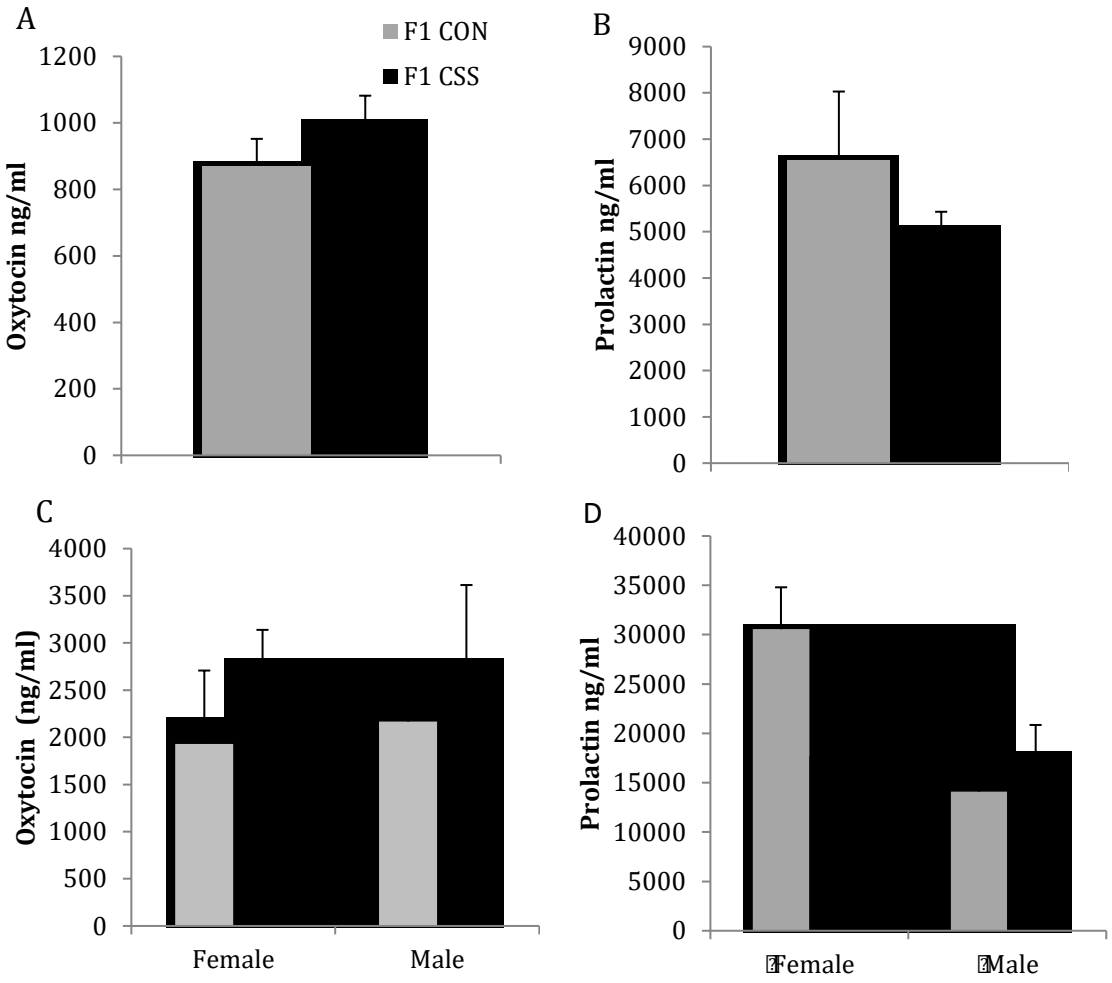


Figure 3.5. Oxytocin and Prolactin levels. *Mean + SEM of serum levels of Oxytocin (A) and Prolactin (B) in CSS and control Dams (lactation d23) and Oxytocin (C) and Prolactin (D) in CSS and control adults. * Indicates a significant effect of treatment (2-tailed t-test, $p < 0.05$). n= 12 control and 13 CSS Dams and for Prolactin n= 7 control and 8 CSS pups.*

3.4.1.6 bdnf and TSH regulation in F1

Serum levels of bdnf were measured in the F2 Dams together with TSH. This revealed no significant differences between CSS and controls at day 23 of lactation in bdnf (2–tailed t-test p=0.78) and TSH (2–tailed t-test p=0.36). In F1 adults bdnf and TSH also did not differ in either females or males (bdnf, 2–tailed t-test male p=0.45; female = 0.93; TSH, male p=0.48; female = 0.48) (**Figure 3.6**).

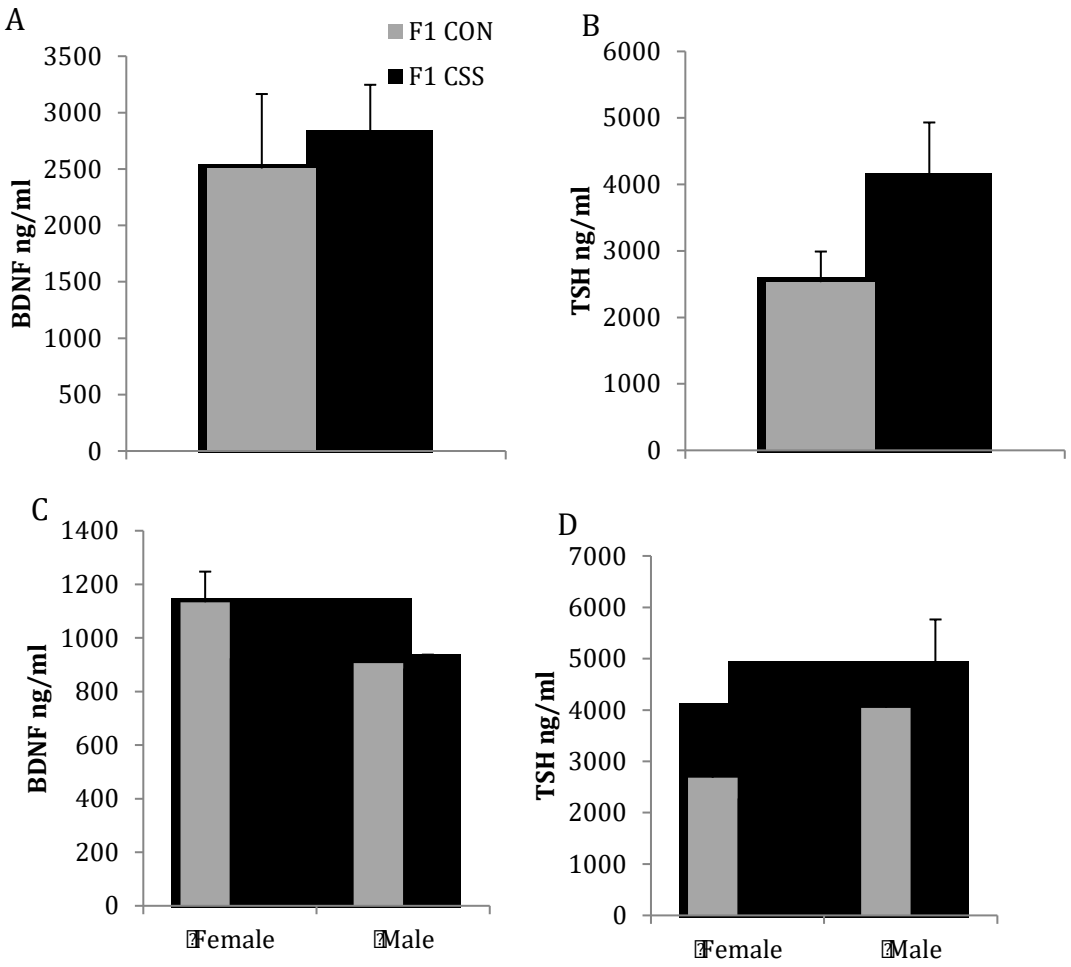


Figure 3.6. **bdnf** ***and TSH levels in F1.*** *Mean + SEM of serum levels of BDNF (A) and TSH (B) in CSS and control Dams (lactation d23) and BDNF (C) and TSH (D) in CSS and control adults. * Indicates a significant effect of treatment (2-tailed t-test, $p < 0.05$). n= 12 control and 13 CSS Dams and for Prolactin n= 7 control and 8 CSS adults.*

3.4.1.7 Regulation of LH, GH and FSH in F1 adults

Serum levels of LH, GH and FSH were measured in the F1 adults. This revealed no significant differences between males and females in either (LH, 2–tailed t-test male p=0.72; female = 0.24; GH, male p=0.91; female = 0.27; FSH, male p=0.4; female = 0.31) (**Figure 3.7**).

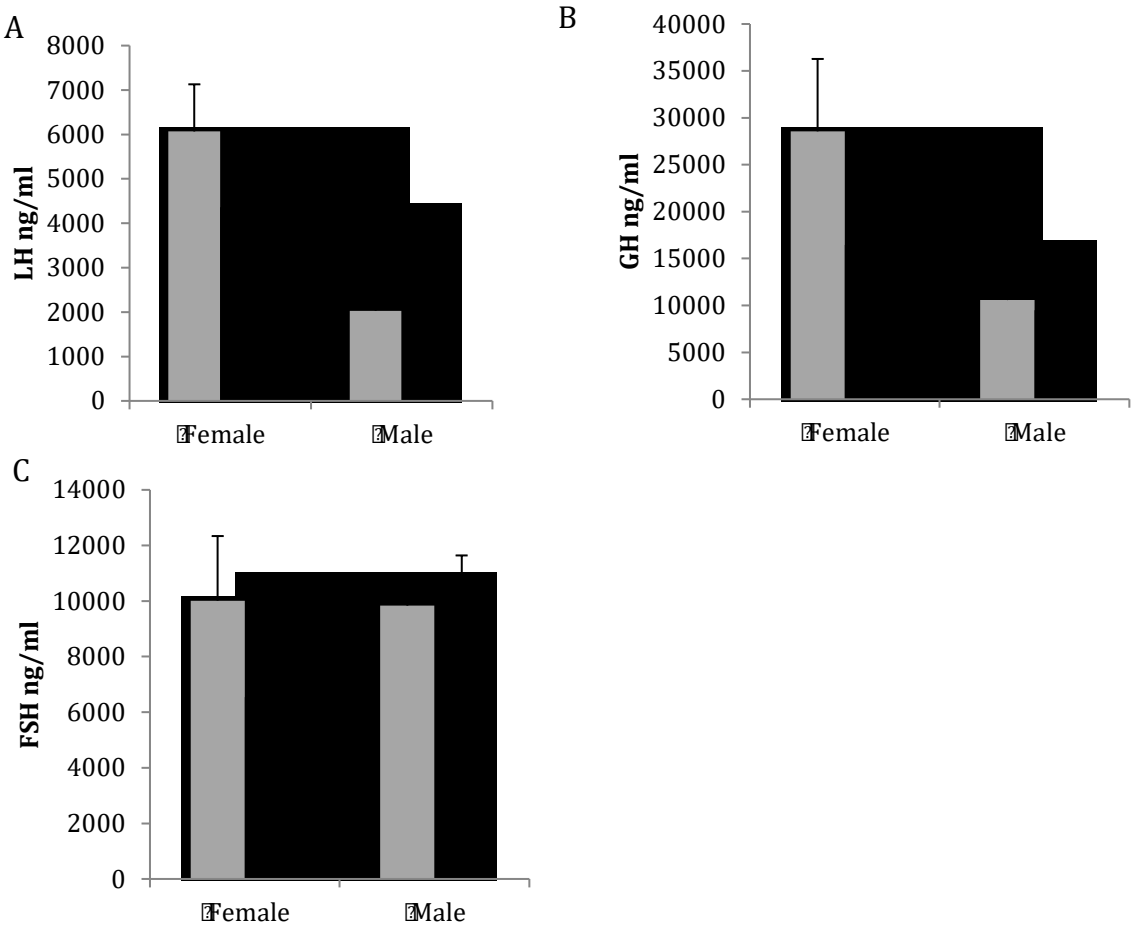


Figure 3.7. LH, GH and FSH levels in F1 adults. *Mean + SEM of serum levels of LH (A) GH (B) and FSH (C) in CSS and control adults. * Indicates a significant effect of treatment (2-tailed t-test, $p < 0.05$). n= 12 control and 9 CSS female pups and n= 12 control and 12 CSS male adults.*

3.4.1.7 Regulation of Alpha-MSH, Substance P, Beta-endorphin and Orexin A in F1

Serum levels of Alpha-MSH, Substance P, Beta-endorphin and Orexin A were measured in the F1 adults. This revealed no significant differences between males and females in either Alpha-MSH, Substance P, Beta-endorphin and Orexin (**Figure 3.8**).

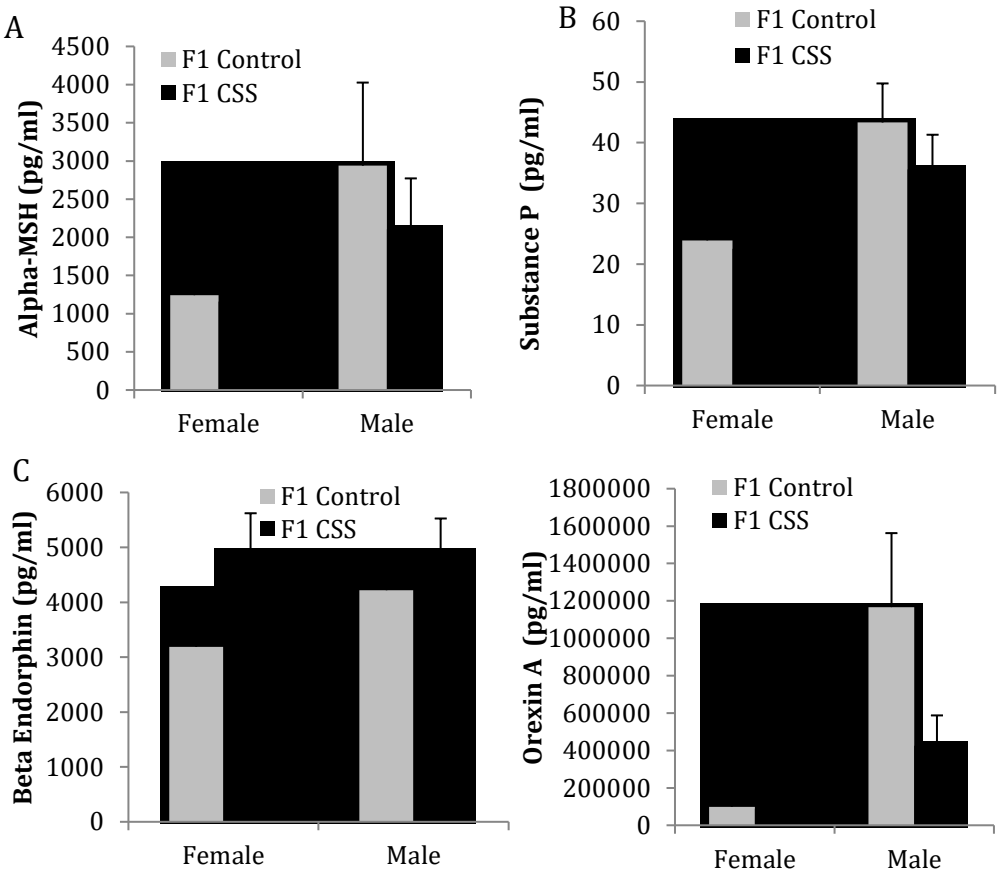


Figure 3.8. Alpha-MSH, Substance P, Beta-endorphin and Orexin A levels in F1. *Mean + SEM of serum levels of alpha-MSH (A) Substance P (B) Beta-endorphin (C) and Orexin A (D) in CSS and control adults. * Indicates a significant effect of treatment (2-tailed t-test, $p < 0.05$). n= 12 control and 9 CSS female and n= 12 control and 12 CSS male rats.*

3.4.2 Regulation of immune markers

A number of immune markers were measured to the effects of CSS and inheritance of different components of the immune system, namely Interferons (specifically, IFN-γ), Interleukins (specifically, IL-2, IL-4, IL-6 and IL-10), Tumour Necrosis Factors (specifically TNF-α). We also tested other immune-regulatory factors such as: GM-CSF, that functions as a cytokine and important in neuronal differentiation of adult neural stem cells and neuroprotection; VEGF, important in blood vessel growth in the CNS and together with neurogenesis, neuroprotection and inhibition of inflammation; Icam-1/CD54 a cell surface protein important in activation of inflammation and blood brain barrier.

3.4.2.1 Inheritance of IFNγ in F1 and F2

Levels of IFNg were too low to measure in all serum samples two different assays on F1 and F2 samples with different concentrations of serum tested and both gave values below the detection level for over 90% of the samples.

3.4.2.2 Inheritance of TNFα in F1 and F2

Levels of IFNg were too low to measure in around 80% of the samples in two different assays with different concentrations of serum used, leaving not enough samples for statistical analyses.

3.4.2.3 Inheritance of Interleukins in F1 and F2

Serum levels of interleukins were measured in F1 and F2 dams. This revealed no significant differences in IL-6 between CSS and controls dams at day 23 of lactation in F1 (2–tailed t-test p=0.73) and F2 (2–tailed t-test p=0.91). There were no significant differences in Il-10 between CSS and controls dams at day 23 of lactation in F1 (2–tailed t-test p=0.64) and F2 (2–tailed t-test p=0.11) (**Figure 3.9**).

For the Il-2 and IL-4 and ELISAs all serum samples produced values below the standard curve so could not be tested (*data not shown*).

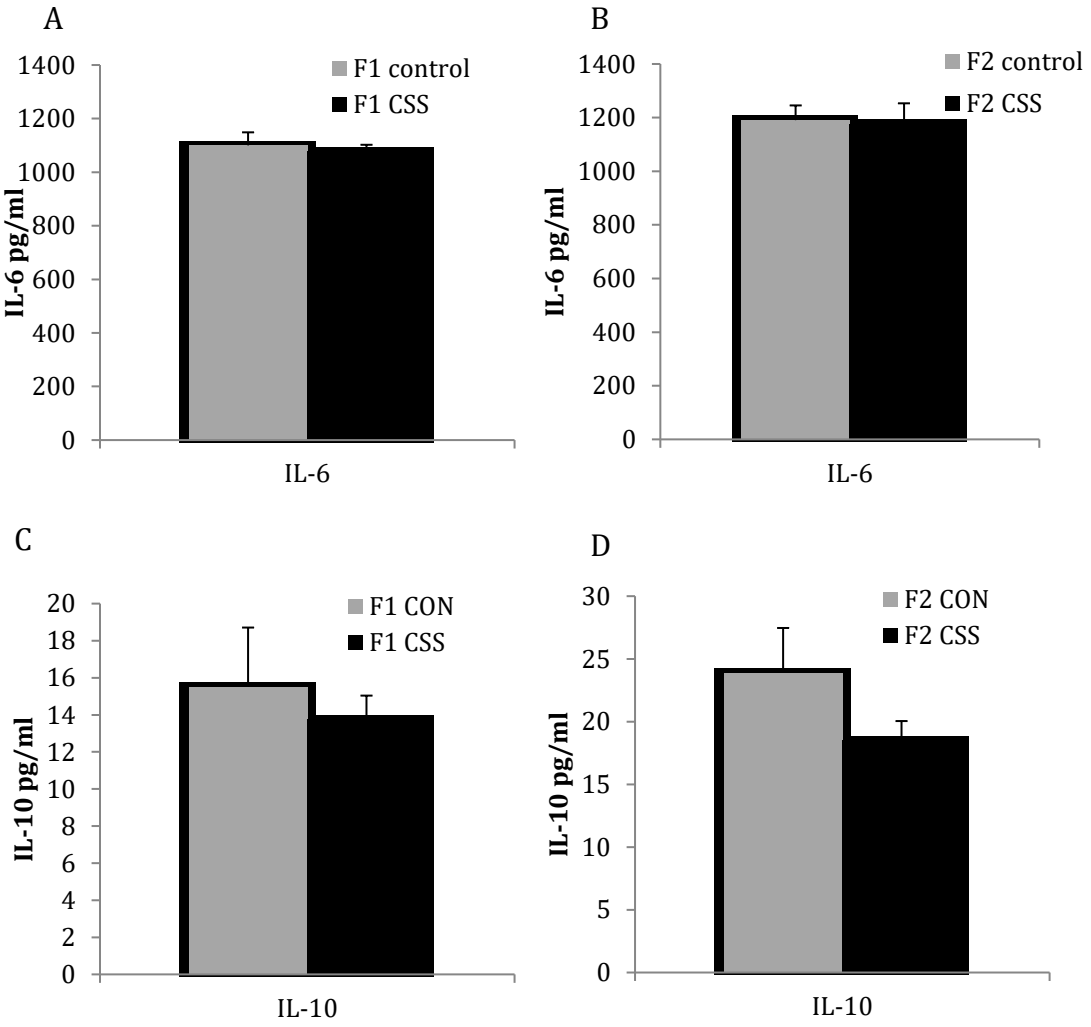


Figure 3.9. IL-6 and IL-10 levels in F1 and F2 dams. *Mean + SEM of serum levels of IL-6 in F1 (A) and F2 (B) dams and IL-10 in F1 (C) and F2 (D) dams between CSS and control. * Indicates a significant effect of treatment (2-tailed t-test, $p < 0.05$). F1 n= 6 control and 8 CSS female and n= 11 control and 17 CSS mal.*

3.4.2.4 Inheritance of VEGF, GM-CSF, Icam-1 in F1 and F2 animals

Serum levels of VEGF, GM-CSF, Icam-1 were measured in F1 and F2 dams. This revealed no significant differences in VEGF between CSS and controls dams at day 23 of lactation in F1 (2–tailed t-test p=0.66) and F2 (2–tailed t-test p=0.56). There were no significant differences in GM-CSF between CSS and controls dams at day 23 of lactation in F1 (2–tailed t-test p=0.69) and F2 (2–tailed t-test p=0.89) (**Figure 3.10**).

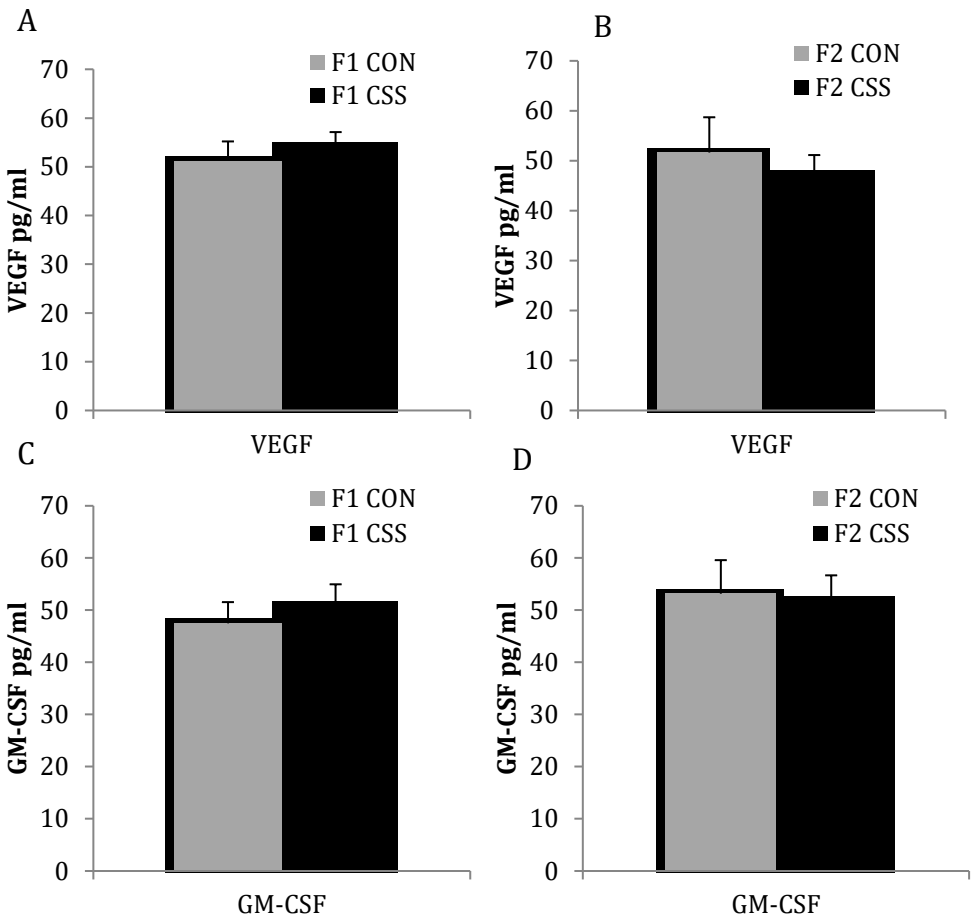


Figure 3.10. VEGF and GM-CSF levels in F1 and F2 dams. *Mean + SEM of serum levels of VEGF in F1 (A) and F2 (B) dams and GM-CSF in F1 (C) and F2 (D) dams between CSS and control. * Indicates a significant effect of treatment (2-tailed t-test, $p < 0.05$). F1 n= 6 control and 8 CSS female pups and n= 11 control and 17 CSS male pups.*

There were no significant differences in Icam-1 between CSS and controls dams at day 23 of lactation in F1 (2–tailed t-test p=0.45) and F2 (2–tailed t-test p=0.1). However, if one considers the direction of Icam-1 in the F1 animals and uses a 1-tailed t-test, then there is a significant (p=0.05) decrease in CSS F2 animals (**Figure 3.11**).

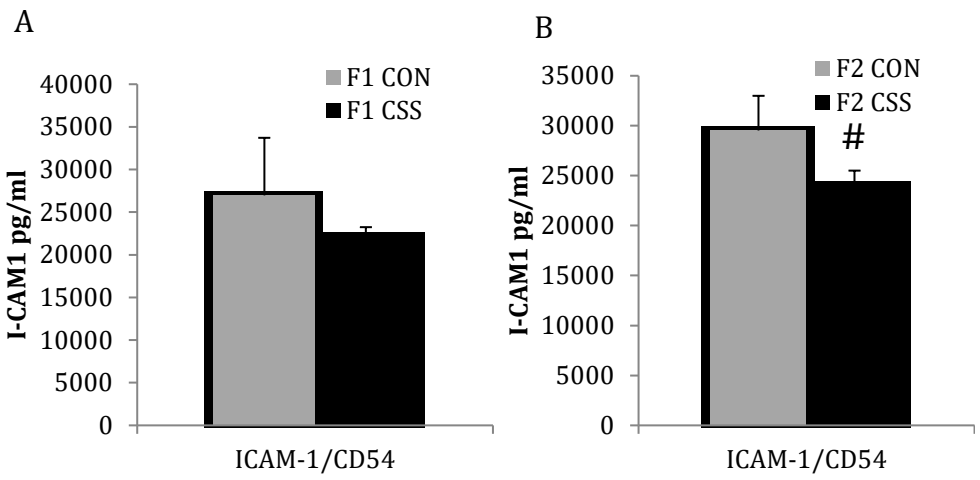


Figure 3.11. Icam-1 levels in F1 and F2 dams. *Mean + SEM of serum levels of VEGF in F1 (A) and F2 (B) dams, GM-CSF Mean + SEM of serum levels of ICAM-1 in F1 (A) and F2 (B) dams between CSS and control. * Indicates a significant effect of treatment (2-tailed t-test, p < 0.05). # Indicates a significant effect of treatment (1-tailed t-test, p < 0.05), n= 6-10 F1 group and 11-13 F2 group). F1 n= 6 control and 8 CSS female pups and n= 11 control and 17 CSS male pups.*

Considering the reduced levels of Icam-1 in F2 CSS rats, and the direction of a reduction in the F1 CSS animals, F0 dams were also studied This revealed no significant differences (1-tailed t-test, p= 0.45) (**Figure 3.12**).

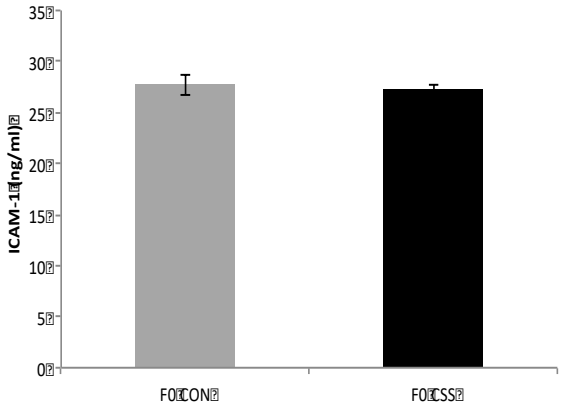


Figure 3.12. Icam-1 levels in F0. *Mean + SEM of serum levels in F0 CSS and control dams of Icam1. n= 10 control, 11 CSS*

Considering the reduced levels of Icam-1 in F2 CSS rats, and the direction of a reduction in the F1 animals we plotted I-Cam1 levels in the mothers and their offspring dams to test

if those levels in the F1 dams associated with subsequence levels in their F2 offspring (**Figure 3.13**). This revealed no significant correlation

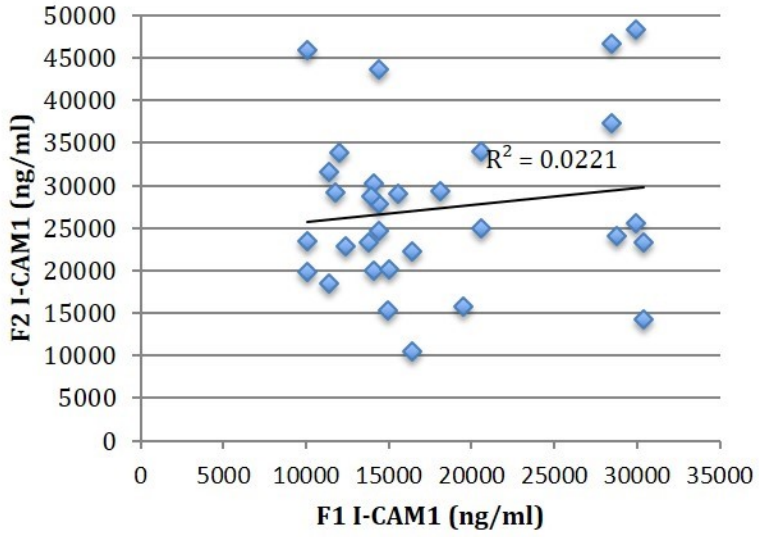


Figure 3.13. Inheritance of I-cam1 between F1 and F2 dams. *Serum levels of I-cam1 in F2 dams (y axis) were plotted against serum levels of I-cam1 in F1 dams.*

3.4.3. Epigenetic regulation of stress related genes in F1 and F2 dams.

The hypothalamus was investigated for the regulation of *Nr3c1* (GR) and *bdnf* in the CSS and control rat dams from the F1 and F2 generations to determine if the differentially regulation HPA axis, judging from the corticosterone changes related to alterations in GR and *bdnf*.

3.4.3.1 Preparation of DNA and optimization of methods

DNA from hypothalamus from F1 and F2 CSS and control rat dams were extracted together with RNA. Tissue punches were extracted and DNA and RNA quantities and quality measured. DNA concentrations were around 30-50ng/ml in 20 µl volume, therefore around 600ng-1ug (**Table 3.1**). However, the first 8 were lower – this is because learning the procedure increased its efficiency. The RNA levels were around 100-300ng/ml in 20 µl volume, therefore around 2-6ug total RNA.

The RNA was reverse transcribed to cDNA for measurement using real-time PCR.

Table 3.1. *Comparisons* ***of total DNA and RNA isolated from rat brain punches using Nanodrop***

	DNA				RNA			
Sample	Total conc. ng	Conc. ng/ml	260/280 purity ng/ml	260/230 purity ng/ml	Total conc. ng	Conc. ng/ml	260/280 purity ng/ml	260/230 purity ng/ml
1:5-1-1	261	8.7	1.77	1.00	2732	136.6	1.55	0.27
2:25-1-1	270	9.0	1.87	0.18	10.614	530.7	1.37	0.31
3:26-1-1	75	2.5	34.06	0.31	4292	214.6	1.60	0.34
4:20-2-1	123	4.1	3.40	1.43	4274	213.7	1.57	0.90
5:21-1-2	24	0.8	-1.87	-1.23	9772	488.6	1.97	0.67
6:10-2-2	108	3.6	2.28	1.38	8330	416.5	1.90	
7:26-1-2	39	1.3	1.87	3.62	6982	349.1	1.71	0.52
8:4-1-1	42	1.4	2.00	1.77	8772	438.6	1.82	0.73
9:1-2-2	12	4.3	2.00		2088	104.4	1.64	
10:1-1-1	420	14.0	1.97	3.39	1766	88.3	1.61	0.31
11:27-2-2	400	20.0	1.53	1.43	956	47.8	1.55	0.25
12:22-2-1	784	39.2	1.46	2.0	119.6	99.6	1.61	0.25
13:27-1-1	1362	26.1	1.61	1.48	6954	347.7	1.66	0.53
14:26-2-1	1292	64.6	1.57	1.40	2672	133.6	1.61	0.30
15:25-2-1	834	41.7	1.46	1.21	1494	74.7	1.46	0.30
16:18-1-1	2114	105.7	1.35	0.07	3094	154.7	1.49	0.31
17:14-1-1	1394	69.7	1.43	0.84	4398	219.9	1.70	0.33
18:17-2-1	550	27.5	1.51	1.15	2172	108.6	1.61	0.29
19:22-1-2	1162	58.1	1.50	1.20	2734	136.7	1.61	0.27
20:27-2-1	800	40.0	1.57	1.40	4312	215.6	1.65	0.35
21:18-2-1	1052	52.6	1.61		1906	95.3	1.47	
22:2-1-2	710	35.5	1.55		5226	261.3	1.62	
23:15-1-1	2668	133.4	1.23		4166	208.3	1.49	
24:21-1-1	1068	36.2	1.53		4026	201.3	1.58	
25:3-1-1	758	37.9	1.79		7810	390.5	1.57	
26:2-1-1	1346	67.3	1.46		4508	225.4	1.44	
27:17-1-1	926	46.3	1.49		2782	139.1	1.57	
28:24-1-1	604	30.2	1.42		8610	430.5	1.77	
29:24-1-2	328	16.4	1.50		2722	136.1	1.53	
30:25-2-1	574	28.7	1.47		8366	418.3	1.75	
31:22-1-1	1152	57.6	1.36		4432	221.6	1.68	
32:11-1-1	306	15.3	1.47		16482	824.1	1.48	
33:11-1	2052	82.1	1.40	-0.90	2464	123.2	1.61	
3426-2	1255	50.2	1.57	-0.42	2760	138.0	1.58	
35:14-2	1237	49.5	1.52	-0.41	2646	132.3	1.66	
36:30-1	1080	43.2	1.48	-0.34	3524	176.2	1.70	
37:16-1	1315	52.6	1.49	-0.44	3148	157.4	1.57	
38:20-1	1437	57.5	1.83	-0.96	3042	152.1	1.71	
39:22-1	795	31.8	1.71	-0.27	10920	546.4	1.49	
40:27-1	710	28.4	1.64	-0.23	4420	221.0	1.70	
41:17-2	447	17.9	1.67	-0.16	4376	218.8	1.69	
42:22-2	627	25.1	1.80	-0.19	2788	139.4	1.69	
43:3-1	927	30.9	1.75		5088	254.4	1.60	
44:24-1	741	24.7	2.03		8322	416.1	1.61	
45:2-1	1245	41.5	1.54		5558	277.9	1.49	
46:24-1	1218	40.6	1.52		7512	375.6	1.54	
47:15-1	1572	52.4	1.54		4844	242.2	1.61	

48:1-2	1662	55.4	1.51	6982	349.1	1.49
49:17-1	1125	37.5	1.74	7948	397.4	1.71
50:25-2	1335	44.5	1.52	8470	423.5	1.63
51:26-1	1344	44.8	1.75	4320	216.0	1.52
52:5-1	2313	77.1	1.43	5778	288.9	1.35
53:4-1	963	32.1	1.61	9350	467.5	1.86
54:27-1	1188	39.6	1.65	4708	235.4	1.61
55:14-1	2130	71.0	1.58	10142	507.1	1.36
56:18-2	2013	67.1	1.49	5416	270.8	1.65
57:20-1	918	30.6	1.90	11458	572.9	1.47
58:1-1	1506	50.2	1.49	8064	403.2	1.74
59:25-1	1179	39.3	1.69	15332	766.6	1.41
60:21-1	1380	46.0	1.57	6934	346.7	1.67

Bisulphite pyrosequencing was used for DNA methylation and real-time PCR for gene expression measurements. Primers for both were optimised. PCR was performed using a "practice" rat bisulphite treated DNA samples at varying temperatures together with negative controls. These were run on 2% agarose gels (**Figure 3.14**). This confirmed that the PCR products were adequately amplified for the *bdnf* (195bp) and the *Nr3c1* (132bp) promoter sequences.

