Antenatal Mood Disturbance and Infant Development: Investigating Neurobiological Mechanisms of Risk

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Abstract

Introduction: Maternal antenatal depression is associated with increased risk of adverse offspring outcomes, which manifest in approximately 20% of infants. However, the mechanisms by which risk is transferred from mother to infant, and the factors determining susceptibility to antenatal mood disturbance, remain poorly understood.

Objectives: The primary objectives of this thesis are to investigate whether:

i. Antenatal depression is associated with alterations of the maternal and infant Hypothalamic-Pituitary-Adrenal (HPA) axis.
ii. The infant serotonin transporter genotype (5-HTTLPR) confers susceptibility to antenatal mood disturbance.

Methods: This thesis is an analysis of two different cohorts. First, 103 pregnant women were recruited in Oxford, UK. Participants' self-reported antenatal mood, and salivary cortisol was assessed in response to a stressor and diurnally. 88 participants were visited two months post-birth. Mothers reported postnatal mood and infant temperament. Infant cortisol responses to inoculation were assessed, as was infant DNA methylation. Analysis of this cohort addresses the first objective of this thesis. Next, data from the ALSPAC cohort was analysed to address the second objective. Maternal-reported antenatal mood and infant behaviour up to 7 years was available, as was 5-HTTLPR genotype data for over 4,000 infants.

Results: Antenatal depression was not associated with increased maternal cortisol during pregnancy. Neither antenatal depression nor cortisol was associated with infant cortisol reactivity or temperament. Antenatal depression predicted increased NR3C1 DNA methylation in males, and decreased BDNF DNA methylation in male and female infants. Infant 5-HTTLPR genotype did not moderate associations between antenatal mood disturbance and behavioural difficulties.

Conclusions: This thesis does not support the theory that antenatal depression exerts influence on infant development via increased activity of the maternal and infant HPA axis; however, changes in infant DNA methylation may be a mediating mechanism. Further, susceptibility to antenatal mood may be more complex than previously thought.

Table of Contents

Table of Tables

Table of Figures

List of Abbreviations

11BHSD2	11 beta hydroxyl steroid dehydrogenase 2
5-HT	Serotonin
5-HTTLPR	Serotonin transporter polymorphism
ACTH	Adrenocorticotrophic hormone
ALSPAC	Avon Longitudinal Study of Parents and Children
ANOVA	Analysis of variance
ANS	Autonomic nervous system
AUC	Area under the curve
BDNF	Gene encoding the Brain-derived neurotropic factor protein
BSCT	Biological sensitivity to context
CAR	Cortisol awakening response
CNS	Central nervous system
CpG	Region of DNA where a cytosine nucleotide appears next to a guanine nucleotide
CREB	Cyclic-AMP response element binding
CRH	Corticotrophin-releasing hormone
dB	Decibels
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DST	Differential susceptibility theory
EPDS	Edinburgh Postnatal Depression Scale
FHR	Fetal heart rate
GR	Glucocorticoid receptor
GxE	Gene by environment
HPA	Hypothalamic-Pituitary-Adrenal
IBQ	Infant Behaviour Questionnaire
ITQ	Infant Temperament Questionnaire
IVF	In vitro fertilisation
log AUC	Log of the area under the curve
mRNA	Messenger ribonucleic acid
NGF-1A	Nerve growth factor 1A
nmol/L	Nano mol per litre
NR3C1	Gene encoding the glucocorticoid receptor
PCR	Polymerase chain reaction
PFC	Prefrontal cortex
PSS	Perceived Stress Scale
sAA	Salivary alpha-amylase
SAM	Sympathetic-adrenal-medulla
SD	Standard deviation
SDQ	Strengths and Difficulties Questionnaire
SLC6A4	Gene encoding the serotonin transporter (Solute carrier family 6, member 4)

SLE	Significant life event
SSRI	Selective Serotonin reuptake inhibitor
STAI	Spielberger State-Trait Anxiety Index
TSST	Trier Social Stress Test
VAS	Vibro-acoustic stimulus
VAS	Visual analogue scale
μL	Microliter

Overview of Thesis

Antenatal mood disturbance, which encompasses feelings of depression, anxiety and perceived stress, increases risk for a number of adverse offspring outcomes, including: poor birth outcomes, behavioural difficulties in childhood, and psychiatric disease in later life. Intriguingly, recent research has highlighted that such associations may be, in part, independent of postnatal mood and shared risk genes between mother and infant. This suggests that i*n utero* biological mechanisms may, to some extent, mediate this association. However, current understanding of potential underlying mechanisms is limited. A prevailing theory in perinatal psychiatry is that such associations may be mediated via increased activity of both the maternal and fetal HPA axis in pregnancy, and such changes in offspring HPA function increase risk for later outcomes. However, evidence linking antenatal mood disturbance with altered maternal and infant HPA function is mixed, and generally limited to diurnal measures. Thus, the primary aim of this thesis was to further characterise associations between maternal antenatal depressive symptoms, and both maternal antenatal and infant HPA function. A further intriguing and repeatedly replicated aspect of the association between antenatal mood disturbance and infant development is that only approximately 20% of exposed infants manifest the adverse outcomes associated with antenatal mood disturbance. However, there is a paucity of understanding as to what factors may confer susceptibility, or indeed resistance to, the effects of antenatal mood. Thus, a secondary aim of this thesis

was to investigate whether infant 5-HTTLPR genotype may confer such susceptibility to antenatal mood disturbance.

Chapter One presents a review of the existing literature, which has identified associations between antenatal mood disturbance and adverse offspring outcomes. Current understanding of underlying biological mechanisms and susceptibility to early environmental influences is outlined.

Chapters, Two, Three and Four present data collected as part of a short-term longitudinal study in Oxford, UK, during 2013-14. 103 pregnant participants were recruited to the study, all were primiparous, had a singleton pregnancy, were not using steroid-based medications and had no medical complications associated with their pregnancy. Participants self-reported levels of antenatal mood during a test session, and were also exposed to an infant distress stimulus. Salivary cortisol and alpha-amylase responses to the stressor were measured (N=103), as were diurnal salivary biomarkers over two days following the test session (n=90). Participants were visited at home approximately two months after birth, and reported postnatal mood and infant temperament (n=88). Infant salivary cortisol responses to inoculation were assessed (n=74), and buccal swabs for DNA methylation analysis were obtained (n=57).

Chapter Two investigated the effects of antenatal depressive-symptoms on maternal salivary cortisol reactivity to a stressor during pregnancy, as well as diurnal salivary cortisol release. Saliva samples were also assayed for alpha-amylase, to address the question of whether effects of antenatal depression on maternal physiology are HPA-specific. There were no effects of antenatal depression on salivary cortisol and alpha-

amylase responses to the stressor. Similarly, there was no effect of antenatal depression on diurnal salivary cortisol. However, participants in late pregnancy with symptoms of depression had higher diurnal measures of salivary alpha-amylase.

Chapter Three was a follow-up of the infants born to the mothers assessed in Chapter Two. The aim of this chapter was to investigate whether maternal antenatal depression had implications for infant salivary cortisol reactivity or temperament. No associations were found between maternal antenatal depression and the examined infant outcomes. Similarly, maternal cortisol reactivity in pregnancy did not predict infant cortisol reactivity. However there was some evidence to suggest that maternal salivary cortisol and alpha-amylase predicted some aspects of temperament. It has previously been suggested the antenatal biological markers of stress may be better predictors of infant outcomes than self-reported measures of mood. This study provides the first evidence in support of this theory.

One potential mechanism by which antenatal mood disturbance may influence infant development is via changes in DNA methylation. DNA samples obtained from the infants described in Chapter Three were used to test this hypothesis in Chapter Four. The aim of this chapter was to investigate the effects of antenatal depression on infant NR3C1 1F and BDNF IV DNA methylation. This study identified that male infants exposed to antenatal depression have increased methylation of the NR3C1 1F promoter region, in line with previous animal and human findings. However, this study also identified a trend to suggest that this association is in the opposite direction for female infants: those exposed to antenatal depression had decreased DNA methylation of this region. This is

the first study to report that the effects of antenatal depression on infant NR3C1 1F methylation may be sexually dimorphic. This is also the first study to report associations between maternal antenatal depression and infant BDNF IV DNA methylation; those exposed infants had decreased methylation of the assessed region.

The second objective of this thesis was to investigate why some infants may be more susceptible to the effect of maternal antenatal mood disturbance than others. A previous study reported that infants homozygous for the short allele of the serotonin transporter were more likely to have negative emotionality at 6 months following antenatal anxiety exposure than those homozygous for the long allele. The aim of Chapter Five was to replicate this finding in a large cohort (ALSPAC), and to investigate whether such effects persisted past infancy and into childhood. However, there was no evidence to suggest that 5-HTTLPR moderated the effects of antenatal depression and anxiety on infant temperament at 6 months, and later behavioural difficulties in childhood. This well-powered direct non-replication highlights the need for large sample sizes in gene-environment interaction studies.

Finally, Chapter Six presents a summary of the results and a discussion of the implications of this research for our understanding of how antenatal mood disturbance impacts on fetal and infant development. This chapter also addresses the methodological considerations that must be taken into account in interpreting these findings. Future directions for research of the impact of maternal antenatal mood on infant development are also discussed.

CHAPTER 1

BACKGROUND

1.1 Introduction

> *'When we hold our babies for the first time, we imagine them clean and new, unmarked by life, when in fact they have already been shaped by the world, and by us. It's a koan of parenthood, one worthy of long contemplation: We are meeting someone we know well for the very first time.'*
>
> *–Annie Murphy Paul, Origins*

Maternal mood disturbance during the perinatal period, which encompasses feelings of depression, anxiety and stress, has clear implications for adverse infant development. It has been well characterised and is widely accepted that maternal postnatal depression poses a significant risk for the child. This is an understandable association, given that symptoms of depression include attention deficits, lapses of concentration and fatigue. It is likely that such symptoms would interfere with a woman's ability to optimally care for her infant. Indeed, a wealth of literature has linked postnatal depression with decreases in maternal sensitivity and responsiveness towards the infant, as well as compromised parenting practices, such as feeding, sleep routines, and vaccinations (Field, 2010).

However, it has become increasingly evident over the last decade that maternal mood disturbance *during* pregnancy also poses a significant risk to fetal and infant development. This risk is indexed by increased rates of preterm birth and low birth weight babies (Wadhwa et al., 1993, Omer et al., 1986), increased behavioural and emotional problems in childhood (O'Connor et al., 2002a), and increased incidence of adolescent psychiatric disease (Rice et al., 2010, Pearson et al., 2013) in the offspring of women with antenatal mood disturbance. This increased risk of adverse development

appears to be independent of postnatal mood (O'Connor et al., 2002a), and shared genetic factors between mother and infant (Rice et al., 2010), suggesting that there is a specific and independent adverse impact of mood disturbance during pregnancy on fetal and infant development.

Given that depression during the antenatal period has been associated with a number of adverse infant outcomes, it is particularly concerning that the prevalence of antenatal depression has been estimated as between 6% and 20%, which is significantly higher than the estimated national average of 6% (Leung and Kaplan, 2009, Gavin et al., 2005, Gaynes et al., 2005). This is perhaps because depression onset is most commonly seen between the ages of 20 and 40, when women are of childbearing age (Marcus and Heringhausen, 2009). An alternative explanation is that pregnancy is a time of increased stress, hormonal change and sleep disturbance, all of which may contribute to increased prevalence of depression. Symptoms of depression during the antenatal period are typical of those seen in the whole population, and include persistent low mood, with associated cognitive, emotional and physical symptoms, such as low self-esteem, disturbed sleep and appetite. However, measures of antenatal depression do not typically assess somatic symptoms of depression, such as disturbed sleep, appetite, and fatigue, since these are often altered during pregnancy regardless of depressive state.

Although this thesis is focused on maternal antenatal depression, the field of perinatal psychiatry often uses the terms ‘depression’, ‘anxiety’ and ‘stress’ interchangeably to reflect disturbances in maternal antenatal mood. This is largely because of the high

degree of comorbidity between depression, anxiety and perceived stress, but also because of the common mechanisms hypothesised to transfer risk from mother to child.

The first section of this chapter is focused on the considerable body of evidence, which has reported significant associations between maternal antenatal mood disturbance and offspring development, including: birth outcomes, behavioural problems in childhood and psychiatric disorder in later life. The second part considers potential pathways by which risk may be transferred from antenatal mood disturbance to the developing fetus, such as shared risk genes, environmental factors and *in utero* biological mechanisms. The final part of the chapter is a discussion of why some infants maybe more susceptible to the effects of antenatal mood disturbance than others, with a particular focus on the 5-HTTLPR polymorphism as a potential susceptibility genotype.

1.1.1 Antenatal mood disturbance and fetal neurobehaviour

Given that antenatal mood disturbance has clear implications for fetal development, one could hypothesise that significant differences between offspring of mothers with and without mood disturbance could be detected very early during development, even as early as *in utero*. Although there are a number of challenges associated with assessing fetal neurobehaviour, it is possible to measure fetal heart rate (FHR), FHR coupling and fetal movement, both at baseline and in response to stimulation. Such measures provide useful information regarding fetal neurodevelopment, but have also been suggested to predict later outcomes, including size at birth (Sandman et al., 2011) and temperament (DiPietro et al., 2008). Existing evidence suggests that the fetuses of mothers with mood

disturbance show altered FHR reactivity. For example, fetuses of anxious mothers have shown increased heart rate responses when their mothers were exposed to a stressful laboratory task (Monk et al., 2000).

An alternative approach to measuring fetal reactivity is by directly applying a novel stimulus (vibro-acoustic stimulus, VAS) to the fetus. Using this approach, it has been documented that fetuses of depressed mothers have higher baseline FHR, slower FHR reaction to a VAS, and a longer recovery period, compared with controls (Allister et al., 2001). There is also evidence that fetuses of depressed and anxious mothers habituate more quickly to a VAS (Dieter et al., 2008). Intriguingly, a study based in a very poor area of rural South India reported a U-shaped relationship between maternal depressive symptoms and FHR responses to a VAS (Fernandes et al., 2014b). That is, fetuses of mothers with both very high and very low levels of depression showed the greatest FHR responses. From these findings it was suggested that in stressful environments, moderate levels of maternal antenatal stress might be required for the optimal development of fetal stress responses. However, this finding is yet to be replicated, and it is currently unknown whether there may be a similar U-shaped relationship between maternal depression and FHR reactivity in a developed country, where the levels and type of stress experienced by women during pregnancy are very different to that in a very poor area of southern India.

There is also evidence to suggest that fetuses of depressed mothers are more active. Dieter et al. (2008) demonstrated increased fetal movements in a group of depressed pregnant women, and the results were strengthened when symptoms of maternal

anxiety were included concurrently in the analyses. Similarly, measures of maternal stress have been associated with increased fetal movements at 24, 30 and 36 weeks of gestation (DiPietro et al., 2002). A very recent study found that fetuses of mothers with negative mood had an advanced neurobehavioral profile (Doyle et al., under review). This advanced neurobehavioral profile was indexed by a lower FHR and a flatter increase in coupling, which is a characteristic of later gestation, compared with control fetuses of the same age. This finding supports the theory that maternal psychological distress during pregnancy increases risk for preterm delivery (Dole et al., 2003), and therefore foetuses of stressed mothers may undergo advanced or rapid development (Pike, 2005, Worthman and Kuzara, 2005).

Thus, antenatal mood disturbance is associated with changes in FHR responses to stimulation and increased fetal movement. As such, it is possible that assessments of fetal neurobehaviour could be used as an early screening tool to detect developmental alterations as a result of exposure to maternal mood disturbance.

1.1.2 Antenatal mood disturbance and birth outcomes

Antenatal mood disturbance is also associated with increased risk of adverse birth outcomes, such as low birth weight and preterm birth. Increased risk for such outcomes is particularly pertinent, because low birth weight and preterm birth are themselves risk factors for later adult disease, such as diabetes, obesity and cardiovascular disease.

Often, a measure of life events during pregnancy is used to index maternal psychological distress during the antenatal period. Indeed, a number of studies have reported

significant associations between major life events during pregnancy and increased risk for preterm birth (Gunter, 1963, Newton and Hunt, 1984, Berkowitz and Kasl, 1983, Mutale et al., 1991, Zhu et al., 2010, Newton et al., 1979) and low birth weight (Newton and Hunt, 1984, Reeb et al., 1987). However non-replications within the literature are evident (Omer et al., 1986, Hedegaard et al., 1996, Stein et al., 1987), and may be attributable to differences in methodology. For example, the above studies vary greatly in sample size (range n=40 to n=5972), participant ethnicity (including African-America, Caucasian, Hispanic and Chinese), point in gestation when life events were measured (range <19 weeks to term) and the measure of life events used (Major Life Events Inventory, Social Re-adjustment Rating Scale, Life Events and Difficulties Scale, the General Health Questionnaire, and self-reported experiences of recent life events).

High self-reported levels of perceived stress during pregnancy have also been reported to increase risk for preterm birth (Nkansah-Amankra et al., 2010, Class et al., 2011, Hedegaard et al., 1996, Pritchard and Teo, 1994, Copper et al., 1996), low birth weight (Sable and Wilkinson, 2000, Zhu et al., 2010, Nkansah-Amankra et al., 2010, Class et al., 2011, Copper et al., 1996) and delivery complications (Norbeck and Tilden, 1983, Da Costa et al., 2000). Fewer studies have specifically reported on maternal symptoms of antenatal depression/anxiety and adverse birth outcomes, and here the findings appear to be more variable (Wadhwa et al., 1993, Newton and Hunt, 1984, Berle et al., 2005, Omer et al., 1986, Dayan et al., 2006). For example, in a sample of 90 women assessed during the third trimester, Wadhawa and colleagues (1993) reported a significant association between antenatal depression/anxiety and low birth weight and gestational age at birth. In a similar study of 113 pregnant women, Omer and colleagues (1986)

reported a significant association between antenatal depression/anxiety and preterm birth. This link between antenatal mood disturbance and preterm birth is strengthened further by the findings from a very large cohort study of 5872 Danish women, which found a strong association between prenatal psychological distress measured at the 30th week of gestation and preterm birth (Hedegaard et al., 1996). However, once again there are some failures to replicate this association (Newton and Hunt, 1984, Berle et al., 2005), though neither of these studies have a sample size to rival that of the study by Hedegaard and colleagues (1996) (n=224 and n=680 respectively). Interestingly, in a large sample of 681 French women, Dayan and colleagues (2006) reported that antenatal depression, but not anxiety, was associated with preterm birth. Therefore, although these initial findings require replication in another large cohort, it is possible that differing effects of antenatal depression and anxiety on birth outcomes could explain discrepancies in the existing literature.