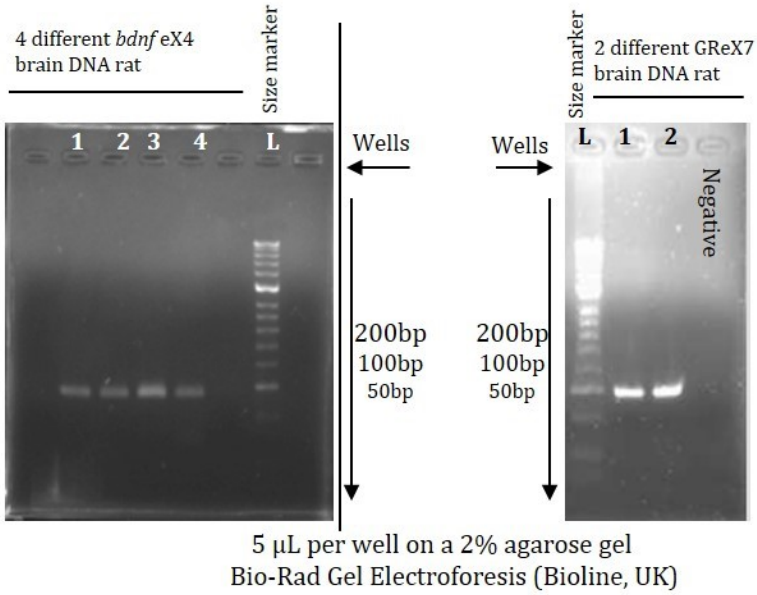


Figure 3.14. Optimisation of PCR for bisulphite pyrosequecning. *PCR products for Bdnf exIV and Nr3c1 run on a 2% agarose gel together with a DNA ladder (Hyperladder 50 bp) and negative.*

Bisulphite pyrosequencing was performed on the PCR products to check that the bisulphite conversion is of high quality and that the sequencing produced readable results. The Pyromark sequencer software gives % methylation at each CpG and colour codes the "calls" depending on its confidence. This can be affected by the quality of the read and the sequencing reaction. Blue is highest confidence and red is the lowest. However, in some reactions such as the rat *Nr3c1* and *Bdnf*, CpGs consistently called as red in repeat experiments still gave highly repeatable results.

Examples of the *Nr3c1* (**Figure 3.15A**) and *Bdnf* (**Figure 3.15B**) pyrosequencing results are shown highlighting when some bases are labeled red.

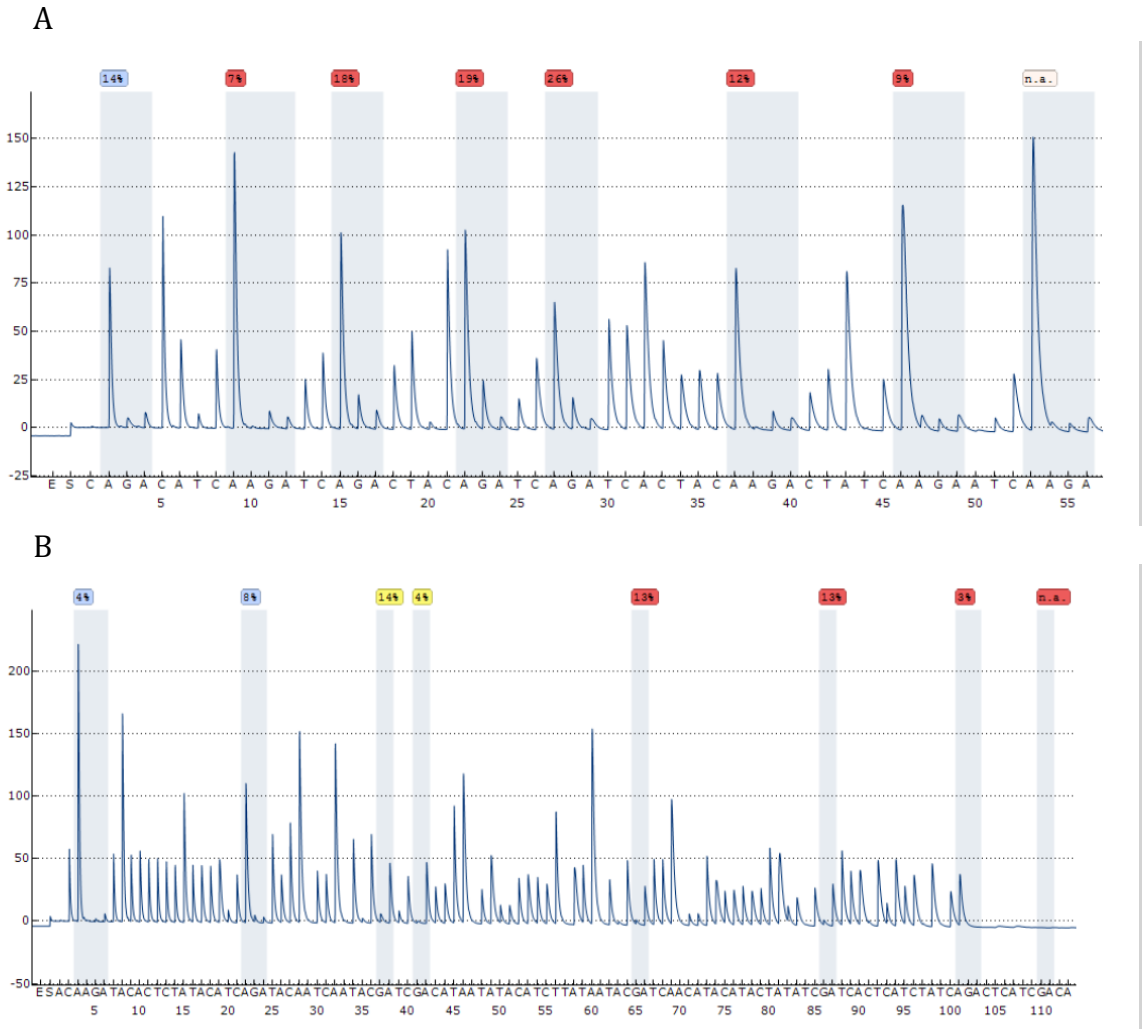


Figure 3.15. Pyromark sequencing of the rat Nr3c1 (A) and bdnf (B) promoters.

Real-time PCRs were performed on cDNAs to check that the cDNA products produced clear melting curves and bands of appropriate sizes to allow measurement of *Nr3c1* expression. The *Nr3c1* primers gave clear results however the *bdnf* primers constantly gave primer dimers and multiple bands which would interfere with the experiment. Three further primers were designed and tested, however the same problems arose. Housekeeping genes (*Beta-actin*, *Hprt* and *Gapdh*) were also measured to allow relative gene expression to be determined (**Figure 3.16**)

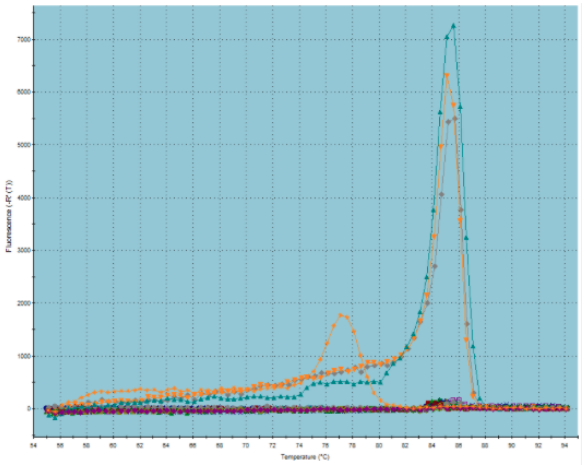


Figure 3.16. Example of a melting curve for relative cDNA expression of Beta-actin.

3.4.3.2 Epigenetic regulation of *Nr3c1* in the transgenerational CSS rat model:

RNA expression and DNA methylation of *Nr3c1* were measured between CSS and control animals form the F1 and F2 generations.

Nr3c1 expression Comparing expression of *Nr3c1* in the hippocampus of F1 CSS and F1 control dams it as found that there was significant increased expression in the CSS (2-tailed t-test, p= 0.02) (This was after removing one control value that was 2.5 times higher than the next highest sample in the controls) (**Figure 3.17**).

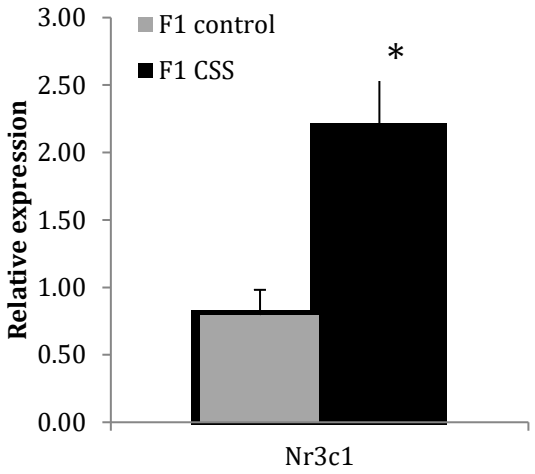


Figure 3.17. Relative expression of Nr3c1 between F1 CSS and control dams. *Mean + SEM of hippocampal Nr3c1 relative expression in F1 CSS and control dams. Significant effects of treatment using a 2-tailedt-test are indicated *P< 0.05. n= 14 control, 14 CSS*

Comparing expression of *Nr3c1* in the hippocampus of F2 CSS and F2 control dams there were no significant changes in expression found (2-tailed t-test, p= 0.56). Removing any outliers did not change the significance (**Figure 3.18**).

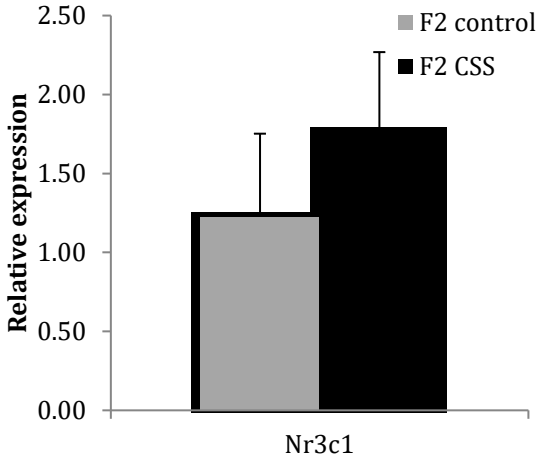


Figure 3.18. Relative expression of Nr3c1 between F1 CSS and control dams. *Mean + SEM of hippocampal Nr3c1 relative expression in F1 CSS and control dams. n= 11 control, 13 CSS*

Nr3c1 methylation: DNA methylation was measured at the promoter 1-7 *Nr3c1* gene (**Figure 3.19**) levels of methylation across the 7 CpG sites in the hippocampus of F1 and F2 CSS and controls.

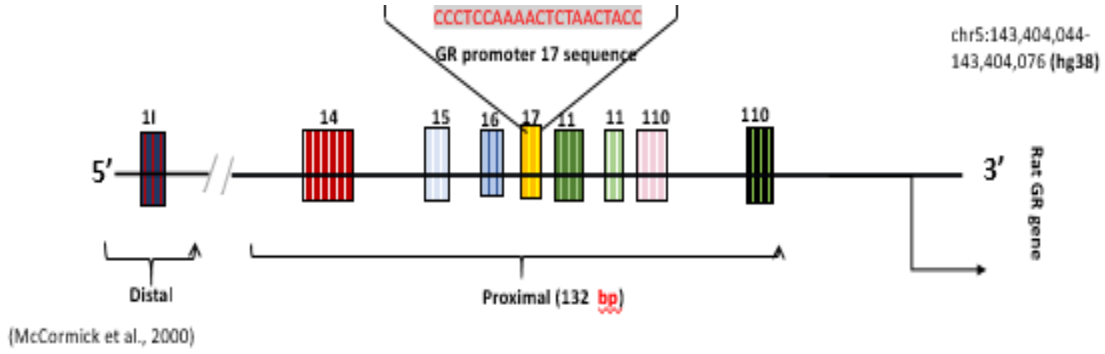


Figure 3.19. A schematic representation of the rat Nr3c1 promoter region. *This contains 9 untranslated 1st exons and promoter regions. Within the exon 1.7 promoter region 7 CpG sites were investigated.*

Measuring DNA methylation within the F1 dams comparing controls and CSS dams significantly lower levels of methylation were found at six of the seven CpGs tested in the CSS compared to the control (2-tailed t-test, CpG1 p= 0.004; CpG2 p=0.009; CpG3 p= 0.004; CpG4 p= 0.003; CpG5 p= 0.001; CpG6 p=0.038; CpG7 p = 0.12). The average methylation off all CpGs was also significantly lower in the F1 CSS group compared to the F1 control dams (2-tailed t-test, p= 0.003) (**Figure 3.20**).

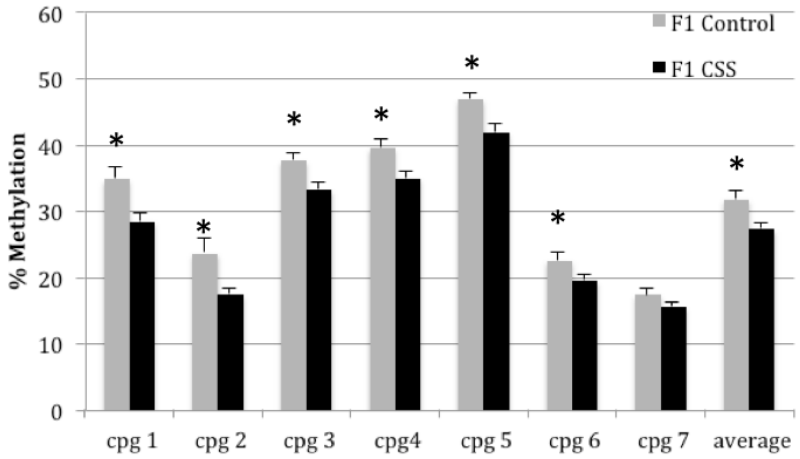


Figure 3.20. Glucocorticoid receptor methylation in F1 CSS and control dams. *Mean + SEM of DNA methylation and at Nr3c1 promoter 1.7 in F1 control and CSS dams. Significant effects of treatment using a 2-tailedt-test are indicated *P< 0.05.n= 14 control, 14 CSS.*

Measuring DNA methylation within the F2 dams comparing controls and CSS dams significantly lower levels of methylation were found at six of the seven CpGs tested in the CSS compared to the control (2-tailed t-test, CpG1 p= 0.74; CpG2 p=0.25; CpG3 p= 0.88; CpG4 p= 0.59; CpG5 p= 0.98; CpG6 p=0.32; CpG7 p = 0.38). The average methylation of all CpGs was also not significantly different between the groups (2-tailed t-test, p= 0.6) (**Figure 3.21**).

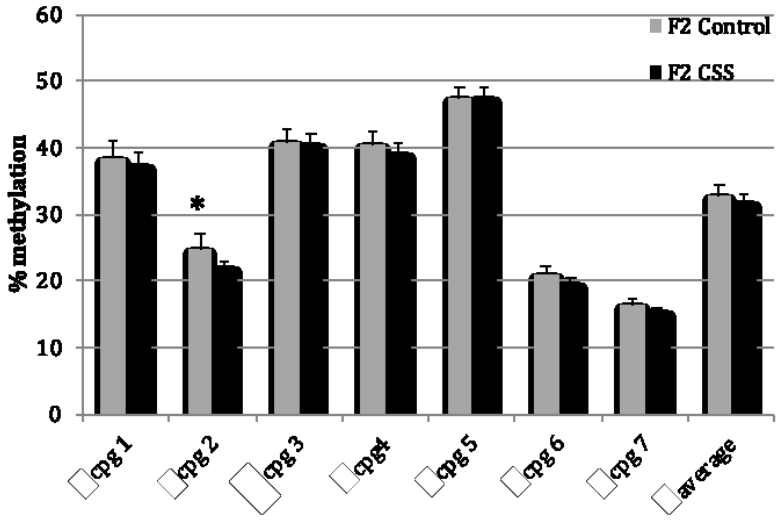


Figure 3.21. Glucocorticoid receptor methylation within F2 control and CSS. *Mean + SEM of DNA methylation and at Nr3c1 promoter 1.7 in F2 control and CSS dams. n= 13 control, 13 CSS.*

Correlation between methylation and expression: DNA methylation at a promoter usually represses gene expression. To test whether promoter 1-7 methylation associates with gene expression, correlations were plotted between F1 and F2 data points this revealed no association using a Pearson's correlation (**Figure 3.22**).

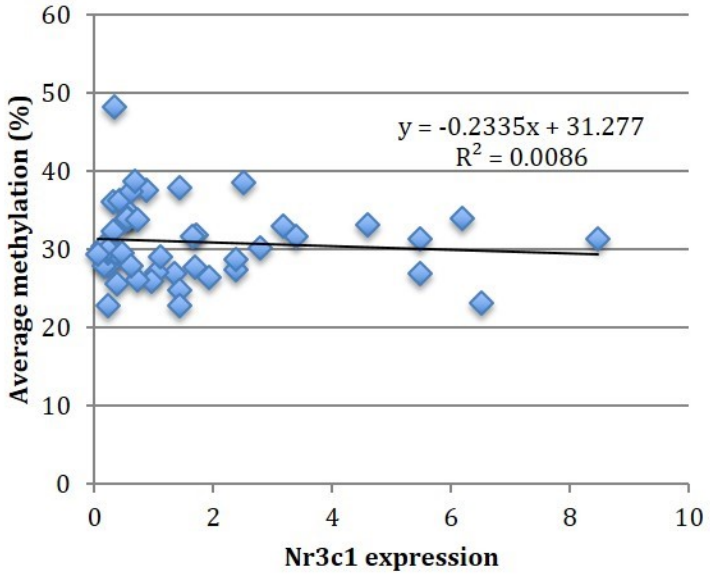


Figure 3.22. Scatter plot of Nr3c1 methylation and expression. *Average methylation and expression from each animal were plotted. n= 53.*

Correlation between Corticosterone and Nr3c1 methylation: DNA methylation at a promoter usually represses gene expression. To test whether promoter 1-7 methylation associates with gene expression, correlations were plotted with all F1 and F2 data points (**Figure 3.23**).

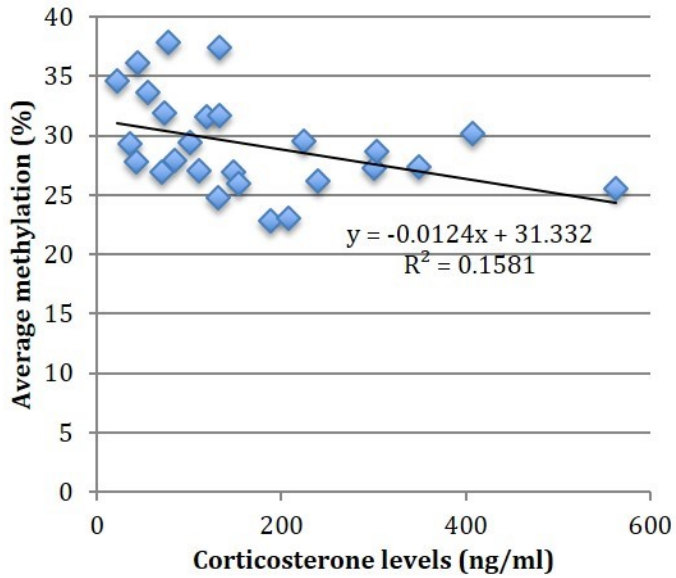


Figure 3.23. Scatter plot of Nr3c1 methylation and corticosterone levels. *Average methylation and serum corticosterone levels from each animal were plotted. N=27.*

3.4.3.3 Epigenetic regulation of *Bdnf* in the transgenerational CSS rat model:

The epigenetic regulation of *Bdnf* in the hippocampus of F1 and F2 CSS was then examined.

Bdnf expression: Expression of *Bdnf* in the hippocampus of F1 and F2 CSS and Control samples was tried, however optimization using three sets of primers still did not give clear enough signals to allow quantification of expression levels (data not shown)

Bdnf methylation: DNA methylation was measured at the promoter IV of the *Bdnf* gene (**Figure 3.24**).

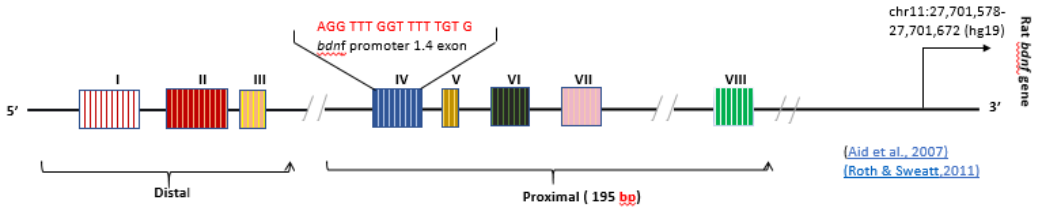


Figure 3.24. A schematic representation of the rat Bdnf promoter region. *This contains 8 untranslated 1st exons and promoter regions. Within the exon IV promoter region 6 CpG sites were investigated.*

Measuring levels of *Bdnf* exon IV DNA methylation within the F1 dams comparing controls and CSS dams revealed significantly lower levels of methylation at one of the six CpGs tested in the CSS compared to the control (2-tailed t-test, CpG1 p= 0.078; CpG2 p=0.025; CpG3 p= 0.25; CpG4 p= 0.8; CpG5 p= 0.86; CpG6 p=0.58). The average methylation off all CpGs was not significantly different (2-tailed t-test, p= 0.13) (**Figure 3.25**).

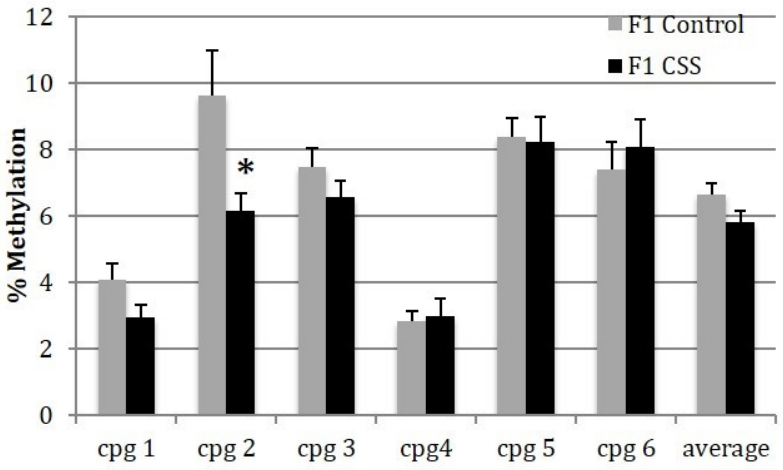


Figure 3.25. Bdnf methylation in F1 CSS and control dams. *Mean + SEM of DNA methylation and at bdnf promoter IV in F1 control and CSS dams. Significant effects using a 2-tailed t-test are indicated *P< 0.05. n= 13 control, 13 CSS.*

Measuring *bdnf* DNA methylation within the F2 dams comparing controls and CSS dams significantly lower levels of methylation were found at six of the seven CpGs tested in the CSS compared to the control (2-tailed t-test, CpG1 p= 0.69; CpG2 p=0.01; CpG3 p= 0.79; CpG4 p= 0.34; CpG5 p= 0.87; CpG6 p=0.71; CpG7 p = 0.57). The average methylation of all CpGs was also not significantly different between the groups (2-tailed t-test, p= 0.57) (**Figure 3.26**).

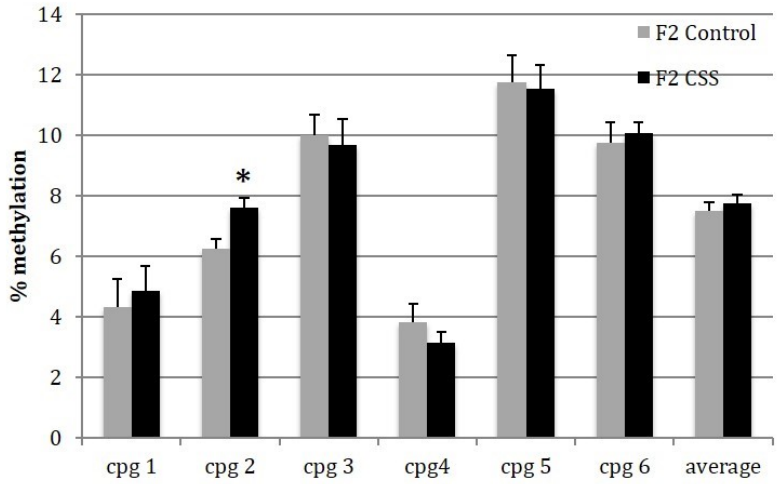


Figure 3.26. bdnf methylation in F2 CSS and control dams. *Mean + SEM of DNA methylation and at bdnf promoter IV in F1 control and CSS dams. n= 12 control, 13 CSS (F1 group).*

3.5 Discussion:

In the CSS intergenerational depression model there are increasingly reduced maternal care-associated behaviours at increasing durations of lactation from F0 to F2, i.e. reduced nursing at days 2, 9 and 16 in F2, compared to day 9 and day 2 only in the F0 and F1 dams respectively (**Figure 1.2**). This gives the pattern of an increasing accumulation of reductions in maternal care through the three generations. There was a similar pattern in maternal anxiety behaviour. As the F2 animals were never in direct contact with the CSS intruder male suggest that it is either the reduced maternal care of the F1 dams that mediate the effects on the F2 maternal care and anxiety behaviour, changes in serum markers that impacted development of the offspring or the inheritance of epigenetic marks.

Testing HPA axis activity revealed reduced corticosterone in the F2 animals as dams and also earlier in life at the pup stage suggesting a permanent reduction in the basal levels of this stress steroid. Interestingly, previous research on the F1 dams showed increased corticosterone levels (Murgatroyd and Nephew, 2013). That we find reduced corticosterone in the F2 dams that showed the reduced maternal care and increased anxiety seems contrary to the idea of increased corticosterone levels and stress and depression. However, numerous studies are now showing increased corticosterone to be associated with more chronic maternal stress and depression (Groer and Morgan, 2007), with increases in corticosterone more confined to women with immediate postnatal stress or shorter lasting depression (Seth et al., 2015). If we consider that the F2 dams showed more prolonged changes in reduced maternal care and anxiety, than the F1s, this would support our finding. Why the corticosterone is reduced may be hypothesized that a possible initial stress-induced corticosterone release form the HPA activation may over time during chronic stress become reduced in order to protect the brain and other physiological processes from prolonged exposure to excess cortisol levels that can be damaging (Fries et al., 2005). This would suggest that the regulation of the HPA axis activity during early life depends on the duration of the stress exposure: in the CSS generations, this may relate to the duration of depressed maternal care and increased

anxiety-related behaviour the pups receive, that is gradually lengthened though the generations. One might therefore expect even stronger stress phenotype in the F4 generation if this were studied, as those pups would have received reduced care and increased maternal stress across the whole of the lactational period.

The importance of maternal care during early life is supported by other studies. For example Capitanio et al. showed that in nursery reared Rhesus monkeys those that were denied access to maternal care have decreased levels of cortisol compared to those monkey raised by their mother (Capitanio et al., 2005) In a human study, infants exposed maternal depression stress showed lower cortisol levels when compared to control populations exposed to moderate levels of early life stress (Essex et al., 2011).

When studying levels of ACTH in the F2 dams and F1 however there were no significant differences. Though ACTH regulates corticosterone they did not correlate. Several studies have shown that the basal levels of these factors often do not directly correlate and are more a measure of acute stresses as cortisol levels may remain after the ACTH stimulation (Christ-Crain et al., 2007; Bruder et al., 2008). Testing the maternal behaviour related hormone prolactin revealed no difference in F2 dams. However, in the F1 adults the stressed females showed significantly reduced serum prolactin. Though this difference did not reach statistical significance in the F1 dams in this study (the study here only had group sizes of 6 for the F1 CSS and control dams), previous findings with larger group sizes of 12 F1 dams did show a significant of decrease in plasma prolactin levels (Carini and Nephew, 2013). F1 dams have also been shown to have reduced *Prolactin* gene expression in the hypothalamus. (Murgatroyd and Nephew, 2013). Together this all suggests long-lasting prolactin changes in the F1 animals that are not carried to the F2 generation. This supports the importance of prolactin in regulating stress response (Torner, 2016) and a human study has found lower levels in women suffering from postnatal depression (Abou-Saleh et al., 1998).

It is interesting that the F2 dams also lacked changes in oxytocin. But again, only groups of 6 animals were used in the analysis. Previous findings from the F1 dams showed reduced levels of *Oxytocin* and *Oxytocin receptor* gene expression in the hypothalamus

and amygdala (Murgatroyd and Nephew, 2013) while F0 dams showed also lower amygdala *Oxytocin* expression (Murgatroyd et al., 2015b) (**Table 1**). The normalisation of oxytocin in the F2s seem to suggest the prolactin and oxytocin endocrine factors do not explain the F2 dam's increased levels of depressed maternal care. This would suggest that these neuroendocrine systems play less of a role in the F2 dam behaviour in contrast to the F1 and F0 dams. Finally, it should be noted that the F1 and F2 values for oxytocin and prolactin do vary considerably. The first multiplex pituitary ELISA plates run during this study had issues with the plate-washer that did not remove all wash buffers, even though the machine registered that it had. It was only later that this was properly serviced and repaired. However, results between samples on the same plate did have relatively uniform values so can be compared.

Other neuropeptides important in behaviour and stress regulation were also tested. This revealed no changes in bdnf in F1 or F2 animals. Substance P, alpha-MSH, Beta-endorphin or Orexin A also did not differ when tested in F1 adults. This suggests these factors have no role in the maternal behavioural changes in the CSS dams.

Thyroid stimulating hormone (TSH) levels did not differ in F1 or F2s and neither did with growth hormone (GH) in F1 adults suggesting these important metabolic regulatory factors are not also important for the generational changes seen. The further growth factors VEGF and GM-CSF also did not significantly differ in either F1 and F2 dams.

Luteinizing hormone (LH) and follicle-stimulating hormone (FSH), important in the hypothalamic–pituitary–gonadal (HPG) axis, did not significantly differ in the F1s. Though there were some HPA differences in the F1s, it appears that the HPG axis was not impacted or that these factors are involved in the decreased maternal care behaviour.

Early stress can induce lasting changes in the immune system that may be important in programming the adverse effects of early life stress on mental health (Carpenter et al., 2010). Analysis of cytokines including IL-6, IL-10, and immune regulatory factors GM-CSF and VEGF in F2 adults revealed no differences in either F1 or F2 dams. However, reduced levels of Icam-1 were found in the F2 CSS dams serum (using a 1-tail t-test), though this

no longer differed in F1 or F0 dams. The 1-tail t-test was justified as reduced Icam-1 levels have been previously reported in the F2 adults (Nephew et al. 2017). Increased Icam-1 levels have been associated with increased stressed-induced cortisol levels (Heinz et al., 2003) which may relate to the lower corticosterone in the F2 animals. A marker of inflammation, I-CAM-1 plays a role in regulating blood-brain-barrier function with reduced levels found in schizophrenia (Schwarz et al., 2000) and depression and suicide. However, another study contrarily found a significant increase in Icam-1 in the dorsolateral prefrontal cortex in major depression in the elderly (Thomas et al., 2002). This reduction in only the F2 dams and adults would support the accumulation of stress theory through the F0 to F2 dams with more chronic behavioural phenotypes and rescued HPA regulation and increased immune components. Further work would be important to investigate the regulation of Icam-1 in the brain of the F0 to F2 dams.

Epigenetic changes were investigated in stress-regulatory genes between the F1 and F2 dams to test whether this mechanism has a role in the intergenerational inheritance of the depressed maternal care and maternal stress behaviours. In the F1 hippocampus there were significant reductions in DNA methylation at most of the CpGs measured in the *Nr3c1* gene in the CSS hippocampi. These F1 CSS dams also has significantly reduced *Nr3c1* RNA expression supporting the role of the DNA methylation in recuing gene expression (though these did not directly negatively correlate). In the F2 dams there were no longer any differences in *Nr3c1* DNA methylation or expression. This suggests that *Nr3c1* methylation is not maintained through the generations or that it is important in the maternal behavioural changes.

Investigation of *Bdnf* epigenetic regulation showed reduced methylation at CpG2, one CpG out of six tested, in the hippocampi of the F1 CSS dams. Interestingly, this same CpG2 showed significant increased methylation in the F2 CSS dams again supporting little level of heritability in CpG methylation between the generations.

There are some limitations to this study. The multiplex ELISA results seems to give variations between plates in overall reads, especially the first runs. It is thought that this may relate to the plate washer that did not seem to remove all the wash buffers before adding the second antibody. This was repaired and the results became more comparable. Therefore, sample values were not compared across plates, only within plates. Regarding the F1 dams, there were group sizes of only 6, compared to 12-14 in the F2 dams and F1 adults. Some of the analytes did not measure so well. For example, IL-2 and IL-4, TNF-alpha, while others did not produce results for all samples. This considerable reduced the numbers for some statistical analyses.

Overall, this study, taken together, reveals decreases in HPA axis dysregulation and increases in the levels of a marker of inflammation through the generations F0-F2 of the CSS. This suggests adaptive mechanisms functioning in regulating of chronic and acute stress, through inheritance of a chronic stressor.

4 CHAPTER 3: RNA seq

4.1 Introduction

The above results from the *Study 1* suggest that *Nr3c1* gene is upregulated following stress in the F1 dams but not the F2 dams. Conversely, levels of I-cam1 protein were upregulated following stress in the F2 dams but not the F1 animals. This suggests variations in gene regulations between the generations, possibly switching from stress to immune regulatory pathways. Genomewide studies allow gaining a bigger picture of the circuits and mechanism in the brain important in the regulation of depression and its heritability across generations.

Numerous studies have shown transcriptome (i.e. genomewide gene expression) changes in brains of animal models following acute (Stankiewicz et al., 2015)and chronic stress (Muhie et al., 2015). For example, Stankiewicz et al. studying hippocampus transcriptomes of mice exposed to chronic social stress found altered regulation in genes involved in the functioning of the vascular system (*Alas2*, *Hbb-b1*, *Hba-a2*, *Hba-a1*), injury response (*Vwf*, *Mgp*, *Cfh*, *Fbln5*, *Col3a1*, *Ctgf*) and inflammation (*S100a8*, *S100a9, Ctla2a*, *Ctla2b*, *Lcn2*, *Lrg1*, *Rsad2*, *Isg20*) supporting that stress may impact the vascular system (Stankiewicz et al., 2015)

However, one finding across many of these studies is that there seem to be relatively little correlation between findings in regards to particular genes that are differentially regulated. This is contrary to the epigenetic studies that focus on candidate genes in which we see many complementary and supporting findings (e.g. Tables 2.4, 2.5, 2.6). One reason might be that the genomewide approaches, in having to account for multiple analyses, do not have the statistical power to identify common genes changes that might not be at large magnitudes. Technical limitations such as tissue heterogeneity and the fact that major depression is a complex disorder involving numerous systems of interacting genes, and single-gene associations of expression that may not reach genome-wide significance. Added to this, is that in human studies there is widespread genetic

difference that further influences gene expression. To combat this, a number of studies looking at the types and functions of differentially regulated genes are beginning to identify common patterns in genes belonging to particular pathways of functions such as immune regulation and neurotransmission.

For example, using RNA-seq, Pantazatos et al. (Pantazatos et al., 2017) examined transcriptomes in the frontal cortex of suicides with major depression, non-suicides with major depression and non-psychiatric controls revealing 35 genes differentially expressed between groups that in the depressed groups consisted of lower expression of immune-related pathways such as chemokine receptor activity, chemotaxis and cytokine biosynthesis, and lower expression of genes involved in oligodendrocyte differentiation, regulation of glutamatergic neurotransmission, and oxytocin receptor expression. Further studies on genomewide expression in lymphocytes have found stress-related alterations in expression levels of immune response genes with various environmental adversities, such as childhood abuse and neglect and post-traumatic stress disorder (Schwaiger et al., 2016; O'Donovan et al., 2011). One of the largest RNAseq study on bloods from 463 major depression and 459 controls people (Mostafavi et al., 2014), when investigating the impact of childhood trauma found no significant results. However, a more recent study on blood transcriptome of childhood trauma combining with gene-set enrichment analyses identified genes and pathways differentially expressed in adults who had experienced emotional abuse and neglect. Specifically, there was a dysregulation in the expression of genes involved in cytokine system pathways including interferon (IFN) α/β and γ signaling (Minelli et al., 2018).

No study has yet investigated heritable transcriptional changes between generational showing transmission of depressive behaviour. It is unclear if key pathways or genes might be passed to subsequent offspring. Perhaps the expression of particular genes might be maintained between generations, maybe highlighting possible epigenetic inheritance mechanisms. Here we investigate hippocampal changes in rats with intergenerational inheritance of depression that allows us to control of genetic

differences and the environmental exposure. Finally, it also allows us the first investigation of possible heritable changes in gene expression following depression.

4.2 Aims

The aim of this chapter is to investigate whether genomewide changes in gene expression occur following CSS in the F1 and F2 dams. This is done using Next generation sequencing to perform RNAseq on hippocampus RNA from F1 and F" CSS and control dams. This will test whether specific genes are regulated or if there are specific functions of genes or shared pathways that might be transgenerational affected by stress. In sum, we hope to find if changes in the regulation of specific key genes are transgenerationally inherited in the brain following stress.

4.3 Objectives

1. Perform RNASeq on 24 hippocampus samples from CSS and control F1 and F2 dams.
2. Test if specific genes are differentially regulated or specific pathways or groups of genes differ between CSS and control.
3. Determine if there are transgenerationally inherited differences in gene expression between the F1 and F2 generation of CSS dams compared to control.

4.4 Methods

Transcriptome changes were measured in the F1 and F2 CSS and control hippocampus. To do this RNA sequenced using next generation sequencing (RNAseq).

4.4.1 RNA samples

The previously extracted RNA **(Table 3.1)** was tested for RNA quality prior to performing RNASeq to allow us to select on the highest quality RNA. The RNA was firstly measured for quality using the bioanalyser (**Figure 4.1** and **Table 4.1**). Those samples with high RIN values and concentrations were selected for RNAseq (add **Table 4.1**), these included 6 of each group of F1 CSS, F1 Control, F2 CSS and F2 Control.

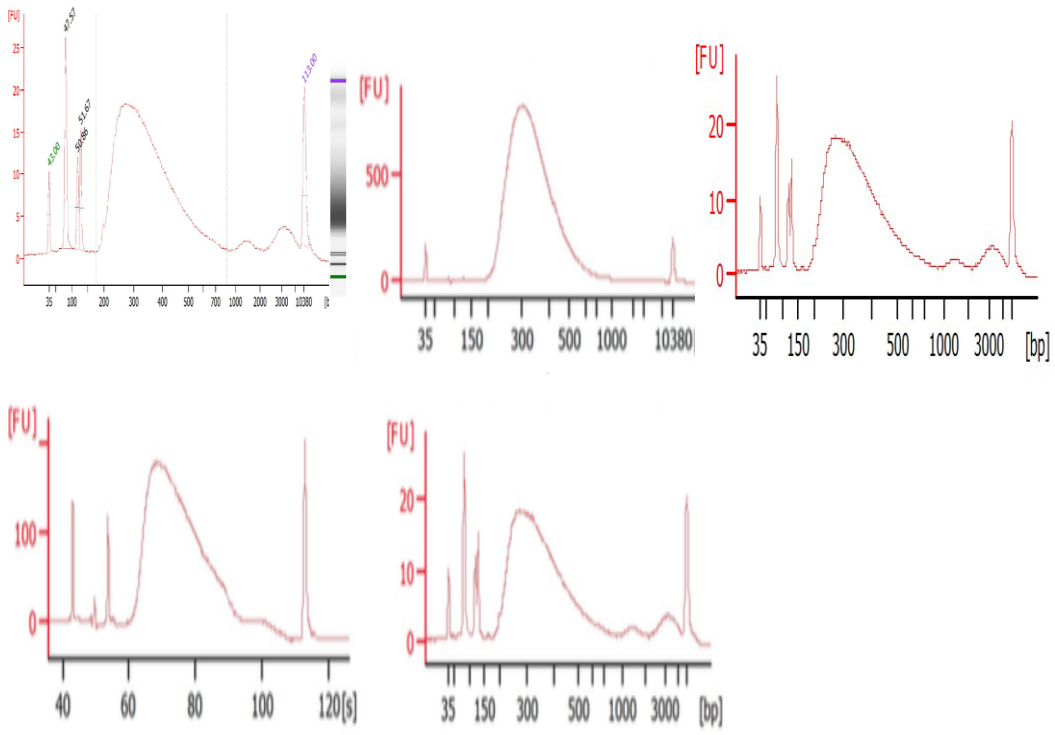


Figure 4.1. Example bioanalyser results of hippocampal RNAs

Table 4.1. RNA quality and quantity analyses. *Bioanalyser and Nanodrop results of hippocampal RNAs to give RIN values and concentrations.*

	Sample	RIN values	Nano-drop Conc. ng/ µl	Bioanalyzer Conc. ng/ µl	Nano-drop Conc. ng/ µl
5.1.11		**8.3**	**136.6**	**21**	**150**
25.1.1	2	8.4	530.7	23	581
26.1.1	3	7.9	214.6	30	263
20.2.1	4	8.2	213.7	15	218
21.1.2	5	8.3	488.6	33	496
10.2.2	6	8.4	416.5	26	416
26.1.2	7	8	349.1	25	351
4.1.1	8	N/A	438.6	5	462
1.2.2	9	7.8	104.4	36	102
1.1.1	10	7.8	88.3	21	90
27.2.2	11	7.4	47.8	15	49
22.2.1	12	7.8	99.6	50	95
27.1.1	13	6.9	347.7	11	355
26.2.1	14	7.5	133.6	37	137
25.2.1	15	8	74.7	21	77
18.1.1	16	7.4	154.7	17	154
14.1.1	17	7.6	219.9	29	231
17.2.1	18	7.9	108.6	29	117
22.1.2	19	6.8	136.7	28	139
27.2.1	20	7.3	215.6	23	221
18.2.1	21	7.4	95.3	19	100
2.1.2	22	7.4	261.3	20	267
15.1.1	23	7	208.3	26	223
21.1.1	24	7.2	201.3	44	213
3.1.1	25	6.4	390.5	57	395
2.1.1	26	5.6	225.4	24	227
17.1.1	27	5.7	139.1	69	212
24.1.1	28	6.7	430.5	30	475
24.1.2	29	5.8	136.1	46	141
25.2.1	30	6.6	418.3	34	446
22.1.1	31	6.3	221.6	58	222
11.1.1	32	4.6	824.1	33	825
11.1	33	5.7	123.2	65	125
26.2	34	6.6	138	113	130
14.2	35	6.9	132.3	91	140
30.1	36	7.5	176.2	210	177
16.1	37	7.9	157.4	67	162
20.1	38	7.4	152.1	34	140
22.1	39	6.5	546.4	35	589

27.1	40	6.5	221	56	226
17.2	41	N/A	218.8	17	222
22.2	42	N/A	139.4	11	139
3.1	43	N/A	254.4	6	386
24.1	44	N/A	419.1	43	455
2.1	45	N/A	277.9	1	307
24.1	46	N/A	375.6	17	440
15.1	47	7.9	242.2	44	254
1.2	48	N/A	349.1	160	375
17.1	49	8.2	397.4	31	419
25.2	50	7.9	423.5	14	450
26.1	51	1	216	20	230
5.1	52	N/A	288.9	94	4.5
4.1	53	N/A	467.5	76	4.2
27.1	54	8.6	235.4	52	34.4
14.1	55	9.1	507.1	65	571
18.2	56	8.7	270.8	44	285
20.1	57	8	572.9	72	623
1.1	58	9	403.2	33	425
25.1	59	N/A	766.6	6	859
21.1	60	8.6	346.7	57	354

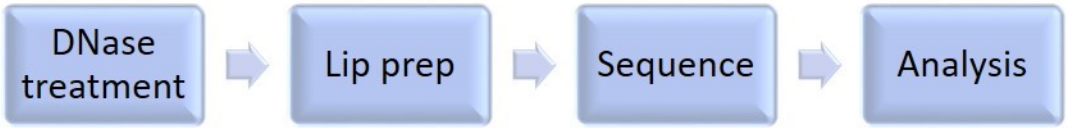
Results from the RNA quality and quantity allowed us to select 48 samples to RNA sequence. We firstly started with 24 and would then perform another 24. However, the library preps for the second run of 24 were not so efficient and so only 24 samples were finally sequenced. Samples selected are shown in (**Table 4.2)**.

Table 4.2. RNA samples selected for RNAseq. *Six samples were selected for each of the 4 groups of F1 control, F1 CSS, F2 control, F2 CSS.*

Groups	RNA number (Animal number)
F1 Control	3, 4, 11, 12, 19, 20
F1 CSS	7, 8, 15, 16, 23, 24
F2 Control	1, 2, 10, 17, 18
F2 CSS	5, 6, 13, 14 , 21 , 22

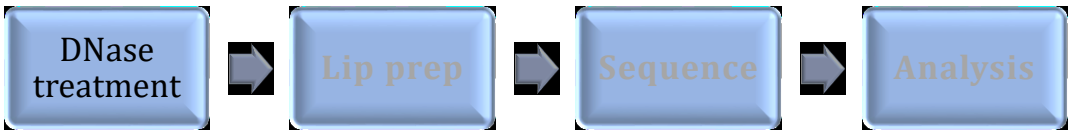
4.4.2 Library preparation and Next Generation Sequencing

The outline of the RNAseq involved DNase treatment of RNA samples, cDNA library preparation, sequencing using the Illumina NextSeq and bioinformatics analyses.



DNase treatment

Turbo DNA-free DNase treatment and removal reagents (Ambion) were used to remove any contaminating DNA from the 48 RNA samples, and to also remove the DNase and divalent cations from the samples (**Figure 4.2**).

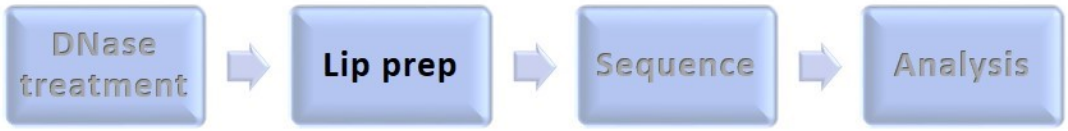


➢ It used to remove any contaminating DNA and the DNASE divalent cations from the samples	Isolate and impurity RNA Convert RNA to cDNA Adaptor ligation PCR amplification Validation and quantification	Denaturing and dilution and libraries loaded Sequence cDNA using Cluster Generation on Illumina	Quality Control- Fast QC Alignment-TopHat Expression quantification and Normalization and distribution-DESeq and SeqMonk List of differentially Genes Pathways.

Figure 4.2. Flowchart of DNase treatment

For each reaction, 200µg of nucleic acid was used per ml. 5.5 µl of TURBO DNase Buffer and 1 µl of TURBO DNase was added to 50 µl of RNA, and the resulting mixture incubated at 37°C for 30 minutes. Briefly, 9 µl of resuspended DNase inactivation reagent was added, mixed and the supernatant was transferred to a fresh tube.

4.3.2.1 RNA library Preparation



It used to remove any contaminating DNA and the DNASE divalent cations from the samples	➢ Isolate and impurity RNA ➢ Convert RNA to cDNA Adaptor ligation PCR amplification ➢ Validation and quantification	Denaturing and dilution and libraries loaded Sequence cDNA using Cluster Generation on Illumina	Quality Control- Fast QC Alignment-TopHat Expression quantification and Normalization and distribution-DESeq and SeqMonk List of differentially Genes Pathways.

In order to carry out the RNA sequencing, the RNA library first had to be prepared for Illumina using the NEBNext Ultra RNA Library Prep kit (New England Biolabs). This involved RNA enrichment, RNA fragmentation, cDNA synthesis, end prep, adaptor ligation, and PCR amplification (**Figure 4.3**).

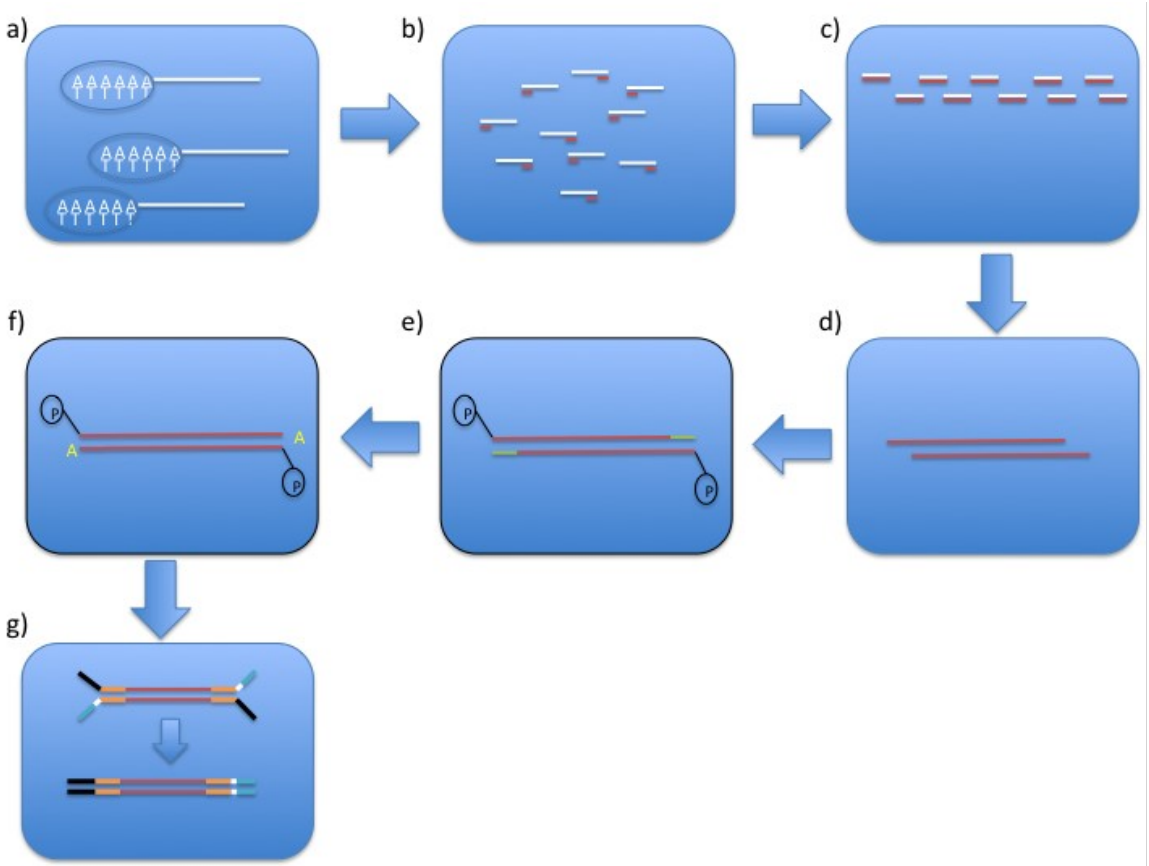


Figure 4.3. Step involved in the RNA Library preparation for illumine Next Generation Sequencing. *a) RNA enrichment (mRNA isolation/rRNA depletion). Poly-A selection using poly-(T) oligo-attached magnetic beads. b) RNA Fragmentation and Random Priming c) First strand cDNA synthesis d) Second strand cDNA synthesis e) End repair and 5' Phoshphorylation e) End repair and 5' Phoshphorylation f) A-tailing i.e. 3' ends adenylated g) Adaptor Ligation followed by PCR amplification*

RNA enrichment

Firstly, the mRNA had to be isolated from the total RNA and bound to the NEBNext Oligo d(T)25 beads. The beads were washed with 100µl of RNA binding buffer, and the tubes containing the beads were placed on a magnetic rack. The supernatant was discarded and 50µl of RNA binding buffer was added to the beads along with 50µl of total RNA sample. Eppendrof PCR for 5 minutes at 65°C was used to allow the poly-mRNA to bind to the beads. The resulting samples were washed, and following this, 50µl of Tris buffer was added and the samples placed in PCR for 2 minutes at 80°C. Another 50µl of RNA binding buffer was added and the samples resuspended and placed on the magnetic rack, to further ensure adequate binding of the poly-mRNA to the beads. The beads were rewashed and all supernatant removed.

RNA fragmentation

The poly-mRNA underwent a fragmentation process to elute the mRNA from the beads. Briefly, 17µl of First Strand Synthesis Reaction Buffer and Random Primer mix (2X) was added to the samples and PCR was carried out at 94°C for 15 minutes. The purified mRNA was collected by transferring 15 µl of supernatant to fresh tubes.

cDNA synthesis

First strand cDNA was synthesized from the fragmented and primed mRNA (15µl). 0.5µl of Murine RNase inhibitor, 1µl of ProtoScript II Reverse Transcriptase, and 3.5µl of nuclease-free water were added to each tube. The resulting samples underwent PCR with the following program: 25°C (10 minutes), 42°C (15 minutes), 70°C (15 minutes).

Second strand cDNA was synthesized by adding the following reagents to the first strand synthesis reaction (20µl): 48µl of nuclease free water, 8 µl of second strand synthesis reaction buffer, 4 µl of second strand synthesis enzyme mix. The resulting 80µl sample underwent PCR for 1 hour at 16°C.

The resulting double stranded cDNA was purified using 1.8X Agencourt 1AMPure XP Beads. 144µl of 1.8X 1AMPure XP Beads was added to the second strand synthesis

reaction, and the sample microcentrifuged before discarding the supernatant. 200µl of 80% ethanol was used to wash the beads. 60µl of 10 mM Tris-HCl PH 8.0 was then added, and 55.5µl of the supernatant was transferred to a clean PCR plate.

End preparation

End Prep of cDNA library was carried out. 6.5 µl of NEBNext end repair reaction buffer and 3 µl of NEBNext end prep enzyme mix were added to each 55.5 µl sample of purified double stranded cDNA. PCR was carried out with the following program: 20°C (30 minutes), 65°C (30 minutes).

Adaptor ligation

Adaptor ligation was then performed. This started by diluting the NEBNext Adaptor for illumina (15 uM) to 1.5 uM with a 10-fold dilution with 10 mM Tris-HCl. 1ul of the diluted NEBNext Adaptor was added to the 65ul end prep reaction, along with 15ul of Blunt/TA ligase master mix and 2.5ul of nuclease free water. The resulting samples underwent PCR at 20°C for 15 minutes. After this, 3µl of USER Enzyme was added and the mixture incubated at 37°C for 15 minutes.

Next the ligation reaction was purified using AMPure XP beads. Firstly, 17µl of nuclease free water was added to each sample. 100µl of resuspended AMPure XP beads was added to each sample, this was mixed and then placed on a magnetic rack, and the supernatant discarded. The samples were washed twice with 200µl of 80% ethanol each time. After this, 52µl of 10 mM Tris-HCl was added to elute the DNA target from the beads. 50µl of supernatant was transferred to a clean PCR tube, and the beads discarded. 50µl of resuspended AMPure XP beads was then added to each 50µl sample, this was mixed and then placed on a magnetic rack, and the supernatant discarded. 200µl of 80% ethanol was used to wash the samples twice. 22 µl of 10 mM Tris-HCl was added to elute the DNA target from the beads. Finally, 20 µl of the supernatant was transferred to a new PCR tube, ready for PCR enrichment of the adaptor ligated DNA.

PCR amplification

2.5µl of an individual appropriate index primer (NEBNext Multiplex Oligos for Illumina Set1 and Set 2) was added to each sample i.e. each sample had a different primer used. Following this, 2.5µl of universal PCR primer was added to each sample, along with 25µl of NEBNext Q5 Hot Start HiFi PCR Master Mix. Samples underwent 13 cycles of PCR as follows: 98°C (30 seconds), 98°C (10 seconds), 65°C (75 seconds), 65°C (5 minutes). It was important to limit the number of PCR cycles to avoid overamplification.

Lastly, the PCR reaction was purified using Agencourt AMPure XP beads. 45 µl of beads was added to each sample and mixed before supernatant discarded. 200 µl of 80% ethanol was then used to wash the samples twice. The DNA target was eluted from the beads by adding 23 µl of 0.1xTE. 20 µl of supernatant was transferred to a clean PCR tube and stored at 20°C.

Validation and quantification

Fragment sizes were evaluated via using a Bioanalyzer. The quality of the samples was assessed using a Bioanalyzer (Agilent high sensitivity assay). 32 µl of nuclease free water was added to each of the samples from the previous step, and they were re-cleaned using Agencourt AMPure XP beads and ethanol as described above. The concentration of DNA in each sample was assessed using the Quibit 3 Fluorometer (Invitrogen).

A scheme of the whole method is given in (**Figure 4.4)**.

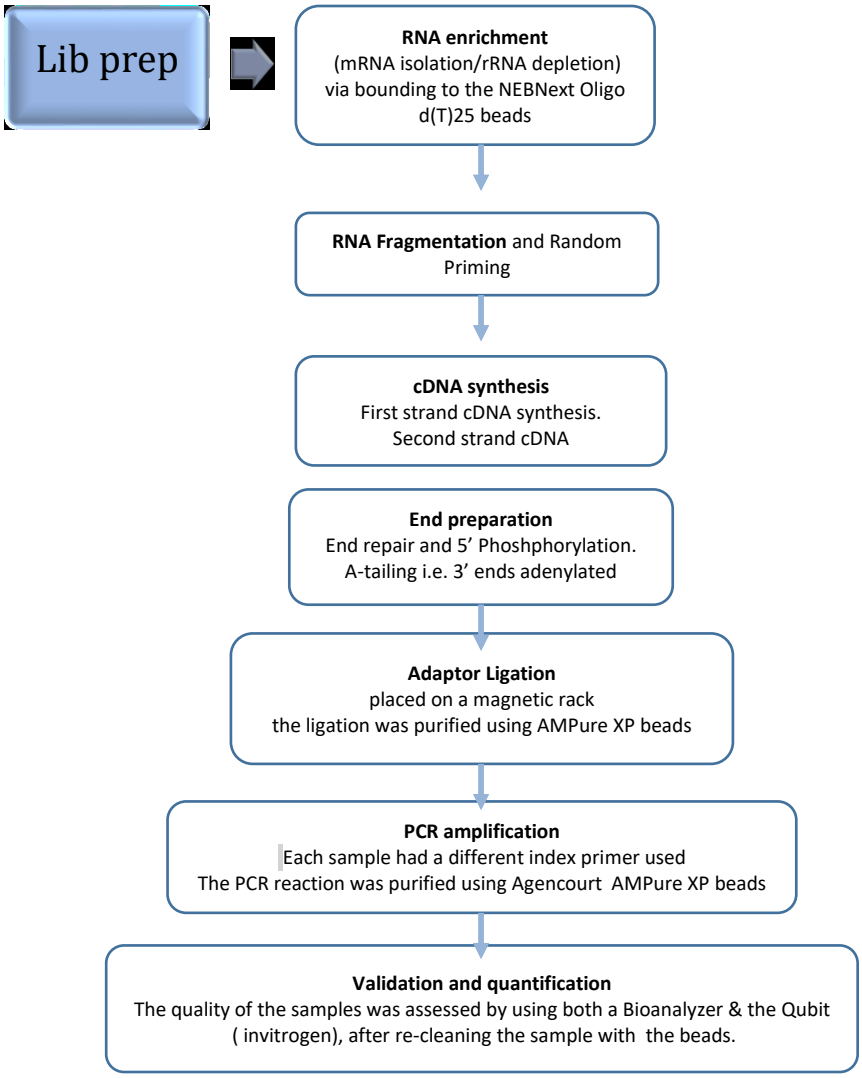
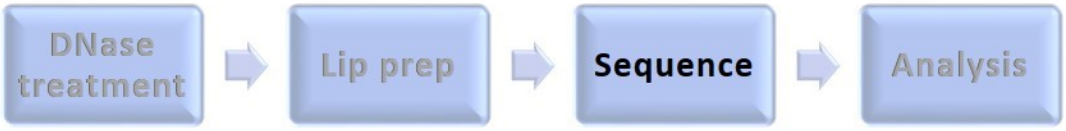


Figure 4.4. Flow chart of RNA lib preparation

4.4.2.2 Next Generation Sequencing



It used to remove any contaminating DNA and the DNASE divalent cations from the samples	Isolate and impurity RNA Convert RNA to cDNA Adaptor ligation PCR amplification Validation and quantification	➢ Denaturing and dilution and libraries loaded ➢ Sequence cDNA using Cluster Generation on Illumina	Quality Control- Fast QC Alignment-TopHat Expression quantification and Normalization and distribution-DESeq and SeqMonk List of differentially genes Pathways.

The prepared DNA libraries were denatured and diluted to a target concentration of 20pM, before sequencing on the Illumina® NextSeq® system. Firstly, 20 µl of 1nM DNA library was prepared, and added to 20 µl of freshly diluted 0.2 N NaOH. The resulting mixture was centrifuged at 280 x g for 1 minute. After 5 minutes incubation at room temperature, 200 mM Tris-HCL (pH 7) was added, and the resulting mixture centrifuged at280 x g for 1 minute. The mixture now contains denatured DNA library. To dilute this to 20pM, 940 µl of prechilled hybridization buffer was added and centrifuged for 280 x g for 1 minute.

After denaturing and dilution, the libraries were loaded onto the reagent cartridge and the sequencing run set up. To do this we used the Illumina NextSeq® 500/550 High Output Kit v2 (150 cycles).

Cluster generation

Before sequencing could occur, the single stranded DNA templates were bridged-amplified, forming clonal clusters within the flow cell. To achieve this, the double stranded DNA from the PCR amplification step had to be broken down into single strands. Firstly, the DNA templates were hybridized to a slide containing a high-density of immobilized forward and reverse primers. 3' extension with DNA polymerase was then used to copy the templates from the hybridized primers. The original templates were broken down, and only the immobilized copies on the flow cell surface remained. Next the immobilized copies looped over to hybridize to adjacent primers, and double stranded DNA bridges formed by DNA polymerase copying the templates. The DNA bridges were then denatured to form two single stranded molecules with the same information as the initial DNA template. Lastly, specific base cleavage was used to remove the reverse DNA strand, and the 3' ends of the immobilized forward strand were blocked to stop them from interfering in the sequencing process. Dense clonal clusters with over 1000 molecules per cluster were made by repeating this procedure on each template through cycles of isothermal denaturation and amplification (**Figure 4.5**).

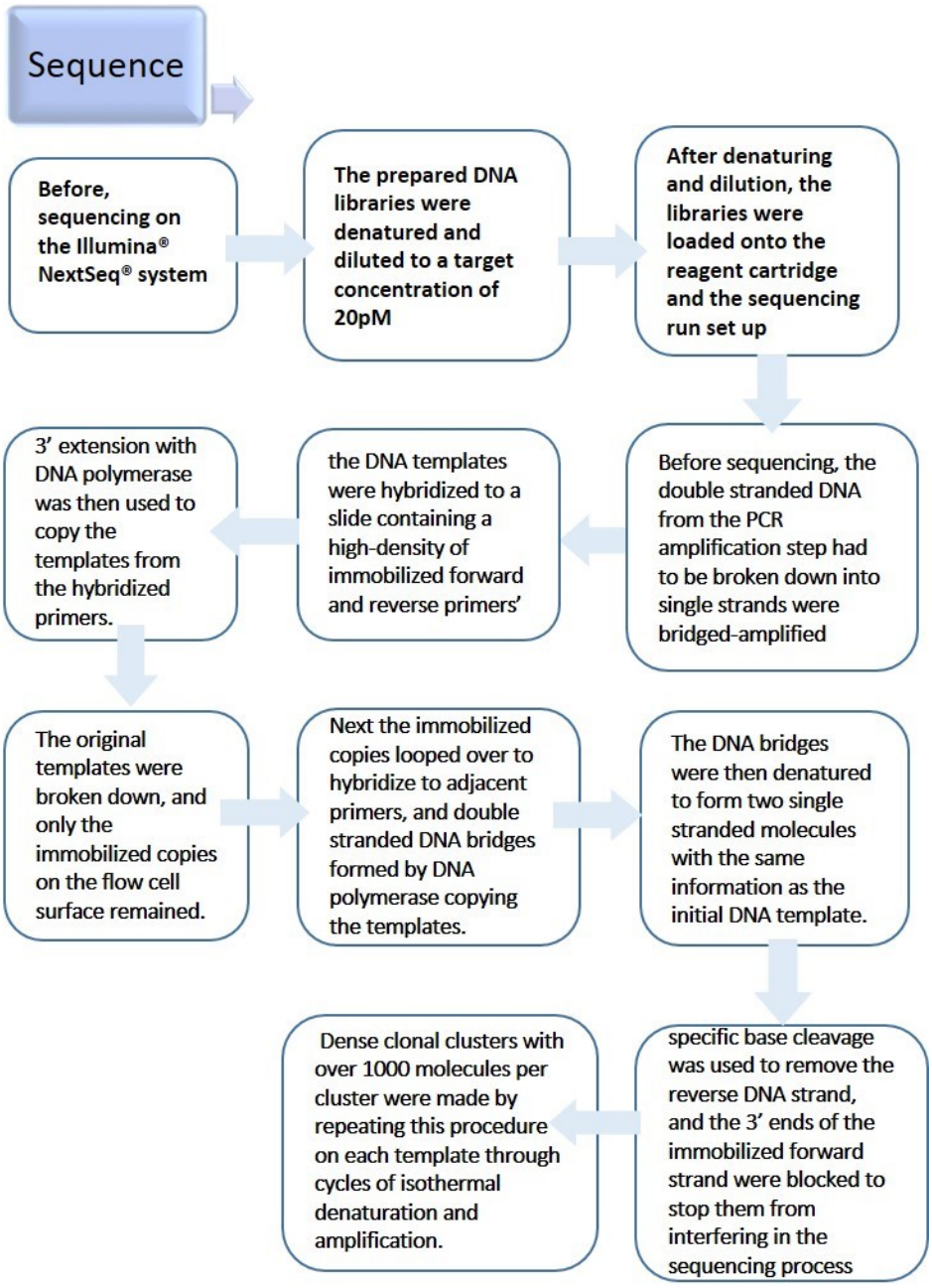
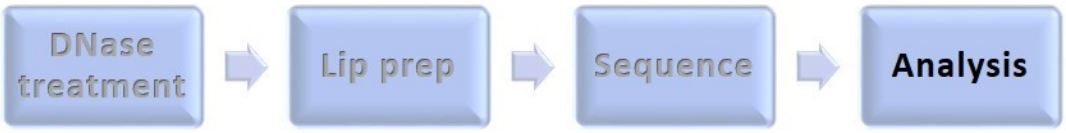


Figure 4.5. Flow chart of Cluster generation

4.4.2.3 Quality Control Analysis

Sequencing data from the Illumina NextSeq run was firstly analysed for sequence quality of reads. The sequences were then aligned to the rat genome and coverage of the reads and assayed to determine the quality of the data and uniformity of the coverage. RNA

expression differences were then tested for between CSS and control samples from the F1 and F2 hippocampi. Any differently regulated RNAs were then analysed for shared ontologies and pathways.



It used to remove any contaminating DNA and the DNASE divalent cations from the samples	Isolate and impurity RNA Convert RNA to cDNA Adaptor ligation PCR amplification Validation and quantification	Denaturing and dilution and libraries loaded Sequence cDNA using Cluster Generation on Illumina	➢ Quality Control- Fast QC ➢ Alignment-TopHat ➢ Expression quantification and Normalization and distribution-DESeq and SeqMonk ➢ List of differentially ➢ genes Pathways.

Quality control of sequence reads

The FastQC program was used for quality control (QC) of the reads of RNA sequence for each library sample to check that the sequence data does not have errors or biases, and that it is of a good standard. This gives the following information: per base sequence quality, per sequence quality scores, per base sequence content, per base GC content, per sequence GC content, per base N content (N=unread bases), sequence length distribution, sequence duplication level, and kmer content for levels of duplication.

Per Base Sequence Quality: The FastQC program gives a quality score, which can be interpreted as the statistical probability of an error in base calling occurring: the higher the quality score, the lower the error probability and hence the more reliable a base call is likely to be. For example, Q40 quality score implies 1 base call out of 10,000 is likely to be incorrect, while a Q30 score means one base call out of 1,000 is likely to be incorrect. This was done for each sample individually and a graph was generated that reports quality scores based on position, for every read. The ideal score is over 28 at each position and a quality score dropping much below 28 should be trimmed at that point as this indicates a

high probability of error that could influence biological conclusions when performing the alignments in the following steps. Using sample 1 as a representative example it can be seen that most of the sequence falls into good to reasonable quality scores (**Figure 4.6**). The quality calls do decrease towards the end of the sequence which is common to see.