Thus, a plethora of research has identified significant associations between antenatal mood disturbance and adverse birth outcomes such as low birth weight and premature birth. However, there are discrepancies in the findings, which may be attributable to the large degree of variation in methodologies employed by these studies. A further explanation may be the differing effects of maternal mood symptoms (e.g. anxiety vs. depression) on fetal development, although this requires further investigation.

1.1.3 Antenatal mood disturbance and later offspring outcomes

As well as proximal effects of maternal antenatal mood disturbance on fetal neurodevelopment and birth outcomes, it is particularly striking that adverse effects of such mood disturbance may persist beyond birth and into later life. Indeed, a growing body of literature suggests that antenatal mood disturbance has implications for adverse offspring outcomes in infancy, childhood, and adolescence.

Infants of prenatally depressed mothers have been reported to have more difficult temperaments (McGrath et al., 2008, Huot et al., 2004) and also to cry more frequently (Milgrom et al., 1995, van der Wal et al., 2007) than control infants. Antenatal depression has also been associated with developmental delays in children at 18 months of age (Deave et al., 2008), and also with externalising and internalising problems up to 4 years of age (de Bruijn et al., 2009). Interestingly, Bruijin *et al* (2009) reported that whilst antenatal depression in the first trimester was associated with internalising problems for males, third trimester depression was linked with both internalising and externalising behaviours in females. These findings have been partly replicated in a different cohort; maternal depression during the third trimester predicted externalising, but not internalising, behaviours at 4-5 and 8-9 years of age (Luoma et al., 2004). Exposure to antenatal depression and anxiety has also been associated with behavioural and emotional problems in childhood (O'Connor et al., 2002a), further strengthening the idea that antenatal mood disturbance impacts negatively on childhood outcomes.

Two studies have reported associations between antenatal stress and anxiety, and increased risk for offspring attention deficit hyperactivity disorder (ADHD) (Van den

Bergh and Marcoen, 2004a, Grizenko et al., 2012). In a study of 72 mothers and their first-born infants, antenatal anxiety during early gestation significantly predicted ADHD symptoms, as well as externalising problems, in 8 and 9-year olds. In a larger retrospective study, with a sample of 71 children with ADHD and their siblings (with no ADHD symptoms), a logistic regression model indicated that mothers were more likely to have experienced high stress during the pregnancy of their ADHD child than their other child (Grizenko et al., 2012).

Further, antenatal mood disturbance has also been reported to increase risk for adolescent depression (Van den Bergh et al., 2008a, Pearson et al., 2013, Pawlby et al., 2009), and antisocial behaviour (Hay et al., 2010). Van den Bergh and colleagues (2008) reported that antenatal anxiety was associated with a flattened daily cortisol profile in 14 and 15-year old offspring, and in females only this was associated with depressive symptoms (n=58). Although perhaps limited by a small sample size, this was the first study to identify that adolescents exposed to antenatal mood disturbance had an unregulated Hypothalamic Pituitary Adrenal (HPA) axis, a common characteristic of depressive disorders. Similarly, in a sample of 127 16-year olds, risk of developing depression was 4.7 times greater for those exposed to antenatal depression than controls (Pawlby et al., 2009). Further supporting evidence comes from a much larger cohort study of over 4,500 mothers and their adolescent offspring (Pearson et al., 2013). In this study, Pearson and colleagues demonstrated that antenatal depression was an independent risk factor for offspring adolescent depression: adolescents were 1.28 times more likely to have depression for each SD increase in maternal antenatal depression. Intriguingly, these effects appeared to be independent of maternal postnatal depression.

Similarly, antenatal depression has been shown to predict violence in adolescents independently of postnatal parental mood (n=120) (Hay et al., 2010).

Thus, accumulating evidence suggests that maternal antenatal mood disturbance has implications for adverse offspring outcomes, which extend into the postnatal period. Indeed, some effects appear to persist into adolescence. However, studies that have followed up offspring into adolescence and adulthood, where the incidence of psychopathology greatly increases, are limited. This is perhaps because the notion that antenatal mood disturbance could potentially have such long lasting effects on offspring outcomes is in its infancy, and few longitudinal studies have yet to reach the stage where the offspring are adolescents/adults. However, on-going and future research will begin to comprehensively document these associations.

1.1.4 Antenatal mood disturbance and offspring development – a summary

The literature summarised above suggests that antenatal mood disturbance has implications for adverse offspring outcomes, which range from disturbances in fetal neurodevelopment, to enduring outcomes such as increased risk for psychopathology in adolescence. However, a number of questions remain regarding these associations. Firstly, the current literature tends to use the terms 'depression', 'anxiety' and 'stress' interchangeably. Therefore, it is difficult to determine whether different psychological symptoms have different implications for offspring outcomes. In order to tease apart the differing effects of these disorders, which often have great overlap in symptomatology and physiology, very large sample sizes are required.

Secondly, the majority of research that has considered antenatal mood disturbance and birth outcomes has often used a measure of major life events to index maternal stress. However, there are a number of problems with using life events to measure maternal stress in this way. For example, such measures are often recorded retrospectively, which introduces problems of misreporting. Further, animal studies have shown that the timing of a stressor during gestation is important for offspring outcomes. For example, rodent studies have shown that prenatal stress in early gestation leads to behavioural impairments and reduced locomotion in offspring (Schneider et al., 2002), whereas exposure to stress in late gestation results in increased offspring exploration (Meek et al., 2000). Further, chronic stress throughout gestation has been associated with offspring anxiety behaviours and exaggerated stress responses (Richardson et al., 2006). Thus, if a woman experiences a significant life event during pregnancy, and is therefore considered 'stressed', it is important to take the timing of the event into account; however many studies do not currently do this. Further, the grouping of women who have experienced life events during pregnancy with women who experience chronic anxiety or depression throughout pregnancy into a single group may not be appropriate.

A further issue with the existing literature is that the majority of studies are observational, and therefore questions remain regarding causality. However, experimental manipulations to induce prenatal stress in humans are clearly unethical and unfeasible. In this instance, animal models become invaluable in determining causality and in attempting to understand underlying mechanisms. However, there are also limitations with translating animal models of prenatal stress to human mood disorder. For example, it is difficult to model chronic feelings of anxiety and depression

using a prenatal stress paradigm in animals, and the developmental windows during gestation differ between humans and rodents.

Thus, it is clear that antenatal mood disturbance has potential implications for adverse offspring outcomes, but further research is required to fully understand the intergenerational transmission of risk. Understanding the pathways by which antenatal mood disturbance impacts on offspring development is a critical step towards developing targeted early interventions, aimed at reducing the transmission of risk and potentially preventing a range of adverse infant outcomes.

1.2 Pathways of risk transmission from antenatal depression to infant development

The idea that risk could be transmitted from mother to fetus during the antenatal period hailed from the pioneering works of David Barker and colleagues. In 1989, Barker reported an association between low birth weight (LBW) and cardiovascular disease and diabetes, such that infants born with LBW were more likely to develop cardiovascular disease and diabetes in adulthood (Barker et al., 1989, Barker, 1999). Although the underlying cause of the LBW was unclear, it was evident that fetal growth within the womb had been compromised, and as a result offspring were at increased risk of disease in adult life. This research highlighted, for the first time, that *in utero* environmental factors could have implications for adult health and disease. Until this time it was widely assumed that a fetus within the intra-uterine environment was protected from outside influences.

Since the initial findings by Barker and colleagues, a number of other environmental influences, such as malnutrition, exposure to toxins, and maternal mood disturbance during pregnancy have also been shown to have implications for fetal development (O'Connor et al., 2003, Talge et al., 2007, Brown et al., 2000, Stein et al., 2009, Van den Bergh et al., 2008a, Barr et al., 2006, Fergusson et al., 1998). However, there are a number of significant challenges in attempting to understand the pathways by which risk is transferred from the antenatal environment to offspring development. First, it is extremely difficult to disentangle the individual effects of shared genes between mother

and infant. Second, the continuation of some environmental exposures into the postnatal period makes the separation of prenatal and postnatal exposures difficult. Finally, research disaggregating the remaining biological mechanisms occurring *in utero* that mediate these effects is in its infancy.

This section begins with a consideration of the fetal programming hypothesis, followed by a discussion of the pathways by which risk could be transferred from maternal antenatal mood disturbance to fetal development. The main focus of this section is the potential biological *in utero* mechanisms that may mediate this risk, with particular attention to changes in the maternal and fetal HPA axis as one potential mediating pathway.

1.2.1 The fetal programming hypothesis

The original work of Barker and colleagues (Barker et al., 1989, Barker, 1999), which suggests that a sub-optimal *in utero* environment results in perturbed fetal development, and increased risk of disease in later life, led to the formation of the 'fetal programming hypothesis'. This theory attempts to explain why a fetus may adapt to changes in the *in utero* environment in such a way that results in increased risk for disease later in life. It proposes that the developing fetus adapts to maternal cues within the womb, and that this adaptation has the aim of ultimately increasing the chance of offspring survival *in the short term*. For example, in the case of LBW, this may be the result of the direction of energy resources away from the body and towards the fetal central nervous system to ensure adequate brain development in situations where there is a shortage of available

nutrients during development. Also, fetal metabolism may be altered so that fewer nutrients are sufficient for survival. While such adaptations are beneficial for fetal survival in the short term, they come at a cost; in this case other organs, such as the heart, will be insufficiently developed at birth, and also metabolism may be permanently altered. While in the short term this does not have implications for offspring health, in adulthood offspring are more likely to have cardiovascular disease and diabetes as a result of these adaptations. Thus, short-term adaptations to maternal cues that are essential for survival may pose a risk to health in later life.

Recently, the fetal programming hypothesis has been expanded to explain, in evolutionary terms, how maternal antenatal stress, depression or anxiety may result in adverse offspring outcomes (Talge et al., 2007, Glover, 2011). It has been suggested that high levels of maternal stress during pregnancy may reflect environmental pressures such as competition for food or conflict with rival groups. As such, the fetus responds to maternal cues that the postnatal environment will be one of adversity, and adapts in a way that would be beneficial for survival. For example, the child may be more likely to be aggressive and have a rapidly distracted attention (Glover, 2011, Talge et al., 2007), which could benefit survival in such a high-stress environment. However, in modern-day developed countries, although mothers experience high levels of stress in pregnancy, it is unlikely that a child will be exposed to such extreme environmental pressures as competition for food and rival groups. Thus, the result is a 'miss-match' between fetal adaptations and the environment, in which case such adaptations become maladaptive. This theory has been used to explain the increased incidence of ADHD symptoms, emotional problems and aggressive behaviour seen in children of prenatally stressed

mothers (Glover, 2011). However, an alternative explanation could be that these symptoms are the price paid for a more reactive HPA axis, which might be necessary for short-term adaptation but becomes maladaptive in later life.

1.2.2 Problems with teasing apart genetic and environmental effects

1.2.2.1 Shared risk genes between mother and infant

The fetal programming hypothesis provides a convincing argument as to why risk may be transferred from maternal mood to fetal development. However, understanding how such risk is transmitted poses a significant challenge to the field of perinatal psychiatry[1]. One possible explanation is via shared risk genes between mother and infant.

Mood disorders, such as depression, have a genetic component (Levinson, 2006); from twin studies it is estimated that heritability of Major Depressive Disorder is 0.33 (95%CI 0.26-0.39) (Sullivan et al., 2000). As such, a number of genotypes have been reported to increase risk or susceptibility to depression, such as the serotonin transporter polymorphism 5-HTTLPR (Caspi et al., 2003), and Val66Met, a single nucleotide polymorphism in the Brain-derived Neurotrophic Factor (BDNF) gene (Ribeiro et al., 2007). However, findings have been inconsistent (Munafo et al., 2009, Risch et al., 2009). A more recent approach to identifying genetic variants associated with depression is via

[1] Some of these issues are described in a briefer format in a review paper written as part of my D.Phil studies (Braithwaite EC, Murphy SE, Ramchandani PG (2014) Prenatal risk factors for offspring depression: A critical review of the evidence and potential mechanisms. *The Journal of Developmental Origins of Health and Disease,* 19:1-12)

genome-wide association studies. Several single nucleotide polymorphisms have reached genome-wide significance in such studies (Hek et al., 2013, Lewis et al., 2010, Wray et al., 2012), however it has been estimated that a very large sample size, upwards of 50,000 cases, would be required to detect specific polymorphisms which cause depression (Hek et al., 2013).

In a very elegant study, Rice *et al.* (2010) capitalized on the use of *in vitro* fertilization (IVF) to design a 'prenatal cross-fostering' study in which pregnant mothers were genetically related or unrelated to their child, in order to disentangle maternally inherited and environmental influences on offspring (Rice et al., 2010). They examined 574 mother-related dyads and 205 mother-unrelated dyads, and found that associations between self-reported stress in pregnancy and offspring birth weight, gestational age and antisocial behaviour were evident in both the mother-related ($\beta=0.207$, $p<0.001$) and mother-unrelated pairs ($\beta=0.211$, $p<0.01$). This is consistent with the idea that psychological stress in pregnancy is an important environmental influence and has implications for offspring outcomes, regardless of shared risk genes between mother and infant. . However, different patterns emerged for associations between antenatal stress and other infant outcomes, such as anxiety symptoms, which appeared to be more genetically driven. In particular, infant symptoms of ADHD were only related to antenatal stress in the mother-related pairs, highlighting that these symptoms may be genetically, rather than environmentally, driven. Interestingly, this finding opposes the theory put forward by Glover (2011), which suggests that infant symptoms of ADHD may be an adaptive response to an adverse *in utero* environment.

However, it is important to note that all the data collected in this study was via postal questionnaires, and neither researchers nor clinicians assessed the mothers and infants. Thus, this data is subject to both mis-reporting and reporter-bias, which could potentially lead to greater associations between variables. A further consideration is that as the participants were undergoing IVF treatment, it is possible that the demographic characteristics of the participants in this study vary compared with parents who conceive naturally. Thus, these findings may not be generalizable to the whole population. Further, it is unknown whether the process of *in vitro* fertilisation induces increased levels of oxidative stress within the cell environment, which may have implications for offspring outcomes regardless of the in utero environment.

Nonetheless, this study offers useful insights into the independent roles of genes and environment in moderating the effects of antenatal mood disturbance on offspring outcomes. These findings highlight that, although shared genes are important in this relationship, there is more to the association between antenatal mood disturbance and adverse offspring outcomes than genetics alone can account for, and therefore the antenatal environment plays a significant role. However, given the limitations associated with the Rice et al. (2010) study, replication is required.

1.2.2.2 Continuation of environmental exposures from the pre- to postnatal period

A further challenge is to dissociate the independent effects of the antenatal and postnatal environment. Often, adverse environmental influences are present during both the

antenatal and postnatal period. For example, antenatal depression is the main risk factor for postnatal depression (Leigh and Milgrom, 2008), and women who smoke whilst pregnant are also likely to smoke during the postnatal period (Cornelius et al., 2012). Further, there is evidence from the animal literature to suggest that antenatal stress has implications for postnatal maternal behaviour, which potentially increases risk for adverse offspring outcomes (Champagne and Meaney, 2006). Thus, it is difficult to discern whether it is the antenatal or postnatal exposure, or both, which poses risk to offspring development.

Another challenge is to understand whether prenatal and postnatal exposures may exert different influences on offspring development. To some extent Rice *et al.* (2010) addressed this question within the IVF study mentioned previously, and found that maternal postnatal stress increased risk for offspring anxiety, whereas prenatal stress presented risk for antisocial behaviour (Rice et al., 2010). Often, such genetically informed study designs are not feasible, and a more powerful method of testing causal effects is through experimental methods; however, there are clear limitations to the use of experimental manipulations of stress in pregnant women. One approach, which has been used to infer causality from prenatal risk factors, is Mendelian randomization. Using this method, genetic variants known to be reliably associated with a modifiable exposure are used to make inferences about those exposures and disease risk (Lewis et al., 2013). An alternative approach is via the use of large cohort studies, where it is possible to statistically partial out the effects of the postnatal environment in order to study independent antenatal effects. O'Connor and colleagues used this method in 2002. They found that maternal antenatal anxiety predicted child emotional and behavioural

problems, and this association remained significant after controlling for postnatal anxiety (O'Connor et al., 2002a). Pearson *et al.* took the same approach when analysing data from the ALSPAC cohort, and found that antenatal depression, independently from postnatal mood, was a significant risk factor for offspring adolescent depression (Pearson et al., 2013). However, it is important to note that other environmental influences during the postnatal period may have impacts on child outcomes, and these effects are not taken into account when controlling for maternal postnatal depression alone. For example, exposure paternal depression during the antenatal and postnatal period has been shown to increase risk for conduct problems and psychopathology in childhood (Ramchandani et al., 2008), however the majority of existing studies have not controlled for postnatal depression. Further, there is evidence to suggest that the effect of maternal postnatal depression on adverse infant outcomes is moderated by the mother's sensitivity towards the infant (Kaplan et al., 2008); such that the adverse outcomes associated with postnatal depression only manifest in infants of mothers with low levels of sensitivity.

Thus, existing evidence suggests that adverse environmental influences during the antenatal period may have implications for altered fetal and infant development. In some cases these effects appear to be independent of the postnatal environment, however often postnatal environmental effects are not sufficiently controlled for. Therefore future studies should extensively assess the postnatal environment, and take this into consideration in the statistical analyses.

1.2.3 Biological mechanisms

The evidence presented above suggests that antenatal mood disturbance increases risk for adverse offspring outcomes, in part, independently of postnatal environmental influences and shared genetics. Therefore, it is likely that biological mechanisms that occur *in utero* mediate at least part of the association between antenatal mood disorder and offspring outcomes. A number of potential mechanisms have been proposed to explain this association, including: increased maternal noradrenaline which may cause vasoconstriction and reduce fetal blood flow, immunological mechanisms which increase maternal inflammation, and changes in maternal HPA function so that the fetus is exposed to high levels of maternal stress hormones. Of course, it is likely that a combination of mechanisms play a role, and that the implicated systems interact with one another; however there is a paucity of research here.

1.2.3.1 Increased maternal sympathetic nervous system activation

Psychological distress activates the sympathetic nervous system (SNS), which results in an increase in the circulating levels of noradrenaline. Noradrenaline does not directly cross the placenta (Giannakoulopoulos et al., 1999); however it is possible that its release could indirectly affect the fetus by initiating vasoconstriction and disrupting uterine blood flow. Supporting evidence from animal research has shown that both acute stress exposure and intravenous infusions of noradrenaline induce a decrease in uterine blood flow (Stevens and Lumbers, 1995, Shnider et al., 1979). Human research in this field is limited, and initial investigations reported associations between antenatal anxiety

and decreased uterine blood flow (Teixeira et al., 1999, Sjostrom et al., 1997). However, notably, there have been a number of failed replication attempts (Kent et al., 2002, Harville et al., 2008, Mendelson et al., 2011, Monk et al., 2012). Discordance in the published literature may be attributable to a number of methodological differences in the existing studies, such as the stage in gestation when uterine blood flow was assessed, the methods used for characterising antenatal mood disturbance, and the uterine blood flow indices reported (e.g. uterine vs. umbilical artery, highest vs. mean resistance value). A further explanation may be that doppler assessments of uterine blood flow are difficult to administer during pregnancy due to a number of factors, for example the position of the placenta, stage of gestation where an accurate reading can be obtained, and the competency of the sonographer. Thus, it is currently unclear from the human literature whether changes in uterine blood flow could mediate associations between antenatal mood disturbance and fetal development.