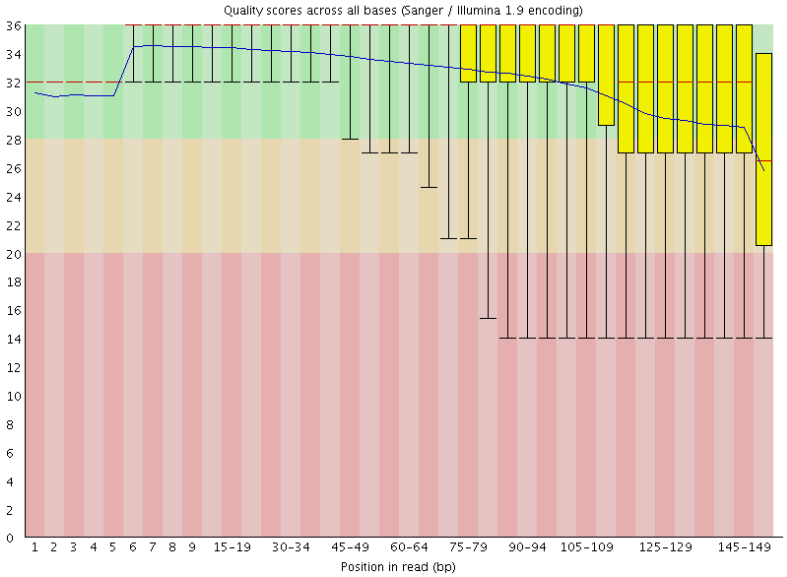


Figure 4.6. Per base Sequence Quality. *Sample 1 is shown as a representative example. For each position in read length a BoxWhisker type plot is shown where the central red line is the median value, the yellow box is the inter-quartile range (25-75%) and the upper and lower whiskers represent the 10% and 90% points. The blue line shows the mean quality. On the y-axis are the quality scores divided into very good quality calls (green), reasonable quality (orange), and poor quality (red).*

Per sequence quality scores: In addition to going over each base one can average the quality of each read instead and show a cumulative plot of the sequence quality of these. Using sample 1 as a representative example this revealed that most reads have an average quality of 34. This is to be considered very good (**Figure 4.7**). All 23 other samples had similar scores

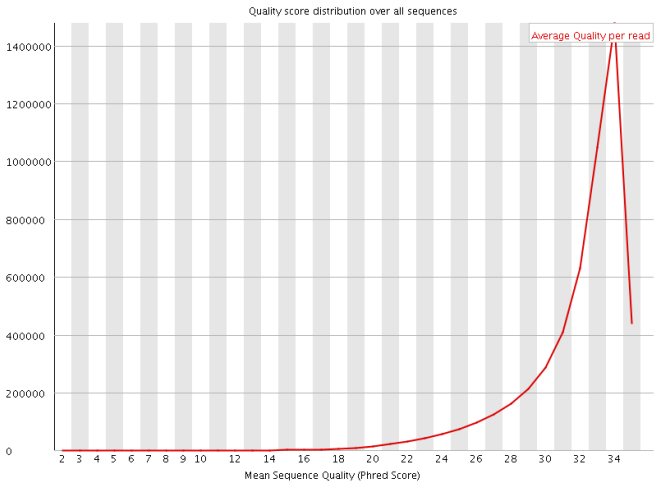


Figure 4.7. Per base quality score over all sequences. *Sample 1 is shown as a representative example. The red line shows the mean sequence against the y-axis of the number of sequences.*

Per base sequence content: The quality of the reads were then again assessed for sequence content with a graph produced for the percent of bases called for each of the four nucleotides at each position across all reads. The proportion of each of the four bases should remain relatively constant over the length of the read, i.e. A (%) = T (%) and G (%) = C(%). For the first 10 nucleotides there is non-normal distribution but this is normal and expected with RNA-Seq. Importantly though our samples show a uniform distribution for the rest of the sequence again supporting that the sequencing data from all the samples are of good quality (**Figure 4.8**).

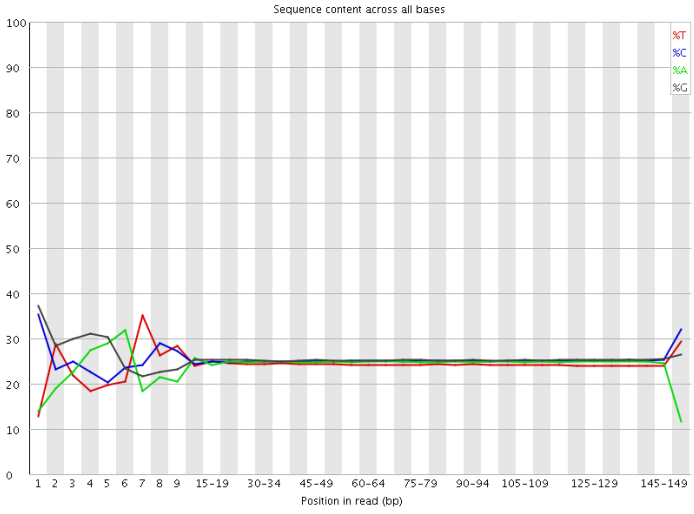


Figure 4. 8. Per base sequence content: *Sample 1 is shown as a representative example. The percent of bases called for each of the four nucleotides (y axis) is plotted against at each position across all reads (x axis).*

Per sequence GC content: The sequences were then tested for GC content. A graph was produced comparing the theoretical distribution of GC content with the observed GC content. These two plots should be very similar: large variation between these 2 plots could signal contamination from another organism with higher GC content: if the distribution is shifted it could mean a systematic bias not dependent on base position of A/T rich sequences: a very low GC content could signal only the mRNA poly-A tails have been sequenced. All of our samples all showed similar theoretical and observed plots with good GC content supporting high quality sequencing (**Figure 4.9**).

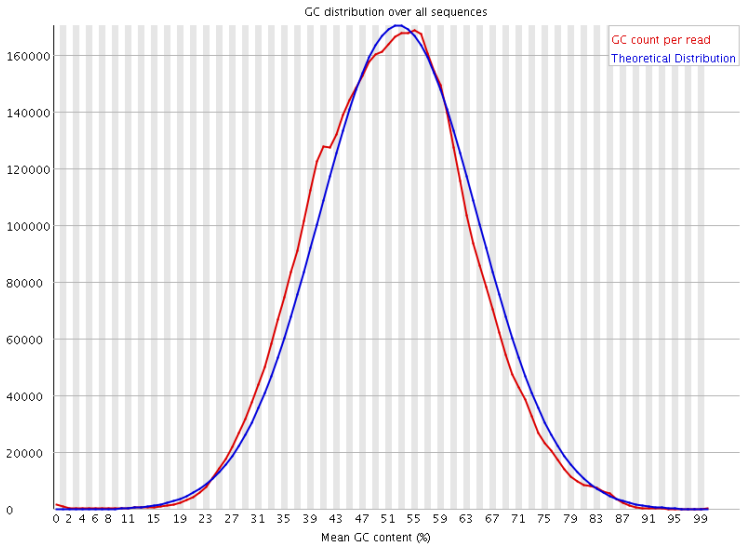


Figure 4.9. Per sequence GC content: *Sample 1 is shown as a representative example: A graph of % Mean GC content (x axis) and number of sequences (y axis) was used to plot Mean GC count per read (red line) and theoretical GC distribution (blue line).*

GC content across all bases: Plotting GC content across all bases supported the previous graph that the sequecnes have a good uniform level of GC at around 50%. The fluctuation in the first 8bp reflect the sequcning of the adaptors which have defined sequecnes (**Figure 4.10**).

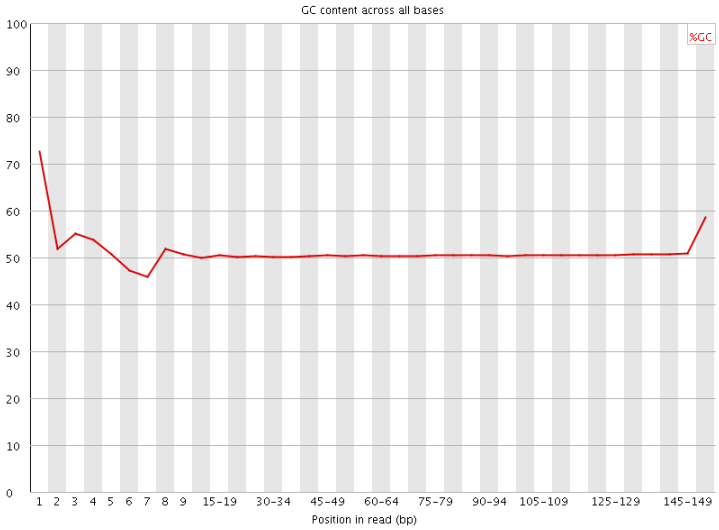


Figure 4.10. GC content across all bases: *Sample 1 is shown as a representative example: A graph of position in read across the sequence (x axis) and % of GC (y axis) was used to plot % mean GC count per read across all the sequences (red line).*

Per base N content: the quantity of unclear base calls (N's) were measured across the sequences in all samples. A plot was produced that shows the fraction of indistinguishable bases as a function of the base position in the reads (**Figure 4.11**). In high quality sequence data this is should be close to zero: increases in these values indicate problems during the sequencing. All our sequences showed close to zero.

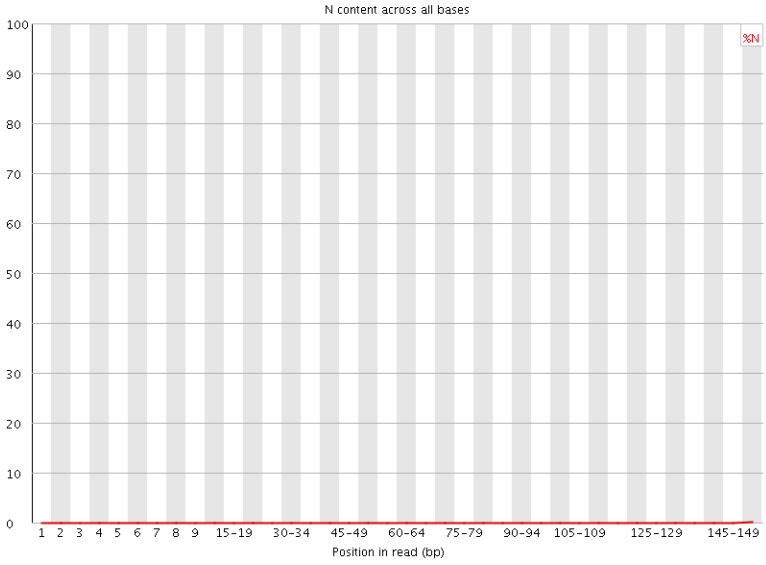


Figure 4.11. Per base N content: *Sample 1 is shown as a representative example: A graph of position in read across the sequence (x axis) and % of unclear base calls (N) (y axis) was used to plot %N per read across all the sequences (red line).*

Sequence Length Distribution: The Sequence length distribution was plotted which showed the distribution of read lengths with a peak at 150 bp as expected (**Figure 4.12**).

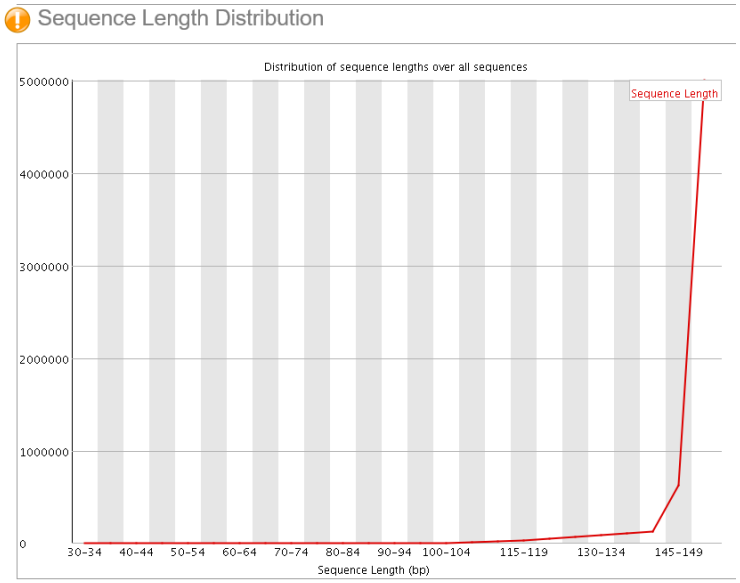


Figure 4.12. Sequence length distribution: *Sample 1 is shown as a representative example: A graph of the number of sequences (y axis) and sequence length was used to plot the sequence across all the sequences (red line).*

Sequence Duplication Levels: The levels of duplicated sequences were then tested for in the sequencing reads. There are generally two sources of duplicate reads: PCR duplication in which library fragments have been over represented due to biased PCR enrichment or truly over represented sequences such as very abundant transcripts in an RNA-Seq library. The former is a concern because PCR duplicates misrepresent the true proportion of sequences in your starting material. The latter is an expected case and not of concern because it does faithfully represent your input. In RNAseq data there is expected to be some duplication due to possibly highly expressed genes but too much of over 10+ could suggest a problem with the experiment. All our samples showed relatively low duplication levels (**Figure 4.13**).

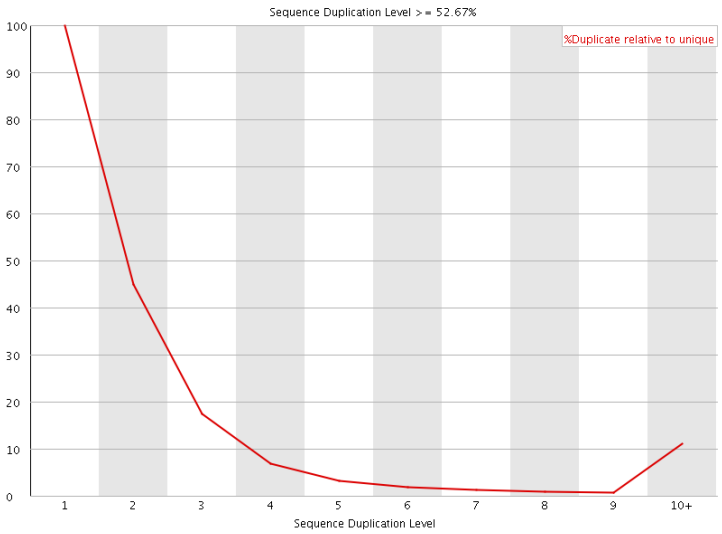


Figure 4.13. Sequence duplication levels: *Sample 1 is shown as a representative example: A graph of the number of % of sequences against the sequence duplication levels (y axis) was used to plot the sequence duplication across all the sequences (red line).*

Kmer content: As a further test for sequence duplication the occurrence of kmers - nucleotide sequences of fixed k length were measured. This plot tests that a small fragment of sequence is not biased to any position as it appears in a library that is diverse. If a certain Kmer is enriched or depleted overall due to a biological reason, and not an error, then any bias should affect all positions equally without any positional bias. The FastQC plots the top 6 most biased Kmers to relay their distribution. Testing the samples these revealed similar patterns across all samples (**Figure 4.14**).

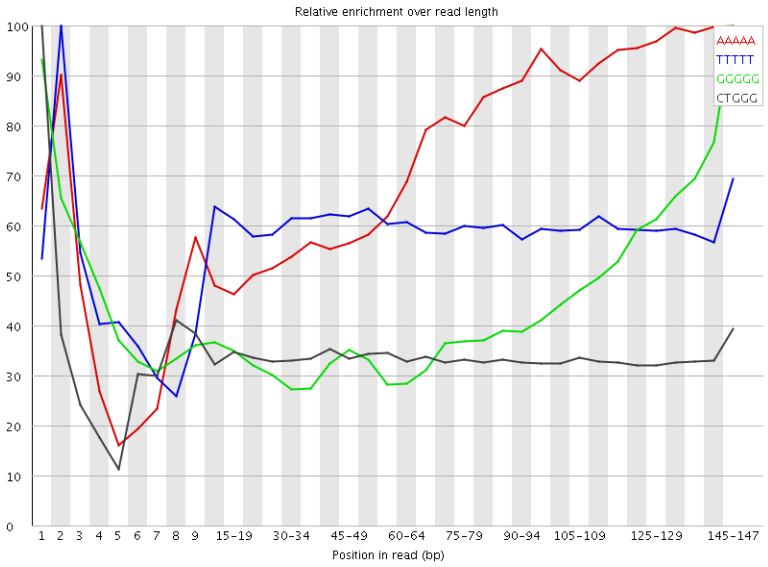


Figure 4.14. Kmer distribution over read length: *Sample 1 is shown as a representative example: A graph of the number of % of sequences(y axis) against position in read (x axis) for 4 Kmers across all the sequences (different colours lines represent different Kmers).*

The FastQ Toolkit program was used to trim all the sequences in all samples to 100 bp.

4.4.2.4 Sequence alignment:

The previous FASTQ files that contain the nucleotide sequence of each read that contain the quality scores etc. were then aligned to a reference rat genome. This produces BAM files. This was done using the TopHat2 spliced alignment software in combination with the Bowtie index available at the Illumina Basespace.

The sequences were then analysed for Length of reads, Number of Reads (Total number of reads passing filter for this sample), percentage of reads passing filter that aligned to the reference (% Total Aligned), percentage of reads that do not align to the reference (% Unaligned), percentage of reads that align to abundant transcripts, such as mitochondrial and ribosomal sequences (% Abundant) and the median coefficient of variation of coverage of the 1000 most highly expressed transcripts. The Ideal value = 0. (Median CV Coverage Uniformity). (See **Table 4.3)** for the summary of these factors for all samples.

Table 4.3. Read length, number of reads, % aligned, % abundant, % unaligned and median CV coverage uniformity for all samples.

Sample ID	Read Length	Number of Reads	% Total Aligned	% Abundant	% Unaligned	Median CV Coverage Uniformity
Sample1	100	19,328,365	88.21%	7.94%	11.79%	0.45
Sample10	100	22,932,906	85.73%	7.36%	14.27%	0.54
Sample11	100	22,339,745	78.33%	26.46%	21.67%	0.51
Sample12	100	26,643,711	81.36%	9.23%	18.64%	0.55
Sample13	100	21,677,960	80.48%	8.71%	19.52%	0.53
Sample15	100	32,068,201	86.82%	15.90%	13.18%	0.47
Sample16	100	25,620,016	77.47%	6.44%	22.53%	0.49
Sample17	100	23,711,773	77.74%	9.66%	22.26%	0.48
Sample18	100	29,515,133	70.87%	9.55%	29.13%	0.60
Sample19	100	23,574,288	84.78%	9.98%	15.22%	0.49
Sample2	100	16,569,892	87.76%	9.43%	12.24%	0.45
Sample20	100	34,179,124	84.53%	8.22%	15.47%	0.50
Sample21	100	30,304,456	84.93%	9.32%	15.07%	0.48
Sample22	100	21,494,834	90.68%	10.06%	9.32%	0.48
Sample23	100	20,487,688	83.47%	7.22%	16.53%	0.46
Sample24	100	20,804,246	80.62%	14.08%	19.38%	0.46
Sample3	100	23,399,765	86.26%	6.70%	13.74%	0.45
Sample4	100	17,060,348	87.03%	8.30%	12.97%	0.48
Sample5	100	21,214,979	88.32%	7.39%	11.68%	0.45
Sample6	100	15,034,051	88.26%	8.44%	11.74%	0.44
Sample7	100	24,907,150	89.15%	10.59%	10.85%	0.49
Sample8	100	21,944,435	89.32%	6.98%	10.68%	0.46

Insert Length Distribution: A Histogram was plotted for all samples (these are from paired reads so from 100bp each read from each end) to show the full distribution of insert sizes determined from the alignments. Importantly, this shows relatively similar pattenrs across all sampels anfd all sampels have insert lengths of around 150-300bp (**Figure 4.15**)

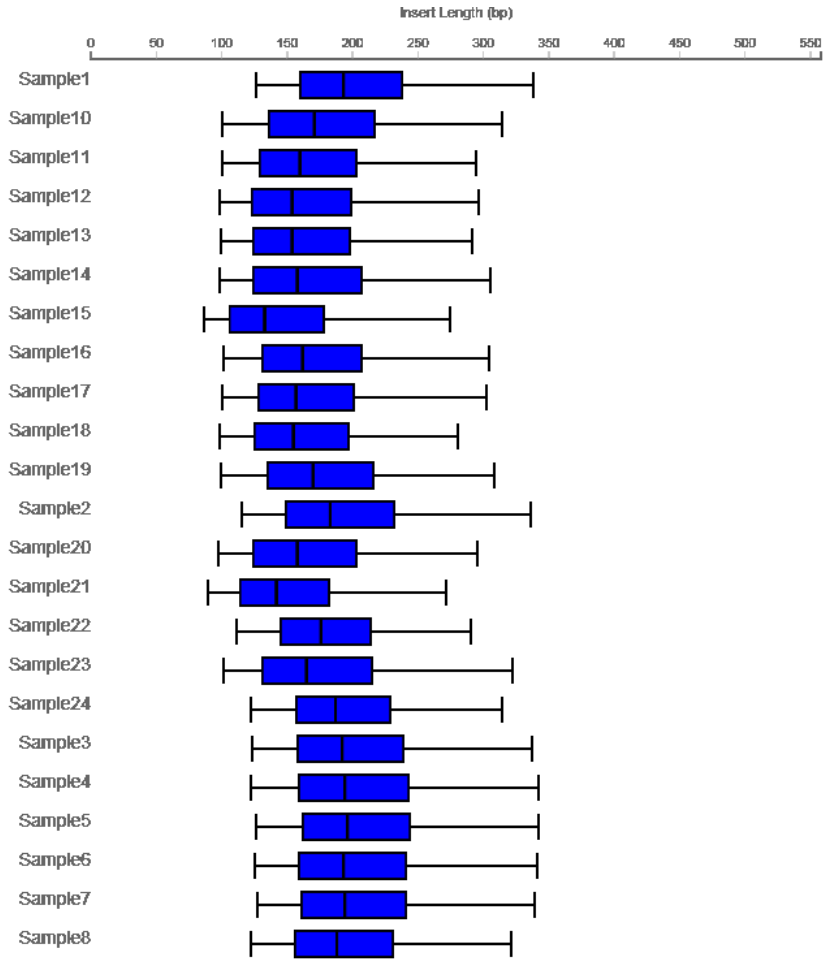


Figure 4.15. Insert Length Distribution Histogram: *The full distribution of insert sizes determined from the alignments for all samples is shown.*

The sequences were analysed using an alignemnt distribution histogram where the numbers of bases (%) are graphed accoring to genomic regions; either coding, untranslated regions (UTR), intronic or intergenic. Importantly all the sampels show a similar distribution and a good coverage of coding and UTR and intergenic. There is less intronic, though there will be some, dependent on intronic splicing (**Figure 4.16**).

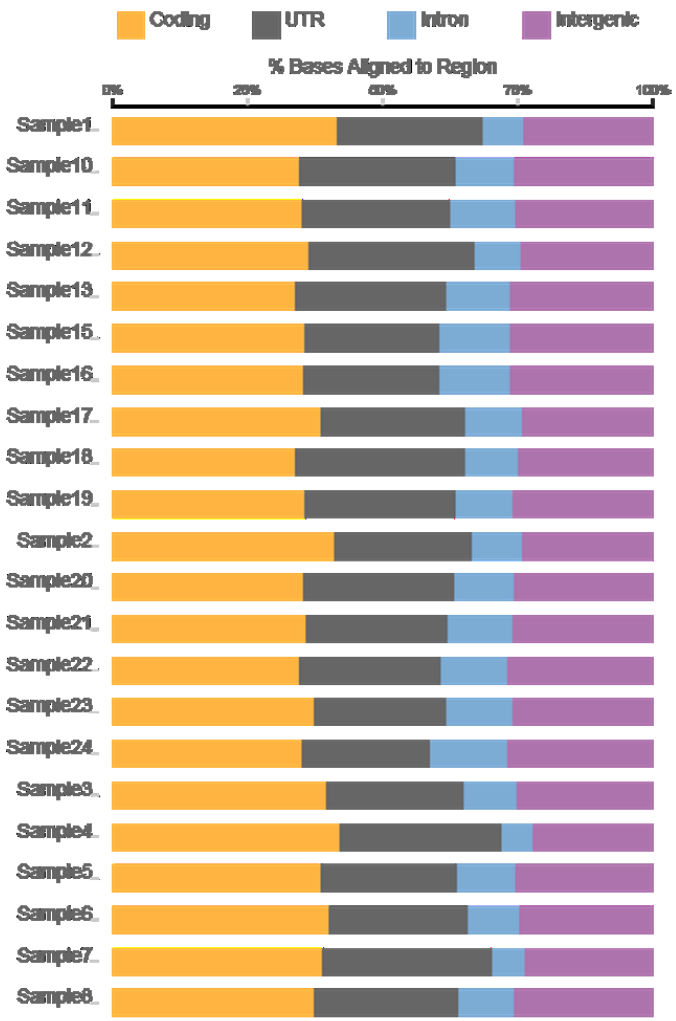


Figure 4.16. Alignment distribution: *All Samples were aligned and plotted for the distribution across coding, UTR, exonic, intronic and intergenic reads.*

Transcript coverage was then plotted for all samples. This produces a graph showing the coverage of a transcript position by that transcript position as reported by CollectRnaSeqMetrics utility from Picard tools. The coverage is normalized for the coverage of that transcript. It is important that a similar pattern is seen for all samples and that there is a clear bell-shaped curve (**Figure 4.17**).

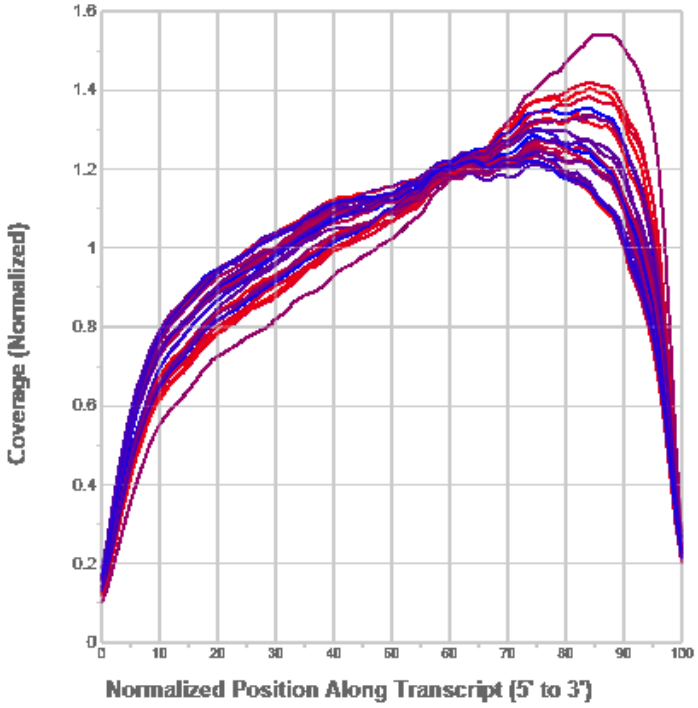


Figure 4.17. Alignment distribution: *All Samples were aligned and plotted for the distribution across coding, UTR, exonic, intronic and intergenic reads.*

The samples were all checked across read lengths, duplicates, aligned reads coverage and numerous other criteria to ensure uniformity across all samples. This did revealed that all samples were indeed comparable, supporting the previous graphs (**Table 4.4**) (**Table 4.5**).

Table 4.4. Alignment distribution: *All Samples were aligned and plotted for the distribution across coding, UTR, exonic, intronic and intergenic reads.*

	Definition	
Read Length	Length of reads	100
Number of Reads	Total number of reads passing filter for this sample	19,328,365
Bases (GB)	The total number of bases for this sample	1.93
Q30 Bases (GB)	The total number of bases with a quality score of 30 or higher	1.75
Insert Length Mean	Mean length of the sequenced fragment. The fragment length is calculated based on the locations that a read pair aligns to the reference. The read mapping information is parsed from the BAM files.	207.33
Insert Length S.D.	Standard deviation of the sequenced fragment length.	67.85
Duplicates (% Reads)	Percentage of paired reads that have duplicates, from a sub-sampled set of 4 million reads. If a panel is selected, only 0.5 million reads are sampled.	16.65%
Total Aligned Reads (% Reads)	Percentage of reads passing filter that aligned to the reference.	89.40%
Abundant Reads (% Reads)	Percentage of reads that align to abundant transcripts, such as mitochondrial and ribosomal sequences.	7.98%

Unaligned Reads (% Reads)	Percentage of reads passing filter that are not aligned to the reference.	10.60%
Reads with spliced alignment (% Aligned Reads)	Percentage of aligned reads with a spliced alignment.	26.68%
Reads aligned at multiple loci (% Aligned Reads)	Percentage of aligned reads to multiple loci.	3.37%
Median CV	The median coefficient of variation of coverage of the 1000 most highly expressed transcripts, as reported by the CollectRnaSeqMetrics utility from Picard tools. Ideal value = 0.0	0.46
Median 3'	The median uniformity of coverage of the 1000 most highly expressed transcripts at the 3' end, as reported by the CollectRnaSeqMetrics utility from Picard tools. 3' bias is calculated per transcript as: mean coverage of the 3' most 100 bases divided by the mean coverage of the whole transcript.	0.35
Median 5'	The median uniformity of coverage of the 1000 most highly expressed transcripts at the 5' end, as reported by the CollectRnaSeqMetrics utility from Picard tools. 5' bias is calculated per transcript as: mean coverage of the 5' most 100 bases divided by the mean coverage of the whole transcript.	0.39
Reads aligned to correct strand	Percentage of reads that align to the correct strand, as reported by the CollectRnaSeqMetrics utility from Picard tools.	0.00%
Homozygous Reference	Number of homozygous reference calls.	4,165,100
Heterozygous	Number of heterozygous variant calls.	12,052
Homozygous variant	Number of homozygous variant calls.	17,991
SNVs	Total number of SNVs (single nucleotide variants) detected for the sample.	28,011
Indels	The number of indels (insertions and deletions) detected for the sample.	2,092
Tn/Tv	Transition rate of SNVs that pass the quality filters divided by transversion rate of SNVs that pass the quality filters. Transitions are interchanges of purines (A, G) or of pyrimidines (C, T). Transversions are interchanges of purine and pyrimidine bases (for example, A to T).	3.00

Table 4.5. Coverage uniformity information: *All Samples were aligned and plotted for the distribution across coding, UTR, exonic, intronic and intergenic reads.*

Statistic	Definition	
Fold Coverage	The total number of bases in the category divided by the size of the entire category.	
% Bases	The total number of bases aligned to this region relative to the total number of aligned bases.	
Region	**Fold Coverage**	**% Bases**
Coding	49.63x	41.56%
UTR	61.93x	26.82%
Intron	0.34x	7.62%

Intergenic	0.35x	23.99%

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Statistic	Definition
Coverage	Gene level coverage. 1X: At least 1X; 10X: At least 10X; 30X: At least 30X; 100X: At least 100X.
Number of Genes	Number of genes covered at the mean base coverage level or deeper.

Coverage	**Number of Genes**
1X	11,283
10X	7,831
30X	4,719
100X	1,733

4.5 Results

4.5.1 RNA expression analysis:

The aligned data in the BAM files were imported into a program called SeqMonk that is able to run r analysis statistical determination of expression differences and DESeq software for visualization of expression analyses. Though this could have been done in the same BaseSpace package as above (which also runs DESeq), Illumina charge costs, whereas SeqMonk is free version of the package.

Within SeqMonk the data from the alignments, were imported and again aligned to the rat genome (Rattus 5.0) in the form of raw counts, were then Log2 transformed to allow better visualization. Preliminary QC analysis was then performed to confirm the quality assessments verified in Basespace and the BAM files. Firstly, a plot was performed to check the proportion of reads in genes falling into exons and the strand-specificity of the library – as we ran a bidirectional sequencing, we need to be certain that both read directions are uniform and that they are uniform across samples. (**Figure 4.18)** shows that all samples show similar percent in gene and exons and 50% on sense strand. They also show 0% rRNA (we purified only for mRNA prior to sequencing) and 0% in Mitochondria that we again selected against.

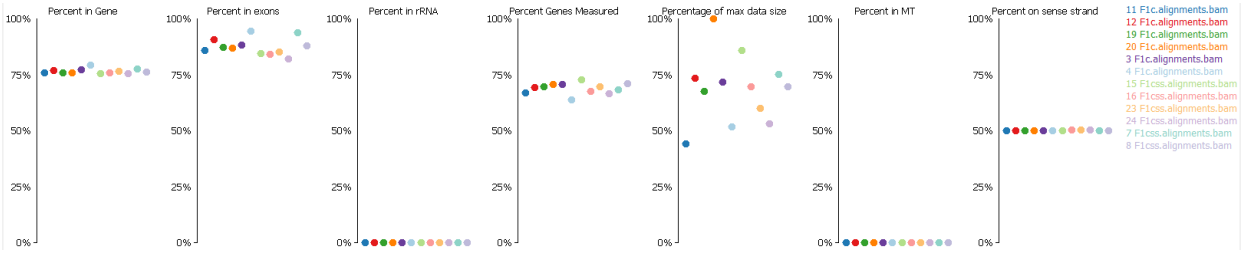

Figure 4.18. Alignment distribution: *All Samples were aligned and plotted for the distribution across percent in gene, percent in exons, percent in rRNA, percent genes measured, percent of max data size, Percent in MT and percent on sense strand.*

Duplication levels within the samples were then also re-checked. This was done by plotting duplication against read density over all exons. This revealed an association with read density and duplication with low density exons having low duplication, which. Importantly it showed that everything was not high duplication or non-continuous duplication (**Figure 4.19**).

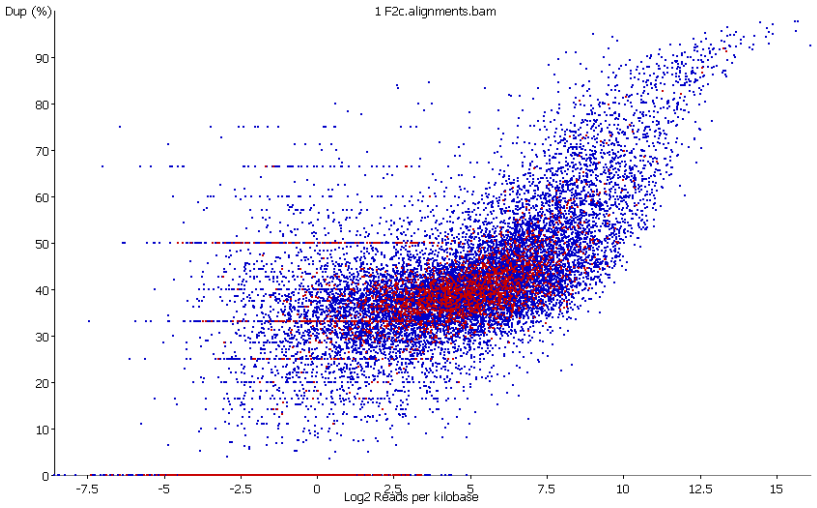

Figure 4.19. Duplication plot: *Sample 1 as representative example is shown. Sequences were plotted for % duplication (y axis) against Log2 reads per KB (x axis).*

Duplication levels within the samples were then also re-checked. This was done by plotting duplication against read density over all exons. This revealed an association with read density and duplication with low-density exons having low duplication. Importantly it showed that everything was not high duplication or non-continuous duplication (**Figure 4.20**).

A distribution plot was then performed to make sure all the log2 counts from the samples were similarly distributed. The idea is that overall distributions of counts should be similar but different individual genes might change. (**Figure 4.20)** shows that the samples are generally similar.