Given the difficulties associated with measuring uterine blood flow in humans, an alternative method for assessing antenatal sympathetic nervous system function may be via the use of the salivary biomarker, alpha-amylase. In recent years, salivary alpha-amylase (sAA) has been proposed as a sensitive biomarker of stress related changes in SNS activity, and there is a growing body of literature to support this idea. Alpha-amylase is an enzyme produced by the salivary glands, which is involved in the initiation of starch break-down in the oral cavity (Nater and Rohleder, 2009). sAA release is known to be initiated by the activation of the SNS system, which controls the salivary glands. Thus, increased sAA concentration is expected during periods of psychological distress, when activation of the autonomic nervous system is high, and indeed a number of studies have

found evidence to support this (Bosch et al., 1996, Chatterton et al., 1997, Skosnik et al., 2000, Bosch et al., 2003, Takai et al., 2004, Takai et al., 2007).

Just one study has assessed the effects of antenatal anxiety during pregnancy on sAA concentrations, and reported a significant association between symptoms of anxiety and increased sAA (Giesbrecht et al., 2012). Thus, sAA may be a useful alternative method for assessing alterations in SNS function in pregnancy as a result of mood disturbance, although further research is required to corroborate this idea.

1.2.3.2 Increased maternal inflammation

A further mechanism, which has been proposed to mediate the link between antenatal mood disturbance and adverse offspring outcomes, is via changes in maternal immune function. There is strong evidence to suggest that depression involves alterations in immunity (Gold and Irwin, 2006, Brod et al., 2014, Zunszain et al., 2013), though there has been recent speculation that symptoms of depression may manifest as a consequence of chronic, low-level inflammation (Berk et al., 2013, Dantzer et al., 2008). Rodent studies have reported increased anxiety and depression behaviours in offspring of dams with increased immune activation during pregnancy (Babri et al., 2014, Khan et al., 2014). Interestingly, adult offspring exposed to high levels of maternal antenatal immune activation also had deficits in cognition and hippocampal long-term potentiation. (Khan et al., 2014).

However, there is a paucity of human studies linking mood disturbance and maternal immune activation during pregnancy. On the other hand, one study has examined the

relationship between maternal antenatal anxiety and adaptive immune responses of infants at 2 and 6 months of age to a hepatitis B vaccine. In this study prenatal anxiety predicted lower hepatitis B titers at 6 months of age, and altered responder cell frequencies to antigen application (O'Connor et al., 2013b). Thus, this initial finding suggests that antenatal anxiety may change the adaptive immunity of the infant. Further, the combination of this finding with the animal research discussed above highlights the possibility that alterations of the maternal and infant immune systems may be a mechanism by which antenatal mood disturbance exerts its influence on offspring development.

1.2.3.3 Increased activity of the maternal HPA axis

The HPA axis forms a major part of the neuroendocrine system and has a number of biological roles, such as increasing blood sugar via gluconeogenesis, suppression of the immune system, and regulation of stress responses. The perception of acute stress initiates the release of corticotrophin-releasing hormone (CRH) from the hypothalamus, which stimulates the anterior pituitary gland to release adrenocorticotrophic hormone (ACTH). This in turn initiates the release of glucocorticoid hormones (mainly cortisol) from the adrenal glands, which act to suppress the release of CRH and ACTH from the hypothalamus and anterior pituitary, via the activation of glucocorticoid receptors (GR) in a negative feedback loop, see Figure 1**.**

Figure 1 Action of the HPA axis in response to a stressful stimulus

Over recent years, salivary biomarkers have become an important tool in neuroendocrinology research, because they are readily accessible and easily obtained. Specifically, salivary cortisol has become a widely used and important tool in measuring responses to stress, as a less invasive indicator of the HPA axis. Salivary cortisol concentrations are highly correlated with plasma cortisol levels (Kirschbaum and Hellhammer, 2007), and reflect the unbound fraction of plasma cortisol, which is thought to be the active portion (Mendel, 1992). Acute exposure to psychological stress has been shown to increase salivary cortisol concentrations, with peaks at approximately 20

minutes following exposure and concentrations return to baseline levels by 40 minutes post-exposure (Hellhammer et al., 2009).

A similar rise in cortisol concentrations is evident after awakening, which is considered to be an endogenous challenge to the HPA axis (Balbo et al., 2010). Cortisol concentrations peak at approximately 30 minutes after awakening, which is known as the cortisol awakening response (CAR), and levels gradually decline throughout the day. Thus, cortisol has a diurnal pattern of release, indexed by high morning and low evening levels.

A number of clinical observations have demonstrated a clear link between cortisol hypersecretion and depression (Mullen et al., 1986, Cowen, 2002). An existing problem in this field is to understand what excess cortisol represents. For example, currently depressed individuals have an increased CAR (Bhagwagar et al., 2005). However, it appears that this cortisol hypersecretion has a trait-like characteristic: waking cortisol hypersecretion is also evident in individuals with a high genetic risk for depression (Mannie et al., 2007, Portella et al., 2005), and also in those recovered from depression (Bhagwagar et al., 2003). This suggests that, while some symptoms of depression may recover following treatment, others such as cortisol hypersecretion may persist after recovery. It is possible that cortisol hypersecretion may have been present before the onset of depression, and therefore may represent a vulnerability factor (Bhagwagar and Cowen, 2008). This is supported by evidence that elevated cortisol secretion may predict a depressive episode (Goodyer et al., 2000, Harris et al., 2000). Chronically high levels of cortisol disrupts neurogenesis, and a structural imaging studies have revealed that

patients with major depressive disorder have hippocampal atrophy, which could be the result of long-term cortisol hyper-secretion (Sheline et al., 1996).

The HPA axis undergoes dramatic changes during the course of pregnancy. This is largely due to the release of corticotrophin releasing hormone (CRH) from the placenta, which increases exponentially during pregnancy resulting in up to a 1000-fold increase in CRH at term (Lindsay and Nieman, 2005). CRH derived from the placenta is physically and biologically identical to hypothalamic-derived CRH (Lindsay and Nieman, 2005, Magiakou et al., 1997). Thus, the increased circulating CRH stimulates the release of excess adrenocorticotrophic hormone (ACTH) from the pituitary gland, which in turn initiates the release of more cortisol from the adrenal glands; this is demonstrated in Figure 2. Thus, as pregnancy progresses, a state of hypercortisolism develops, whereby plasma concentrations of cortisol are 2-3 fold higher by term than in non-pregnancy (Mastorakos and Ilias, 2000). As a result of increased circulating plasma cortisol and ACTH, a gradual hypertrophy of the adrenal and pituitary glands is evident (Mastorakos and Ilias, 2003).

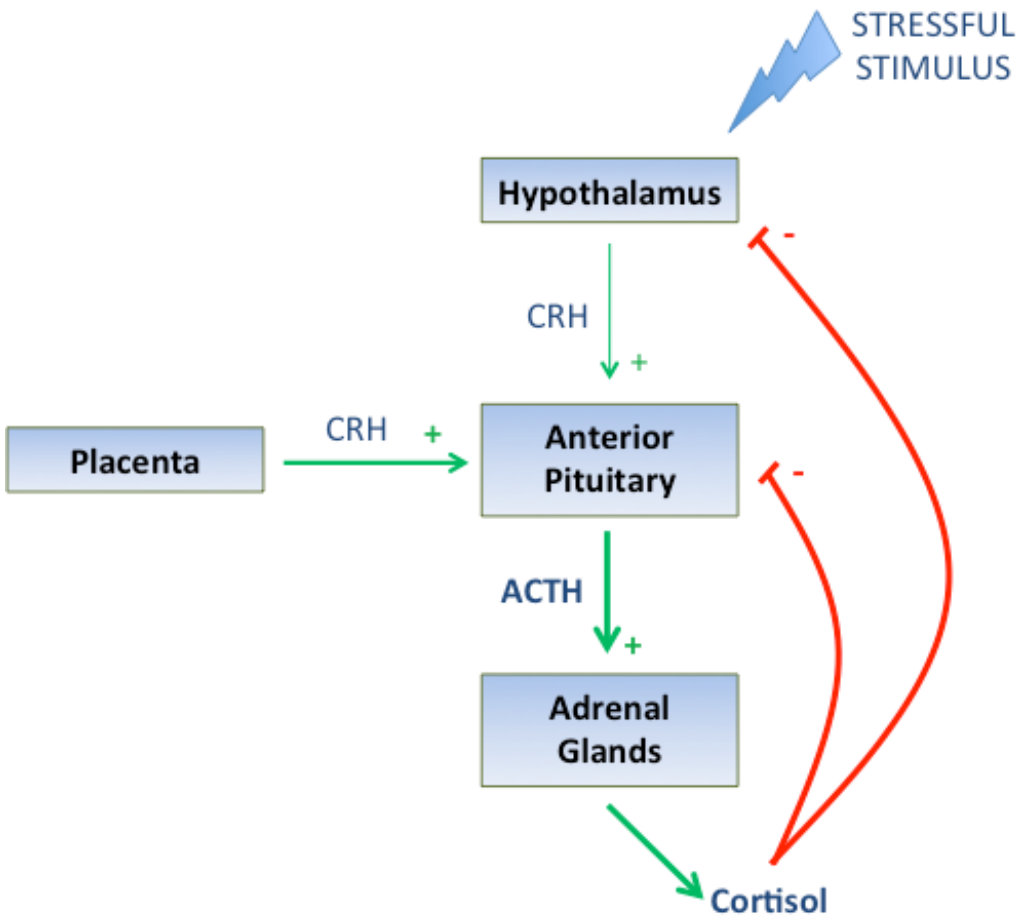

Figure 2 Changes to the HPA axis during pregnancy

As the HPA axis undergoes such a dramatic change during pregnancy, it is currently unclear whether pregnant women with symptoms of depression also hyper-secrete cortisol. This is because baseline cortisol levels rise throughout pregnancy; therefore detecting increases in cortisol as a result of depressive symptoms in pregnancy becomes difficult.

It has been proposed that a possible biological mechanism by which antenatal mood disturbance impacts on fetal development and increases risk for adverse offspring

outcomes may be via increased maternal glucocorticoids leading to altered programming of the fetal HPA axis. This theory suggests that antenatal mood disturbance is associated with increased circulating maternal glucocorticoids, mainly cortisol. This excess cortisol crosses the placental barrier resulting in increased circulating fetal cortisol. As a result, the development of the fetal HPA axis is altered, and when the infant is born their HPA axis is permanently over reactive. This hyperactive HPA axis then predisposes the infant to behavioural problems in childhood, and to psychiatric disorder in adulthood (Talge et al., 2007, Glover, 2011). This idea is depicted in Figure 3.

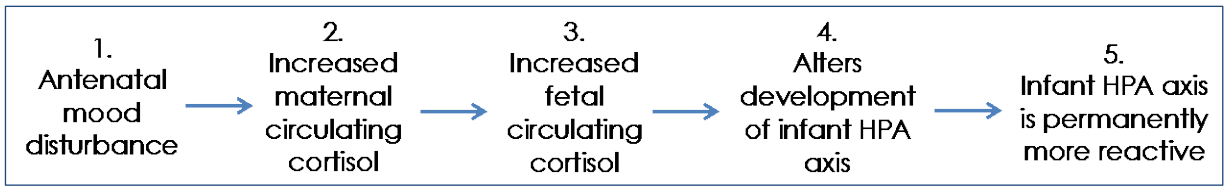

Figure 3 The effects of antenatal mood disturbance on maternal and fetal HPA function. Based on Talge *et al* (2007), Glover *et al* (2011).

There is some initial evidence to support this theory. For example, salivary cortisol reactivity to an acute stressor had been reported to be increased in participants with depressive-symptoms in early pregnancy (Murphy et al., 2014). However, a study in later pregnancy reported that participants did not show increases in salivary cortisol in response to a stressful laboratory task, and only those participants with co-morbid symptoms of depression and anxiety had increased salivary cortisol concentrations across the test session (Evans et al., 2008). Symptoms of antenatal depression and

anxiety have also been associated with increases in diurnal salivary cortisol (Obel et al., 2005, O'Connor et al., 2013a), though there has been a failed replication attempt (Hellgren et al., 2013).

Further, maternal and fetal cortisol levels have been reported to be correlated (O'Donnell et al., 2012). Normally, maternal cortisol is metabolised at the placental barrier by the enzyme 11B-HSD2, so that just approximately 10% of active maternal cortisol enters fetal circulation (Benediktsson et al., 1997). However, maternal antenatal anxiety has been shown to be associated with a down regulation of the placental 11B-HSD2 enzyme, so that more active maternal cortisol enters fetal circulation (Glover et al., 2009a). Thus, it is proposed that the increased cortisol in the fetal circulation may alter the development of the fetal HPA axis. Indeed, there is evidence from both the animal and human literature that exposure to early life stress results in increased methylation of the glucocorticoid receptor gene (NR3C1) (McGowan et al., 2009, Weaver et al., 2001, Oberlander et al., 2008), resulting in an associated decrease in hippocampal GR expression (McGowan et al., 2009, Weaver et al., 2001). Activation of the hippocampal GR activates the negative feedback loop of the HPA axis, therefore a decrease in GR expression should be associated with exaggerated cortisol stress responses as a result of less-negative feedback. In support of this idea, there is evidence to suggest that infants and adolescents born to depressed mothers show exaggerated responses to stress (Davis et al., 2011a, Brennan et al., 2008, O'Connor et al., 2005, Van den Bergh et al., 2008b). Further, such increased cortisol stress responses have been related to symptoms of depression in adolescence (Van den Bergh et al., 2008b).

However, there are clearly a number of gaps in the evidence to support this theory. For example, data associating depressive symptoms and hypercortisolism in pregnancy is mixed (Obel et al., 2005, O'Connor et al., 2013a, Hellgren et al., 2013), and generally limited to diurnal measures of cortisol rather than reactivity of the HPA axis. Further, there is a paucity of evidence linking maternal HPA function in pregnancy with infant HPA function, and the underlying mechanisms remain elusive.

1.2.4 Pathways of risk transmission – a summary

It is extremely challenging to dissociate the pathways that mediate the relationship between antenatal mood disturbance and offspring outcomes, and it is likely that both shared genetic factors between mother and infant, and continuation of exposure from the pre- to postnatal period, contribute. Nonetheless, it is also clear that *in utero* biological mechanisms account, at least in part, for this association. The majority of the research has focused on alterations of the infant HPA axis. However, it is likely that a number of systems are affected in a complex manner as a result of exposure to antenatal mood disorder.

1.3 Susceptibility to the antenatal environment

Despite the clear link between antenatal mood disturbance and increased risk of adverse infant outcomes, it is important to recognise that not all offspring are affected uniformly by exposure to mood disturbance in pregnancy. It is estimated that just 20% of exposed offspring will experience the adverse outcomes that are associated with antenatal mood disturbance, such as behavioural and emotional problems in childhood (O'Connor et al., 2002a). Therefore, an important unanswered question in this field is why some infants may be more susceptible to the effects of antenatal mood disturbance than others. The identification of such vulnerable individuals is essential for understanding the mechanisms by which antenatal mood disturbance may impact on fetal and infant development, and has the potential to inform targeted prenatal interventions.

The final section of this chapter begins with an outline of two theories regarding vulnerability and susceptibility to environmental influences, the 'diathesis-stress model' and the 'differential susceptibility hypothesis'. A discussion of genetic susceptibility factors follows, with a particular emphasis on the role of the serotonin transporter polymorphism (5-HTTLPR), as this is the most extensively studied genetic polymorphism in relation to susceptibility to environmental influences.

1.3.1 Diathesis-stress model

The diathesis-stress model proposes that there are certain individuals who are vulnerable to unfavourable outcomes when exposed to adverse environmental

influences, whereas other individuals are resilient (Gottesman and Shields, 1967, Monroe and Simons, 1991b). Such diathesis, or predisposition, interacts with environmental stress to result in unfavourable outcomes. As such, the theory attempts to explain how biological or genetic traits interact with the environment to produce disorders, such as depression, anxiety or schizophrenia. An inherent assumption of this theory, however, is that both the vulnerable and resilient individuals will have the same outcome in favourable environments. Figure 4 demonstrates predicted trajectories of vulnerable and resilient individuals under positive and negative environmental influences.

1.3.2 Theory of differential susceptibility

The theory of differential susceptibility (DST), defined by Belsky and colleagues (Belsky, 1997, Belsky et al., 2007, Ellis and Boyce, 2011), builds on ideas posed by the diathesis-stress model. However, rather than describing different individuals as 'vulnerable' and 'resilient', they use the terms 'susceptible' and 'fixed'. That is, the DST states that those individuals, who are susceptible to adverse environmental influences, are also equally and simultaneously susceptible to positive environmental influences. On the other hand, 'fixed' individuals are less affected by both positive and negative environments. Thus, those individuals described as susceptible are more responsive and adaptive to the environment regardless of whether it is positive or negative. The predicted trajectories for both type of individual are demonstrated in Figure 4.

It should be noted that another distinct, but not mutually exclusive, theory was developed which claimed that individuals should vary in their developmental plasticity and susceptibility to environmental influence; the biological-sensitivity-to-context (BSTC) theorem (Boyce and Ellis, 2005, Ellis et al., 2005). Although the focus of these two theories is very similar, they do have subtle differences. The DST emphasises the role of nature in shaping individual differences in plasticity; that is pre-existing factors such as susceptibility genes or phenotypes. On the other hand, the BSTC is more focused on the role of the environment in shaping susceptibility. Further, the DST defines no specific hypotheses about susceptibility factors or mediating mechanisms, whereas these empirical questions are central to the BSTC. Nonetheless, both models predict that some children, and perhaps adults, will be more susceptible than others to both the adverse and beneficial effects of unsupportive and supportive environments, respectively.

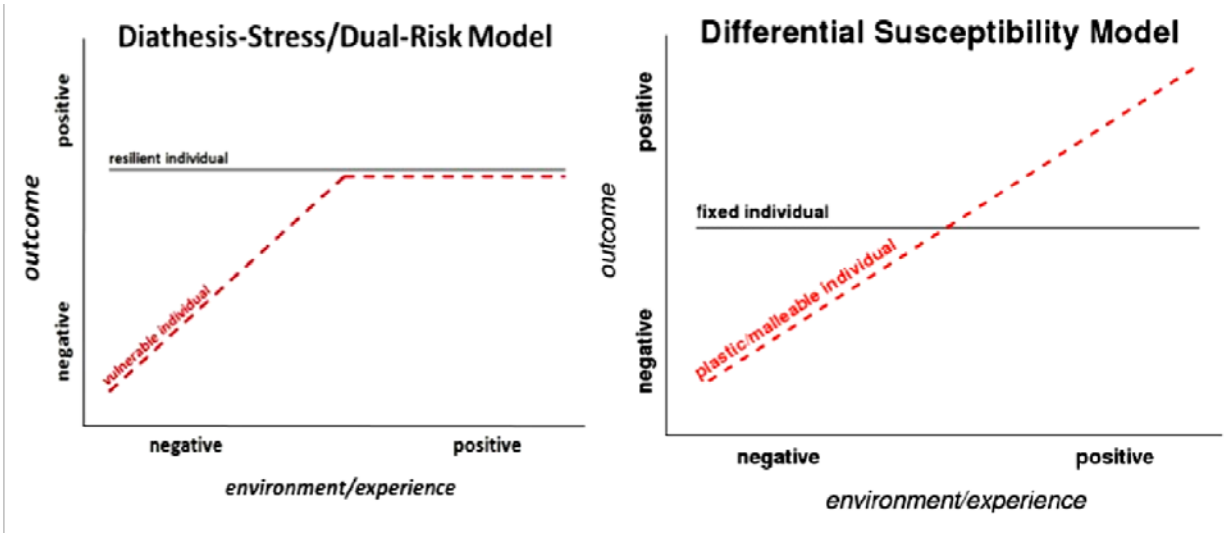

Figure 4 Graphical representation of the diathesis-stress theory and differential susceptibility model. Adapted from Ellis et al. (2001, pp 9 Figure 1). The x-axis represents the variation of the environmental influence/experience from negative to positive, whereas the y-axis is a scale of the outcome, from positive to negative. In the diathesis-stress model (left), the black line represents the resilient individual whose outcome remains consistent regardless of environmental influence. On the other hand, the vulnerable individual, represented by the red line, performs poorly in the negative environment, but the same as the resilient individual in the positive environment. In the differential susceptibility model (right), like the resilient individual, the fixed individual (black line) has the same outcome regardless of environmental input. However the plastic/susceptible individual (red line) performs both poorly in the negative environment and well in the positive environment.

1.3.3 Susceptibility factors

A number of different factors have been identified that define susceptibility to environmental influences, and it has been suggested that susceptibility may be defined at three levels: genetic, phenotypic and endophenotypic. Much research has focused on susceptibility to early postnatal environmental influences, as young infants are thought to be more susceptible to change as a result of environmental influences than older

children or adults. For example, a proposed genetic susceptibility factor, the DRD4 7-repeat polymorphism, has been shown to moderate the effects of maternal sensitivity: infants with the 7-repeat allele had more externalising behaviours when exposed to maternal insensitivity than infants without the 7-repeat allele (Bakermans-Kranenburg and van Ijzendoorn, 2006).

Also, infant temperament (particularly negative or unregulated temperament) has been shown to define susceptibility to postnatal environmental influences. Most studies here have focused on parenting style as an environmental measure. For example, Belsky et al. (1998) showed that parenting was a stronger predictor of externalising problems in highly negative infants only, and also Rubin et al. (1998) found that infants with an unregulated temperament were more likely to have externalising behaviours when their mothers demonstrated high levels of negative dominance than infants with a regular temperament. More recent studies have considered the moderating role of infant temperament in light of the differential susceptibility hypothesis: those infants with a high-reactive or negative temperament are simultaneously more vulnerable to the adverse effects of negative parenting, and more susceptible to the beneficial effects of positive parenting (Van Aken et al., 2007b).

1.3.4 Offspring susceptibility to antenatal mood

Thus, when considering why some infants manifest the adverse effects associated with antenatal mood disturbance while others don't, it may be beneficial to consider the role of susceptibility factors. Indeed, one genetic polymorphism, 5-HTTLPR, has been

extensively studied and proposed to confer susceptibility to a number of environmental influences, including antenatal mood disturbance.

1.3.4.1 The Serotonin transporter polymorphism, 5-HTTLPR

5-hydroxytriptamine (5-HT), also known as serotonin, is a monoamine neurotransmitter that is biochemically derived from the amino acid tryptophan. Although the majority of serotonin in the body is located in the gut, serotonin is also synthesised by serotonergic neurons of the central nervous system (CNS). The primary source of serotonin in the CNS are the neurons of the raphe nuclei which are located in the brain stem (Frazer and Hensler, 1999). Axonal projections from the raphe nuclei extend to almost every part of the brain, and therefore serotonergic systems play a pivotal role in regulating a number of CNS functions, including: the circadian rhythm, appetite, aggression, mood, cognition, memory and learning (Sodhi and Sanders-Bush, 2004). Serotonin dysregulation has also been implicated in a number of psychiatric disorders, such as depression, anxiety and schizophrenia, and pharmacological therapies that target the serotonergic system are often used to treat these disorders. For example, selective serotonin re-uptake inhibitors (SSRI's) are a widely prescribed class of antidepressants.

At the synaptic cleft, 5-HT is released by serotonergic neurons, which activates post-synaptic 5-HT receptors, resulting in excitatory or inhibitory post-synaptic potentials. The action of serotonin is then terminated by the re-uptake of the neurotransmitter into the pre-synaptic terminal by the serotonin transporter. Figure 5 depicts this process.

The gene that codes for the serotonin transporter protein is called SLC6A4 (Solute Carrier Family 6, member 4), and it is located on the long arm of chromosome 17 (17q11.1-q12). The SLC6A4 gene has 2 allelic variants; a short allele (S) and a long allele (L). This is as a result of an insertion/deletion polymorphism in the promoter region of the gene; the area which controls transcription rate. The long allele contains 16 repeat elements, whereas the short allele is missing a 43 base-pair, and therefore only has 14 repeat elements (Nakamura et al., 2000). As a result of this polymorphism, the transcriptional efficiency of the short allele is reduced. So, the short allele has a slow rate of transcription, whereas the long allele has a fast rate of transcription. This means that those individuals with two copies of the short allele (SS genotype) have decreased expression of the serotonin transporter at the pre-synaptic membrane, which results in a slower clearance of 5-HT from the synaptic cleft and a prolonged activation of the postsynaptic neuron (Lesch et al., 1996). On the other hand, those individuals with two copies of the long allele (LL genotype) have a high expression of 5-HTT at the pre-synaptic membrane, and therefore clearance of 5-HT form the cleft is fast, and the activation of the post-synaptic neuron is terminated quickly. Heterozygous individuals (SL genotype) have an intermediate expression level of 5-HTT, and therefore an intermediate rate of clearance of 5-HT from the cleft. This is demonstrated in Figure 6. However, it is important to note that a positron emission tomography study in humans found no relationship between the serotonin transporter genotype and availability of 5-HTT (Murthy et al., 2010). Thus, the 5-HTTLPR genotype may not be directly associated with human brain serotonin levels, as previously thought.

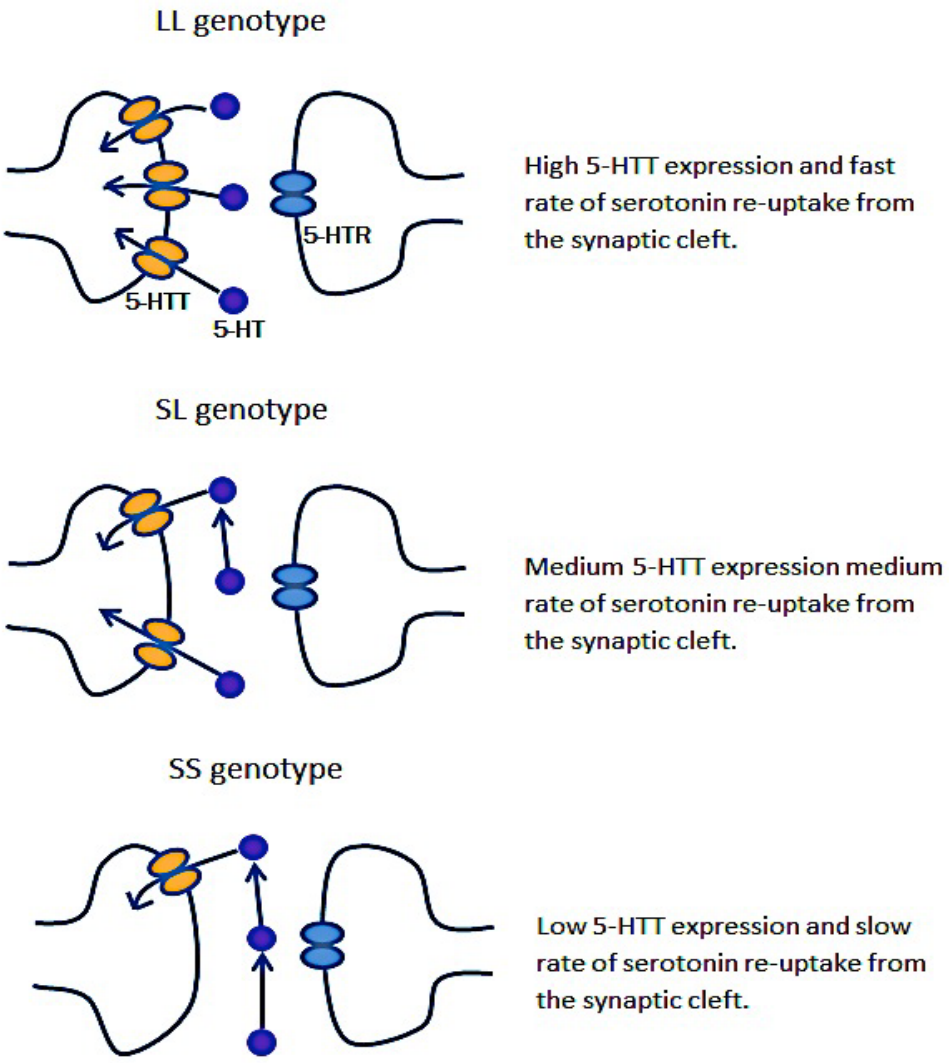

Figure 5 Relationship between 5-HTT expression level and rate of clearance of 5-HT from the synaptic cleft

The SLC6A4 genotyping is complicated slightly by the presence of another polymorphism within the long allele. There is a single nucleotide substitution (A→G) in the promoter region of the L allele, which results in a functional transcription factor binding site. As a result, a transcription factor binds to the promoter region, which reduces the rate of transcription (Hu et al., 2006). The long allele with the polymorphism is termed L_G, and can be functionally grouped with the S allele, as both have slow rates of transcription. The long allele without the polymorphism is termed L_A. Thus, the triallelic genotypes can be categorised into functional groups as followed:

- High expression level of 5-HTT: L_AL_A,
- Medium expression level of 5-HTT: L_AS, L_AL_G,
- Low expression level of 5-HTT: SS, SL_G, L_GL_G.

Biallelic coding of the 5-HTTLPR genotype (SS, SL, LL) was primarily used in early studies of this gene, whereas more recent studies have used the triallelic coding.

1.3.4.2 5-HTTLPR as a susceptibility factor

The first study to highlight the role of 5-HTTLPR as a moderator of environmental influences, reported that childhood maltreatment and stressful life events (SLEs) interacted with the serotonin transporter genotype to increase risk of depression in adulthood (Caspi et al., 2003). Specifically, when exposed to childhood maltreatment or SLEs, individuals with one or two copies of the S allele had increased risk of developing depression and were more likely to commit suicide compared to those with two L allele

(Caspi et al., 2003). From these findings, Caspi and colleagues concluded that the S allele might confer susceptibility to adverse environmental influences, whereas the L allele may have a protective function, in line with the diathesis-stress model. Since its publication in 2003, this paper has been cited over 900[2] times, and many researchers have investigated the role of this genotype in moderating the effects of both negative and positive environmental influences on adverse and positive outcomes.

However, failure to replicate the original 5-HTTLPR x SLE interaction by a number of studies has led to significant debate over the validity of the original gene by environment (GXE) finding. Recently, two meta-analyses have investigated this interaction and have concluded that Caspi's 5-HTTLPR x SLE finding is consistent with a false-positive and that the 5-HTTLPR genotype does not moderate the association between early life stress and depressive symptoms (Munafo et al., 2009, Risch et al., 2009). Several researchers have responded to the findings of the meta-analysis by questioning the methodological approach used (Koenen and Galea, 2009, Lotrich and Lenze, 2009, Rutter et al., 2009), and therefore this debate continues.

1.3.4.3 5-HTTLPR confers susceptibility to antenatal maternal mood

Although there are questions regarding the validity of 5-HTTLPR as a moderator of stress on depressive outcomes, a plethora of neuroimaging, rodent, primate and human studies have investigated the role of this genotype in moderating a range of both positive and negative environmental influences on behavioural outcomes. Thus, there is a wealth

[2] Data taken from http://www.ncbi.nlm.nih.gov on 30.09.2014

of literature pertaining to 5-HTTLPR as a susceptibility factor, and therefore it is reasonable to question whether 5-HTTLPR may moderate the effects of antenatal mood disturbance on later child outcomes.

Indeed, previous studies have investigated this, and the 5-HTTLPR polymorphism has been found to moderate the effects of antenatal anxiety: infants with two copies of the S allele were more likely to have negative emotionality at 6 months (Pluess et al., 2011) and behavioural difficulties up to three years (Oberlander et al., 2008, Babineau et al., 2014) following antenatal anxiety exposure. However, a number of outstanding questions remain regarding this moderation effect. For example, it is currently unknown whether such effects may persist past infancy and into childhood, and whether 5-HTTLPR may also moderate the effects of antenatal depression as well as anxiety.

1.3.5 Susceptibility to the antenatal environment – a summary

The idea that individuals differ in susceptibility to environmental influences has existed for many years, and a number of theoretical models have been proposed to explain such differences in susceptibility. It is clear that not all infants are uniformly affected by disturbances in antenatal mood, as adverse outcomes associated with antenatal mood disturbance only manifest in approximately 20% of exposed infants. Therefore, applying models that attempt to explain differences in susceptibility is a logical step in understanding the mechanisms by which antenatal mood disturbance impacts on offspring development.

1.4 Synthesis

Antenatal depression increases risk for a number of adverse offspring outcomes, an association that seems to be somewhat independent of maternal postnatal mood and shared risk genes between mother and infant. Understanding the pathways by which antenatal depression impacts on fetal and infant development is a key step towards the development of antenatal interventions aimed at decreasing the adverse effects of antenatal mood disturbance on infant development.

There are two key questions in the field of perinatal psychiatry that remain unanswered regarding associations between antenatal mood disturbance and infant development. First, the biological mechanisms, which may mediate this association, remain poorly understood. Second, it is currently unknown why some infants may be more susceptible to the effects of antenatal mood disturbance than others. The research presented in this thesis addresses two specific hypotheses that are embedded within these two unanswered questions:

1. Maternal antenatal depression is associated with alterations in maternal and infant HPA function.
2. The serotonin transporter polymorphism (5-HTTLPR) confers infant susceptibility to maternal antenatal depression.

The research presented in Chapters 2, 3 and 4 are derived from a short-term longitudinal study based in Oxford, UK. During 2013-2014, 103 pregnant women were recruited and assessed during pregnancy, and followed up during the postnatal period when their

infant was 2 months old. The aim of this study is to investigate whether maternal antenatal depression results in changes in both the maternal and infant HPA axis.

Data from the antenatal part of this study is presented in Chapter 2. The primary aim of Chapter 2 is to investigate whether women with symptoms of depression during pregnancy have exaggerated salivary cortisol reactivity to an acute stressor and also raised diurnal salivary cortisol levels. The secondary aim of this chapter is to investigate whether such changes in salivary biomarkers are HPA-specific, by also assessing salivary alpha-amylase. It is hypothesised that pregnant women with current symptoms of depression will have exaggerated salivary cortisol and alpha-amylase reactivity to a stressor, and raised diurnal levels of salivary cortisol and alpha-amylase, compared to non-depressed control participants.

In Chapter 3, data from the postnatal section of this study is presented. The aim of this part of the study is to investigate whether infants of women with symptoms of depression during pregnancy have exaggerated cortisol responses to inoculation at 2 months of age and more reactive temperaments. It is hypothesised that infants of prenatally depressed mothers will have increased salivary cortisol responses to inoculation, and show greater activity and distress to limitations, than infants of non-depressed mothers. A second hypothesis is that maternal antenatal cortisol reactivity will be a stronger predictor of infant cortisol reactivity than self-reported antenatal depressive symptoms.

At the postnatal follow up visit, DNA samples were also obtained from 57 of the infants, and methylation was analysed at two key genes involved in stress responses and

implicated in neuropsychiatric disorders: the glucocorticoid receptor gene (NR3C1) and the brain-derived neurotrophic factor gene (BDNF). This data is presented in Chapter 4. The aim of this chapter is to investigate whether exposure to maternal antenatal depression results in increased infant NR3C1 and BDNF DNA methylation, and whether such increases in DNA methylation predict infant cortisol responses to inoculation and temperament. It is hypothesised that those infants exposed to antenatal depression will have increased NR3C1 and BDNF methylation, and that methylation of these genes will predict infant cortisol reactivity.

In Chapter 5, the second hypothesis of this thesis is addressed. This study is an analysis of data collected as part of the Avon Longitudinal Study of Parents and Children (ALSPAC). ALSPAC is a large prospective birth cohort where over 14,000 pregnant women from the Avon area around Bristol, UK, were recruited during 1991/2. Comprehensive assessments of participants were carried out during pregnancy, and offspring have been regularly followed up. The aim of this study is to investigate whether the effects of antenatal depression and anxiety on infant behaviour are moderated by the 5-HTTLPR genotype. It is hypothesised that those infants with two copies of the short allele have more difficult temperaments at 6 months, and more behavioural problems during childhood when exposed to antenatal anxiety or depression, than those infants with two copies of the long allele.

CHAPTER 2

INVESTIGATING THE EFFECTS OF MATERNAL ANTENATAL DEPRESSIVE SYMPTOMS ON SALIVARY CORTISOL AND ALPHA-AMYLASE REACTIVITY TO INFANT DISTRESS, AND DIURNAL SALIVARY CORTISOL AND ALPHA-AMYLASE

2 Overview of Chapter

This chapter is an investigation of how symptoms of depression during pregnancy may influence acute salivary cortisol and alpha-amylase responses to a stressful stimulus, and diurnal salivary cortisol and alpha-amylase release. It has been suggested that a biological mechanism by which antenatal mood disturbance confers risk for fetal development is via changes in the maternal and fetal HPA axes. However, there is currently little evidence to suggest that reactivity of the HPA axis is altered in pregnant women experiencing symptoms of depression, as existing research is generally limited to diurnal measures of maternal cortisol. Therefore, the aim of this chapter is to test whether women with symptoms of depression during pregnancy have exaggerated salivary cortisol responses to an infant distress video, and also raised diurnal levels. Salivary alpha-amylase was also assessed to address the question of whether any differences in biological markers of stress may be HPA-specific.