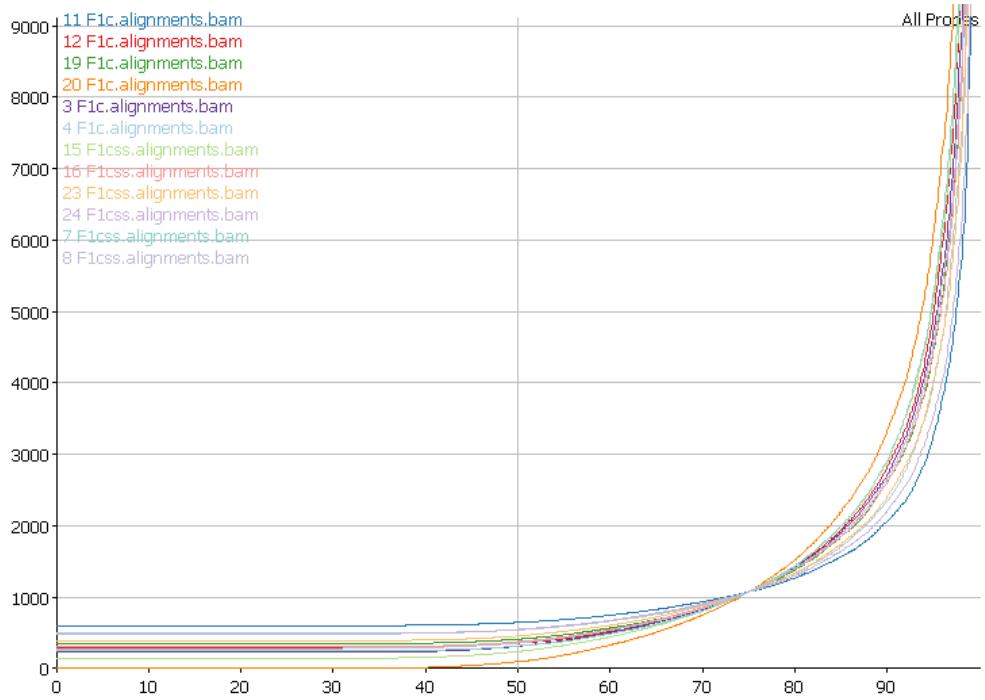

Figure 4.20. Distribution plot of reads across samples: *Numbers of sequences (y axis) were plotted against percentage (x axis) for each sample.*

Finally, a cluster tree of the samples was performed to test that certain samples might not be highly unrelated to the others. This revealed that the samples were generally related (**Figure 4.21**).

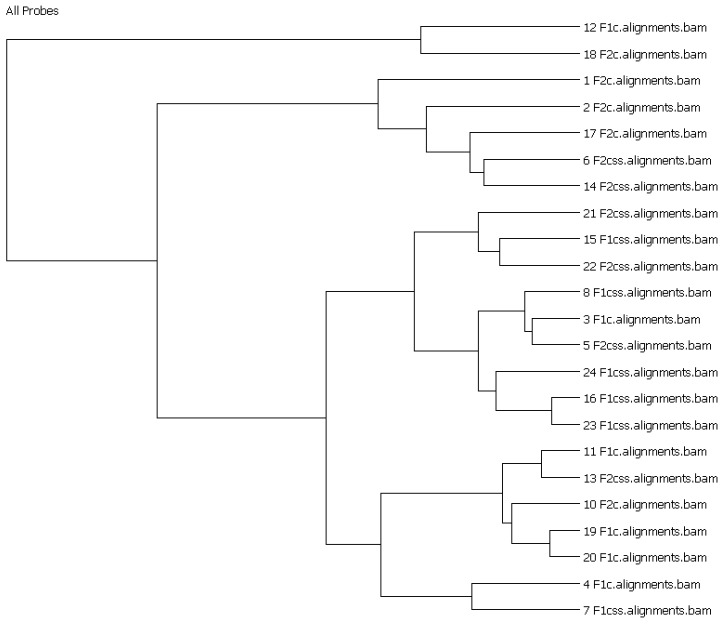

Figure 4.21. Cluster tree analysis of the samples: *A cluster tree was performed of the different samples.*

All the samples were then placed into set groups for F1 control, F1 CSS, F2 control, F2 CSS (**Table 4.2)** to allow comparison for RNA expression changes using DESeq, an R program for expression analysis.

4.5.2 Differences in expression between F1 control and F1 CSS:

DESeq was used to compare F1 control with F1 CSS. Intensity-difference statistics were used, using a p-value cutoff of 0.05 and applying multiple testing correction. All genes significantly different are given in **(Table 4.6)**.

Table 4.6. Gene significantly different between F1 control and F1 CSS: *Probe names and gene names together with significant differences are given.*

Probe	Gene name	Location	Diff – p-value	Function of coded protein
ApoE	apolipoprotein E	Chr1:43236641-43269644	0.001783995	packaging cholesterol & fat through blood stream with fat.
Slc6a5	solute carrier family 6 (neurotransmitter transporter, glycine), member 5	Chr1:81878373-81881182	1.1460418E-8	neurotransmitter
Map3k11	mitogen-activated protein 11	Chr1:106335558-106385226	0.021159545	
Fth1	ferritin heavy polypeptide 1	Chr1:227978441-227991739	5.5415276E-4	Hemochromatosis, CNS
Fam111a	family with sequence similarity 111, member A	Chr1:232979054-232981410	0.0026425656	Gracile bone dysplasia
Scd	stearoyl-coenzyme A desaturase 4,key enzyme	Chr1:271502294-271515353	0.032811735	Fatty acid metabolism
Map1b	microtubule-associated protein 1B	Chr2:48837124-48930483	0.032811735	Neurogenesis,
Car3	carbonic anhydrase 3	Chr2:107900583-107909291	3.4972896E-8	Normal growth

Sertm1	serine rich and transmembrane domain containing 1	Chr2:163721932-163741483	0.0029803626	
Sfrp2	secreted frizzled-related protein 2	Chr2:202136233-202143777	0.0037722779	
S100a10	S100 calcium binding protein A10 (calpactin)	Chr2:210966310-210975004	0.03962063	Cell-cell progression & differentiation
Celsr2	cadherin, EGF LAG seven-pass G-type receptor 2	Chr2:230654782-230677391	0.0057067005	Bind cells, growth factors.
Ak1	adenylate kinase 1	Chr3:16995039-17002961	0.001783995	Energy metabolism homeostasis
Fjx1	four jointed box 1	Chr3:98940094-98941443	0.004252445	Growth & differentiation
Snhg11	Small Nucleolar RNA Host Gene 11	Chr3:160736978-160743135	0.0026595236	Host gene
Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	Chr3:170577777-170578880	0.001783995	Transcription factor, inflammation
Cadps2	Ca2+-dependent activator protein for secretion 2	Chr4:50109970-50640382	0.042915583	
Iqub	IQ motif and ubiquitin domain containing	Chr4:51258446-51365176	0.029059142	Formation & maintenance
Retsat	retinol saturase (all trans retinol 13,14 reductase)	Chr4:165235748-165244511	2.497757E-5	Vitamin –A Metabolism pathways
Tmsb10	thymosin, beta 10	Chr4:165645783-165646842	0.0334597	Infection bacterial disease
Ccdc77	coiled-coil domain containing 77	Chr4:219949770-219972305	0.009776219	TF & gene expression
Pnisr	PNN interacting serine/arginine-rich	Chr5:40646030-40672914	0.0015030955	Splicing factors
Rnf11l	RING finger protein 11	Chr5:133132389-133132853	5.1929953E-4	
Ncdn	Neurochondrin	Chr5:148539983-148549743	0.014347231	Transcription Antigen-process

Ptpru	protein tyrosine phosphatase, receptor type, U	Chr5:153601583-153675513	0.011278439	Early neurodevelopment & cell growth
Plch2	phospholipase C, eta 2	Chr5:175842863-175871527	0.03259342	Phospholipase pathways & phosphyt metabolism
Eml5	echinoderm microtubule associated protein like 5	Chr6:131991613-132118730	0.034290496	CNS expression & glial cells
Calm1	calmodulin 2 neurotransmitters	Chr6:133444274-133454391	0.0046323547	Binding to calciumions growth cell
Serpina3n	calmodulin 3, proteinase inhibitor	Chr6:137283364-137292168	0.0028383373	Cancer, Alzheimer's disease
Hsp90aa1	Heat shock protein 90α	Chr6:144182014-144187540	8.0430077E-4	Interacting with co-chaperone Cancer growth
Nts	Neurotensin	Chr7:44140236-44150124	7.497003E-12	Enodcrine fat luteinizing
Nov	nephroblastoma overexpressed	Chr7:95015159-95022176	0.003180026	Cardiovascular Cancer Skeletal development
Mpped1	metallophosphoesterase domain containing 1	Chr7:124671298-124740991	3.674019E-4	Transport & post protein Aniridia
Mettl7a	Methyl transferase like 7 a	Chr7:139783755-139790601	0.019388966	Innate immune system
Nr4a1	Nuclear receptor 4 group a member 1, steroid thyroid	Chr7:140713176-140721070	6.482955E-4	Cancer fetal disease endocrine disease
Vps13c	Vascular protein sorting 13C	Chr8:78012253-78174561	0.0037190893	Parkinson disease autosomal recessive
Plscr4	Phospholipid scramblase 4	Chr8:99364963-99403085	0.00829274	Binding calcium ions
Trank1	tetratricopeptide repeat and ankyrin repeat containing 1	Chr8:118916156-118993803	0.005603083	Hydrolase activity
Cmc1	Cox assembly mitochondrial protein 1	Chr8:125679760-125737762	0.036573105	Metocondorial enzyme copper chaperon
Lyzl4	Lysozyme like 4	Chr8:129406685-129411111	0.005825823	hydrolasis

Nktr	natural killer tumor recognition sequence	Chr8:129548839-129572815	0.003918177	
Slc5a7	Soluble carrier family 5 choline transporter, member 7	Chr9:4343096-4379724	0.012006678	Neuronal disease fetal eye muscle
Aox1	Aldehyde oxidative 1	Chr9:64726637-64804786	0.031017216	Nephrological metabolism disease
Hba2	Hemoglobin alpha, adult chain 1	Chr10:15484461-15485305	0.046643484	Blood endocrine
Hba-a2	Hemoglobin alpha, adult chain 2	Chr10:15497886-15498744	0.031017216	Blood fetal immune liver
Dnah9	Dynein heavy chain 9	Chr10:52103139-52465432	0.034090836	Fetal ear respiratory cardiovascular
Wsb1	WD repeat & SOCS box-containing 1, atigen	Chr10:65046088-65062151	0.027572677	Innate & adaptive immunity
Luc7l3	LUC7- like 3 (s.cerevisiae)	Chr10:81823960-81859932	0.0051783216	mRNA binding
Spp1	Osteopontin	Chr14:6653093-6658953	6.1179094E-6	Cytokines upregulated bone muscles cancer disease
Rasl11b	RAS-like, family 11, member B	Chr14:36378166-36382381	0.00829274	Transmembrance & transporter activity
Cc2d2a	Coiled-coil and C2 domain-containing protein 2A, calcium binding	Chr14:71922811-71997340	0.012006678	Fetal respiratory endocrine bone liver eye
Wfs1	wolframin ER transmembrane glycoprotein	Chr14:78613738-78630689	0.0	Wolframe, metabolism. Fetal ear Endocrine. bbb. NS
Sncg	synuclein, gamma, nurogenerative	Chr16:9049951-9054488	1.9992008E-11	Alzheimer tumors breast
Fam129c	Family with sequence similarity 129, member C	Chr16:19887776-19899604	4.153117E-4	Immune system
Tenm3	Teneurin-3 , transmemberance glycoprotein	Chr16:46462637-46657495	0.0029803626	Eye disease, NS
Dctd	Deoxycytidylate deaminase	Chr16:46903570-46933233	0.01870843	Nucleotides metabolism, Glass syndrome fetal

Kbtbd11	Kelch repeat & BTB domain containing 11	Chr16:79275079-79276980	0.045282472	Endocrine, bone, lung, liver, muscles, skin
Rgs14	A member of the regulation of G- protein signaling	Chr17:11887270-11901352	0.0014981425	Long memory & learning in Hippocampus
Rreb		Chr17:29453342-29579484	0.025306202	Zinc thyroid
Rock1	Coiled-coil- containing protein Kinase 1	Chr18:1292383-1432455	0.027572677	Angiogenesis cancer
Grp	gastrin releasing peptide	Chr18:60759080-60772845	0.011574095	CNS binding receptors gast trac lung disease
Mbp	myelin basic protein	Chr18:78473296-78504226	0.032811735	Bone marrow immune neuronal disease
RT1-M6-2	RT1 class, Locus M6, gene 2, peptide antigen signaling receptor binding	Chr20:4113595-4116511	4.681462E-10	Immune & process endogenous
RT1-N2	ras responsive element binding protein 1	Chr20:5245094-5248485	0.027572677	germlines
Col11a2	Collagen type X1 alpha 2 chain	Chr20:5909495-5938415	0.017659966	Found between cells in space surrounding to make muscles
Col6a1	Collagen type V1 alpha 1 chain	Chr20:14817563-14835866	1.3494605E-10	strength & growth
Tmsb4x	Thymosin beta-4	ChrX:28985069-28987071	0.006946334	Polymerization cell proliferation homeostasis bone endocrine cancer immune
Klhl34	Kelch Like Family Member 34	ChrX:40264786-40266723	0.034739614	
Ccnt	Cylcin T1	ChrX:114723350-114993826	2.5451824E-5	Endocrine Immune regulation TF &chromatin acetylation regulation tumor growth

To visualize the significantly expressed genes in table data a scatter plot was made plotting F1 control expression against F1 CSS expression. Genes that were significantly differentially expressed are highlighted (**Figure 4.22**).

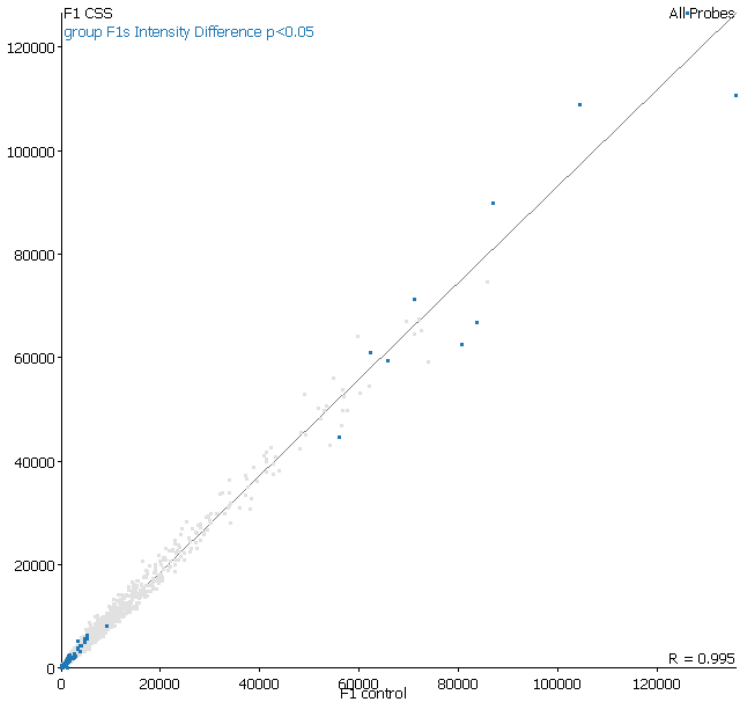

Figure 4.22. Scatter plot of expression count differences between F1 control and F1 CSS. *Genes significantly differently expressed between F1 CSS (y axis) and F1 control (x axis) are highlighted.*

To visualize the significantly expressed genes in table a heat map was produced that allowed to see if the CSS and control groups clearly differ across all genes (**Figure 4.23**).

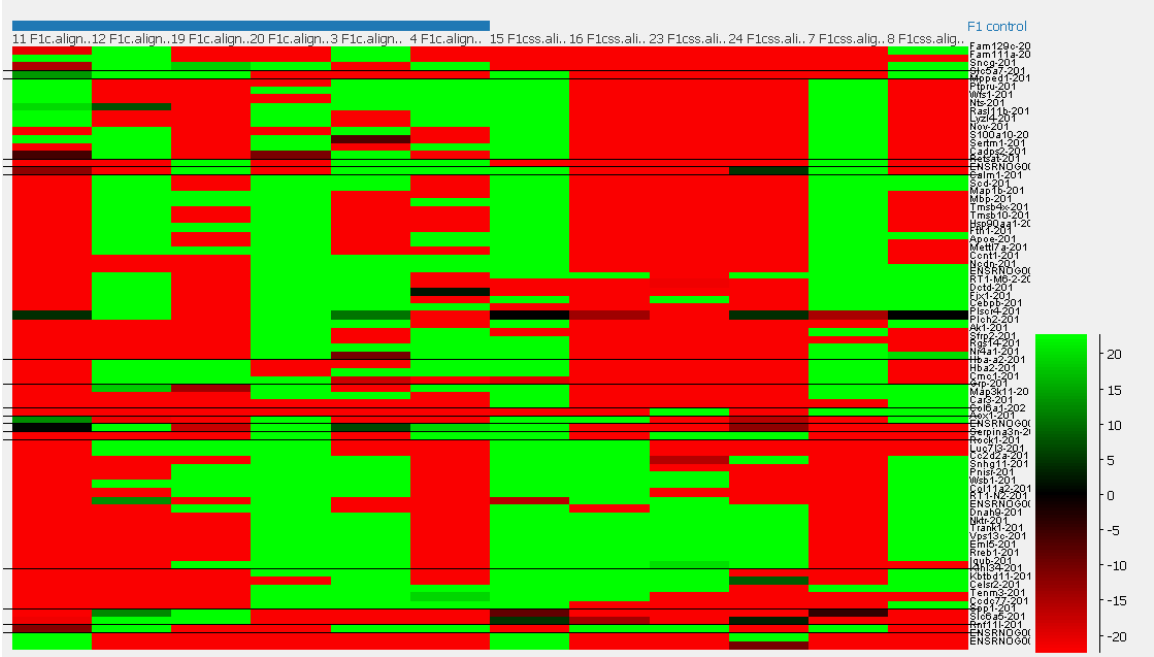

Figure 4.23. Heat plot of differentially regulated genes between F1 control and F1 CSS. *A heat map shows expression, low (red) too high (green), of all significantly expressed genes in the control (highlighted by blue line) and the CSS.*

4.5.3 Differences in expression between F2 control and F2 CSS:

DESeq was used to compare F2 control with F2 CSS based. Intensity-based statistics were used, using a p-value cutoff of 0.05 and applying multiple testing correction. This revealed a number of differentially regulated genes (**Table 4.7**) (**Figure 4.24**).

Table 4.7. Gene significantly different between F2 control and F2 CSS: *Probe names and gene names together with significant differences are given.*

Probe	Gene name	Location	Function of coded protein	Diff – p-value
Apoe	apolipoprotein E	Chr1:81878373-81881182	Packaging cholesterol and other fats for transport	0.0
Hbb	Beta-globin	Chr1:175134674-175136088	Component of hemoglobin in red blood cells	0.0033
Smg1	SMG1 homolog, phosphatidylinositol 3-kinase-related kinase (C. elegans)	Chr1:194739745-194836241	Involved in Nonsense Mediated MRNA Decay	0.0088
Fth1	ferritin heavy polypeptide 1	Chr1:232979054-232981410	Component of ferritin, the intracellular iron storage protein	1.416E-10

Mpeg1	macrophage expressed gene 1	Chr1:235887051-235889195	membrane protein of macrophages, thought to be involved in the killing of intracellular bacteria	0.0088
Fam111a	family with sequence similarity 111, member A	Chr1:236163691-236179257	Promotes S-phase entry and DNA synthesis	0.0039
S100a9	S100 calcium binding protein A9 (calgranulin B)	Chr2:209531412-209533744	Plays a prominent role in the regulation of inflammatory processes and immune response.	0.0157
Nktr	natural killer tumor recognition protein	Chr8:129548839-129572815	A tumor-recognition complex involved in the function of NK cells	0.0256
Slc5a7	Solute Carrier Family 5 Member 7	Chr9:4343096-4379724	Transmembrane transporter that imports choline from the extracellular space into the neuron	2.3322E-8
Hba-a2	Hba-a2 hemoglobin alpha, adult chain 2	Chr10:15497886-15498744	Component of haemoglobin	2.5722E-4
Sncg	Synuclein gamma	Chr16:9049951-9054488	Plays a role in neurofilament network integrity.	0.0
Fam129c	family with sequence similarity 129, member C Niban-like protein 2	Chr16:19887776-19899604	Expressed in early stages of B-cell differentiation,	4.5840E-7
RT1-M6-2	RT1 class I, locus M6, gene 2	Chr20:4113595-4116511	Predicted antigen binding and immune processes	2.572E-4
Pcsk1n	Proprotein convertase subtilisin/kexin type 1 inhibitor	ChrX:16104506-16107925	May function in the control of the neuroendocrine secretory pathways.	0.0346

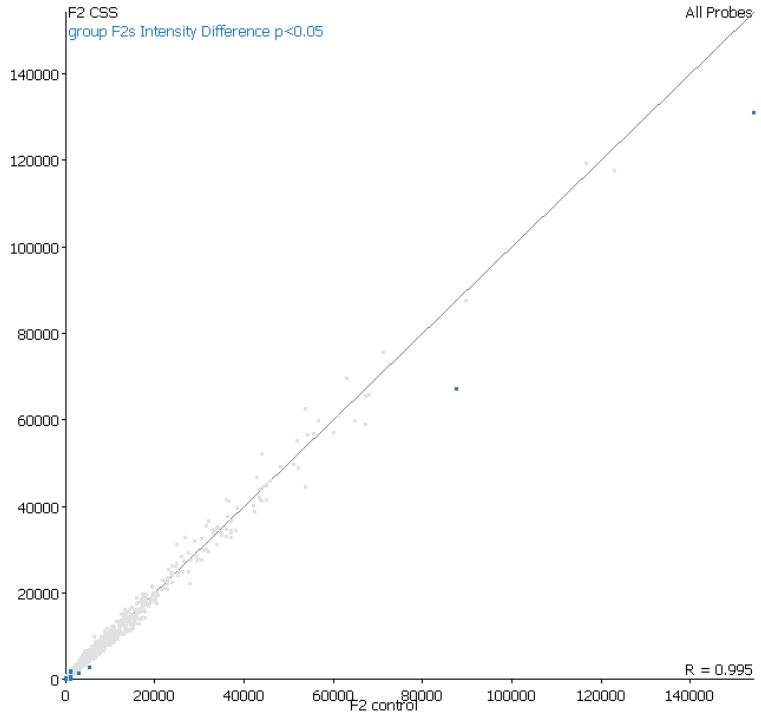

Figure 4.24. Scatter plot of expression count differences between F2 control and F2 CSS. *Genes significantly differently expressed between F1 CSS (y axis) and F1 control (x axis) are highlighted.*

4.5.4 Gene ontology analysis

To test whether certain classes of proteins, coded by the genes differentially expressed between CSS and Control software was used. The PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System is able to classify proteins (and their genes) according to family of evolutionarily related proteins or subfamilies of related proteins that also have the similar function. Gene coding proteins can aso be related by molecular function, their biological processes or if they are involved in a same pathway suggesting relationships between the interacting molecules (Mi et al., 2016).

Inputting the genes identified as differentially expressed between the F1 control and F1 CSS groups it appears that most of the genes code for proteins involved in biological regulation (**Figure 4.25A**), are receptors and calcium binding proteins (**Figure 4.25B**) and have a molecular function as binding (**Figure 4.25C**).

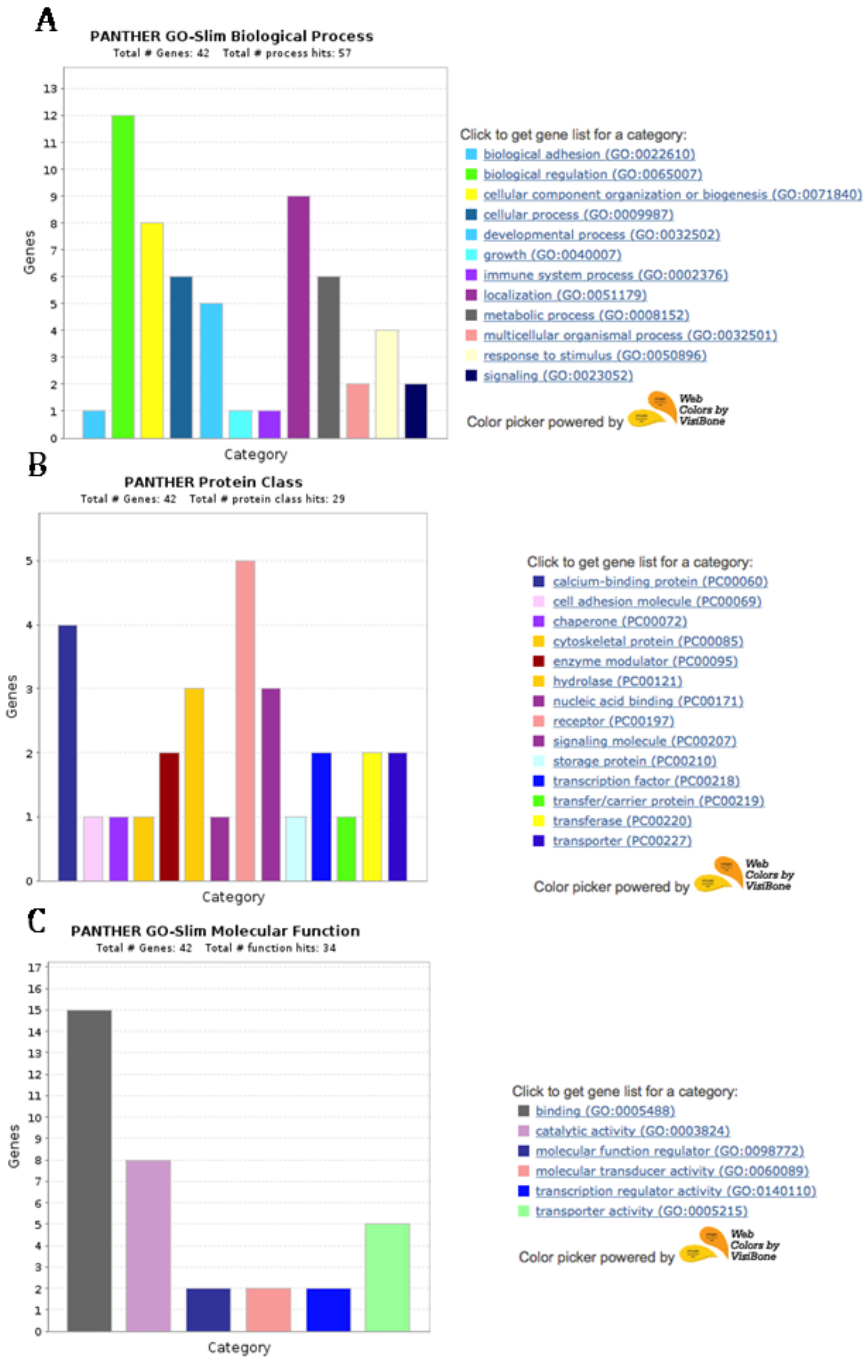

Figure 4.25. Gene Ontology analysis of the genes differentially expressed between F1 CSS and Control. *Genes significantly differently grouped according to Biological Processes (A) Protein class (B) and molecular function (C).*

When testing the genes identified as differentially expressed between the F2 control and F2 CSS groups it appears that most of the genes code for proteins involved in metabolic

processes (**Figure 4.26A**). There is no clear protein class (**Figure 4.26B**) but again have a molecular function as binding (**Figure 4.26C**).

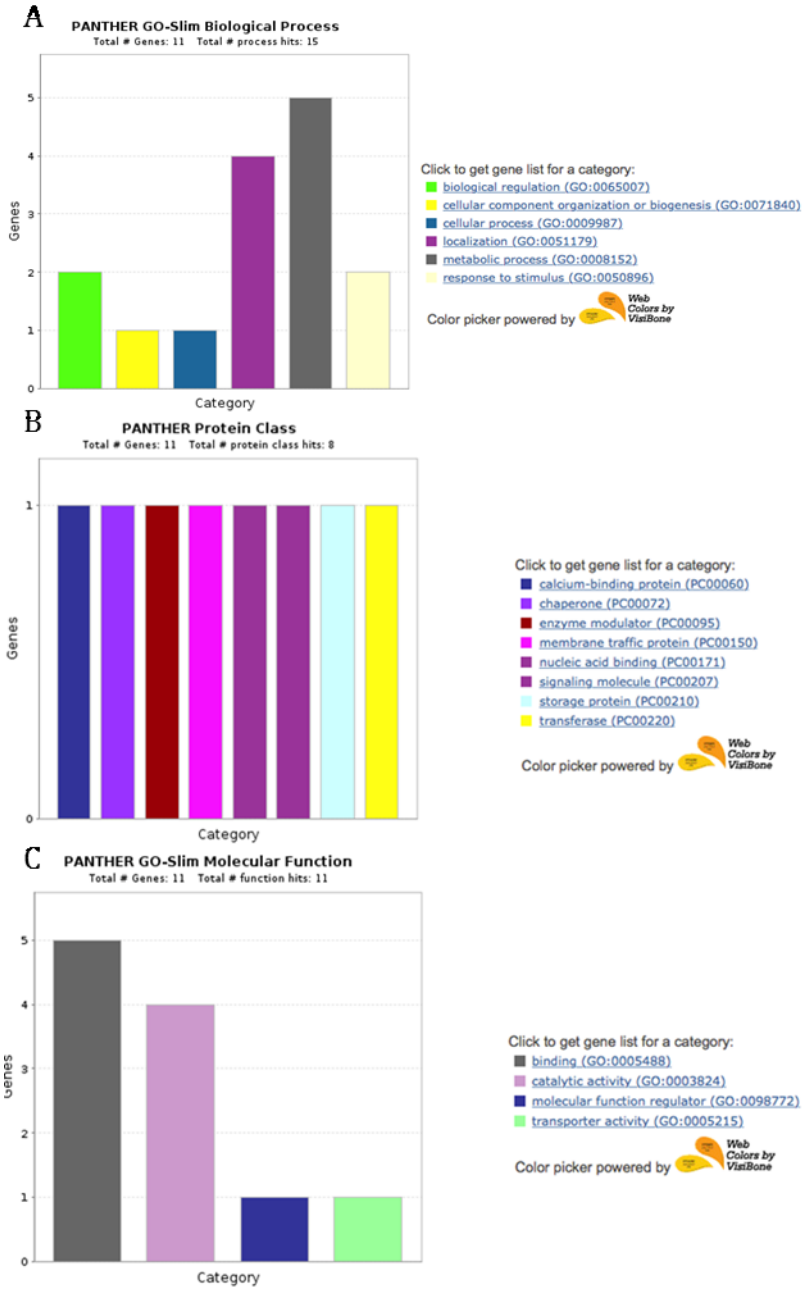

Figure 4.26. Gene Ontology analysis of the genes differentially expressed between F2 CSS and Control. *Genes significantly differently grouped according to Biological Processes (A) Protein class (B) and molecular function (C).*

Gene ontology analysis was performed using another software called GProfiler. (Reimand et al., 2016) Inputting the genes identified as differentially expressed between the F1 control and F1 CSS groups it appears that most of the genes code for proteins involved in

biological regulation (**Figure 4.27A**), are receptors and calcium binding proteins (**Figure 4.27B**) and have a molecular function as binding (**Figure 4.27C**).

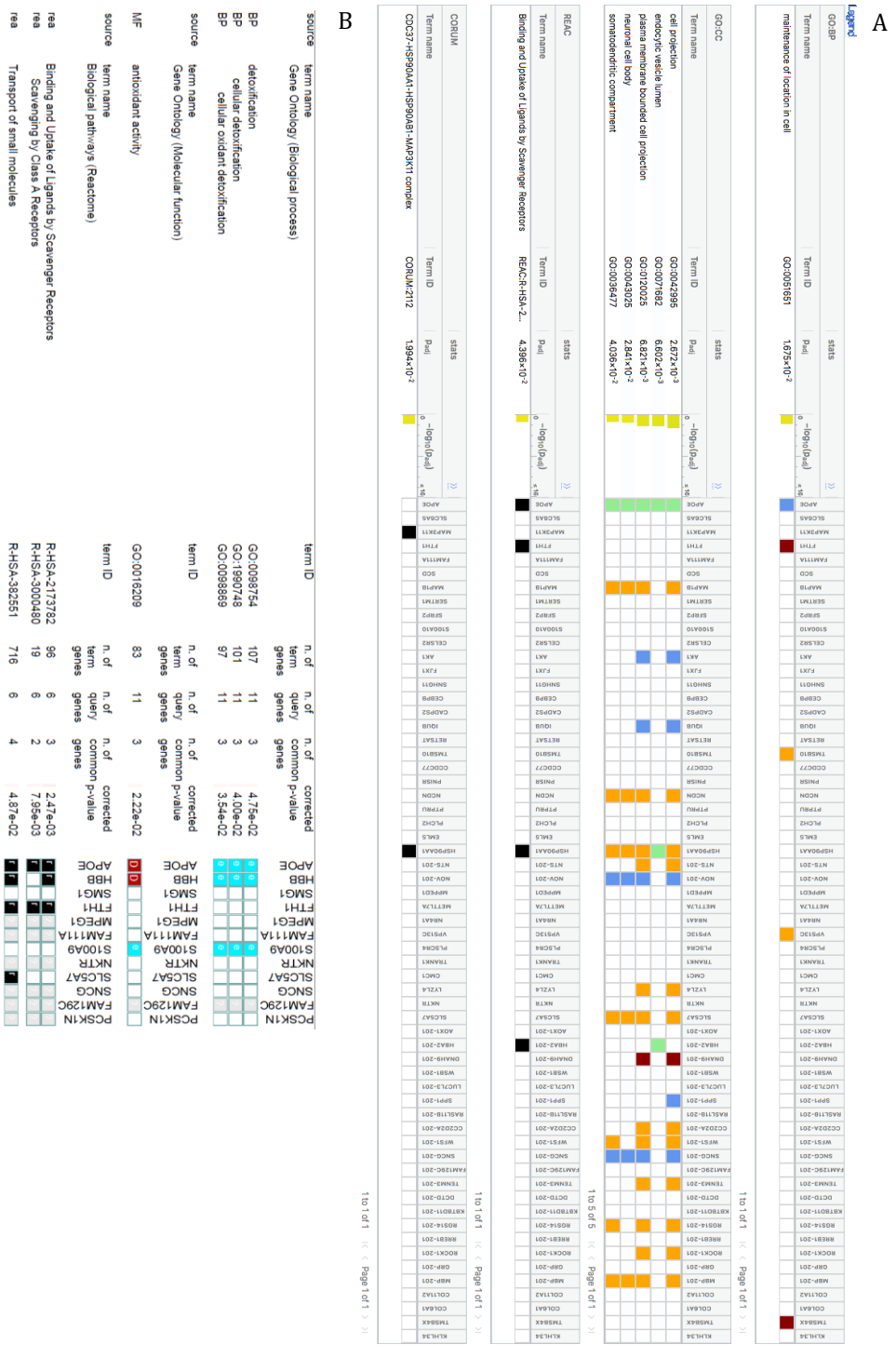

Figure 4.27. Gene Ontology analysis using GProfiler. *Was used to investigate genes differentially expressed between F1 CSS and Control (A) and F2 CSS and Control (B).*

4.6 Discussion

The results of *Study 1* suggest that between F1 and F2 generations there is a shift from HPA dysregulation to more immune changes. To investigate genomewide changes in gene expression in the F1 and F2 CS dams hippocampi were tested using RNASeq. Groups of 6 each of F1 CSS and controls and F2 CSS and controls were tested. A further 6 of each group were also prepared for RNAseq, to increase numbers to 12, however the library preps were not efficient and a repeat of the method again led to low yield. Reasons included a malfunctioning PCR machine and using ice at the wrong step. However, the results for the first 24 samples revealed high quality sequencing across the samples that allowed comparisons of differential gene regulation across the samples and groups.