This study is an analysis of data collected as part of a short-term longitudinal study based in Oxford, UK, during 2013/14. 103 pregnant women were recruited to the study during either the second or third trimester of pregnancy. Salivary cortisol and alpha-amylase samples were obtained in response to an infant distress video (N=103), and diurnal salivary cortisol and alpha-amylase samples were taken at home over two working days (n=90). Maternal antenatal mood and self-reported psychological responses to the infant distress stimulus were also measured.

This chapter begins with an overview of studies that have investigated cortisol responses to acute stress in pregnancy, as well as research that has considered the relationship between maternal antenatal mood disturbance and cortisol. The role of the autonomic nervous system (ANS) in responding to stress, the use of salivary alpha-amylase measures in neuroendocrinology research, and our current understanding of the relationships between salivary alpha-amylase, depression and pregnancy are then discussed. A description of the cohort and methods used in this study is presented in the methods section, and results of this study follow. This chapter concludes with a discussion of the findings of this study, the strengths and limitations, and the conclusions of this research.

This is the first study to investigate the effects of maternal antenatal depressive symptoms on both HPA and ANS responses to a stressor, and diurnal biomarkers within the same cohort of participants. Understanding the biological processes by which antenatal mood disturbance may impact on fetal and infant development is of great importance when considering child health outcomes and intervention targets. Thus, the results of this study contribute to a clinically relevant body of literature.

2.1 Introduction

The HPA axis has an important role in regulating responses to acute stress and this system undergoes dramatic changes during the course of pregnancy. This is largely due to the release of corticotrophin releasing hormone (CRH) from the placenta, which increases exponentially during pregnancy resulting in up to a 1000-fold increase in CRH at term (Lindsay and Nieman, 2005). It is currently unclear whether pregnant women with symptoms of depression hyper-secrete cortisol in response to acute stress compared with non-depressed controls, as cortisol concentrations in pregnancy may be already near ceiling levels.

However, antenatal mood disturbance has been implicated in alterations of diurnal cortisol patterns, suggesting that there may be an effect of mood on dynamic responses of this system during pregnancy. Current research suggests that women who report stressful life events and pregnancy worries have significantly higher evening cortisol than those who do not (Obel et al., 2005). Self-reported stress has been linked with lower morning cortisol levels and a flatter diurnal decline (Suglia et al., 2010), and symptoms of depression and anxiety have also been associated with a flatter diurnal cortisol pattern (Kivlighan et al., 2008, O'Connor et al., 2013a, O'Keane et al., 2011).

Recently, O'Connor *et al* (2013) reported a modest but significant association between depression and elevated cortisol in the 2nd and 3rd trimester in a group of 101 participants, which was a result of decreased morning cortisol levels and

diminished diurnal decline. These effects were evident for self-reported depression, but stronger when using diagnostic data from a clinical interview (O'Connor et al., 2013a). Taken together, these findings suggest that during pregnancy, although total cortisol levels are elevated, the diurnal pattern of cortisol release is maintained (see Figure 7). However, symptoms of mood disturbance during pregnancy may be associated with alterations in the diurnal pattern of cortisol release.

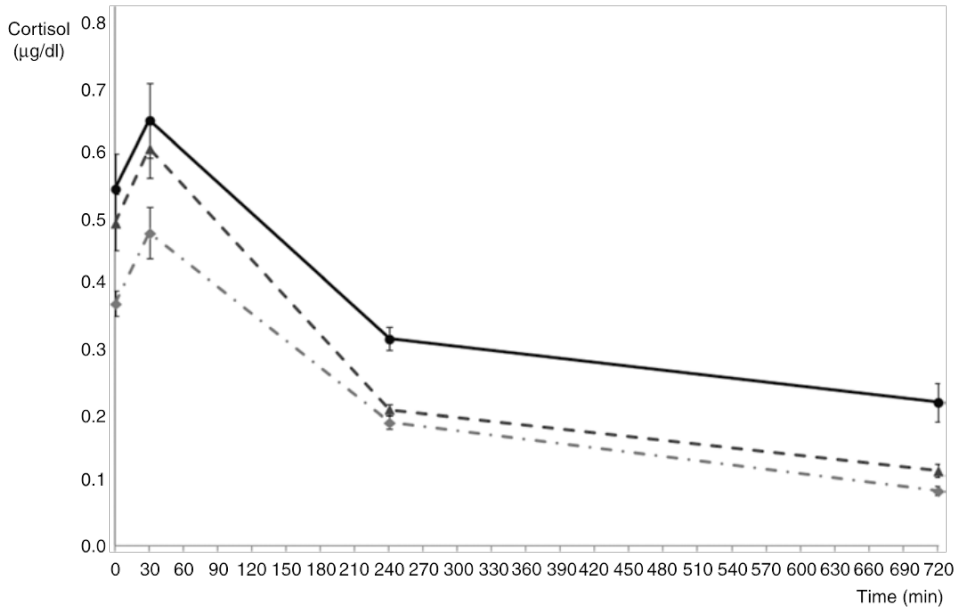

Figure 6 Diurnal cortisol profiles during pregnancy (N=91). The dashed grey line represents the 1st trimester, the dashed black line represents the 2nd trimester, and the solid black line represents the 3rd trimester. There is a clear increase in cortisol concentrations as gestation progresses, however the diurnal cortisol pattern is maintained. Adapted from Hompes et al. (2013).

2.1.1 HPA responses to challenge during pregnancy

The majority of studies investigating responses to acute stress in pregnancy have focused on blood pressure and heart rate outcomes, arguably because these

measures are readily accessible and non-invasive. A well-replicated finding is that cardiovascular responses to acute stress attenuate as gestation progresses (Matthews and Rodin, 1992, Entringer et al., 2010).

Since the HPA axis undergoes such a dramatic change during pregnancy, it is reasonable to suggest that reactivity of this system to challenge may also be somewhat altered compared to the non-pregnancy state. Generally, two different types of challenge to the HPA axis are used in research: the cortisol awakening response (CAR) is considered an endogenous challenge to the system (Balbo et al., 2010), whereas exposure to an acute stressor is considered an exogenous challenge.

2.1.1.1 The cortisol awakening response

In non-pregnant populations, changes to the CAR are considered an endophenotype of depression. For example, there is a clear elevation of the CAR in moderately depressed patients (Bhagwagar et al., 2005), and also in those recovered from depression (Bhagwagar and Cowen, 2008, Bhagwagar et al., 2003), and individuals at high genetic risk of depression (Portella et al., 2005, Mannie et al., 2007).

Although pregnancy is associated with hyper-cortisol secretion, the CAR remains intact (de Weerth and Buitelaar, 2005, Giesbrecht et al., 2012), but its magnitude attenuates as gestation progresses (Buss et al., 2009, Entringer et al., 2010). There is also evidence that mood disturbance during pregnancy has implications for the CAR. In sample of 603 pregnant women, Obel and colleagues (2005) reported that women who experienced a significant number of stressful life events had a blunted CAR during early pregnancy; however this effect was not seen during late pregnancy. In a

smaller sample of 101 women, O'Connor *et al* (2013) also reported a smaller CAR in women experiencing symptoms of depression. However, Hellgren and colleagues (2013) failed to replicate this finding. They assessed 134 participants during the 3rd trimester who were categorised into 3 groups: never depressed (n=57), those with a previous history of depression (n=39) and currently depressed (n=39), and failed to find any significant group differences in the CAR. Similarly, Pluess *et al* (2012) failed to show that negative life events were related to the CAR, however positive life events significantly predicted lower cortisol levels immediately after awakening ($p<0.05$). Thus, the evidence linking antenatal mood disturbance with alterations of the CAR is mixed, with evidence both for and against a diminished CAR. However, a high degree of variation in the methods used to measure psychological distress in pregnancy, as well as a range of participants from different ethnic and socio-demographic backgrounds, of differing parity and previous histories of mental health problems may explain the differing findings. It is also worth noting that, while there is some evidence of a diminished CAR in pregnant women experiencing current symptoms of mood disturbance, this is in fact opposite to the direction of effect found in non-pregnant populations where depression is associated with an increased CAR. More details of these studies are available in Table 1.

Table 1 Summary of studies that have investigated the CAR in pregnancy

Study	N	Trim-ester	Main Finding of study
Obel *et al* (2005) *Psychoneuroendocrinology*	603	1st and 3rd	In late pregnancy, women exposed to more than one life event had 27% higher evening cortisol (95%CI: 1-59%) and women with worries about pregnancy complications had 27% higher evening levels (95%CI: 2-57%). There was no difference in morning cortisol levels. In early pregnancy, women who reported stressful life events did not have higher evening cortisol levels, but did

			have a blunted CAR.
DeWeerth *et al* (2005) *Psychoneuroendocrinology*	119	3rd	The CAR was present during pregnancy, and the absolute mean increase in cortisol at 30 min post-awakening was larger during pregnancy. Both in the pregnant and non-pregnant states, the magnitude of the cortisol awakening response was not related to time of awakening, or to the anticipation of a working vs. non-working day.
Buss *et al* (2009) *Am J Obstet Gynecol*	101	2nd and 3rd	A clear CAR was observed in the pregnant participants ($p<0.001$), however this attenuated over the course of gestation ($p<0.001$). A larger CAR in later pregnancy, and a lack of attenuation of the CAR over gestation was associated with a significantly shorter gestational length ($p<0.05$).
Entringer *et al* (2010) *Stress*	148	2nd and 3rd	The CAR was present during both the 2nd and 3rd trimester, and was also significantly attenuated in later compared to earlier gestation ($p<0.01$).
Pluess *et al* (2012) *Psychoneuroendocrinology*	60	3rd	Negative life events were unrelated to the CAR during the 3rd trimester, however positive life events significantly predicted lower cortisol levels immediately after awakening ($p<0.05$), but did not predict cortisol levels at 30, 45 and 60 minutes post awakening.
Giesbrecht *et al* (2012) *Psychoneuroendocrinology*	83	1st, 2nd or 3rd	The CAR was present in the 1st, 2nd and 3rd trimester. There was a robust within-person association between negative mood and cortisol: for every 1% increase in negative mood there was a corresponding 1.9% increase in cortisol.
O'Connor *et al* (2013) *Biol Psychiatry*	101	2nd and 3rd	There were modest but significant associations between depression and elevated cortisol ($p<0.05$). This was due to a decreased CAR and diminished diurnal decline. The effects were strongest for diagnostic data from clinical interview, but also evident using a self-report measure of depression (EPDS). These effects were independent of socio-demographic factors and sleep disturbance.
Hellgren *et al* (2013) *Psychoneuroendocrinology*	134	3rd	Participants were split into 3 groups: never depressed (n=57), previous history of depression (n=39) and currently depressed (n=39). Depression was characterised using the EPDS. A mixed model analysis failed to yield any significant group differences in the CAR.

CAR: Cortisol awakening response; EPDS: Edinburgh Postnatal Depression Scale

2.1.1.2 Cortisol reactivity to an acute stressor

A number of paradigms have been used to induce acute stress in pregnant participants, including: the Stroop Test, the Cold Hand Test, public speaking and mental arithmetic. Baseline measures of salivary cortisol increase as pregnancy progresses (Nierop et al., 2008), and as with the CAR, there is some evidence to suggest that cortisol reactivity to acute stress is diminished as pregnancy progresses (Kammerer et al., 2002, De Weerth et al., 2007, Entringer et al., 2010). However, two studies have reported the maintenance of salivary cortisol responses to acute stress in pregnant women without mood disturbance (De Weerth et al., 2007, Nierop et al., 2006). More details of these studies are available in Table 2.

As noted by De Weerth *et al.* (2007), an important consideration for studies of cortisol reactivity in pregnancy is the time of day of assessment. As described above, serum concentrations of cortisol increase to term due to excessive release of CRH from the placenta. However, the diurnal cortisol pattern is maintained throughout pregnancy. Therefore, testing stress reactivity during the morning may create a 'ceiling effect', whereby detecting cortisol responses to stress is near impossible. This may explain the discrepancy in the literature, and why some studies failed to find significant cortisol responses to acute stress in pregnant participants as time of assessment was not taken into account. A further consideration is that some stressors may be more potent than others in inducing a salivary cortisol response, and if so then the findings from these previous studies cannot be directly compared.

Table 2 Summary of studies that have investigated salivary cortisol and alpha-amylase responses to acute stress in pregnancy

Study	N	Trimester	Stressor	Main Finding of study
Hartikainen-Sorri *et al* (1991) *Obstet Gynecol*	17	3rd	90dB white noise (15 min)	There was no significant effects noise exposure on salivary cortisol concentration.
Kammerer *et al* (2002) *BMC Pregnancy Childbirth*	10	3rd	Cold pressor test (1 min)	The control group (n=10) showed a highly significant salivary cortisol response 20 mins following the test ($p<0.001$), whereas the pregnant group(n=10) showed no significant mean increase in salivary cortisol.
Saisto *et al* (2004) *Acta Obstet Gynecol Scand*	40	3rd	Cold pressor test (0.75-5 min: subjects withdrew arm when pain was intolerable)	20 pregnant women with fear of labour were compared to 20 pregnant women without fear of labour. There was no significant increase in cortisol following the CPT in either group. .
Nierop *et al* (2006) *J Clin Endocrinol Metab*	60	2nd (30) 3rd (30)	Trier Social Stress Test	Pregnant women in the 3rd trimester had significantly higher baseline cortisol and α-amylase measures compared with 2nd trimester pregnant women ($p<0.001$) and non-pregnant controls (n=30)($p<0.001$). The stress protocol induced a significant cortisol response in all groups ($p<0.001$), and there was no significant difference between the groups ($p=0.75$). The stress also induced a significant α-amylase response in all groups ($p<0.001$), however there was a significant group*time effect, with the lowest response in the 3rd trimester group, and the highest in the non-pregnant group.
De Werth *et al* (2007) *Acta Obstet Gynecol Scand*	120 pregnant and 31 non-pregnant controls.	3rd	Public speaking (27min) and Mental arithmetic (8 min)	The stressors induced a significant increase in salivary cortisol ($p<0.01$), and in all three CV variables ($p<0.001$) in the pregnant participants. Stress reactivity was strongly related to time of day of testing; pregnant participants tested during the morning had diminished stress responses (n=18), and were excluded from analysis. Baseline measures of HR, BP and salivary cortisol were higher for the pregnant group compared to controls, and the magnitude of the stress response was decreased compared to the controls ($p<0.05$)

Evans *et al* (2008) *Arch Womens Ment Health*	180	3rd	Stroop Task	Participants were split into three groups: control (n=121), depressed (n=16), anxious (n=34) and co-morbid depressed and anxious (n=9). None of the participants showed a significant increase in salivary cortisol in response to the Stroop task, however those participants with co-morbid depression and anxiety had increased salivary cortisol concentrations across the test session compared with the other three groups.
Entringer *et al* (2010) *Stress*	148	2nd and 3rd	TSST (5 min) and Mental arithmetic (5 min)	In the pregnant group (n=148) and the control group (n=30), the TSST failed to elicit a significant increase in salivary cortisol concentration The pregnant group had a higher baseline of these measures ($p<0.001$).
Murphy *et al Arch Womens Ment Health*	53	2nd	Infant distress stimulus (6 min)	Participants were classified into a depressed symptom group (n=14) and a non-depressed control group (n=39). Both groups showed similar increases in state anxiety in response to the infant distress, however only the group with symptoms of depression showed a significant increase in salivary cortisol in response to the stressor ($p<0.01$).

TSST: Trier Social Stress Test

Just three studies have investigated the impact of mood disturbance, specifically depressive symptoms, during pregnancy on responses to acute stress: one study measured cardiovascular responses and two assessed salivary cortisol. Pearson and colleagues (2012) exposed 72 pregnant participants to an infant distress stimulus for 15 minutes, which included images and audio clips of distressed young infants. They found that women with symptoms of depression during pregnancy had larger BP responses to the stimulus compared with non-depressed controls ($p<0.01$), and this was evident in both the first and third trimester.

In a similar laboratory-based study, Evans et al. (2008) exposed 180 women in late pregnancy to the Stroop task and measured salivary cortisol reactivity. None of the participants showed an increase in salivary cortisol in response to the task, which

may be attributable to already high cortisol levels in late pregnancy, and therefore it was not possible to measure dynamic changes. On the other hand it may be that the Stroop task is not an effective probe of the HPA axis. One interesting finding from this study was that those participants with symptoms of depression (n=16) or anxiety (n=34) did not have significantly higher cortisol levels than the control participants (n=121). However, pregnant women with comorbid depression and anxiety (n=9) had significantly higher salivary cortisol levels across the test session than all other groups. Although the small sample sizes of the subgroups should be considered when interpreting the results, this finding suggests that in a pregnant population salivary cortisol concentrations may be closely related to symptom severity. Thus, increases in HPA activity may be only present in those with co-morbid depression and anxiety symptoms.

In a related study of 53 women in the first or early second trimester, Murphy and colleagues (2014) used a 6-minute infant distress video, which included a number of video clips of young infants in distress, to investigate salivary cortisol responses. In this study, participants were classified into a depressive-symptom group (n=14) and a non-depressed control group (n=39). Interestingly, both groups showed a similar increase in state anxiety in response to the infant distress film, however only the group with symptoms of depression showed a significant salivary cortisol response ($p<0.01$).

Thus, the results from these three initial studies suggest that symptoms of depression during pregnancy may be associated with increased cardiovascular and HPA responses to infant distress in early pregnancy. However, it is currently unknown whether such increased HPA responses to infant distress in depressed women are

maintained across gestation, given that cortisol concentrations increase as pregnancy progresses. Further, evidence suggests that salivary cortisol concentrations in late pregnancy may be related to symptom severity, and increases in cortisol may only be evident in those women with co-morbid depression and anxiety.

2.1.2 The Autonomic nervous system

The autonomic nervous system (ANS) is also involved in responses to acute stress. Activation of the ANS results in increased cardiovascular output, including increased blood pressure and heart rate, and also an increase in blood sugar level. Catecholamine hormones, such as adrenaline and noradrenaline, are released, which also act to prepare the body for fighting or flighting. For example, catecholamine release results in the dilation of muscular blood vessels, auditory exclusion, tunnel vision and inhibition of digestion.

As discussed in Chapter 1, salivary alpha-amylase (sAA) has been reported to be an indirect marker of sympathetic nervous system (SNS) activity. Like cortisol, sAA has a diurnal pattern of release; in a sample of 76 healthy volunteers, sAA was shown to have a pronounced decrease in concentration within 60 minutes of awakening, followed by a steady increase in concentration over the course of the day (Nater et al., 2007); notably the opposite diurnal profile to cortisol. Interestingly, sAA concentrations were not associated with momentary changes in mood, however chronic stress was associated with increases in sAA over the course of the day. Similarly, initial studies suggest that sAA concentrations are also raised in people who are experiencing a current episode of depression (Ishitobi et al., 2010), and sAA

responses to acute stress have been shown to be elevated in women with major depressive disorder (Tanaka et al., 2012).

The diurnal profile of sAA release has been replicated in a sample of pregnant participants (Giesbrecht et al., 2012), although sAA concentrations have been shown to be increased over the course of the day compared with non-pregnant women (Abrao et al., 2014). Giesbrecht and colleagues (2012) also reported that, after controlling for obstetric factors, trait anxiety was significantly associated with increased diurnal sAA in the 2nd and 3rd trimester (N=83). Just one study has tested sAA responses to acute stress in pregnancy. Nierop *et al.* (2006) reported significant rises in sAA in 30 second- and 30 third-trimester women who underwent the TSST. Although increases in sAA were evident, the magnitude of the response was attenuated compared to a group of non-pregnant controls.

Thus the use of sAA as an indirect measure of ANS activity is a relatively new concept, and initial research suggests that it could be an alternative salivary biomarker for mood disturbance. However, it is currently unknown how symptoms of depression in pregnancy may influence both diurnal sAA concentrations, and responses to a stressor.

2.1.3 Summary

It has been suggested that an attenuation of stress responses during pregnancy, which is supported by both the human and animal literature (Nisell et al., 1985, Eneroth-Grimfors et al., 1988, Matthews and Rodin, 1992, McCubbin et al., 1996, Greenwood et al., 1998, Woisetschlager et al., 2000, Monk et al., 2000, Monk et al.,

2001, Monk et al., 2003, DiPietro et al., 2003, Rohde et al., 1983, Neumann et al., 1998, Neumann et al., 2000), may be an adaptive process, as over exposure to maternal stress hormones and alterations in cardiovascular output, including uterine blood flow, may disrupt normal fetal development (Talge et al., 2007, Glover, 2011). On the other had, it may be that salivary cortisol responses to an acute stressor are difficult to measure in later pregnancy because baseline cortisol levels are so high. Thus, it appears that responses to acute stress are attenuated.

Nonetheless, the findings presented above indicate that the usual attenuation of salivary cortisol responses to acute stress throughout pregnancy may be perturbed by antenatal mood disorder, which could potentially have implications for fetal development. Thus, this could be one pathway by which antenatal mood disturbance exerts influence on fetal development.

2.1.4 Rationale

Evidence linking maternal antenatal mood disturbance with changes in diurnal cortisol has provided mixed results. It is possible that maternal antenatal mood disturbance may be related to changes in HPA reactivity to an acute stressor, rather than increased cortisol over the course of the day. Thus, this chapter investigates whether symptoms of depression during pregnancy may alter maternal cortisol reactivity to an acute stressor. To investigate whether changes in HPA function may be specific to stress reactivity, diurnal salivary cortisol was also assessed. A further unanswered question is whether any changes to biological markers of stress as a result of depressive symptoms may be HPA-specific. As such, saliva samples collected in this study were also assayed for alpha-amylase, a biological marker of sympathetic

nervous system activity. Elucidating the effects of mood disturbance on maternal physiology is an important step in both understanding the risk that antenatal mood disturbance poses to fetal development, and also in identifying new intervention targets.

2.1.5 Aims and Hypotheses

2.1.5.1 Aim

The aim of this study was to investigate the impact of maternal antenatal mood disturbance on reactivity of the HPA axis and sympathetic nervous system to an acute stressor, as well as diurnal measures of salivary biomarkers.

2.1.5.2 Primary Objectives

The primary objective of this study was to investigate how antenatal symptoms of depression may influence responses to infant distress. Specifically;

i. Investigate whether pregnant participants with symptoms of depression have greater salivary cortisol and alpha-amylase responses to an infant distress stimulus than non-depressed controls.
ii. Examine whether responses to infant distress attenuate as gestation progresses in both groups of participants.

2.1.5.3 Primary Hypotheses

i. Pregnant women with symptoms of depression during pregnancy will have increased salivary cortisol and alpha-amylase responses to a video depicting infant distress compared to non-depressed control participants.
ii. The effects of antenatal depression on cortisol and alpha-amylase responses to infant distress will attenuate as gestation progresses.

2.1.5.4 Secondary Objective

The secondary objective of this study was to investigate how antenatal mood disturbance may impact the diurnal pattern of salivary cortisol and alpha-amylase release.

2.1.5.5 Secondary Hypothesis

i. Participants with symptoms of depression during pregnancy will have higher diurnal salivary cortisol and alpha-amylase levels than non-depressed control participants.

2.2 Methods

2.2.1 Participants

103 pregnant women were recruited into the study through a number of recruitment methods. Posters advertising the study were placed at various locations around Oxfordshire, Buckinghamshire and Berkshire, including the Women's Centre at the John Radcliffe Hospital, the maternity unit at Milton Keynes Hospital, General Practitioners' surgeries, the waiting room of the Infant Parent Perinatal Service, churches, schools, shops and community centres. Community midwives who expressed an interest in the project were given leaflets to distribute to potential participants. The study was advertised on a number of websites, including: Daily Info, Gumtree, Facebook, Netmums and the Perinatal Psychopathology and Offspring Development (PPOD) research group website. 73 Oxford University colleges and departments were emailed and asked to circulate information about the study, in the form of a short paragraph, and an advert for the study was published in the Oxford University Gazette. Antenatal teachers in Oxfordshire, Berkshire and Buckinghamshire were emailed and asked to circulate information about the study to their clients, and leaflets advertising the study were at the National Childbirth Trust (NCT) nearly new sales at the following branches: Oxford, Henley and District, Abingdon, Bicester, and Aylesbury, Thame and District.

122 participants were initially screened for the study. 10 women decided not to take part in the study following screening, and 9 were excluded because they did not meet the inclusion criteria (5 already had children, 3 were using steroid-based medications and one lived too far away to take part). Therefore 103 pregnant women took part in this study. All participants were primiparous, more than 14 weeks pregnant, had a

singleton pregnancy, were over the age of 18, had no medical complications associated with their pregnancy and were not currently taking any steroid-based medications. More details of this cohort are available in Table 3. All participants were reimbursed for their time and travel expenses. This research study was reviewed and approved by the Research Ethics Committee South Central Oxford B (REF: 12/SC/0473), and all participants provided informed consent.

2.2.2 Procedure

Potential participants who were interested in taking part in the study made contact by email or telephone. They were provided with a copy of the participant information sheet that contained details about what the study involved (see appendices, page 298), a screening questionnaire, which contained questions about the inclusion criteria and the Edinburgh Postnatal Depression Scale (EPDS). They were also sent a contact information sheet. Potential participants were informed that if, after reading the participant information sheet, they would like to take part in the study, they should complete and return the screening questionnaire and contact information sheet.

Eligible participants were invited to take part in the study, and booked in for the antenatal session, which took place either at the Department of Psychiatry, Warneford Hospital, or at the participant's home. These sessions all took place between the hours of 1pm and 7pm, and lasted approximately 90 minutes. Participants were asked to sign a consent form, and then to complete the antenatal questionnaire, which contained questions about their demographic characteristics and current levels of mood. Participants were then asked to watch a short film

depicting distressed young infants, all under the age of 6 months. The film was 6 minutes in length and included 8 short clips of crying infants. The clips were taken from online sources with permission from the owners. This video has been used in a previous study (Murphy et al., 2014) and found to induce significant salivary cortisol responses in a group of late first/early second trimester pregnant women with symptoms of depression. During the film participants were asked to wear headphones, sit quietly and watch the film.

Saliva samples were collected at five time points during the test session using saliva collection aids and plastic cryovials (Salimetrics, UK). Two samples were taken before the film, approximately 20 minutes apart. The third sample was taken immediately following the end of the film, and the fourth and fifth samples were taken 10 and 20 minutes following the end of the film, respectively. Samples were taken at 10 and 20 minutes post-film, because following administration of an acute stressor, salivary alpha-amylase concentrations peak at approximately 10 minutes post-stimulus and salivary cortisol concentrations peak at approximately 20 minutes post-stimulus (Engert et al., 2011). Before and after the film participants rated their mood and also their psychological response to the film on three visual analogue scales (see appendices, page 298).

Participants were asked to collect six further saliva samples at home over two working days (three per day) within two weeks of the antenatal session. On each day samples were collected immediately after awakening, 30 minutes after awakening and 12 hours after awakening. Participants were provided with six cryovials, six saliva collection aids, and a stamped addressed envelope to return the samples. 90 participants (87.37% of the total sample) completed and returned the diurnal saliva

samples, which were stored at -20°C until analysis. The attrition rate for the depressive-symptom group (16.67%) was higher than the control group (11.39%). Notably, there was a higher rate of attrition from the third trimester depressive-symptom (20%) group than the other three groups (control second trimester: 12.2%, depressive-symptom second trimester: 11.11%, control third trimester 10.53%).

2.2.3 Measures

2.2.3.1 Maternal mood

Edinburgh Postnatal Depression Scale (EPDS). The EPDS is the most widely used self-report questionnaire to identify symptoms of depression during the perinatal period. The scale consists of 10 items that describe common symptoms of depression; however the scale does not include somatic symptoms of depression, such as change in appetite and fatigue, which are commonly experienced in pregnancy. Each item is scored from 0 to 3, and the scale has a maximum score of 30. A score of 13 or above is indicative of clinical levels of depression, however for research purposes a cut off score of 10 is frequently used to identify a group 'at risk' of depression (Murray and Cox, 1990, Felice et al., 2004, Adouard et al., 2005, Adewuya et al., 2006, Bergink et al., 2011). A recent study has shown that using a cut off of 10 in the second and third trimester of pregnancy provides a good balance between sensitivity (70-79%), specificity (96-97%) and positive predictive value (39-51%) (Bergink et al., 2011). Thus, in this study, participants who scored 10 or above on the EPDS comprised the 'depression-symptom' group, whereas participants who scored 9 and below were the control group.

Spielberger Trait Anxiety Inventory (STAI). The STAI is commonly used to identify symptoms of anxiety, and contains 20 items that identify trait anxiety, such as 'I worry too much over something that doesn't matter' and 'I am a steady person'. Participants were required to rate their response on a 4-point scale from 'almost never' to 'almost always'. The maximum score for this questionnaire is 60, and higher scores indicate higher levels of anxiety. The STAI has good test-retest reliability coefficients (.65 to .75) over a 2-month interval, and internal consistency coefficients have ranged from .86 to .95 (Spielberger et al., 1983).

Perceived Stress Scale (PSS). The PSS is a widely used psychological instrument for assessing the perception of stress (Cohen et al., 1983). It is a 10-item questionnaire, which is designed to measure to the degree to which situations in one's life may be appraised as 'stressful'. The scale contains a number of direct questions about current levels of perceived stress, such as 'In the last week, how often have you felt that things were going your way?' However, the scale also contains questions about how unpredictable and uncontrollable respondents find their lives, such as 'In the last week, how often have you been upset because of things which happened unexpectedly?' Participants are required to rate their responses on a 5-point scale from 'Never' to 'Very Often'. The maximum score on this scale is 40, and higher scores indicate higher levels of perceived stress. This scale has established reliability and validity (Cohen et al., 1983).

2.2.3.2 Maternal psychological response to infant distress

The following measures were administered both pre- and post-film.

Spielberger State Anxiety Inventory (SSAI). This is a 20-item questionnaire used to identify symptoms of state anxiety, such as 'I feel calm', 'I feel tense' and 'I am worried'. Participants are required to rate their responses on a 4-point scale from 'not at all' to 'very much'. The maximum score on this questionnaire is 60, and higher scores indicate higher levels of state anxiety. As with the Spielberger Trait Anxiety Inventory, the SSAI has also shown good internal consistency (0.86-0.95) and test re-test reliability (0.65-0.75) (Spielberger et al., 1983).

Positive and Negative Affect Schedule (PANAS). The PANAS is a 20-item self-report measure of positive and negative affect (Watson et al., 1988). Positive affect represents the extent to which an individual experiences pleasurable engagement with the environment, whereas negative affect represents subjective distress and un-pleasurable engagement with the environment. Thus, emotions such as enthusiasm and alertness are associated with positive affect, whereas lethargy and sadness are associated with negative affect (Watson and Clark, 1984). The scale contains 10 words that relate to positive affect such as 'excited' and 'proud', and 10 that relate to negative affect such as 'disinterested' and 'nervous'. Participants are required to rate to what extent they currently feel each emotion or feeling on a 5-point scale from 'slightly or not at all' to 'extremely'. Scores on the positive words are summed to create the positive affect score, and scores on the negative words are summed to give the negative affect score. Higher scores indicate higher levels of positive and/or negative affect. The scales have shown high internal consistency and are stable over a 2-month period (Watson et al., 1988).

Visual analogue scales. After the film, participants were also asked to complete three visual analogue scales, rating 'how much did you want to comfort the baby?', 'how

upsetting did you find the film?' and 'how good do you think you would be at comforting the baby?'.

2.2.3.3 Salivary biochemical assays

Cortisol. Salivary cortisol concentrations were quantified using a direct double-antibody radioimmunoassay (RIA) with utilisation of 125I-cortisol as the ligand. Assay kits were sourced from Salimetrics, UK, and analysis was carried out in accordance with the manufacturers instructions. Samples were analysed in singlets, and the minimum detectable concentration was 0.2nmol/l when a 0.1ml volume was assayed. For analysis of the diurnal samples, the log of the area under the curve (log AUC) of the diurnal cortisol samples was used. This was calculated using the following equation:

$$\text{Log AUC} = (0.5\ (0.5\ (\text{logD1}+\text{logD4}) + 0.5\ (\text{logD2} + \text{logD5})) + 23/0.5\ (0.5(\text{logD2} + \text{logD5}) + 0.5(\text{logD3}+\text{logD6}))$$

$$= (0.5(\text{log mean awakening} + \text{log mean 30 minutes post awakening}))+(11.5(\text{log mean 30 minutes post awakening} + \text{log mean 12 hours post awakening})$$

Alpha-amylase. The salivary alpha-amylase test determines the amount of alpha-amylase activity present in each sample. It works by using the chromogenic substance 2-chloro-p-nitrophenol linked with maltotriose. The alpha-amylase present in saliva breaks down this substance and yields 2-chloro-p-nitrophenol, which is yellow and can be measured spectrophotometrically at 405nm. The amount of alpha-amylase present in the sample is directly proportional to the increase in

absorbance at 405nm. The protocol used here differs from that recommended by the manufacturer in two ways; firstly, the volumes added to the 96-well plate were reduced to prevent sample loss during the mixing phase, but the ratio of substrate to sample was maintained at that recommended by the manufacturer (40:1). Secondly, the method of calculation of the alpha-amylase activity was changed from the manufactures recommendation of using their pre-prepared equation. This was partly as a consequence of changing the sample volume, which would alter the path length from that given in the equation, but also the equation does not take into account test to test variability due to age of substrate and other experimental errors. Therefore, the amount of amylase activity was calculated using a six point standard curve (one per plate) made from a serial dilution of the high control provided by the manufacturer (with a concentration 285 units/ml +/-71). To reduce plate-to-plate variation, all reagents were from the same lot and were pooled before use; the same standard curve was used throughout the experiment.

Briefly the protocol was as follows: Saliva samples were diluted 1:200 in assay diluent. 5ul of diluted saliva was added to a well of a 96 well plate (16 wells were run at a time). 200ul of pre-warmed 37^{o}C substrate solution (2-chloro-p-nitrophenol linked with maltotriose) was added with a multichannel pipette. The plate was immediately transferred to a heated spectrophotometer and mixed. Optical density readings were taken at 1 and 3 minutes and the change in optical density was calculated by subtracting the first from the second reading. Amount of amylase activity present in the samples was calculated using the standard curve equation. Following optimisation of the assay, this method was tested for reliability and found

to be highly replicable (intra-assay coefficient of variance=3.73, inter-assay coefficient of variance=9.55).

2.2.4 Statistical Analysis

The demographic characteristics of the two groups were compared using t-test and chi squared tests. Pearson's bivariate correlations were used to assess associations between demographic variables, and salivary cortisol and alpha-amylase measures. Repeated measures ANOVAs were used to assess changes in self-reported measures of mood, salivary cortisol and alpha-amylase in response to the infant distress film. Time was used as a within-subjects effect, and group (depressive symptom *vs.* control) and trimester as between-subjects effects. Repeated measures ANOVAs were also used to assess diurnal changes in salivary cortisol and alpha amylase levels, and a log AUC of both diurnal cortisol and alpha-amylase was used as an index of total diurnal levels and assessed using a univariate ANOVA.

2.3 Results

2.3.1 Descriptive statistics

Participant demographics are shown below in Table 3. This primarily Caucasian sample of women was highly educated, had a mean age of 31 and all participants were primiparous. At the time of assessment, the mean gestational length was 187 days (approx. 6.23 months). 50 (48.5%) women were in their second trimester and 53 (51.5%) were in their third trimester.