Gene expression differences between the F1 CSS and control animals were compared using intensity differences and treating the groups as replicates using a p-value cut-off of 0.05 and applying multiple testing correction. This revealed 75 genes differentially regulated between CSS and control (**Table 4.6**). Investigating the genes using gene ontology analyses revealed that most of the genes were receptors and calcium binding proteins that had molecular functions as binding. Calcium binding proteins are particularly important in neuronal functioning and depression (Rajkowska et al., 2007). This family of proteins found in a variety of tissues particularly the brain and neurons that use calcium-mediated signaling functions and calcium influx as part of depolarisation. The most well described calcium binding proteins are parvalbumin, calbindin-D28K, calretinin, calmodulin, calcineurin, and the S100 family (Eyles et al., 2002) and these have well-described roles in stress and depression, for example (Uher and Bob, 2012). Genes differentially regulated include S100 calcium binding protein A10 (calpactin, S100A), Ca2+-dependent activator protein for secretion 2 (Cadps2), calmodulin 1, calmodulin 2, S100 calcium binding protein A9 (Nktr, calgranulin B). S100, for example has been found to be reduced in the cerebral spinal fluid in linked to increasing symptoms of depression (Seo et al., 2017). Calmodulin 1 and 2 have been linked to neurodevelopmental disorders e.g. (Fillman et al., 2014) , and calgranulin to depression and stress (Fillman et al., 2014).

There are also a high number of hydolases including Dnah9, Mpped1, Ptpru, Dctd, Spp1 and Plch2. Hydrolases are one of the largest enzyme classes and play crucial roles in many developmental processes including. One of the other important groups are the receptors including Col6a1, Nr4a1 nuclear receptor, Ptpru protein phosphatase receptor and the G-protein coupled receptors Celsr2 and Sfrp2. G Protein-Coupled Receptors have well-described roles in psychiatric disorders (Catapano and Manji, 2007).

Regarding pathways, the main pathway in which most genes fitted was the Cholecystokinin (CCKR) signaling map, including Nr4a1, Rock1, Map3k11 and Calm1. Though important in control of the intestine, CCK has been strongly linked to depression and suicide with one study finding 22 times higher levels of this hormones in suicide attempters (Jahangard et al., 2018). There are also genes linked to Inflammation mediated by chemokine and cytokine signaling pathways including Col6a1, Rgs14 and Rock1, supporting the role of inflammation in the CSS model. Finally, it is interesting when comparing the previously mentioned study of Stankiewicz et al. on hippocampus transcriptomes of mice exposed to chronic social stress, that we also found altered regulation of proteins involved in the vascular system (i.e. Hba1 and Hba-a2), injury response (e.g. Col6a1) and inflammation (e.g. S100 proteins Rgs14 and Rock1) supporting their conclusions that stress may impact the vascular system (Stankiewicz et al., 2015).

Of other interest are the roles of Calmodulin and Neurotensin in the control of prolactin secretion. Considering that prolactin was reduced in the F1 dams it is important that Calmodulin is altered in expression. Calmodulin is highly important in the regulation of the *Prolactin* gene expression (White and Bancroft, 1987; Merritt et al., 1983) as is Neurotensin (Enjalbert et al., 1982) suggesting perhaps there are gene pathways disrupted in the regulation of this hormone.

Comparing F2 CSS and control dams only 15 genes were found to be differently regulated. This included genes involved in metabolic processes, such as *Smg1*, *Slc5a7*, *Pcsk1n*, *Hbb*, *Apoe* and *S100a9*. Such metabolic processes are highly important in brain function and development. Importantly, there are also genes involved in inflammation regulation

including *Fam111a*, *S100a9* and calcium binding (*Nktras* and *S100a9*) as in the F2 animals suggesting some similar pathways.

Finally, when comparing genes differently expressed between F1 and F2 CSS and control dams we find a startling number of genes similarly regulated in both. In fact 9 of the 15 genes differently regulated in the F2 animals were also differently regulated between the F1 CSS and control dams. This included *ApoE*, *Hba-a2*, *Fam111a*, *Fth1*, *Nkt1*, *SLc5a7*, *Sncg*, *Fam129c* and *RT1-M6-2*. A related S100 protein, *S100a10*, was also differently regulated. This suggests some level of inheritance of the differently expressed genes in the F1 animals. Importantly, many of these genes have also been linked to stress and depression. *ApoE*, an important marker for Alzheimer's disease, has been shown in a meta-analysis of major depression to be a susceptibility gene (López-León et al., 2008). The *Hba-a2* gene has been found to be differently expressed in prefrontal cortex of rats showing increased levels of depression (Yamamoto et al., 2015). Polymorphisms in the *SLC5A7* gene have been associated with infant stress responsivity (Jones et al., 2018). Gamma-Synuclein (Sncg) expression in the hippocampus has been linked to depressive-like behaviour in a rat model of depression (Jeannotte et al., 2009).

Finally, alterations in *S100A10* (also known as p11) are linked to major depression and in the therapeutic actions of antidepressants (Svenningsson et al., 2013; Svenningsson et al., 2006) with a decrease in mRNA and protein in the brains of humans with depression, suicide victims, and a mouse model of depression (Dwivedi et al., 2005; Svenningsson et al., 2006; Anisman et al., 2008; Alexander et al., 2010). (Leckman et al., 2004; Dwivedi, 2009) suggesting a crucial role for this gene in the development of depression.

The major limitations in this study are that the numbers of animals in each group could be increased. It was planned to perform 48 RNAseq experiments, but the final 24 library preparations could not be performed as firstly a PCR machine failed to run properly and then there was a mistake with putting samples on ice at the wrong step. Due to the costs of the library prep kits and associated chemicals we could not re-perform these. However, the high quality of the first 24 samples has allowed analysis.

Overall, the results show that in F1 CSS and control animals there are a relatively larger number of genes differentially regulated than between the F2 CSS and control dams. In the F1s there are genes involved in calcium binding and protein-coupled receptors whereas in the F2s there are genes involved in inflammation. The most striking finding is that two-thirds of the genes (if you include *S100a10*) differently regulated in the F2 were also changes in the F1s. Many of these genes have been previously linked to the stress and the development of depression. This seems to suggest a level of inheritance of expression of key genes involved in the regulation of depression from F1 to F2 dams supporting a possible mechanism for the intergenerational inheritance of the maternal anxiety and depressed maternal care.

5 Study 4: Investigation of epigenetic changes in stress-related genes in mothers and infants exposed to stress and maternal depression.

5.1 Introduction

Results from the Study 1 of the transgenerational CSS rat study highlighted epigenetic regulation of *Nr3c1* and *BDNF* with maternal depression. Specifically, *Nr3c1* and *BDNF* methylation and expression were differentially changed in F1 exposed to postnatal depression in comparison to the F2 animals exposed to prenatal and postnatal expression. Results from the literature review in Chapter 1 shows that these two genes are widely regulated has identified key stress-related genes that are epigenetically regulated by stress and associated with depression.

Studies on *Nr3c1* DNA methylation at promoter 1-7 have shown that stress in prenatal and postnatal stages both induce increased methylation in the offspring. Interestingly, (**Table 2.1)** (*prenatal stress) reveals do differences between prenatal and postnatal in this methylation change. However, a study by Murgatroyd et al. were the first to show an interaction between prenatal and postnatal maternal depression on infant methylation. Specifically, they showed that prenatal and postnatal maternal depression individually increased *Nr3c1* methylation. Importantly though, exposure to prenatal depression protected the infant from the effects of postnatal depression in terms of increased methylation and anxiety and depressive symptoms (Murgatroyd et al., 2015a).

Studies on *FKBP5* DNA methylation, as previously described, again highlight similar epigenetic changes, namely increases in DNA methylation at intron 7 in human studies, of stress exposure and depression (**Table 1.4**). However, there are few studies on prenatal stress and few studies contrast pre and postnatal stages. Similarly, in the *BDNF* studies there is only one report on prenatal stress and child methylation (**Table 1.5)**. However, no studies have yet tested methylation in mothers suffering maternal depression in either *NR3C1*, *FKBP5* or *BDNF*.

One consideration is the difference between studying epigenetic changes in blood and brain. The rodent studies, including in Chapter 3 *Study 1*, generally investigate brain material. However, the majority of the human studies have focused on blood. If one considers that epigenetic changes alter gene expression and the depression should involve gene expression changes in the brain, the question is how relevant is blood to study. Considering that many of the findings in (**Tables 1.3, 1.4, 1.5)** detailing the result from studies of DNA methylation and stress report their measures in blood and that many of the findings support each other, the importance of using blood DNA for the investigation of stress-related epigenetic changes is important.

Using two individual human studies this study will be the first to test whether DNA methylation in mothers at *NR3C1*, *FKBP5* and *BDNF* associate with their level of either prenatal or postnatal depression. Testing in two different studies will allow us to test the robustness of any associations. We then test the role of perceived stress at prenatal and postnatal period on the methylation of the above genes in the mothers using discrimination and pregnancy anxiety.

Finally, we test to what extent either prenatal or postnatal maternal depression impacts DNA methylation at *NR3C1*, *FKBP5* and *BDNF* in their offspring.

5.2 Aims:

This study will investigate epigenetic changes at candidate genes in a human study mothers with maternal depression and stress and infants exposed to maternal prenatal and postnatal depression. Specifically we hypothesis that epigenetic changes in candidate stress-related genes, highlighted from the rat studies, are similarly regulated in human studies.

5.3. Objectives:

1. Investigate epigenetic changes in stress-related genes in mothers exposed to stress.
2. Investigate epigenetic changes in stress-related genes in mothers suffering from prenatal and postnatal maternal depression
3. Test whether infants exposed to maternal depression or maternal stress show epigenetic changes in stress-related genes

5.4 RESULTS

5.4.1 Epigenetic regulation of stress-related genes with stress and in depression during pregnancy in a Latio cohort

Latino mothers living in the US were asked to complete a questionnaire for depression (IDAS-GD) and perceived discrimination (EDS). Blood DNA was measured for methylation at *NR3C1, FKBP5* and *BDNF*.

5.4.1.1 *NR3C1* methylation in the Latino mothers study

PCRs were performed on bisulphite-treated DNA from the Latino mothers study. This was pyrosequenced and 4 CpG sites were investigated (**Figure 5.1**), identical to those CpGs studied in previous studies (**Table 1.3**, **Figure 1.7**).

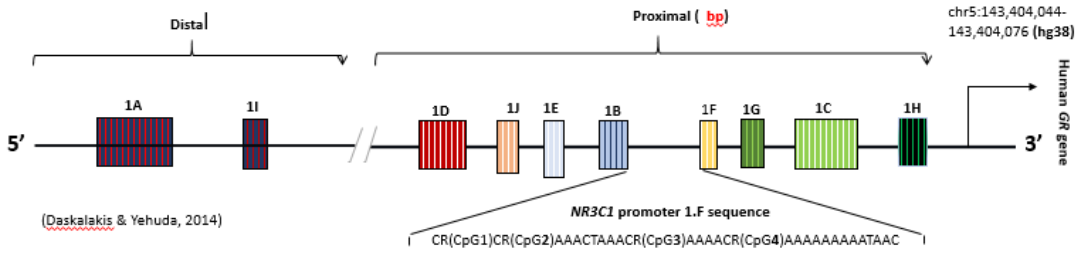

Figure 5.1. A schematic representation of the human the NR3C1 exon 1-F promoter region showing sequence analysed by bisulphite sequencing and the numbered CpGs.

An example of a pyrosequencing run demonstrating methylation levels across the 4 CpG sites in the *NR3C1* promoter is shown in (**Figure 5.2)**.

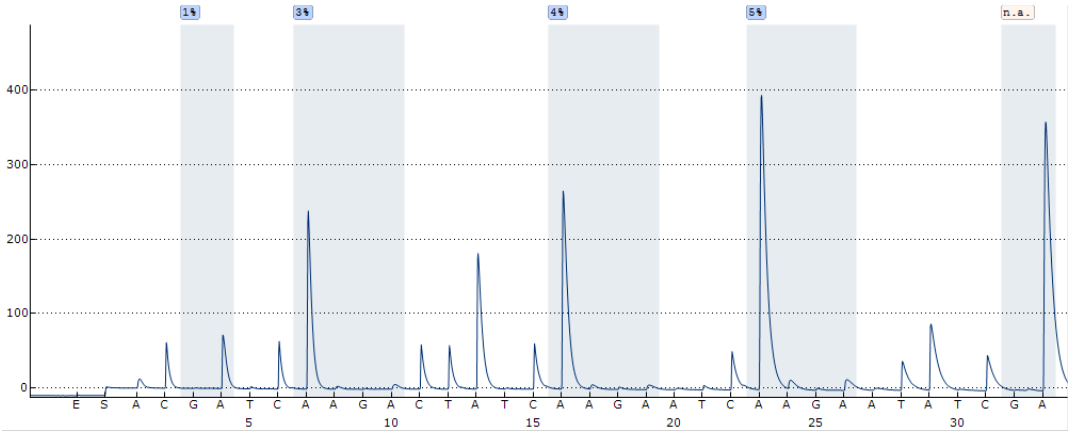

Figure 5.2. Bisulphite pyrosequencing of NR3C1

Levels of methylation across the mothers at the 4 CpG sites in the *NR3C1* promoter in the Latino mothers cohort were: CpG1, 1.36%; CpG2, 3.608%; CpG3, 4.615%; CpG4, 5.32% and average methylation across the CpGs was 2.48% (**Figure 5.3**).

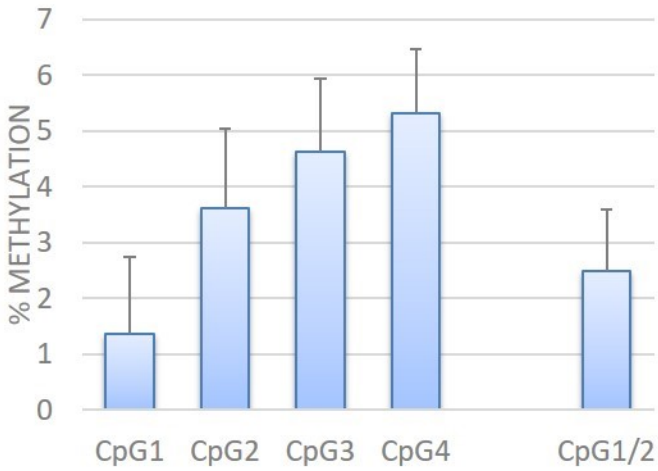

Figure 5.3. NR3C1 methylation in the Latino mothers study. *Percentage average + Standard Deviation of DNA methylation at each of the 4 CPGs are shown together with the average methylation of all CpGs within an individual.*

5.4.1.2 *NR3C1* methylation and discrimination

A correlation was performed between DNA methylation across the CpGs for average methylation and EDS (Everyday discrimination scale, see 2.2.3) during pregnancy (eds1) and postnatally (eds2) and across pre- and postnatal periods (eds total) to determine if there is an association between methylation and stress in the form of discrimination. This revealed a negative correlation between *NR3C1* methylation and total EDS score, i.e. the

higher the amount of average methylation at *NR3C1* the higher the level of perceived discrimination (**Table 5.1**).

Table 5.1. Correlation of average NR3C1 methylation and EDS in the Latino mothers study.

		GR_AVG	eds_1	eds_2	eds_total
GR_AVG	Pearson Correlation	1	-0.111	-0.116	-.163*
	Sig. (2-tailed)		0.176	0.157	0.046
	N	150	150	150	150

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

A correlation was then done between DNA methylation at the individual CpGs and EDS determine if there is an association between methylation and stress at particular CpGs. This revealed there was a negative correlation between CpG1 methylation and prenatal, postnatal and total EDS scores, with higher levels of methylation and higher levels of stress

Table 5.2. Correlation of NR3C1 methylation at individual CpGs and EDS in the Latino mothers study.

		GR_cpg1	GR_cpg2	GR_cpg3	GR_cpg4	eds_1	eds_2	eds_total
GR_cpg1	Pearson Correlation	1	.272**	.285**	.306**	-.171*	-.167*	-.176*
	Sig. (2-tailed)		0.001	0.000	0.000	0.037	0.041	0.032
	N	150	150	150	150	150	150	150
GR_cpg2	Pearson Correlation	.272**	1	.616**	.398**	-0.074	-0.086	-0.132
	Sig. (2-tailed)	0.001		0.000	0.000	0.370	0.295	0.107
	N	150	150	150	150	150	150	150
GR_cpg3	Pearson Correlation	.285**	.616**	1	.445**	-0.033	-0.039	-0.126
	Sig. (2-tailed)	0.000	0.000		0.000	0.689	0.634	0.126
	N	150	150	150	150	150	150	150
GR_cpg4	Pearson Correlation	.306**	.398**	.445**	1	-0.048	-0.044	-0.038

	Sig. (2-tailed)	0.000	0.000	0.000		0.560	0.593	0.646
	N	150	150	150	150	150	150	150

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

A scatter plot was produced to show the significant negative correlation between *NR3C1* CpG1 methylation and EDS (**Figure 5.4**).

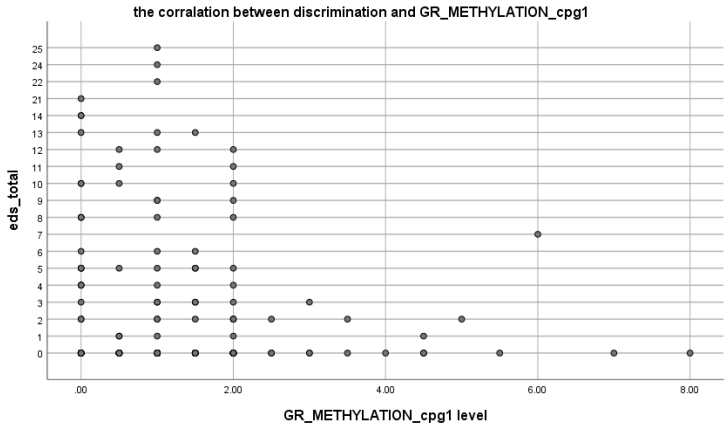

Figure 5.4. A scatter plot of percentage DNA methylation at CpG1 and EDS

5.4.1.3 *NR3C1* methylation and depression

A correlation was performed between DNA methylation and IDAS (Inventory of depression and anxiety scale, see 2.3.4) to determine if there is an association between methylation and prenatal (IDAS1) and postnatal (IDAS) or total depression. This revealed no significant a negative correlation between average methylation at *NR3C1* methylation and IDAS score (**Table 5.3**) or methylation at individual CpGs (**Table 5.4**).

Table 5.3. Correlation of NR3C1 methylation and prenatal (idas1) and postnatal (idas2) or total (idas total) maternal **depression in the Latino mothers study**

		GR_AVG	idas1	idas2	idas_total
GR_AVG	Pearson Correlation	1	-0.055	0.010	-0.057
	Sig. (2-tailed)		0.507	0.902	0.486

N	150	150	150	150	

Table 5.4. Correlation of NR3C1 methylation at specific CpGs and prenatal (idas1) and postnatal (idas2) or total (idas total) maternal depression in the Latino mothers study

		GR_cpg1	GR_cpg2	GR_cpg3	GR_cpg4	idas1	idas2	idas_total
GR_cpg1	Pearson Correlation	1	.272**	.285**	.306**	-0.051	0.056	-0.034
	Sig. (2-tailed)		0.001	0.000	0.000	0.538	0.495	0.682
	N	150	150	150	150	150	150	150
GR_cpg2	Pearson Correlation	.272**	1	.616**	.398**	-0.032	0.022	-0.048
	Sig. (2-tailed)	0.001		0.000	0.000	0.698	0.788	0.558
	N	150	150	150	150	150	150	150
GR_cpg3	Pearson Correlation	.285**	.616**	1	.445**	-0.062	-0.080	-0.033
	Sig. (2-tailed)	0.000	0.000		0.000	0.449	0.329	0.690
	N	150	150	150	150	150	150	150
GR_cpg4	Pearson Correlation	.306**	.398**	.445**	1	-0.006	0.038	-0.047
	Sig. (2-tailed)	0.000	0.000	0.000		0.938	0.642	0.566
	N	150	150	150	150	150	150	150

**. Correlation is significant at the 0.01 level (2-tailed).

5.4.1.4 *FKBP5* methylation in the Latino mothers study

DNA methylation was measured at two sites within a regulatory region in intron 7 of the *FKBP5* gene. These sites were found by Klengel and colleagues (Klengel et al., 2013) to exhibit lower levels of methylation in adults with a history of childhood adversity and have been investigated in several previous studies (See **Table 1.5, Figure 1.8)** (**Figure 5.5**).

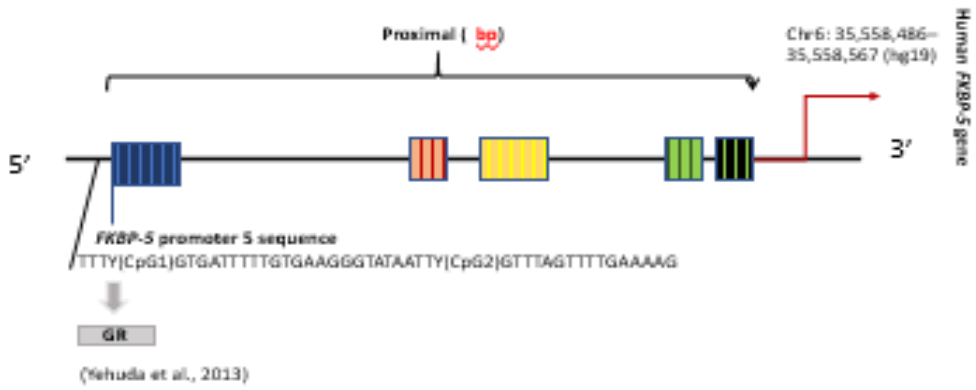

Figure 5.5. A schematic representation of the human FKBP5 intron 7 region showing sequence analysed by bisulphite sequencing and the numbered CpGs.

An example of a pyrosequencing run demonstrating methylation levels across the 2 CpG sites in the *FKBP5* promoter is shown in (**Figure 5.6)**.

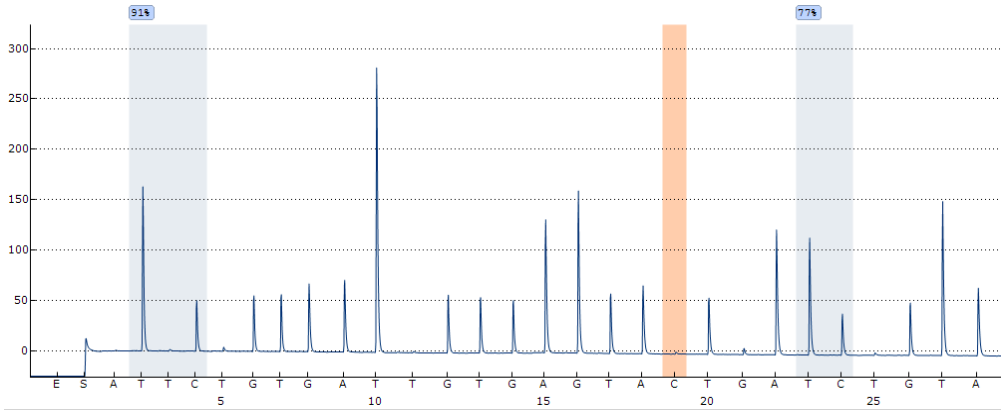

Figure 5.6. Bisulphite pyrosequencing of FKBP5

Levels of methylation across the mothers at the 2 CpG sites in the *FKBP5* intron 7 in the Latino mothers cohort were: CpG1, 98.98%; CpG2, 92.18%. Average methylation across the CpGs was 95.58% (**Figure 5.7**).

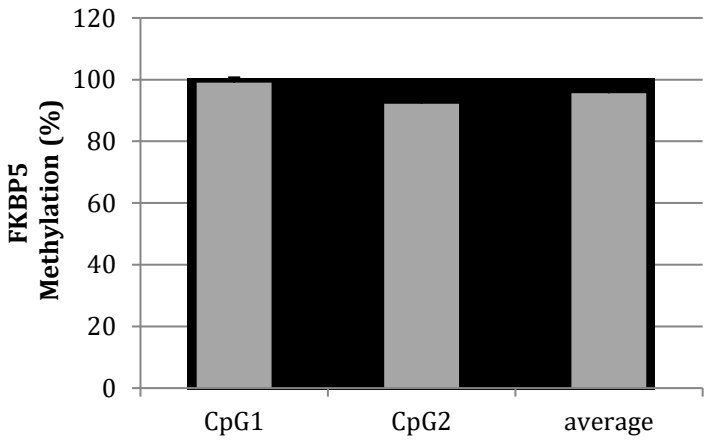

Figure 5.7. FKBP5 methylation in the Latino mothers study. *Percentage average + Standard Deviation of DNA methylation at each of the 4 CPGs are shown together with the average methylation of all CpGs within an individual.*

5.4.1.5 FKBP5 methylation and discrimination

A correlation was performed between average DNA methylation across the CpGs and EDS prenatal (eds1) and postnatally (eds2) to determine if there is an association between methylation and discrimination. This revealed no significant correlations (**Table 5.5**).

Table 5.5. Correlation of average FKBP5 methylation and EDS in the Latino mothers study

		*FKBP5*_AVG	eds_1	eds_2	eds_total
*FKBP5*_AVG	Pearson Correlation	1	-0.056	-0.105	-0.033
	Sig. (2-tailed)		0.493	0.203	0.691
	N	150	150	150	150

A correlation was then performed between DNA methylation at the individual CpGs and EDS. This also revealed no correlation (**Table 5.6**).

Table 5.6. Correlation of FKBP5 methylation at individual CpGs and EDS in the Latino mothers study

		FKBP5_ cpg1	*FKBP5_* cpg2	eds_1	eds_2	eds_total
FKBP5 _cpg1	Pearson Correlation	1	.282**	-0.092	-0.070	-0.040
	Sig. (2-tailed)		0.000	0.263	0.391	0.626
	N	150	150	150	150	150

*FKBP5_* cpg2	Pearson Correlation	.282**	1	-0.020	-0.094	-0.019
	Sig. (2-tailed)	0.000		0.804	0.252	0.815
	N	150	150	150	150	150

5.4.1.6 FKBP5 methylation and depression

A correlation was performed between DNA methylation and IDAS (Inventory of depression and anxiety scale, see 2.3.4) to determine if there is an association between methylation and prenatal (IDAS1) and postnatal (IDAS) or total depression. This revealed no significant a negative correlation between average methylation at *FKBP5* methylation and IDAS score (**Table 5.7**) or methylation at individual CpGs (**Table 5.8**).

Table 5.7. Correlation of FKBP5 methylation and prenatal (idas1) and postnatal (idas2) or total (idas total) maternal depression in the Latino mothers study

		*FKBP5*_AVG	idas1	idas2	idas_total
*FKBP5*_AVG	Pearson Correlation	1	-0.114	-0.005	0.043
	Sig. (2-tailed)		0.164	0.950	0.599
	N	150	150	150	150

Table 5.8. Correlation of FKBP5 methylation and prenatal (idas1) and postnatal (idas2) or total (idas total) maternal depression in the Latino mothers study

		*FKBP5_* cpg1	*FKBP5* _cpg2	idas1	idas2	idas_total
FKBP5 _cpg1	Pearson Correlation	1	.282**	-0.077	-0.115	0.044
	Sig. (2-tailed)		0.000	0.350	0.160	0.593
	N	150	150	150	150	150
FKBP5 _cpg2	Pearson Correlation	.282**	1	-0.103	0.058	0.031
	Sig. (2-tailed)	0.000		0.211	0.480	0.710
	N	150	150	150	150	150

5.4.1.7 BDNF methylation in the Latino mothers study

DNA methylation was measured across promoter 1-4 of the *BDNF* gene. Seven CpG sites were investigated and identical to those CpGs studied in previous studies (See **Table 1.5, Figure 1.8)** and at a homologous region to that investigated in the rat studies (**Figure 1.9**) (**Figure 5.8**).

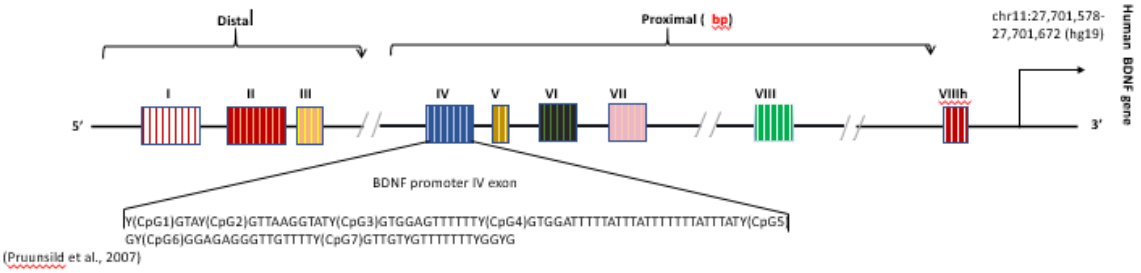

Figure 5.8. A schematic representation of the human BDNF exon IV promoter region showing sequence analysed by bisulphite sequencing and the numbered CpGs.

An example of a pyrosequencing run demonstrating methylation levels across the 10 CpG sites in the *BDNF* promoter IV are shown in (**Figure 5.9)**. Importantly, not all samples gave good quality reads for all CpGs in the pyrosequencing, therefore only the first 7 CpGs were analysed for which most samples had results for.

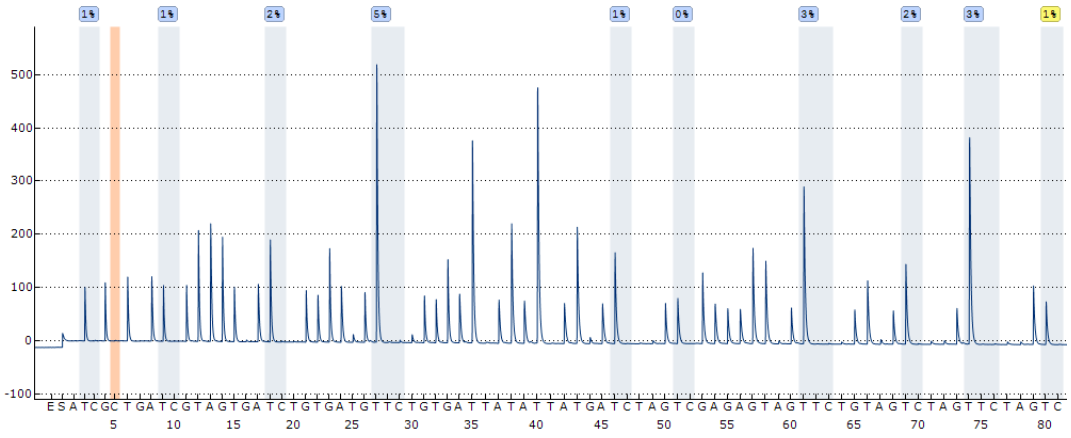

Figure 5.9. Bisulphite pyrosequencing of BDNF

Levels of methylation across the mothers at the 7 CpG sites in the *BDNF* promoter IV in the Latino mothers cohort were: CpG1, 4.03%; CpG2, 2.49%; CpG3, 2.88%; CpG4, 7.28%; CpG5, 3.22%; CpG6 2.2%; CpG7, 5.77% and average methylation across the 7 CpGs was 3.97% (**Figure 5.10**).

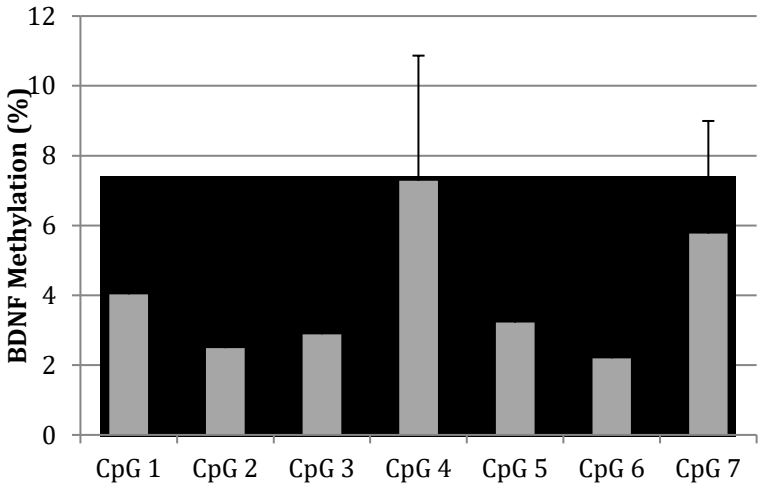

Figure 5.10. BDNF methylation in the Latino mothers study. *Percentage average + Standard Deviation of DNA methylation at each of the 4 CPGs are shown together with the average methylation of all CpGs within an individual.*

5.4.1.8 BDNF methylation and prenatal (T1) and postnatal (T2) discrimination

A correlation was performed between average DNA methylation at *BDNF* and prenatal EDS (eds 1), postnatal eds (eds 2) and total eds to determine if there is an association between methylation and discrimination. This revealed no significant correlations (**Table 5.9**).

Table 5.9. Correlation of average BDNF and EDS in the Latino mothers study

		*BDNF*_AVG	eds_1	eds_2	eds_total
*BDNF*_AVG	Pearson Correlation	1	-0.048	-0.063	-0.044
	Sig. (2-tailed)		0.562	0.444	0.595
	N	149	149	149	149

A correlation was then done between DNA methylation at the individual CpGs and EDS determine if specific CpGs might correlate. This also revealed no correlations between CpG methylation and EDS score (**Table 5.10**).

Table 5.10. Correlation of BDNF methylation at individual CpGs and EDS in the Latino mothers study

		BDNF_cpg1	*BDNF_cpg2*	*BDNF_cpg3*	*BDNF_cpg4*	*BDNF_cpg5*	*BDNF_cpg6*	*BDNF_cpg7*	eds_1	eds_2	eds_total
BDNF_cpg1	Pearson Correlation	1	0.058	0.026	-0.142	-0.034	-0.048	-0.117	0.046	0.104	0.122
	Sig. (2-tailed)		0.485	0.749	0.084	0.682	0.562	0.159	0.581	0.205	0.140
	N	149	149	149	149	149	149	147	149	149	149
BDNF_cpg2	Pearson Correlation	0.058	1	-0.107	-0.091	0.113	-0.022	-0.152	0.030	0.036	0.069
	Sig. (2-tailed)	0.485		0.195	0.269	0.170	0.786	0.067	0.716	0.664	0.404
	N	149	149	149	149	149	149	147	149	149	149
BDNF_cpg3	Pearson Correlation	0.026	-0.107	1	0.125	.247**	.170*	-0.013	-0.034	-0.031	-0.056
	Sig. (2-tailed)	0.749	0.195		0.128	0.002	0.038	0.875	0.679	0.706	0.494
	N	149	149	149	149	149	149	147	149	149	149
BDNF_cpg4	Pearson Correlation	-0.142	-0.091	0.125	1	0.110	.170*	.212**	0.034	0.006	-0.014
	Sig. (2-tailed)	0.084	0.269	0.128		0.183	0.038	0.010	0.677	0.943	0.861
	N	149	149	149	149	149	149	147	149	149	149
BDNF_cpg5	Pearson Correlation	-0.034	0.113	.247**	0.110	1	.195*	-0.009	-0.003	-0.045	-0.022
	Sig. (2-tailed)	0.682	0.170	0.002	0.183		0.017	0.913	0.975	0.587	0.791
	N	149	149	149	149	149	149	147	149	149	149
BDNF_cpg6	Pearson Correlation	-0.048	-0.022	.170*	.170*	.195*	1	.231**	-0.094	-0.080	-0.057
	Sig. (2-tailed)	0.562	0.786	0.038	0.038	0.017		0.005	0.253	0.333	0.488
	N	149	149	149	149	149	149	147	149	149	149
BDNF_cpg7	Pearson Correlation	-0.117	-0.152	-0.013	.212**	-0.009	.231**	1	-0.118	-.175*	-0.149
	Sig. (2-tailed)	0.159	0.067	0.875	0.010	0.913	0.005		0.154	0.034	0.072
	N	147	147	147	147	147	147	147	147	147	147

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

5.4.1.9 *BDNF* methylation and depression

A correlation was performed between *BDNF* DNA methylation and IDAS to determine if there is an association between methylation and prenatal (IDAS1) and postnatal (IDAS) or total depression. This revealed no correlations between average methylation at *BDNF* methylation and IDAS score (**Table 5.11**) or methylation at individual CpGs (**Table 5.12**).