Table 3 Demographic characteristics of the sample by group and trimester

	Second trimester		**Third trimester**	
Demographic Variables	Control Group (N=41)	Depressive symptom group (N=9)	Control Group (N=38)	Depressive symptom group (N=15)
Age (m, SD)	31.0 (5.27)	31.3 (3.16)	31.9 (4.07)	31.9 (5.12)
Education (n,%)				
GCSE/O-level	1 (2.4)	-	-	-
A-level	1 (2.4)	1 (11.1)	1 (2.6)	-
Undergraduate degree	16 (39)	5 (55.6)	13 (34.2)	2 (13.3)
NVQ	2 (4.9)	-	2 (5.3)	3 (20.0)
Postgraduate degree	21 (51.2)	3 (33.3)	22 (57.9)	10 (66.7)
Ethnicity (n,%)				
Caucasian	39 (95.2)	7 (77.8)	37 (97.4)	11 (73.3)
Black	-	-	-	1 (6.7)
Asian	1 (2.4)	1 (11.1)	1 (2.6)	2 (13.3)
Chinese	-	1 (11.1)	-	1 (6.7)
Mixed Race	1 (2.4)	-	-	-
Alcohol units/week (n,%)				
None	35 (85.4)	6 (66.7)	32 (84.2)	13 (86.7)
1-5	6 (14.6)	3 (33.3)	6 (15.8)	2 (13.3)
Cigarettes/week (n,%)				
None	31 (75.6)	9 (100)	34 (89.5)	12 (80)
Did not respond	10 (24.4)	-	4 (10.5)	3 (20)
Weeks of gestation (mean, SD)	20.2 (3.9)	20.8 (4.6)	33.7 (9.9)	33.6 (4.9)
Planned Pregnancy (n, %)	36 (87.8)	9 (100)	31 (81.6)	12 (80)

Previous history of metal health problems (n,%)	7 (17.1)	4 (44.4)	10 (26.3)	8 (53.3)
Antenatal mood (mean, SD)				
EPDS (depression)	3.61 (2.78)	12.44 (3.32)	4.32 (3.19)	14.27 (3.51)
TAI (trait anxiety)	31.44 (6.97)	45.22 (6.30)	33.89 (7.51)	48.13 (7.95)
PSS (perceived stress)	8.27 (4.77)	17.89 (7.57)	9.63 (5.21)	19.67 (6.11)

EPDS: Edinburgh Postnatal Depression Scale, STAI: Spielberger Trait Anxiety Index, PSS: Perceived Stress Scale

The demographic characteristics of the groups were compared:

a) There was no significant difference between groups in maternal age ($t_{(101)}$=-0.229, p=0.819).
b) There was no significant difference between groups in educational qualifications ($\chi^2_{(4)}$=2.271, p=0.686).
c) The control group consisted of more Caucasian participants than the depressive-symptom group ($\chi^2_{(6)}$=15.750, p=0.015).
d) There was no significant difference between groups in alcohol consumption ($\chi^2_{(1)}$=0.425, p=0.514).
e) There was no significant difference between the groups in the proportion of planned pregnancies ($\chi^2_{(1)}$=0.107, p=0.744).
f) There were no significant group differences in previous diagnoses of mental health disorders ($\chi^2_{(3)}$=0.752, p=0.861).
g) There was no significant difference between groups in days of gestation ($t_{(101)}$=-1.640, p=0.104).
h) As expected, participants of the depressive-symptom group had significantly higher scores on the EPDS than the control group ($t_{(101)}$=-13.286, p<0.001).
i) Participants of the depressive-symptom group scored significantly higher on the trait anxiety scale ($t_{(101)}$=-8.462, p<0.001).

j) Participants of the depressive symptom group also scored significantly higher on the perceived stress questionnaire ($t_{(101)}$=-8.000, p<0.001).

2.3.1.1 Maternal antenatal depressive symptoms

Scores on the EPDS ranged from 0 to 22, with a mean of 6.19 and standard deviation of 5.13. A histogram of the distribution of EPDS scores in this sample is presented in Figure 8, and the cut-off point used to establish the two groups is depicted. The EPDS scores were not normally distributed (p=0.002), and were skewed to the left (-0.482). EPDS scores were also highly and significantly correlated with trait anxiety (r=0.767,p<0.001) and perceived stress (r=0.847,p<0.001) scores.

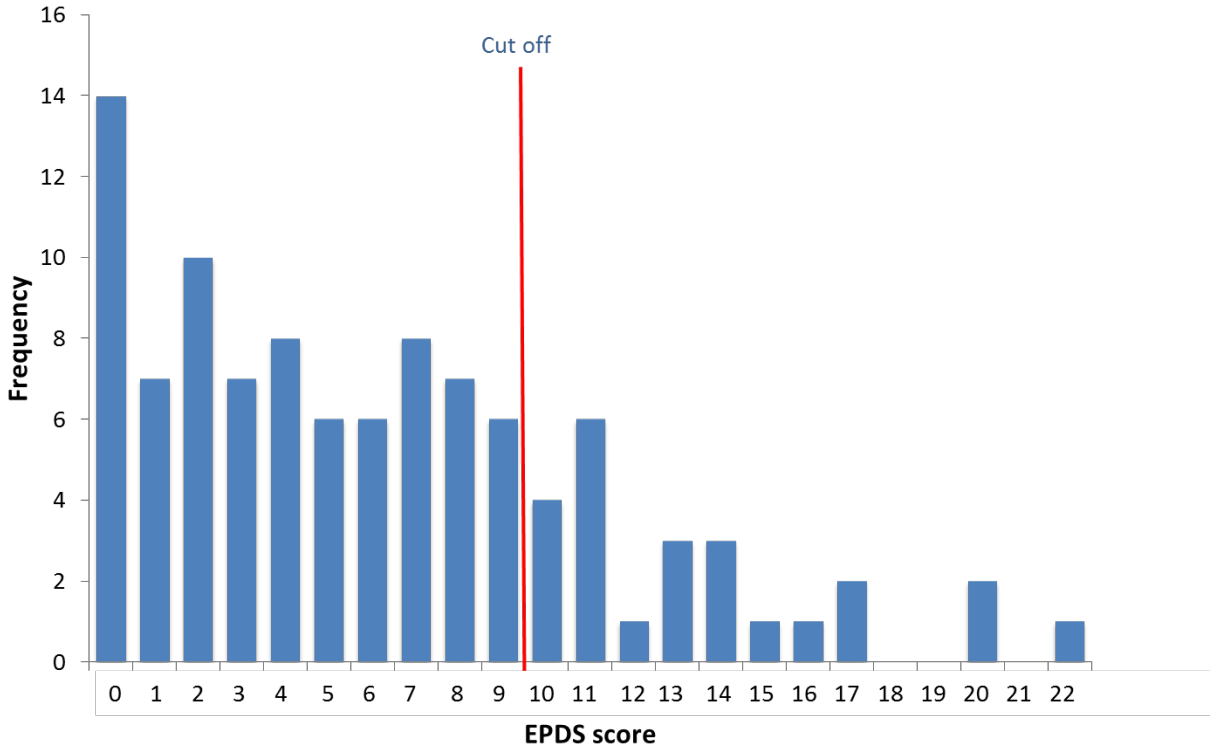

Figure 7 Histogram of the distribution of maternal antenatal EPDS scores in this sample of 103 pregnant women, and the cut-off point used to identify a 'depression-symptom' group.

2.3.2 Correlations

Associations between the maternal demographic characteristics were assessed using Pearson's bivariate correlations. Although measures of maternal mood were all highly and significantly correlated, there were no significant correlations between any of the other demographic variables.

Correlations between maternal demographic variables and cortisol/alpha-amylase measures were also assessed. Gestation was significantly correlated with salivary cortisol measures across the test session, and also with evening cortisol (all p's<0.001), reflecting an increase in cortisol concentration with an increase in gestation. Also, salivary cortisol and alpha-amylase measures during the test session were significantly correlated with maternal education (all p's < 0.05), suggesting that higher educational qualifications were associated with higher salivary cortisol levels, and lower salivary alpha-amylase levels. However, associations with maternal educational qualifications did not remain significant following a correction for multiple testing. No other maternal demographics characteristics were significantly correlated with any salivary cortisol or alpha-amylase measures.

2.3.3 Responses to the infant distress stimulus

2.3.3.1 Psychological responses to the infant distress film

At baseline, participants in the depressive symptom group had significantly higher scores on state anxiety scale ($F_{(1)}$=32.13, p<0.001) and on the negative PANAS scale ($F_{(1)}$=13.44, p<0.001), and significantly lower scores on the positive PANAS scale ($F_{(1)}$=12.41, p<0.01) than the control participants. Baseline scores are shown in Table 4.

Table 4 Psychological responses to the infant distress film

	Control Group (n=79)		Depressive-symptom Group (n=24)	
	Pre-film	Post-film	Pre-film	Post-film
State Anxiety (mean, SD)	24.94 (4.78)	32.57 (9.80	33.96 (10.08)	44.50 (10.47)
Positive Affect (mean, SD)	34.89 (7.24)	34.17 (7.68)	28.83 (8.16)	26.65 (8.38)
Negative Affect (mean, SD)	11.20 (1.84)	13.43 (4.54	13.42 (3.45)	16.35 (5.51)
VAS "How much did you want to comfort the baby?" (mean, SD)		8.01 (2.27)		7.40 (2.32)
VAS "How upsetting did you find the film?" (mean, SD)		5.16 (2.34)		4.98 (2.40)
VAS "How good do you think you would be at comforting the baby?" (mean, SD)		6.66 (1.72)		6.03 (2.71)

VAS: visual analogue scale

A repeated measures ANOVA was used to assess changes in mood scores from pre- to post-film. There was a significant main effect of time reflecting an increase in state anxiety following film viewing in both groups ($F_{(1)}$=65.45, p<0.001). There was also a main effect of group ($F_{(1)}$=36.27, p<0.001), which reflects the relatively increased state anxiety scores at both time points in the depression-symptom group. However,

there was no significant effect of trimester ($F_{(1)}$=2.163, p=0.145), suggesting that psychological responses to the video remain stable across the second and third trimesters. Further, there was no significant interaction between time and group ($F_{(1)}$=1.63, p=0.205), suggesting that the two groups did not differ in the magnitude of change in state anxiety from pre- to post-film, see Table 4.

Similarly, there was a main effect of time and group on positive and negative affect scores (all p's<0.05). This reflects a decrease in positive affect and an increase in negative affect from pre- to post-film in both groups. It also reflects the relatively increased negative affect scores and decreased positive affect scores at both time points in the depression-symptom group. However, there was no significant interaction between time and group for both positive affect ($F_{(1)}$=1.23, p=0.270), and negative affect ($F_{(1)}$=0.113, p=0.738), again showing that the two groups did not differ in the magnitude of change from pre to post film. There was also a significant group*trimester interaction for the negative affect scores ($F_{(1)}$=4.63, p=0.034). Further investigation revealed that for the third trimester participants, there was a main effect of group ($F_{(1)}$=15.91, p<0.001), such that those with symptoms of depression scored higher for negative affect, but this was not significant for the second trimester participants ($F_{(1)}$=0.339, p=0.563).

On the three visual analogue scales, there were no significant differences between the groups on how they rated their desire to comfort the babies in the film ($F_{(1)}$=0.142, p=0.288), how upsetting they found the film ($F_{(1)}$=0.350, p=0.555), or how good they thought they would be at comforting the babies in the film ($F_{(1)}$=0.802, p=0.373). There were also no significant effects of trimester (all p's>0.05) or a trimester*group interaction (all p's>0.05).

2.3.3.2 Salivary cortisol responses to the infant distress film

Cortisol outliers that were more than three standard deviations from the mean were excluded (11 of 515 data points excluded). Descriptive statistics of the cortisol measures at each time point are shown in Table 5.

Table 5 Descriptive statistics for cortisol measures taken at each time point in response to the film

Measure	N	Minimum	Maximum	Mean	SD
Baseline 1	100	0.52	15.94	5.03	3.73
Baseline 2	100	0.60	12.55	4.46	3.22
Immediately post-film	101	0.49	16.50	4.62	3.48
10 minutes post-film	101	0.35	12.32	4.21	2.98
20 minutes post-film	102	0.28	14.76	4.28	3.32

A repeated measures ANOVA was used to assess change in cortisol concentrations over the test session. There was no significant within-subjects effect of time ($F_{(3)}$=0.697, p=0.557), which reflects no significant change in cortisol concentrations in response to the film. Similarly, there were no significant interactions between time, trimester and group (all p's>0.05), suggesting that participants from both groups and both the second and third trimester did not show a change in salivary cortisol across the test session.

There was a main effect of time of day on cortisol concentrations ($F_{(1)}$=4.288, p=0.050). A negative correlation between time of day of assessment and baseline cortisol (r=-0.543, p<0.001) suggests that as the day progresses baseline cortisol

levels decrease, which reflects the diurnal cortisol decline. There was also a significant between-subjects effect of trimester on salivary cortisol ($F_{(1)}$=4.305, p=0.049), reflecting higher cortisol concentrations in the third trimester participants. However, there was no significant effect of group ($F_{(1)}$=0.646, p=0.430), and no significant interaction between group and trimester ($F_{(1)}$=3.724, p=0.203). This is demonstrated graphically in Figure 9.

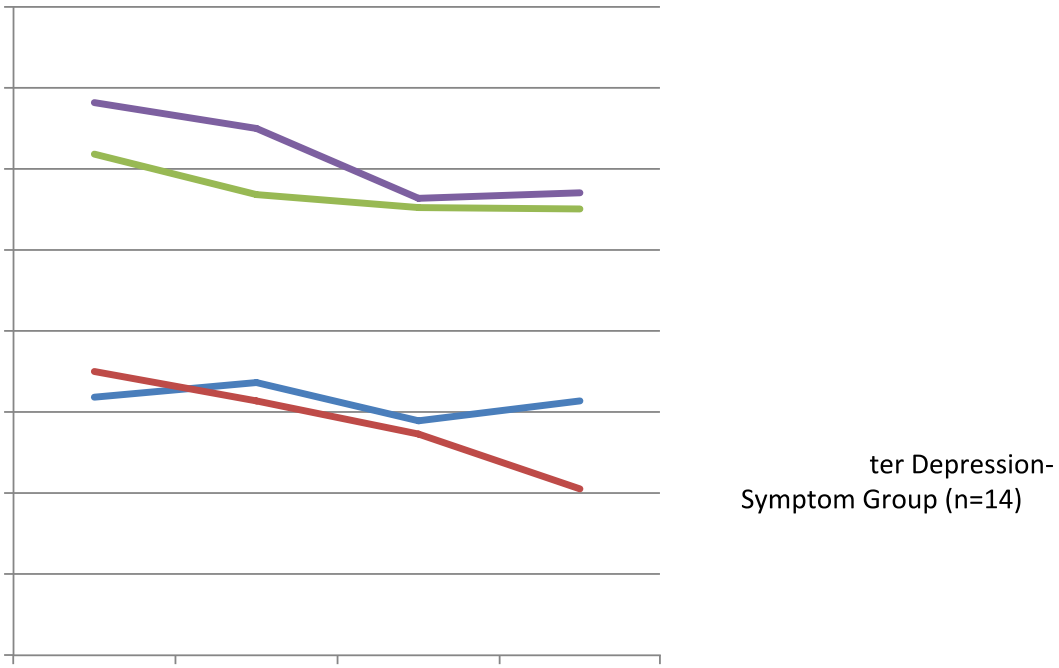

Figure 8 Salivary cortisol responses to infant distress film

2.3.3.3 Salivary alpha-amylase responses to infant distress film

Alpha-amylase data points were excluded if the optical density value was outside of the range defined by the standard curve (5 samples excluded), and outliers that were more than 3 standard deviations above the mean were also excluded (6 samples

excluded). Descriptive statistics for the alpha-amylase measures at each time point are displayed in Table 6.

Table 6 Descriptive statistics for alpha-amylase measures taken at each time point in response to the film

Measure	N	Minimum	Maximum	Mean	SD
Baseline 1	101	8.14	330.07	113.62	74.44
Baseline 2	103	4.86	314.64	106.93	73.61
Immediately post-film	101	8.21	324.21	109.79	68.63
10 minutes post-film	99	9.29	240.86	102.87	59.48
20 minutes post-film	99	6.21	287.14	105.53	64.42

A repeated measures ANOVA revealed that there was no significant effect of time ($F_{(3)}$=1.809, p=0.146), reflecting no change in salivary alpha-amylase across the test session. There were also no significant interactions between time, group and trimester (all p's>0.05), suggesting that participants of both groups and both trimesters did not show a significant change in salivary alpha-amylase over the test session.

Further, there were no significant between-subjects effects of group ($F_{(1)}$=1.613, p=0.207), or trimester ($F_{(1)}$=0.029, p=0.865), or a group*trimester interaction ($F_{(1)}$=0.647, p=0.423). This is demonstrated graphically in Figure 10. There was also no significant effect of time of day of assessment ($F_{(1)}$=1.150, p=0.286).

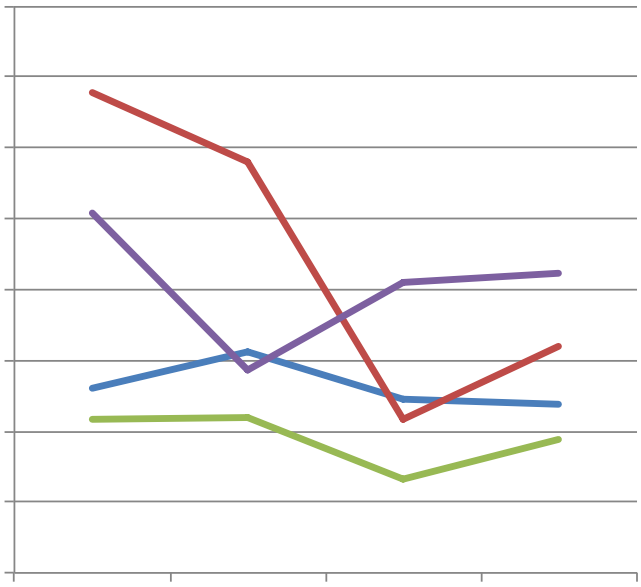

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Figure 9 Salivary alpha-amylase responses to the infant distress film

2.3.4 Diurnal salivary biomarkers

2.3.4.1 Salivary cortisol

Cortisol outliers that were more than three standard deviations from the mean were excluded (9 of 540 data points were excluded). Descriptive statistics for cortisol measures at each time point are shown in Table 7.

Table 7 Descriptive statistics for the diurnal cortisol measures

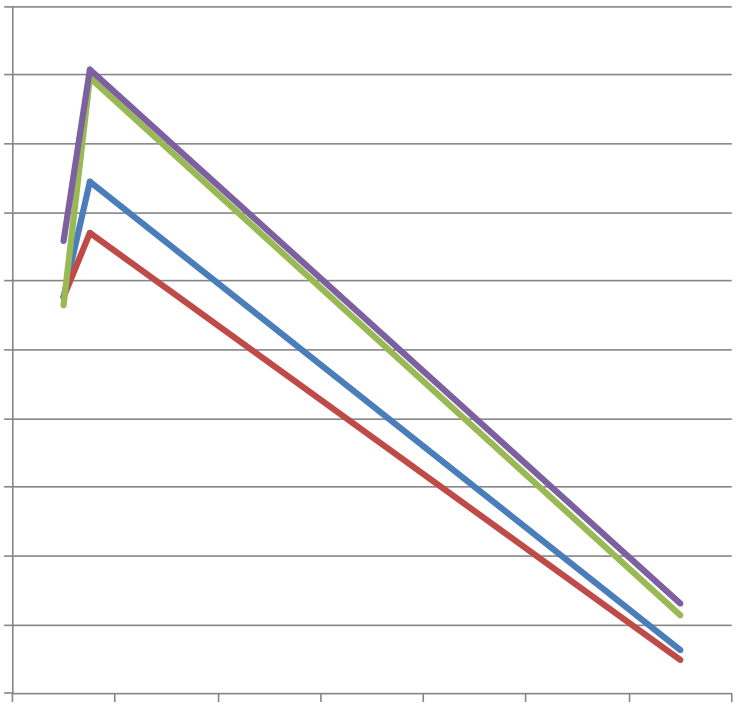
Measure	N	Minimum	Maximum	Mean	SD
Day 1 awakening	90	1.54	31.51	12.26	6.55
Day 1 30 minutes post-awakening	89	1.11	53.05	15.75	10.16
Day 1 12 hours post-awakening	89	0.27	8.14	1.86	1.54
Day 2 awakening	88	2.34	26.77	10.78	5.81
Day 2 30 minutes post-awakening	89	1.2	46.8	16.58	9.52
Day 2 12 hours post-awakening	87	0.13	5.26	1.70	1.17

A repeated-measured ANOVA was used to investigate changes in diurnal cortisol concentrations. There was a significant within-subjects effect of time ($F_{(2)}$=125.38, $p<0.001$), reflecting an increase in cortisol from awakening to 30 minutes post-awakening ($t_{(90)}$=-5.710, $p<0.001$), followed by a decrease in salivary cortisol from 30 minutes post-awakening to 12 hours post-awakening ($t_{(89)}$=17.546, $p<0.001$). However, there were no significant interactions between time, group and trimester (all p's>0.05), suggesting that all participants showed the typical diurnal cortisol pattern.