Table 5.11. Correlation of BDNF methylation and prenatal (idas1) and postnatal (idas2) or total (idas total) maternal depression in the Latino mothers study

		*BDNF*_AVG	idas1	idas2	idas_total
*BDNF*_AVG	Pearson Correlation	1	0.002	-0.017	0.038
	Sig. (2-tailed)		0.976	0.839	0.642
	N	149	149	149	149

Table 5.12. Correlation of BDNF methylation and prenatal (idas1) and postnatal (idas2) or total (idas total) maternal depression in the Latino mothers study

		*BDNF*_cpg1	*BDNF*_cpg2	*BDNF*_cpg3	*BDNF*_cpg4	*BDNF*_cpg5	*BDNF*_cpg6	*BDNF*_cpg7	idas1	idas2	idas_total
*BDNF*_cpg1	Pearson Correlation	1	0.058	0.026	-0.142	-0.034	-0.048	-0.117	-0.047	-0.040	-0.032
	Sig. (2-tailed)		0.485	0.749	0.084	0.682	0.562	0.159	0.570	0.627	0.698
	N	149	149	149	149	149	149	147	149	149	149
*BDNF*_cpg2	Pearson Correlation	0.058	1	-0.107	-0.091	0.113	-0.022	-0.152	-0.048	-0.048	0.080
	Sig. (2-tailed)	0.485		0.195	0.269	0.170	0.786	0.067	0.564	0.563	0.332
	N	149	149	149	149	149	149	147	149	149	149
*BDNF*_cpg3	Pearson Correlation	0.026	-0.107	1	0.125	.247**	.170*	-0.013	0.026	-0.016	0.105
	Sig. (2-tailed)	0.749	0.195		0.128	0.002	0.038	0.875	0.748	0.851	0.204
	N	149	149	149	149	149	149	147	149	149	149
*BDNF*_cpg4	Pearson Correlation	-0.142	-0.091	0.125	1	0.110	.170*	.212**	0.041	0.046	0.023

	Sig. (2-tailed)	0.084	0.269	0.128		0.183	0.038	0.010	0.619	0.578	0.783
	N	149	149	149	149	149	149	147	149	149	149
BDNF _cpg5	Pearson Correlation	-0.034	0.113	.247**	0.110	1	.195*	-0.009	-0.076	-0.022	0.010
	Sig. (2-tailed)	0.682	0.170	0.002	0.183		0.017	0.913	0.358	0.792	0.908
	N	149	149	149	149	149	149	147	149	149	149
BDNF _cpg6	Pearson Correlation	-0.048	-0.022	.170*	.170*	.195*	1	.231**	0.067	-0.022	0.019
	Sig. (2-tailed)	0.562	0.786	0.038	0.038	0.017		0.005	0.418	0.792	0.819
	N	149	149	149	149	149	149	147	149	149	149
BDNF _cpg7	Pearson Correlation	-0.117	-0.152	-0.013	.212**	-0.009	.231**	1	0.022	0.010	-0.021
	Sig. (2-tailed)	0.159	0.067	0.875	0.010	0.913	0.005		0.790	0.902	0.800
	N	147	147	147	147	147	147	147	147	147	147

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

5.4.2 Epigenetic regulation of *NR3C1* in mother with prenatal and postnatal depression and anxiety in their offspring.

Mothers living in the Wirral, UK at 20wks of pregnancy (prenatal) and 5 weeks following birth (postnatal) were asked to complete a questionnaire for depression (EPDS) and anxiety (see methods). Saliva DNA was measured for methylation at *NR3C1* in mothers and their offspring. In the mothers DNA was taken at the age of 27.6 years and in infants at the age of 3 years.

Levels of methylation across the mothers at the 4 CpG sites were: CpG1, 1.22%; CpG2, 3.513%; CpG3, 4.784%; CpG4, 7.22% and average methylation across the CpGs was 2.36%. In the infants methylation was CpG1, 1.77%; CpG2, 3.64%; CpG3, 5.07%; CpG4, 9.29% and average methylation across the CpGs was 2.7% (**Figure 5.11**).

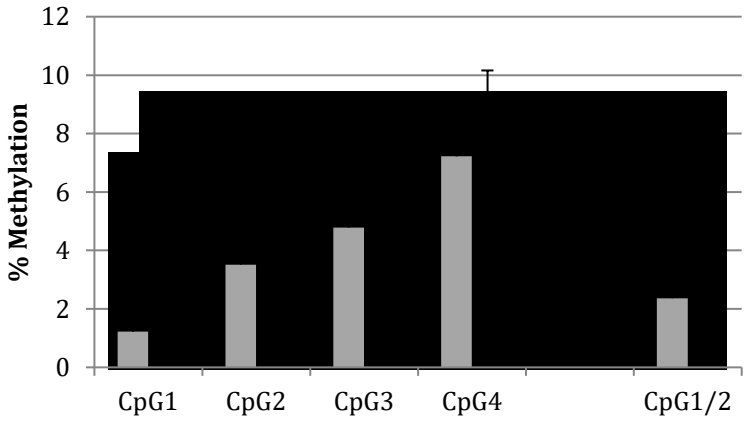

Figure 5.11. NR3C1 methylation in the WCHADS mothers and their offspring. *Percentage average + Standard Deviation of DNA methylation at each of the 4 CPGs are shown together with the average methylation of all CpGs within an individual.*

5.4.2.1 Maternal prenatal anxiety and depression and *NR3C1* methylation: A correlation was performed between DNA methylation across the CpGs for average methylation in mothers who had suffered from prenatal depression and anxiety. This revealed no significant correlation (**Table 5.13**).

Table 5.13. Correlation of average NR3C1 methylation with prenatal anxiety and depression the WCHADS mothers

		Mother CpG12	prenatal anxiety	Prenatal depression
Mother CpG12	Pearson Correlation	1	-0.044	-0.155
	Sig. (2-tailed)		0.608	0.067

**. Correlation is significant at the 0.01 level (2-tailed).

A correlation was then done between DNA methylation at the individual CpGs and EPDS determine if there is an association between methylation and depression. This also revealed no correlations between CpG methylation and EDS score (**Table 5.14**).

Table 5.14. Correlation of NR3C1 methylation at individual CpGs in mothers who suffered from prenatal anxiety and depression

		Mum CpG1	Mum CpG2	Mum CpG3	Mum CpG4	Prenatal depression	Prenatal anxiety
Mum CpG1	Pearson Correlation	1	-0.002	-0.039	-.187*	-0.156	-0.097

	Sig. (2-tailed)		0.981	0.641	0.026	0.066	0.256
	N	143	143	143	143	140	140
Mum CpG2	Pearson Correlation	-0.002	1	0.075	0.094	-0.105	-0.010
	Sig. (2-tailed)	0.981		0.372	0.265	0.215	0.910
	N	143	143	143	143	140	140
Mum CpG3	Pearson Correlation	-0.039	0.075	1	.234**	-0.035	0.032
	Sig. (2-tailed)	0.641	0.372		0.005	0.686	0.704
	N	143	143	143	143	140	140
Mum CpG4	Pearson Correlation	-.187*	0.094	.234**	1	0.020	0.010
	Sig. (2-tailed)	0.026	0.265	0.005		0.819	0.906
	N	143	143	143	143	140	140

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

5.4.2.2 Maternal postnatal depression and *NR3C1* methylation: A correlation was performed between DNA methylation across the CpGs for average methylation and postnatal anxiety and depression. This revealed no significant correlation (**Table 5.15**).

Table 5.15. Correlation of average NR3C1 methylation with postnatal anxiety and depression the WCHADS mothers

		Mother CpG12	postnatal anxiety	Postnatal depression
Mother CpG12	Pearson Correlation	1	-0.016	-0.012
	Sig. (2-tailed)		0.863	0.893
	N			132

A correlation was then done between DNA methylation at the individual CpGs and EPDS determine if there is an association between methylation and depression. This also revealed no correlations between CpG methylation and EPDS scores (**Figure 5.16**).

Table 5.16. Correlation of NR3C1 methylation at individual CpGs in mothers who suffered from postnatal anxiety and depression

		Mum CpG1	Mum CpG2	Mum CpG3	Mum CpG4	Postnatal depression	postnatal anxiety
Mum CpG1	Pearson Correlation	1	-0.002	-0.039	-.187*	-0.069	-0.120

	Sig. (2-tailed)		0.981	0.641	0.026	0.434	0.182
	N	143	143	143	143	132	125
Mum CpG2	Pearson Correlation	-0.002	1	0.075	0.094	-0.020	0.036
	Sig. (2-tailed)	0.981		0.372	0.265	0.822	0.687
	N	143	143	143	143	132	125
Mum CpG3	Pearson Correlation	-0.039	0.075	1	.234**	0.002	0.094
	Sig. (2-tailed)	0.641	0.372		0.005	0.985	0.295
	N	143	143	143	143	132	125
Mum CpG4	Pearson Correlation	-.187*	0.094	.234**	1	0.128	0.172
	Sig. (2-tailed)	0.026	0.265	0.005		0.145	0.055
	N	143	143	143	143	132	125

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

5.4.2.3 Maternal stress and *NR3C1* methylation: A correlation was performed between DNA methylation across the CpGs for average methylation and life events in pregnancy to determine if there is an association between methylation and depression. This revealed no significant correlation (**Figure 5.17**).

Table 5.17. Correlation of NR3C1 methylation in mothers who suffered from life events during pregnancy

		Mum CpG1	Mum CpG2	Mum CpG3	Mum CpG4	Mum CpG12	life events in pregnancy
Mum CpG1	Pearson Correlation	1	-0.002	-0.039	-.187*	.574**	-0.112
	Sig. (2-tailed)		0.981	0.641	0.026	0.000	0.185
	N	143	143	143	143	143	143
Mum CpG2	Pearson Correlation	-0.002	1	0.075	0.094	.791**	-0.098
	Sig. (2-tailed)	0.981		0.372	0.265	0.000	0.245
	N	143	143	143	143	143	143
Mum CpG3	Pearson Correlation	-0.039	0.075	1	.234**	0.041	-0.093
	Sig. (2-tailed)	0.641	0.372		0.005	0.631	0.271
	N	143	143	143	143	143	143
Mum CpG4	Pearson Correlation	-.187*	0.094	.234**	1	-0.021	-0.005
	Sig. (2-tailed)	0.026	0.265	0.005		0.803	0.952
	N	143	143	143	143	143	143

Mum CpG12	Pearson Correlation	.574**	.791**	0.041	-0.021	1	-0.136
	Sig. (2-tailed)	0.000	0.000	0.631	0.803		0.106
	N	143	143	143	143	143	143

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

5.4.2.4 Methylation of *NR3C1* in infants exposed to prenatal depression and anxiety

A correlation was performed between DNA methylation across the CpGs for average methylation and EPDS to determine if there is an association between methylation and depression. This revealed no significant correlation (**Table 5.18**).

Table 5.18. Correlation of NR3C1 methylation at individual CpGs in infants exposed to life events in pregnancy

		GR CpG 1/2	Prenatal anxiety	Prenatal depression
GR CpG 1/2	Pearson Correlation	1	0.113	0.054
	Sig. (2-tailed)		0.081	0.409
	N		238	238

**. Correlation is significant at the 0.01 level (2-tailed).

A correlation was performed between infant DNA methylation across the CpGs for average methylation and prenatal anxiety and depression. This revealed no significant correlation in any of the CpGs with prenatal anxiety and depression (**Figure 5.19**).

Table 5.19. Correlation of NR3C1 methylation at individual CpGs in infants exposed to prenatal anxiety and depression

		GR CpG 1	GR CpG 2	GR CpG 3	GR CpG 4	Prenatal depression	Prenatal anxiety
GR CpG 1	Pearson Correlation	1	0.100	0.051	-0.049	0.034	0.085
	Sig. (2-tailed)		0.119	0.429	0.444	0.600	0.193
	N		244	244	244	239	239
GR CpG 2	Pearson Correlation		1	.213**	0.017	0.036	0.099
	Sig. (2-tailed)			0.001	0.788	0.582	0.127
	N				244	239	239
GR CpG 3	Pearson Correlation				.436**	0.038	0.031
	Sig. (2-tailed)				0.000	0.559	0.633

	N					239	239
GR CpG 4	Pearson Correlation					-0.022	-0.064
	Sig. (2-tailed)					0.732	0.328
	N					239	239

**. Correlation is significant at the 0.01 level (2-tailed).

5.4.2.4 Methylation of *NR3C1* in infants exposed to postnatal depression and anxiety

A correlation was performed between DNA methylation across the CpGs for average methylation and EDS to determine if there is an association between methylation and depression. This revealed no significant correlation (**Table 5.20**).

Table 5.20. Correlation of NR3C1 methylation at individual CpGs in infants exposed to postnatal anxiety and depression

		GR CpG 1	GR CpG 2	GR CpG 3	GR CpG 4	Postnatal depression	Postnatal anxiety
GR CpG 1	Pearson Correlation	1	0.100	0.051	-0.049	0.060	0.124
	Sig. (2-tailed)		0.119	0.429	0.444	0.404	0.088
	N		244	244	244	197	189
GR CpG 2	Pearson Correlation		1	.213**	0.017	-0.068	-0.018
	Sig. (2-tailed)			0.001	0.788	0.340	0.805
	N			244	244	197	189
GR CpG 3	Pearson Correlation			1	.436**	-0.013	0.055
	Sig. (2-tailed)				0.000	0.857	0.449
	N				244	197	189
GR CpG 4	Pearson Correlation				1	0.000	0.044
	Sig. (2-tailed)					0.995	0.550
	N					197	189

**. Correlation is significant at the 0.01 level (2-tailed).

5.4.2.4 Methylation of *NR3C1* in infants exposed to life events during pregnancy

A correlation was performed between DNA methylation across the CpGs for average methylation and life events during pregnancy. This revealed no significant correlations (**Table 5.21**).

Table 5.21. Correlation of NR3C1 methylation at individual CpGs in infants exposed to life events in pregnancy

		CpG1	CpG2	CpG3	CpG4	CpG12	life events in pregnancy
CpG1	Pearson Correlation	1	-0.002	-0.039	-.187*	.574**	-0.112
	Sig. (2-tailed)		0.981	0.641	0.026	0.000	0.185
	N		143	143	143	143	143
CpG2	Pearson Correlation		1	0.075	0.094	.791**	-0.098
	Sig. (2-tailed)			0.372	0.265	0.000	0.245
	N			143	143	143	143
CpG3	Pearson Correlation			1	.234**	0.041	-0.093
	Sig. (2-tailed)				0.005	0.631	0.271
	N				143	143	143
CpG4	Pearson Correlation				1	-0.021	-0.005
	Sig. (2-tailed)					0.803	0.952
	N					143	143
CpG12	Pearson Correlation					1	-0.136
	Sig. (2-tailed)						0.106
	N						143

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

5.4.2.6 Correlation between mother and infant levels of methylation: Correlations were performed between DNA methylation across the CpGs in *NR3C1* between mothers and their offspring to determine to what degree methylation levels might be inherited. This revealed that the average level of methylation did not correlate (**Figure 5.22**), but the level of methylation at CpG3 in the mother significantly negatively correlated with DNA methylation at CpG2 in their offspring (**Table 5.23**).

Table 5.22. Correlation of average NR3C1 methylation in mothers and their infants

		GR CpG 1/2	Mun_GRCpG12
GR CpG 1/2	Pearson Correlation	1	0.099

	Sig. (2-tailed)		0.283
	N	243	120

Table 5.23. Correlation of NR3C1 methylation at individual CpGs in mothers and their infants

		GR CpG 1	GR CpG 2	GR CpG 3	GR CpG 4
Mum_GRCpG1	Pearson Correlation	-0.032	0.021	0.004	0.064
	Sig. (2-tailed)	0.728	0.823	0.967	0.486
	N	120	120	120	120
Mum_GRCpG2	Pearson Correlation	0.172	-0.034	-0.066	-0.068
	Sig. (2-tailed)	0.061	0.712	0.474	0.460
	N	120	120	120	120
Mum_GRCpG3	Pearson Correlation	-0.004	-.219*	-0.108	-0.099
	Sig. (2-tailed)	0.964	0.016	0.242	0.282
	N	120	120	120	120
Mum_GRCpG4	Pearson Correlation	0.057	-0.149	-0.022	-0.008
	Sig. (2-tailed)	0.535	0.105	0.812	0.933
	N	120	120	120	120

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

5.5 Discussion

This study tested the hypothesis that one of the mechanisms by which stress is able to increase risk for depression is by programming changes in the neuroendocrine regulation of stress control through the epigenetic programming of stress regulatory genes.

The results from *Study 1* supported that offspring exposed to reduced postnatal care and increased maternal stress show increased methylation at the *Nr3c1* gene and *BDNF* gene promoters. That these offspring then show reduced maternal care and increased maternal anxiety suggests that methylation at these genes could be important. This reduced methylation is no longer present in the F2 generation exposed to both prenatal and postnatal stress.

It was therefore tested whether similar epigenetic changes might occur in humans. Specifically, if prenatal or postnatal maternal depression associated with changes in *NR3C1*, *FKBP5* or *BDNF* methylation. Also, whether stress at prenatal or postnatal stages impacted methylation. Finally, it was tested whether infants exposed to postnatal stress, or prenatal stress, show changes in *NR3C1* methylation.

We firstly tested for associations between DNA methylation at the stress related genes and perceived discrimination during the prenatal and postnatal periods in Latina women in the US, then with depression. Two hypotheses are that this may serve as a risk for the effects of discrimination on mother and child; or this may relate to the impact of discrimination on the development of psychiatric disorders including maternal depression, major depression and anxiety. Indeed there is a very high rate of stress-related illness in the Latina ethnic group in the US (Romero et al., 2007; Anderson and Mayes, 2010; Santos et al., 2018). Pearson correlations were used to test associations. The *NR3C1* exon 1F methylation it was firstly found that all individual CpGs correlated with each other, i.e. higher methylation at any one of the 4 CpGs associated with higher methylation at the three other sites. Examining perceived discrimination there was a statistically significantly negative association with average *NR3C1* methylation and EDS, i.e. increased methylation with reduced total EDS. Testing the CpGs individually it was seen that increased CpG1 methylation correlated with reduced total EDS, prenatal EDS

and postnatal EDS. There were no associations with *NR3C1* methylation and IDAS either total, prenatal or postnatal.

Epigenetic regulation at *NR3C1* exon 1F is key in several processes of the stress-response regulation and differences in methylation in relation to social environment and stress have been reported in numerous studies (**Table 1.5).** In studies focused on psychological distress, human studies have reported some varied results in terms of *NR3C1* exon 17 methylation with one showing increased methylation (Dammann et al., 2011) and two finding decreased methylation (Alt et al., 2010; Yehuda et al., 2015), while three reported no change (Alt et al., 2010; Steiger et al., 2013; Yehuda et al., 2015). In studies of early life adversity, one found that children exposed to maltreatment, showed decreased methylation at one (corresponding to the CpG1 in this study) and increased methylation at neighbouring CpGs (Romens et al., 2015), while a study on maternal and paternal experience of the Holocaust showed gender differences with decreased and increased methylation, respectively (Daskalakis and Yehuda, 2014) (**Figure 5.12**). The *NR3C1* methylation association with EDS could represent a risk factor and possibly highlight stress related factors as biomarkers for mothers at risk of the adverse effects of discrimination on themselves, and possibly, on their offspring, which would need to be tested.

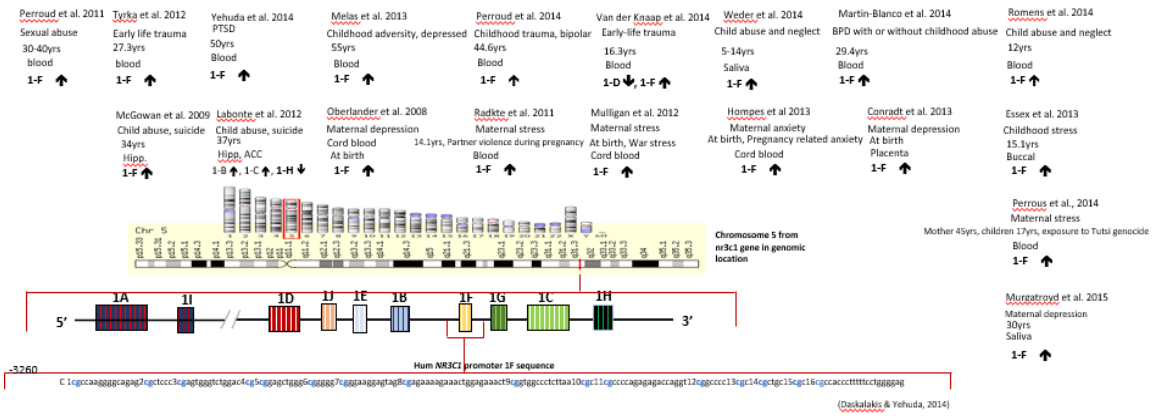

Figure 5.12. Schematic of the NR3C1 promoter with human studies of methylation at exon 1-7.*Individual studies are shown demonstrating increases or decreases in methylation in relation to stress exposure and depression.*

Another important modulator of glucocorticoid signaling in response to stress is *FKBP5.* In this study using Pearson correlations we found no associations with methylation at this gene in either prenatal or postnatal EDS or IDAS. However, analyzing the methylation data using ZIP regression (see below in limitations) did reveal negative correlations. Numerous studies of *FKBP5* methylation have demonstrated positive links with stress, particularly PTSD, though the Tyrka et al. study on childhood maltreatment showed decreases in methylation (Tyrka et al., 2015) (**Figure 5.13**). Importantly, no study has investigated prenatal and postnatal depression.

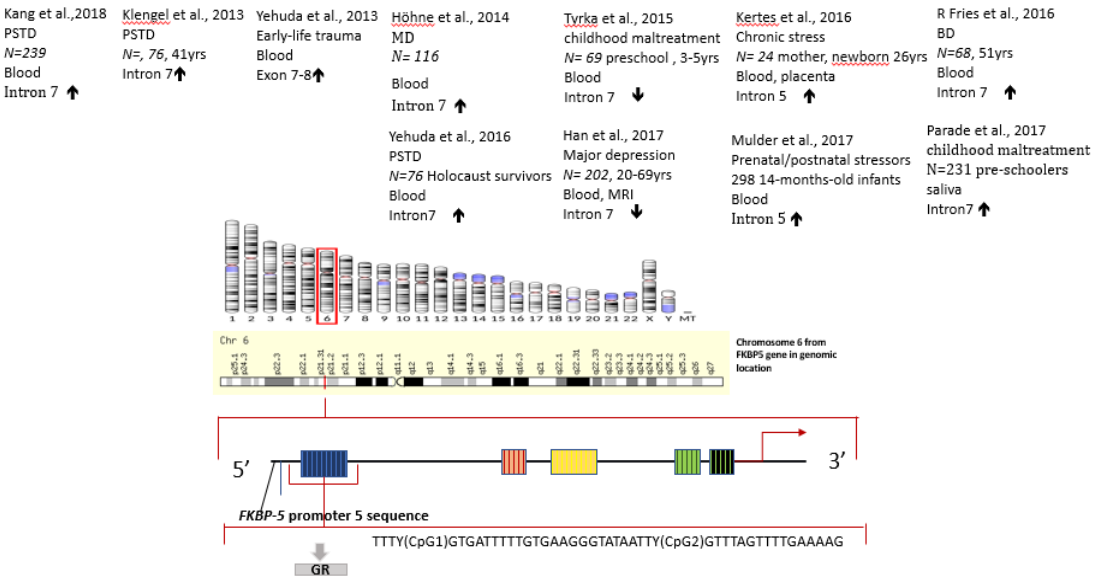

Figure 5.13. Schematic of the FKBP5 intron 7 promoter showing human studies. *Individual studies are shown demonstrating increases or decreases in methylation in relation to stress exposure and depression.*

BDNF methylation was positively associated with postnatal EDS at one CpG site out of the 7 measured. This positive association might suggest that depression in these mothers could have resulted in a decrease in *BDNF* expression. As *BDNF* is important in neurogenesis, this reduction could affect neural development in areas important for the perception and processing of social stimuli, resulting in the higher EDS scores. Reduced levels of *BDNF* in the serum have been found in a study of postnatal depression comparing

36 mothers with depression and 36 mothers without (Gazal et al., 2012) and numerous studies similarly report increases in *BDNF* methylation in depression (**Figure 5.14**).

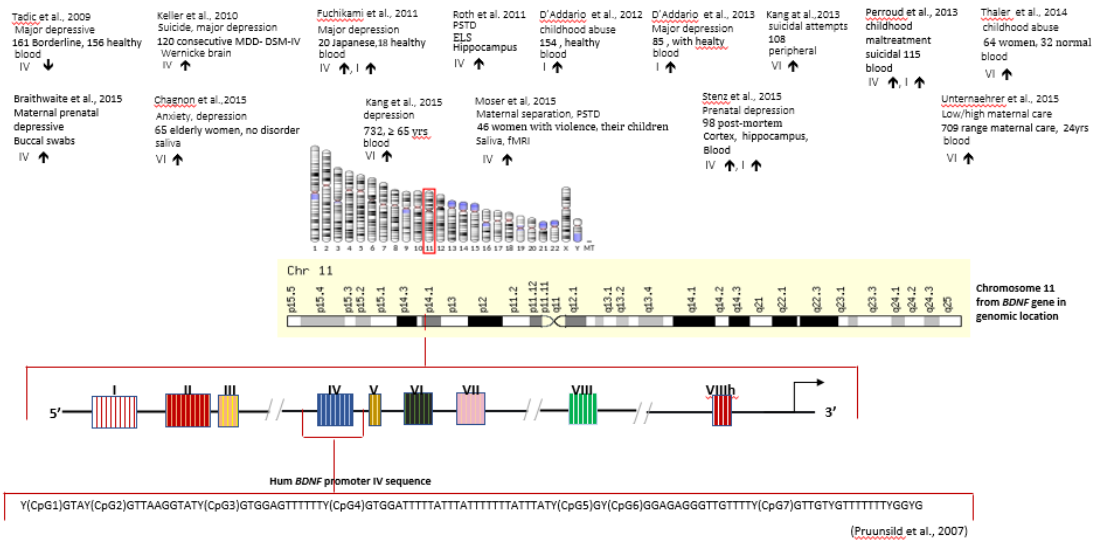

Figure 5.14. Schematic of the BDNF exon 4 promoter showing results of human studies. *Individual studies are shown demonstrating increases or decreases in methylation in relation to stress exposure and depression.*

In the WCHAS study *NR3C1* methylation in mothers with levels of prenatal and postnatal depression were measured. Average methylation did not associate with prenatal or postnatal anxiety though there was a non-significant trend (p=0.055) of increased methylation at one of the CpGs positively correlating with increased postnatal anxiety. There was another non-significant (p=0.066) negative correlation with prenatal depression, but no association with postnatal depression. The trend for a negative association of *NR3C1* methylation with prenatal depression would seem to support the negative association also seen in the Latina cohort.

In DNA methylation from the WCHAS infants there were no associations between *NR3C1* methylation and any of the maternal measures of prenatal anxiety or depression, postnatal anxiety or depression or life events. Interestingly, when correlating DNA methylation between mothers and infants, though there were no associations between average methylation, it was found that increased CpG3 methylation in mothers significantly associated with decreased CpG2 methylation in the infants, while increased

mother CpG2 methylation showed a non-significant trend (p=0.061) for increased CpG1 methylation in the infants.

Some limitations need to be taken into consideration while interpreting the results of this *Study 3*. Regarding statistical analyses, for the Latino study, Pearson correlations were used. However, this did not take into account the number of zero counts in the EDS and IDAS scores or did it adjust for covariates. The methylation data above has been tested using a ZIP regression, which is a zero-inflated Poisson models, adjusting for covariates and multiple-test comparisons to further model count data that has an excess of zero counts. This again showed discrimination negatively associated with methylation at CpG sites within the glucocorticoid receptor (*NR3C1*) but in addition found significant negative associations with CpGs in *BDNF* and *FKBP5* (Argentieri et al., 2017; Santos et al., 2018; Alyamani and Murgatroyd, 2018). For this study within this thesis, however I was unable to master these statistical tests to be able to confidently repeat on my own.

This study importantly focused only on defined regions of the stress-related genes (identified from the ***Literature Review***) at a few key CpGs to avoid possible impact of multiple analyses. However, there may be other neighbouring CpG sites or regions that might be important to investigate. Importantly, in contrast to the rat analyses in *Study 1* and *Study 2* on hippocampus the methylation analysed here was from peripheral cell samples of blood and saliva. These tissues are heterogeneous, which may account for some of the variability in methylation. Although there are some studies suggesting methylation levels in blood and post mortem brain are often substantially correlated (Walton et al., 2015; Nemoda et al., 2015; Smith et al., 2015), it cannot be certain to what degree DNA methylation in peripheral tissues reflects methylation in brain regions. However, the findings of *Study 1* and *Study 2* in highlighting the involvement of immune systems might support some link.

Overall, this *Study 3* supports that there are some epigenetic changes in *nr3c1* methylation that correlate with maternal stress that follow some of the findings from the rat methylation results in *Study 1*, suggesting that this could be a marker for maternal stress and depression.

6 Study 5: Explore components in milk that are altered by exposure to depression in rats.

6.1 Introduction

Key factors and nutrients in breast milk (**Table 1.6**) are important in infant neurodevelopment and regulation of child behavioural development (Martin et al., 2016a). However, stress and depression have been shown to reduce the levels of milk production, possibly through impacting hormones such as oxytocin (Dewey, 2001); (Hatton et al., 2005). A study by (Kordus, 2014) Mansbacher et al. (2012) on 10,000 children and their mothers showed an indirect correlation between breastfeeding and behavioural disorders which were less likely to occur the longer the duration of breastfeeding. This is supported by other longitudinal studies such as (Oddy and McMahon, 2011) who showed that those children who had been breastfed for over half a year were less aggressive than those who were not breastfed. Though it seems that milk components have an effect on infant behaviour and that stress can influence milk components, it is not clear what lactational mechanisms, if any, could link maternal stress to infant behaviour.

There is hypothesis, termed lactroctine programming, that hormones from the mother, ingested through milk, are able bind to receptors within the young triggering hormonal signaling cascades in the offspring (**Figure 1.7**). One candidate is cortisol or corticosterone. Numerous studies in rats and mice have shown that increasing corticosterone consumption to pups through mother's milk or artificially is able to regulate sensitivity of the HPA axis, improve spatial memory and reduce anxiety (Casolini et al., 1997; Catalani et al., 2000). Startlingly, opposite results have been found in human studies, by which infants ingesting higher levels of cortisol consequently showed greater anxiety and stress related behaviours (Grey et al., 2013).

A number of key hormones know to regulate behaviour are found in breast milk. The question is whether these are regulated in milk through maternal stress. For example,

oxytocin and prolactin levels in milk might change in response to depression that may influence milk production or impact behaviour of the offspring.

Immune factors are found in abundance in breast milk where they are important for the development of the infant immune system (Garofalo, 2010) as well as neurodevelopment (Koo et al., 2014). Importantly, maternal health influences the levels of inflammatory factors in the milk. Whitaker et al, on a study of 134 mothers found that high pre-pregnancy BMI and excessive gestational weight gain correlated with increased levels of the pro-inflammatory marker CRP in their breast milk (Whitaker et al., 2017). One study on immune factor levels in the milk of 481 mothers was able to demonstrate that differences in the individual immune composition of the mother's milk influenced early life infant health outcomes, namely eczema and food allergy risk (Munblit et al., 2017). One possible mechanism is the existence of gut receptors for numerous milk hormones and cytokines that support the notion that proteins in breast milk remain bioactive and influence the offspring. However, there is still very little understood as to the consequences of infants receiving varying concentrations of breast milk inflammatory markers or hormones. However, the presence of such a mechanism of transfer from the mother to infant could be important in the intergenerational transmission of stress.

In this study we measured cortisol, endocrine factors and immune markers in the milk from the CSS rats and rats exposed to acute stress to understand the role of maternal depression and milk components.

6.2 Aims

The overall aim of this study is to examine stress-related endocrine and immune factors in milk following stress and its possible role in the inheritance of stress and depression. We further aimed to develop a method for measuring these factors in rat milk, many of which have not been examined before in milk.

6.3 Objectives

1. Test whether maternal stress and depression influence milk production.
2. Explore whether endocrine factors are altered in milk following maternal depression and stress.
3. Investigate whether immune factors are altered in milk following maternal depression and stress.
4. Test the relationships between maternal serum levels of immune and endocrine factors relate to milk levels.
5. Test the relationships between milk levels of endocrine and immune factors relate to serum levels of those factors in offspring.

6.4 Methods

6.4.1 Milk samples

Animal model: Rats from the trans-generation inheritance of CSS study were used to collect milk studies. Collected by Dr Ben Nephew, this was done by allowing the pups to suckle from the mother and then killing the pups and emptying the stomachs for the milk

6.4.2 Analysis of milk factors using ELISA

The samples were centrifuged at 1500×g for 10 min at 10 °C (Beckman coulter Avanti J-26 XP centrifuge, rotor JA-25.15) to separate the solid part of the milk from the clearer supernatant (milk serum). The milk serum was then tested using ELISA.

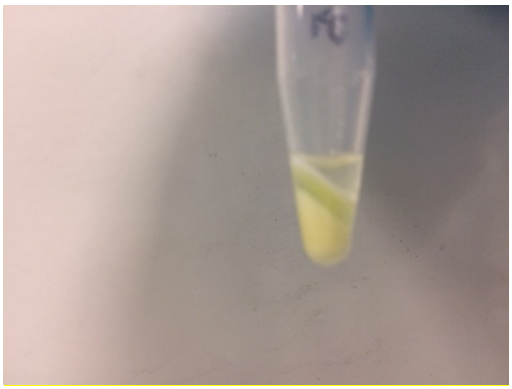

Figure 6.1. Rat milk samples showing solid and small amount of milk plasma

6.4.3 ELISA

6.4.3.1(Single-plex)

Oxytocin, corticosterone, I-CAM, bdnf, and GR were quantified individually from milk serum using single-plex ELISA. The same procedure, was carried out for each of these substances. A 96 –well microplate was used. 100 µl of provided standards and 50 µl of polyclonal antibody to the desired substance (i.e. oxytocin, or corticosterone etc) were added to 100 µl of each sample. The plate was mixed gently, covered, and incubated in a

dark fridge at 4°C for 24 hours. Following this, the well contents were removed and wells washed three times using the supplied wash solution. 200 µl of pNpp substrate (p-nitrophenyl phosphate) was added to each well to allow colour development. After incubation for 1 hour at room temperature, 50 µl of stop solution was added to stop the reaction and development of colour. To complete, the optical density of each well was read at 405nm wavelength using the Synergy HT micropate reader (Biotex). Standard curves were then made (**Figure 6.2**) using GraphPad and values for the samples calculated

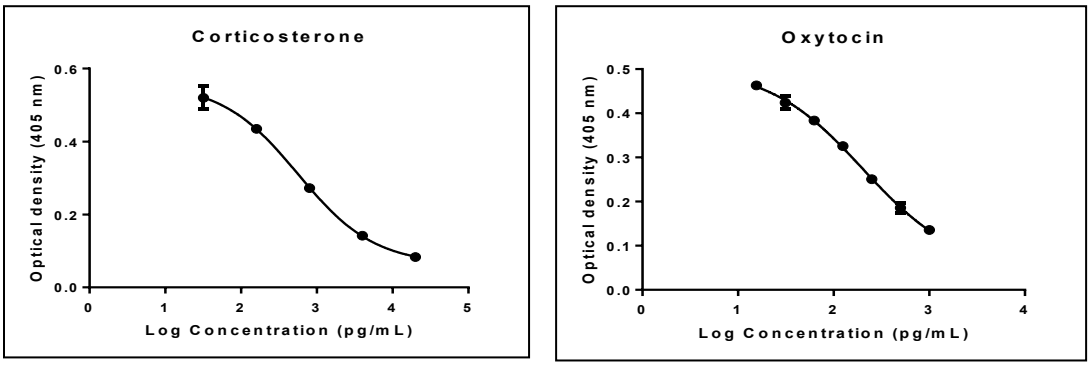

Figure 6.2. Standard curves for Oxytocin and Corticosterone for measuring milk levels

6.4.3.2 ELISA (multiplex)

A MILLIPLEX® MAP Rat Cytokine/ Chemokine Magnetic Bead Panel was used to quantify the following factors were from milk serum: IL-1β, IL-4, IL-6, IL-10, TNFα, IFNϒ. Icam-1, and from TIMP-1, were also measured from another Muliplex plate **(**See **Table 2.1)**. The method involved, washed a 96-well filter plate was with 200 µl of supplied wash buffer, removed by vacuum filtration. 225 µl of assay buffer, 25 µl of provided standards, 25 µl of sample (1:2, 1:5, 1:8) diluted and 25 µl of fluorescent coded magnetic beads were added to each sample well. The plate was then incubated for 16-18 hours on plate shaker a t2-8°C , and then the contents removed and wells washed twice with 200 µl wash buffer (Automatic plate washer for magnetic beads **Bio Tek** system). 25 µl of biotinylated detection antibodies were then added to each well. The plate was then sealed and covered with foil, and incubated on a plate shaker for 1 hour at room temperature. 25 µl of Streptavidin-phycoerythrin was then added to each well before covering with foil and

incubating on a plate shaker for 30 minutes at room temperature. The well contents were removed again, and wells washed 2 times with 200 µl wash buffer (Automatic plate washer for magnetic beads Bio Tek system). Lastly, 125 µl of sheath fluid (xMAP®) was added to each well, and the plate read on luminex (100 µl, 50 beads per bead set). See (**Figure 6.3)** for a schematic overview of the experimental method.

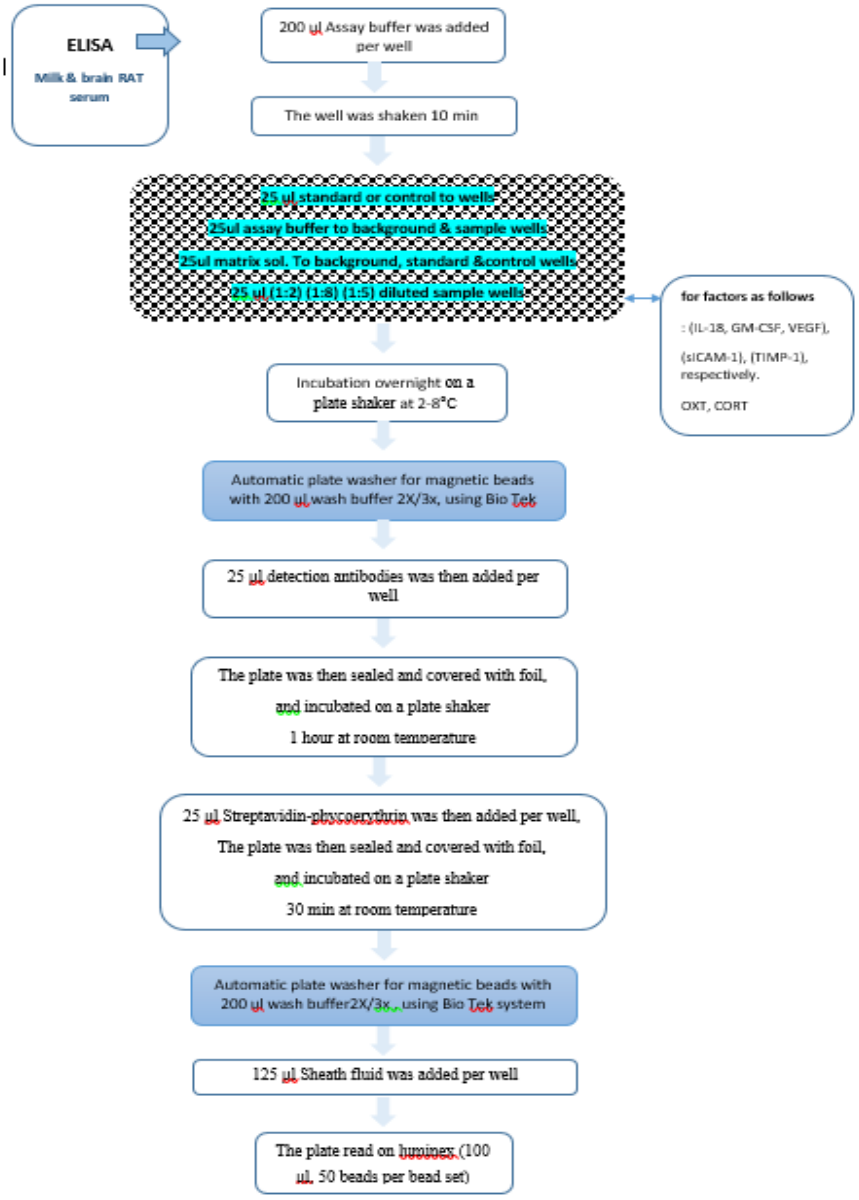

Figure 6.3. Schematic for the milk ELISA method outline

6.5 Results

6.5.1 Milk corticosterone levels and stress:

Levels of corticosterone were measured in the milk taken at day 16 from F2 pups, i.e. milk produced from the F1 dams. This revealed significantly lower corticosterone in the CSS group (2-tail t-test p = 0.05) (**Figure 6.4**).

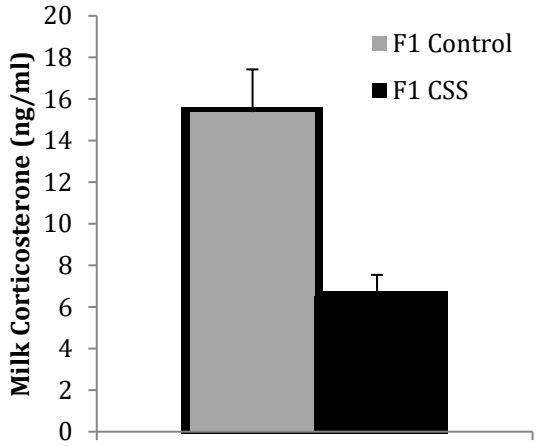

Figure 6.4. Corticosterone levels in milk from F1 Dams. *Mean + SEM of milk corticosterone levels in F2 pups as a measure of F1 milk corticosterone in control (n=8) and CSS (n=7). * 2-tailed t-test p= 0.05.*

As there was a difference in CSS corticosterone in milk (**Figure 6.4**) and a trend in serum we therefore tested if there was a correlation between milk levels of corticosterone and serum levels in the mother if the raised corticosterone levels in the serum of the F1 mothers are transferred to the milk. A Pearson correlation revealed a significant negative correlation between serum corticosterone levels of F1 Dams and levels of corticosterone in the milk (P=0.048) (**Table 6.1**).

We then tested if levels of corticosterone in the milk a pup drinks are associated with serum levels of corticosterone in the pups. A Pearson correlation revealed no significant association between milk corticosterone levels and serum corticosterone levels in the F2 rats receiving the milk (**Table 6.1**).

Table 6.1. Pearson correlations of corticosterone in F1 milk, F1 serum and F2 serum

Correlations

		LogF1_Serum	LogF2_Serum	F1_MilkA
LogF1_Serum	Pearson Correlation	1	.241	-.460*
	Sig. (2-tailed)		.199	.048
	N	30	30	19
LogF2_Serum	Pearson Correlation	.241	1	-.011
	Sig. (2-tailed)	.199		.965
	N	30	30	19

We tested the effects of acute stress on levels of corticosterone in milk. This was done in F0 dams exposed to CSS and acute restraint stress. This revealed that while CSS and control dams did not differ (2-tailed t-test, p= 0.48) and corticosterone levels did not differ in control dams following stress (2-tailed t-test, p= 0.54) there was a significant decrease in corticosterone in the CSS rats following an acute stressor (2-tailed t-test, p= 0.046) (**Figure 6.5**).

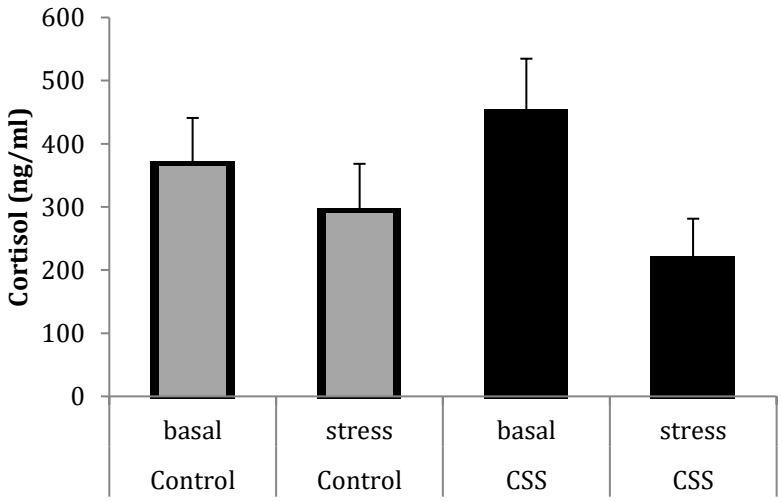

Figure 6.5. Corticosterone levels in milk from CSS F0 Dams exposed to acute stress. *Mean + SEM of milk levels of corticosterone in F1 adults of F0 Dams exposed to CSS and acute stress. n=9, 8, 7 and 8 respectively.* 2-tailed t-test p= 0.0.5*

6.5.2 Milk neuroendocrine levels in CSS and following acute stress

We tested the effects of acute stress on levels of oxytocin in milk. This was done in F0 dams exposed to CSS and acute restraint stress. Importantly, over half the samples gave values below the last point on the standard curve so could not be included. With limited sample numbers tough this revealed that while CSS and control dams did not differ (2-tailed t-test, p= 0.44) and oxytocin levels did not differ in control dams following stress (2-tailed t-test, p= 0.32) there was a trend towards a decrease in oxytocin in the CSS rats following an acute stressor (2-tailed t-test, p= 0.08) (**Figure 6.6**).

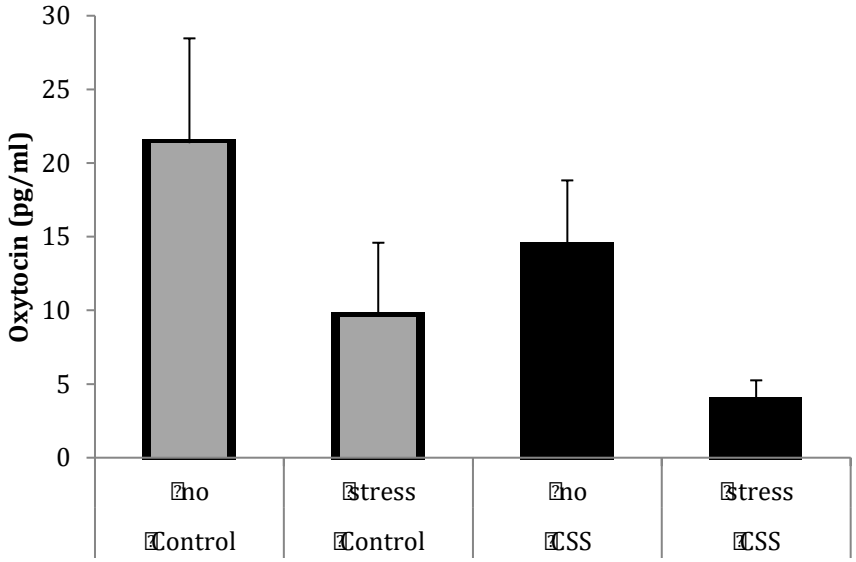

Figure 6.6. Corticosterone levels in milk from CSS F0 Dams exposed to acute stress. *Mean + SEM of milk levels of oxytocin in F1 adults of F0 Dams exposed to CSS and acute stress. n=6, 4, 5 and 2, respectively.*

6.5.3 Milk bdnf levels:

Levels of bdnf were measured in the milk taken at day 16 from F2 pups however; this failed to produce results above background for any samples.

6.5.4 Milk immune levels:

Levels of cytokines IL-4, IL-1B, IL-6, IL-10, IFN-gamma and TNF-alpha were measured in the milk taken at day 16 from F2 pups. From 50 samples, IL-4 games only 3 samples above background, IL-1B had 2, IL-6 gave 13, IL-10 gave 2 values, IFN-gamma produced 6 readings and TNF-alpha gave measurable readings for 9 samples, neither enough to allow statistical analyses.

6.6 Discussion

This study investigated levels of endocrine and immune factors in milk for the F1 CSS and control dams. This revealed that the F1 CSS dams (i.e. the F2 mothers) had lower corticosterone levels in the milk. Interestingly, this negatively correlated with corticosterone levels in their serum and between groups. A number of studies have shown that stressors can lead to alterations in milk cortisol/corticosterone levels (see 1.5.7.5). However, this is the first study to show trans-generational effects of stress, i.e. an indirect stressor and maternal depression, leads to changes in milk corticosterone. Interestingly, corticosterone levels in the F0 dams did not differ following the CSS suggesting that reduced maternal care as infants is important in programming such changes, no chronic stress in itself.

Several studies in rats have shown that ingestion of glucocorticoids via mother's milk has is able to program effects of stress in offspring that persist into adulthood. For example, there are studies by which rat dams that consumed glucocorticoids in their water, increased glucocorticoids in their blood and their milk. As juveniles, individuals who ingested elevated glucocorticoids through their mother's milk showed better memory and coping behaviour during stressors, together with decreased anxiety and altered HPA axis response to stress (Casolini et al., 1997; Catalani et al., 2000; Meerlo et al., 2001).

This association of increased milk corticosterone and reduced stress in the offspring is not found in humans, with two studies showing the opposite effects. A study of 257 Breastfeeding mothers with higher plasma cortisol concentrations, used as a proxy for milk cortisol concentrations, rated their infants as significantly more fearful than did breastfeeding mothers with lower plasma cortisol concentrations (Glynn et al., 2007). Analysis of cortisol concentrations in milk among 52 breastfeeding mothers revealed that higher concentrations of cortisol in breastmilk associated with increased fear, sadness, anger and frustration in infants (Grey et al., 2013).

Interestingly we found a significant negative correlation between serum corticosterone levels of F1 Dams and levels of corticosterone in the milk suggesting that mothers with

reduced levels of the stress steroid produce release higher levels in the milk. Perhaps this supports the rodent studies linking higher milk corticosterone levels with reduced stress in their offspring. We did not find association between milk corticosterone levels and offspring serum corticosterone levels. Though this was only basal levels, perhaps levels in response to stress or as adults might then differ and would be important to measure.

Investigating oxytocin we found levels in milk of around 5-20 ng/ml, similar to levels of around 18.9 ng/mL reported in human studies (Mishra et al., 2014). Interestingly, this was reduced in the F0 CSS dams exposed to acute stress. Though the numbers of animals in each group were relatively small, it is interesting that oxytocin in milk is reduced following stress. Though no other study appears to have tested for this studies in monkeys suggest that oxytocin delivered to the new-born in the perinatal period can have significant and positive impacts on the brain and behaviour (Simpson et al., 2014).

Testing ELISAs for bdnf, prolactin, interleukins and growth factors failed to produce results about background, or enough results above background to allow for analyses **6.4.4**

To further detect, possibly novel, proteins, it was planned for the samples to be analysed using proteomics. This would have been in collaboration with the WPH Proteomics Facility RTP at the University of Warwick following similar published protocol (Hettinga et al., 2015) with peptide and protein identification performed by analysing all MSMS spectra with MaxQuant v.1.2.2.5. However, due to the ELISA results, which showed we still needed to standardise the protein measure protocol, this was not performed.

It was also planned that mothers from the Latino Maternal Depression study (in collaboration with Santos Hudson, University of South Carolina) would supply milk samples that would be shipped on ice to the MMU. However, this was unable to be organised in time.

The major limitations of this *Study 4* is that the milk ELISA protocol still needs to be standardised. The milk samples seemed to vary just by looking at them, with some slightly different shades of creamy-white and with different levels of solids that gave different amounts of milk plasma following centrifugation. One possibility is to test different

methods of homogenisation before centrifugation, though it is unclear to what extent this might interfere with readings if releasing more insoluble proteins.

In sum, this *Study 4* shows that levels of stress and maternal-behaviour related hormones are affected by stress in the CSS model providing support for another mechanism for the intergenerational inheritance of depressed maternal care and anxiety.

7 Final Discussion

Stress during early life such as exposure to prenatal and to postnatal depression and receiving reduced levels of parental care can produce long-lasting behavioural effects on the offspring. Such long-term disruptions in stress-related behaviours have been seen in both human and rodent studies in offspring exposed to a variety early-life stressors such as maternal depression (Murgatroyd et al., 2015a). Importantly, offspring exposed to early life stress have increased susceptibility to maternal depression themselves (Murgatroyd and Nephew, 2013). This suggests a mechanism by which stress could be trans-generationally inherited through maternal stress. However it is unclear what the molecular mechanisms could be that might drive this transmission of maternal stress between generations.

The overall aim for this thesis was, using animal models and human studies, to address and test hypotheses for molecular mechanisms underlying the intergenerational transmission of maternal care behaviour, stress and depression.

A literature review was firstly performed in which to explore evidence for possible mechanisms and identify key factors. The revealed several hypothetical mechanisms by maternal depression might be able to increase the risk of developing stress-related behavioural disorders in the offspring. Firstly, changes in endocrine factors important in regulation of the HPA axis, depression and maternal behaviour could be altered in the mother that effect prenatal environment of the offspring in utero or postnatal levels of maternal care or milk it receives. Growth factors and hormones important in metabolism are key to neurodevelopment, changes in which could lead to subsequent changes in behaviour and stress regulation. Immune factors are also crucially linked to stress and changes in inflammatory factors in the mother can impact prenatal development or postnatal environment through changing maternal behaviour or being passed through milk. Epigenetic mechanisms are important for regulating long-term changes in gene expression. Numerous studies have identified epigenetic changes at regulatory regions of genes important for stress regulation either through exposure to stress postnatally,

prenatally, in adolescence of adulthood. Most studies have focused on a few key genes, namely *NR3C1, FKBP5* and *BDNF*. The literature review also highlighted that these genes were similarly regulated in both rodent models and human studies supporting conserved mechanisms. Related to the epigenetic findings, it was also clear that gene expression is regulated following early life stress and though key genes for stress have been investigated it appears that numerous other pathways might be involved. Finally, maternal depression and stress might alter maternal milk composition that the offspring then receives and reacts to. Studies were found that showed key factors important in HPA regulation were found in milk and altered by maternal stress and that changes in these factors in milk can induce behaviour changes in offspring.

Together, the review found strong evidence of key mechanisms that importantly were supported by bother human and rodent findings. It is also clear that these mechanisms also overlap if epigenetic changes link to gene transcription changes that regulate immune or endocrine components that in turn control impact prenatal or postnatal environments important for development (Alyamani and Murgatroyd, 2018).

In *Study 1* an animal model was tested for the generation inheritance of endocrine, immune, epigenetic and gene regulatory changes in three generations of rats in which the mother of the first generation was exposed to stress. This revealed that stress factors important in the HPA axis were increasingly dysregulated. The F0 dams show no changes in corticosterone, the F1 dams show an increase and here we find a decrease. This supports hypotheses that chronic stress exposure over prenatal and postnatal periods can dampen the HPA axis. Hormones involved in maternal behaviour and depression were not affected and neither were factors important in growth and metabolism. Testing a collection of immune factors revealed the presence of one factor, I-CAM1 that became increasingly reduced in the F2 animals in pups and dams. This factor is important in immune regulation and blood brain barrier development suggesting an increasing role of inflammation regulation with the inheritance of the depressed maternal care. Epigenetic changes at *Nr3c1* and *bdnf* were found in the F1 dams but not the F2s that followed gene

expression changes. This suggests that with the inheritance of maternal stress there are shifts in the epigenetic regulation of specific genes.

In sum, *Study 1* demonstrates generational changes in a key stress steroid involved in the HPA axis and a factor important in immune function supporting mechanisms for these in regulating the transmission of maternal stress (Nephew et al., 2017).

In *Study 2*, transcriptome analyses found widespread gene expression changes in the hippocampus of the F1 dams and fewer differentially regulated genes in the F2s. Gene ontology analyses revealed the most genes coded for receptors and calcium binding proteins that are known to be important in stress regulation and have been highly linked dot depression in both rodent and human studies. Interestingly, in the F2 dams, two-thirds of genes differentially expressed were also altered in the F1s, supporting a high degree of inheritance of gene expression changes following stress. This has never been investigated before and suggests key pathways important in the transmission of stress (Alyamani et al. *in preparation for publication*).

This thesis then investigated whether mechanisms found in rodents translated to human studies. In *Study 3*, two human studies of mothers tested for levels of prenatal and postnatal maternal depression and stress were investigated for epigenetic changes in stress genes identified in the literature review. These are the first investigations of methylation at stress-related genes in mothers with prenatal and postnatal maternal depression. In a Latino study of mothers with prenatal and postnatal measures of depression and exposure to stress in the form of perceived discrimination, there were changes in DNA methylation at *NR3C1* following stress but not with depression. *BDNF* methylation also associated with stress while KFBP5 was not associated with stress or depression. Investigation of *NR3C1* in a second study in the UK of mothers measured for depression and anxiety together with their offspring revealed a non-significant trends, negative with prenatal depression and positive with postnatal anxiety supporting again a role of *NR3C1* epigenetic regulation in mothers with perinatal stress and depression. There were no correlations between exposure to prenatal or postnatal depression in infants and *NR3C1* methylation though there were some significant associations between

maternal and infant methylation supporting a level of inheritance of epigenetic marks at the *NR3C1* gene.

Together, results from *Study 3* demonstrate that there are some epigenetic associations with maternal depression at the *NR3C1* gene. This supports some of the findings from the F1 dams in *Study 1* and highlights key conserved me mechanisms in the role of epigenetic regulation at this gene in maternal depression (Santos et al., 2018).

Finally, this thesis explored the hypothesis that key endocrine and immune factors might be altered in milk following stress. In *Study 4*, Measuring factors in milk of dams inheriting depressed maternal care and following stress revealed reductions in corticosterone and oxytocin. This supports that maternal stress and depression can influence endocrine factors in the milk. Some research suggests that infants receiving milk with different levels of cortisol or corticosterone can develop altered stress-related behaviours. The results from *Study 4* suggest that lactocrine programming is another key mechanism important in intergenerational inheritance of stress (Nephew et al., 2017) **(Table 7.1).**

Table 7.1. Overview of stress, endocrine, immune and epigenetic changes across generations together with maternal care in the CSS intergenerational stress model

Mechanisms	F0	F1	F2
Maternal care	d2- , d9 ↓ , d16-	D2 ↓ , d9-, d12-	D2 ↓ ,d9 ↓ , d16↓
Maternal restless	d2- , d9 ↑ , d16-	d2 ↑ , d9-, d12-	d2 ↑ , d9 , d16 ↑
Milk intake	d2 - ,d9 ↓ , d16↓	d2 ↓ , d9-, d12-	d2- , d9-, d12-
HPA regulation, corticosterone, ACTH	-	↑ CORT ↓ ACTH	↓ CORT d23 ↓ ACTH d23
Maternal-related hormone	-	↓ Oxytocin ↓ Prolactin	-

Growth hormones and reproductive hormones	- -	-FSH, -GH -VEGF, -GM-CSF	-
Immune factors	- -	-Direct reduction	↓ Icam-1
Behaviour-related neuropeptides	- -	-α-MSH, -β-Endorphin, -Substance P, -Orexin A -	- -Bdnf
Epigenetics regulation	- -	↓ *Nr3c1* ↓ *Bdnf*	- ↑ *Bdnf*
Endocrine and immune factors in milk	- - -	↑ CORT	↓ CORT - -

Overall, this thesis, investigating a rodent model and translating some results to human studies, together with literature research reveals key evidence and support for the role of neuroendocrine and immune changes, epigenetic alterations driving changes in regulation of stress-related genes and transcriptome alteration and modifications in endocrine factors in breast milk the intergenerational inheritance of stress (**Figure 7.1**).

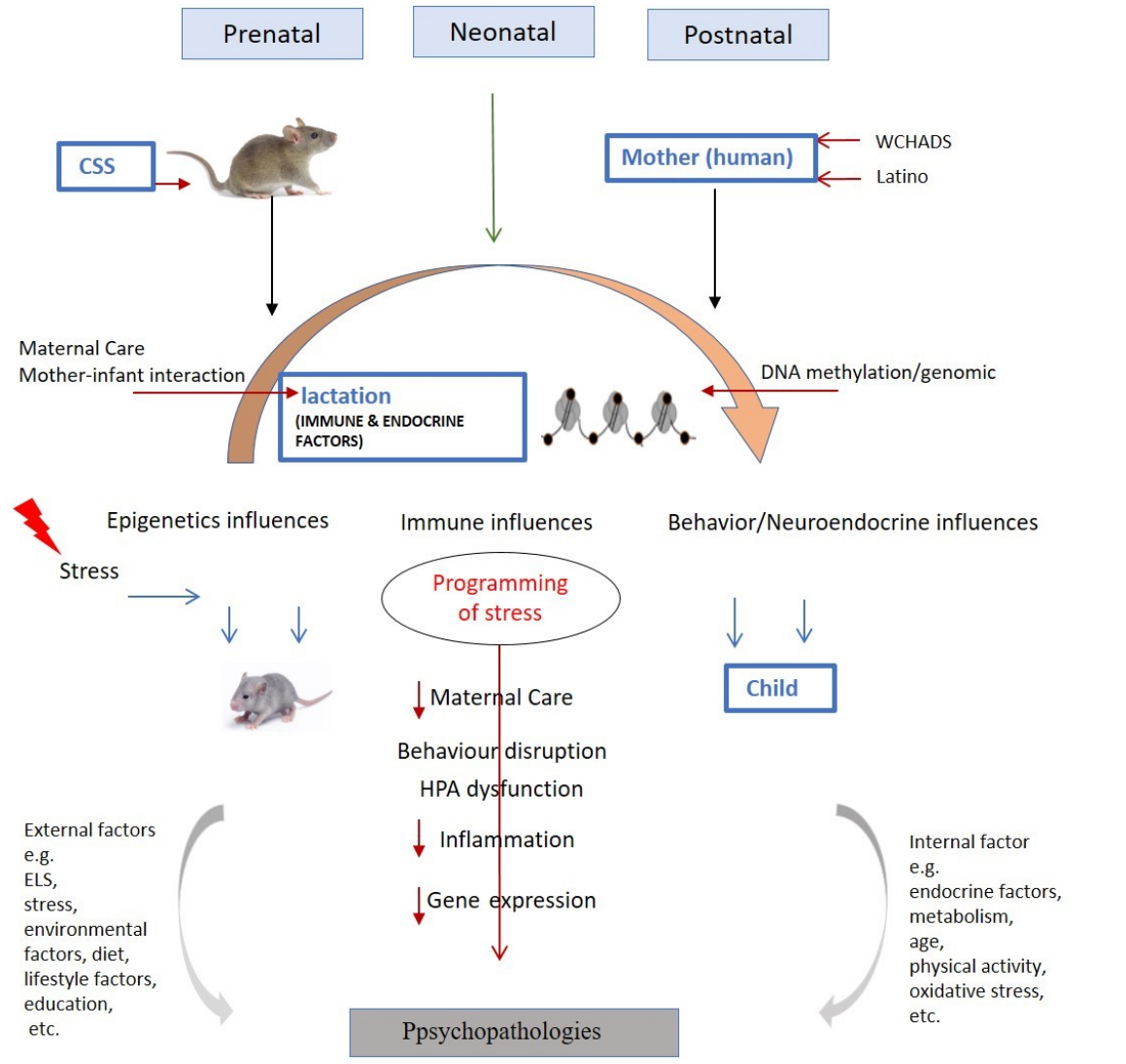

Figure 7.1. Summary of the Investigating Mechanisms Underlying Intergenerational Inheritance of Depression

7.1. Further research

The results of this thesis support the role of endocrine and immune factors, epigenetic, gene regulatory and milk hormones in the transmission of stress from mother to offspring that in turn develop depressed maternal care themselves. These processes serve as mechanisms for intergenerational inheritance of stress.

Some limitations of this research in *Study 1* are that some group numbers, particularly for some of the F1 measures could have been larger. Many of the immune factors measured by multiplex ELISA were too low for the standard curve and so were unable to be quantified. Therefore single-plex could have been used for more of these. However, even that cannot be guaranteed as TNF-alpha for example still did not give results when also used in single-plex. Epigenetic regulation of *Nr3c1* and *bdnf* were only measured in Dam hippocampus, but would be interesting to measure in pups and adults. Also in testing in males would allow to see if the changes were sex-specific. Testing in other tissues such as other hypothalamus, important for the HPA axis would be interesting and also blood cells like in the human analyses of *Study 3*.

Another aspect of the CSS model is that only F2 maternal care was measured. For trans-generational analyses (**1.1**) it would be important to measure if any changes from the CSS were inherited to the F3 generation and perhaps even the F4. From the *Study 1* conclusions, it might be expected that the reductions in maternal care and stress u become even stronger.

In *Study 2* the major limitation is the relatively low number of animals. Though many studies also include similar numbers, the relative variation see in the ELISA support that higher group sizes might increase the power of the findings, either reducing the number of gens found in the F1s or perhaps other related genes. He results from Study 1 show epigenetic changes are important and it would be interesting to test for this genomewide. There are methods such as genomewide bisulphite sequencing, i.e. an extension of the gene-specific method used in *Study 1*, that would allow to see the extent of changes and if particular classes of genes are regulated. If this was done on the hippocampus that one could see how much epigenetic regulation relates to gene expression. It would also allow

to see how much epigenetic marking is inherited through the generations across the genome. If the F0 dams were also tested together with F3 then it would enable to see if a key set of genes are maintained and if those genes shared between F1 and F2 are affected in F0 and passed on to F3.

The human studies in *Study 3* investigated maternal depression in two different cohorts and in one of the cohorts DNA from the infants are studied. This was on blood and saliva DNA as brain material is obviously impossible to work on in maternal depression. The numbers in the groups in the maternal depression is relatively low as some studies have investigated over a thousand, however I assume it can be difficult to recruit many mothers at similar stages of pregnancy in one area that can be similarly measured. As in Study 2, it would be interesting to also look genomewide for expression if the same types of genes identified in the rat model might also differ in humans, such as calcium binding proteins and receptors. Also extending the epigenetic studies genomewide would, give a large picture of any changes as perhaps there are also classes of genes with shared functions that might be epigenetically marked. As child DNA is also available from the WCHADS performing the studies on mother and infant would allow to test for the level of epigenetic inheritance.

In *Study 4* levels of corticosterone and oxytocin were measured. The main limitation of this was the relatively small numbers in groups owing to the fact that many samples did not give readings higher than background. It would be important to increase the sensitivity of this method for measuring milk. Relatively few studies have investigated rat milk. It was planned to perform proteomics on the samples using LC-MS-MS, which has been done on human and cow milk, but not rat. Rat milk has smaller quantities and also more difficult to collect. The results from the ELISA though suggested that the proteomics might not be suitable until methods could be developed to allow larger quantities of milk plasma that could be better detected for proteins. It was also planned to investigate human milk from the Latina study, however proved difficult to organize. Future work would therefore be good to test further factors in milk, if the method could be improved,

and test whether stress and maternal depression impacts milk corticosterone and oxytocin in a similar way to the rats.

Overall, future work would be interested to scale up from these experiments to look at proteomics, transcriptomics and epigeneomics in more generations in animal models and in larger human studies.

7.2. Conclusion

The results of this thesis support the role of molecular mechanisms in the transmission of stress from mother to offspring that also develop depressed maternal care themselves. Serum levels of endocrine factors important in stress regulation and maternal behaviour together with an important immune regulatory factors appear to play important roles. Epigenetic mechanisms regulate key stress-regulatory genes and transcriptome changes in important genes with shared functions are important factors in the brain. Finally, stress and maternal-related hormones in the milk are a further potential mechanism involved in the transmission of stress through generations. Translating studies to humans, it was seen that similar epigenetic changes are also see supporting the importance of animal studies in gaining insights to factors underlying the development of stress-related disorders in humans.

Appendix I

Mean scores for each question of the Everyday Discriminatien scale questionare (EDS)

EDS questions	Mean score at T1 (SD)	Mean score at T2 (SD)
1. You are treated with less courtesy	0,59 (1,08)	0,42 (0,90)
2. You are treated with less respect	0,47 (0,96)	0,30 (0,78)
3. You receive poorer service at restaurants or stores	0,46 (0,93)	0,30 (0,70)
4. People act as if they think you are not smart	0,47 (0,89)	0,22 (0,63)
5. People act as if they are afraid of you	0,09 (0,49)	0,06 (0,31)
6. People act as if they think you are dishonest	0,15 (0,52)	0,09 (0,36)
7. People act as if they are better than you	0,63 (1,15)	0,29 (0,70)
8. You are called names or insulted	0,19 (0,62)	0,11 (0,41)
9. You are threatened or harassed	0,12 (0,95)	0,04 (0,22)
Mean score per question	0,35 (0,84)	0,20 (0,13)
Mean of the total score	3,09 (5,08)	1,83 (3,79)

Appendix II

Publications

Nephew, B., Carini, L., Sallah, S., Cotino, C., Alyamani, R., Pittet, F., Bradburn, S. and Murgatroyd, C. Intergenerational accumulation of impairments in maternal behavior following postnatal social stress. (2017) *Psychoneuroendocrinology*, 82 pp. 98-106.

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