There were no significant between subjects effects of group ($F_{(1)}$=0.003, p=0.956), however the effect of trimester tended towards significance ($F_{(1)}$=3.629, p=0.060),

reflecting higher cortisol levels in the third trimester participants. There were no significant interactions between group and trimester. These results are presented graphically in Figure 11.

As an index of total cortisol levels, the log AUC of diurnal cortisol was also considered using a univariate ANOVA. Here, there was a main effect of trimester ($F_{(1)}$=12.359, p<0.001), which reflects higher cortisol levels in the third trimester participants. However, there was no significant effect of group ($F_{(1)}$=0.599, p=0.441), or a group*trimester interaction ($F_{(1)}$=1.631, p=0.205).



up (n=15)

Figure 10 Diurnal salivary cortisol

2.3.4.2 Salivary alpha-amylase

Alpha-amylase data points were excluded if the optical density value was outside of the range defined by the standard curve (50 samples), and alpha-amylase outliers that were more than three standard deviations outside the mean were excluded (10 samples). Descriptive statistics of the diurnal alpha-amylase measures are presented in Table 8.

Table 8 Descriptive statistics for the diurnal alpha-amylase measures

Measure	N	Minimum	Maximum	Mean	SD
Day 1 awakening	68	10.67	337.33	101.38	75.95
Day 1 30 minutes post-awakening	72	7.47	202.47	59.38	44.03
Day 1 12 hours post-awakening	73	4.57	313.93	116.13	66.65
Day 2 awakening	71	7.93	327.13	99.99	73.65
Day 2 30 minutes post-awakening	69	4.93	216.07	57.74	45.38
Day 2 12 hours post-awakening	72	5.80	369.71	115.60	79.40

There was a significant within-subjects effect of time ($F_{(2)}$=32.24, p<0.001), which reflected a decrease in salivary alpha-amylase concentrations from awakening to 30 minutes post-awakening ($t_{(74)}$=6.289, p<0.001), and an increase in concentrations from 30 minutes post-awakening to 12 hours post-awakening ($t_{(75)}$=-8.802, p<0.001). There was also a significant interaction between time and group ($F_{(2)}$=4.707, p=0.010). Further investigation revealed that on awakening, participants in the depression-symptom group had significantly higher alpha-amylase concentrations than the control group ($t_{(73)}$=-2.737, p=0.008), however at

30 minutes and 12 hours post-awakening there was no significant difference between the groups (p's>0.05).

The between-subjects effect of trimester was non-significant ($F_{(1)}$=0.258, p=0.613), and the effect of group tended towards significance ($F_{(1)}$=3.220, p=0.077). However, there was a significant interaction between group and trimester ($F_{(1)}$=4.046, p=0.040). To further investigate this interaction effect, second and third trimester participants were considered separately. In the second trimester participants, the effect of group was not significant ($F_{(1)}$=0.019, p=0.892), however group was significant for the third trimester participants ($F_{(1)}$=10.924, p=0.002). This suggests that in late pregnancy, women with symptoms of depression have higher levels of salivary alpha-amylase across the day than women without symptoms of depression. The results are depicted graphically in Figure 12.

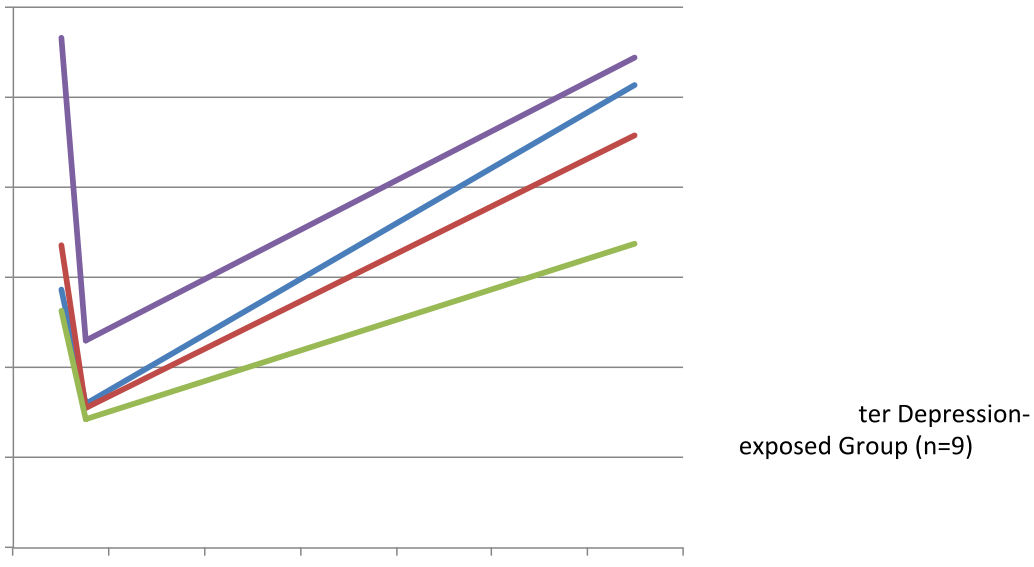


Figure 11 Diurnal salivary alpha-amylase

Log AUC of diurnal alpha-amylase was also considered as an index of total alpha-amylase levels. There was no significant effect of group ($F_{(1)}$=2.660, p=0.107) or trimester ($F_{(1)}$=0.473, p=0.494), however the group*trimester interaction approached significance ($F_{(1)}$=3.935, p=0.051). Further investigation revealed that in the third trimester participants, those with symptoms of depression had significantly higher log AUC alpha-amylase than control participants ($F_{(1)}$=6.912, p=0.013), but there was no significant group difference in the second trimester participants ($F_{(1)}$=0.060, p=0.808). This is presented graphically in Figure 13.

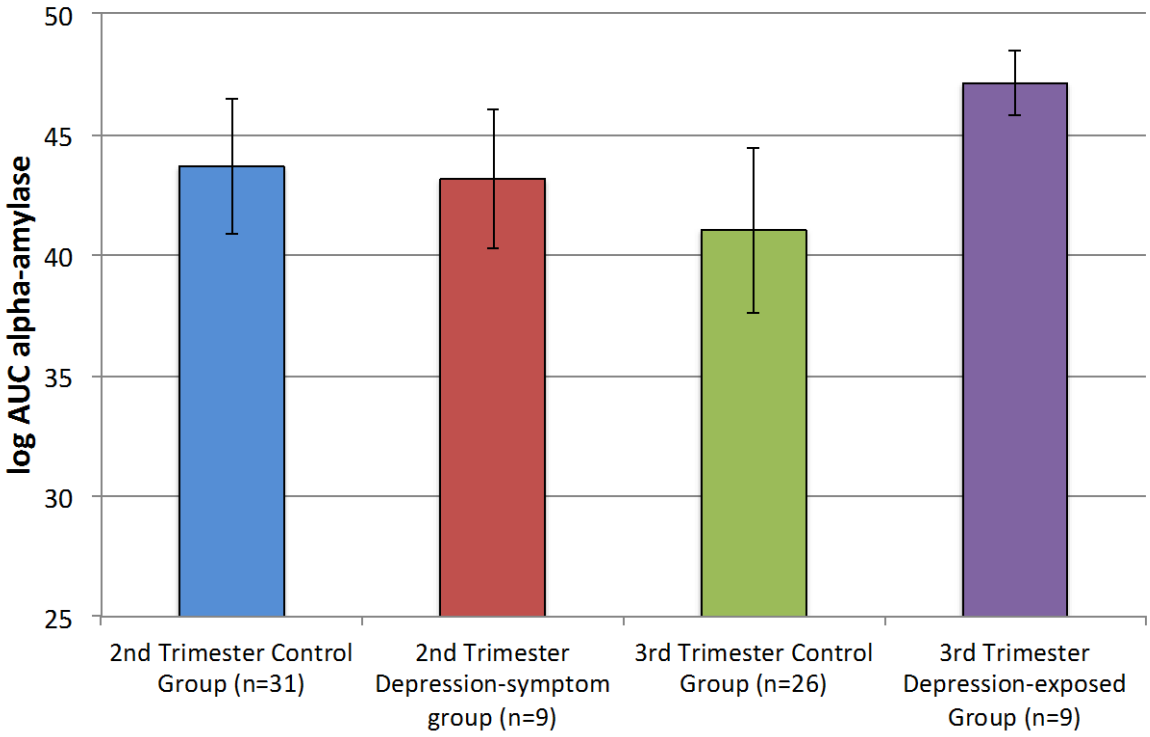


Figure 12 Log AUC of diurnal alpha-amylase, split by group and trimester

2.3.5 Summary of Findings

Responses to infant distress film

- *Psychological*
    - Participants showed significant increases in state anxiety and negative affect, and a decrease in positive affect, in response to the film.
    - There were no significant effects of depression or trimester on mood responses.
- *Salivary cortisol*
    - There was no change in salivary cortisol across the test session.
    - Participants in the third trimester had significantly higher salivary cortisol concentrations than participants of the second trimester.
    - There was no association between antenatal depression and salivary cortisol.
- *Salivary alpha-amylase*
    - There was no change in salivary alpha-amylase across the test session.
    - There were no associations between antenatal depression, trimester, and salivary alpha-amylase.

Diurnal biomarkers of stress

- *Salivary cortisol*
    - Both groups of participants showed a typical pattern of diurnal cortisol release.

- There were no significant effects of depression or trimester on cortisol samples taken across the day.
- The log AUC of diurnal cortisol was significantly greater for third trimester participants than second trimester participants.

- *Salivary alpha-amylase*
  - Both groups showed a typical pattern of diurnal salivary alpha-amylase release.
  - Participants with symptoms of depression had significantly higher salivary alpha-amylase concentrations immediately after awakening than control participants.
  - Third trimester participants with symptoms of depression had significantly higher salivary alpha-amylase concentrations across the day, and also a higher log AUC than third trimester control participants. There were no effects of depression in the second trimester participants.

2.4 Discussion

This study was an investigation into the effects of depressive symptoms during pregnancy on salivary cortisol and alpha-amylase responses to an acute stressor, and on diurnal salivary cortisol and alpha-amylase levels. The main finding of this study is that women with symptoms of depression did not show exaggerated salivary cortisol or alpha-amylase responses to an infant distress stimulus compared to the control group. Indeed, the infant distress film failed to induce a significant biological stress response in this group of pregnant women, despite increases in self-reported state anxiety.

A further finding from this study is that pregnant women with symptoms of antenatal depression did not have increased diurnal salivary cortisol concentrations compared with the control group. However, those participants with symptoms of depression in late pregnancy had increased salivary alpha-amylase levels.

The lack of association in this study between antenatal depression symptomatology and salivary cortisol does not support the hypothesis that pregnant women with symptoms of depression have an overactive HPA axis, and therefore, this may not be a mechanism by which symptoms of depression during pregnancy impact upon fetal and infant development. Thus, the findings of this study suggest that perhaps other biological mechanisms are involved, including, for example, changes in sympathetic nervous system activity.

2.4.1 Responses to the film

A current theory suggests that one potential pathway by which antenatal mood disturbance may impact on fetal and infant development is via programming of both the maternal and fetal HPA axis during pregnancy. There is already some evidence to support this theory: maternal and fetal cortisol levels are correlated (Glover et al., 2009b), and there is evidence that infants born to mothers who were distressed in the antenatal period have increased cortisol responses to acute stress (Davis et al., 2011a, Leung et al., 2010, Oberlander et al., 2008). However, the evidence linking maternal mood disturbance with increased cortisol levels during pregnancy is less clear. Previous studies have provided mixed results (Obel et al., 2005, O'Connor et al., 2013a, Pluess et al., 2012, Hellgren et al., 2013) and are generally limited to diurnal measures. As such, this study was an attempt to further add to this body of literature by measuring salivary cortisol responses to a stressor thought to be particularly potent during pregnancy (infant distress), as well as assessing an alternative salivary biomarker of stress; alpha-amylase.

This study is an extension of previous work, which demonstrated that in a group of late first/early second trimester women, those with symptoms of depression had significantly greater salivary cortisol responses to an infant distress video than a group of non-depressed control participants (Murphy et al., 2014). However, the current study failed to reproduce these findings in a group of second and third trimester participants. One possible explanation for the contradictory findings is that as gestation progresses, serum cortisol levels rise so that by term serum cortisol concentrations are four times higher than in the non-pregnancy state. As such, detecting changes in cortisol in response to an acute stressor in later pregnancy

becomes difficult, as baseline cortisol concentrations may be very close to ceiling levels. Similarly, the infant distress video failed to induce a significant increase from baseline in salivary alpha-amylase in this group of pregnant participants. Though notably, it appeared that those participants with symptoms of depression had more reactive salivary alpha-amylase levels across the test session than the control participants. Just one previous study has investigated salivary alpha-amylase responses to acute stress in a group of pregnant women: Nierop and colleagues (2006) reported increased salivary alpha-amylase in response to a psychological stress test, which included a period of public speaking and mental arithmetic. However, a significant limitation of the Nierop study was the use of cotton salivettes to collect maternal saliva, as there is evidence to suggest that this method of saliva collection can inflate salivary alpha-amylase concentrations (Rohleder and Nater, 2009). The current study, however, used the passive drool method, which avoids the stimulation of saliva production. Thus, differing methodologies could explain the lack of effect found in this study.

A further possible explanation is that the infant distress video was not sufficiently potent to induce a significant biological stress response. Although interestingly, participants reported that they found the film distressing, which was reflected by higher state anxiety and negative affect scores following the film. Previous studies of stress reactivity in pregnancy have typically used the Trier Social Stress Test to probe antenatal HPA function, and have reported significant increases in salivary cortisol in response to this type of stressor (de Weerth and Buitelaar, 2005, Nierop et al., 2006). Notably, physical stressors, such as the cold hand test, have failed to induce similar cortisol responses in pregnancy (Kammerer et al., 2002, Saisto et al., 2004). An

important consideration for this research is that perhaps the type of increased social anxiety induced by the TSST is a particularly good probe for inducing HPA stress responses. On the other hand, one could argue that the infant distress video used in this study did not induce feelings of social anxiety, which may be accompanied for example by an increased heart rate, blood pressure and HPA activation. But instead, the film was a psychologically disturbing stressor, and although participants reported increases in state anxiety, this type of stress may not be a suitable probe of HPA function.

In consideration of this point, when studying symptoms of antenatal depression and anxiety in a laboratory setting, using a stimulus such as infant distress may be more appropriate than the TSST, because it may be more applicable to real-life situations. For example, antenatal depression and anxiety is associated with long-term psychological distress, therefore testing physiological responses using a psychologically distressing stimulus may be more appropriate than using a stimulus that is an efficient probe of the HPA axis.

2.4.2 Diurnal salivary biomarkers

A secondary aim of this study was to investigate how maternal antenatal depression may influence diurnal measures of salivary cortisol and alpha-amylase. It is very clear from the results of this chapter that the diurnal pattern of salivary cortisol and alpha-amylase is maintained across pregnancy. Diurnal salivary cortisol is indexed by a peak in concentrations at 30 minutes post-awakening, followed by a gradual diurnal decline. Diurnal sAA has the opposite pattern of effect: a dip in concentrations after

awakening followed by a gradual diurnal incline. These typical patterns of release were evident in both second and third trimester participants.

As expected there was a significant effect of trimester on salivary cortisol concentrations, which reflects increased salivary cortisol concentrations in the third trimester participants. This is consistent with a number of previous findings (Obel et al., 2005, Entringer et al., 2010, Nierop et al., 2006). However, there were no significant effects of maternal antenatal depressive symptoms on diurnal salivary cortisol. This is inconsistent with a recent finding by O'Connor and colleagues, who reported that antenatal depression was associated with a blunted cortisol awakening response and a diminished diurnal decline. A possible explanation for the differing results is that participants from the O'Connor (2013) study were drawn from a high-risk sample, where 23% of participants had a clinical diagnosis of depression. On the other hand the group of participants in the current study were recruited from a low-risk community sample. Levels of depressive symptoms were low to moderate and only a small number of participants (3%) had been diagnosed with a current depressive disorder. It is possible that there would have been significant group differences in diurnal cortisol had this sample consisted of a high-risk group of women with moderate to severe levels of depression.

As sAA has only recently been identified as an alternative biomarker of stress reactivity, this is just the second study to investigate diurnal levels of sAA in pregnancy. A particularly striking characteristic of the diurnal sAA results is that the range of concentrations appears to be very large (5.8–369.7 Units/ml). Indeed this range is greater than the previous study to report on diurnal sAA in pregnancy (10.19–45.87 Units/ml) (Giesbrecht et al., 2012). Although, a study of diurnal sAA in

a non-pregnant healthy population reported a range of 6 –235 Units/ml (Nater et al., 2007), which is more similar to the concentrations presented in this chapter. Further, the previous study of sAA in pregnancy reported a significant association between trait anxiety and increased sAA concentrations across the day, however this association was not replicated in the current study. There were no significant correlations between self-reported depressive symptoms and sAA. However, the current study did identify a novel finding, that those participants in later pregnancy with symptoms of depression had higher sAA concentrations across the day than control participants. Conversely, depression did not have a significant effect on diurnal sAA in second trimester participants. Thus, as sAA has been suggested to be an indirect marker of sympathetic nervous activity (Rohleder and Nater, 2009), the results from this study suggest that in later pregnancy, participants with symptoms of depression may have increased sympathetic nervous system activation, which could be related to vasoconstriction and reduced fetal blood flow. This could potentially explain associations between antenatal depression and obstetric outcomes such as low birth weight and premature birth (Nkansah-Amankra et al., 2010, Class et al., 2011, Hedegaard et al., 1996, Pritchard and Teo, 1994, Copper et al., 1996).

2.4.3 Strengths and Limitations

This study has a number of strengths, including a moderate sample size, measures of diurnal HPA and autonomic system function, as well as responses to an acute stressor, and a range of validated self-report measures to assess maternal antenatal mood. This allowed the direct testing of whether physiological changes in depressed

pregnant women are HPA-specific, an important question when considering fetal programming effects and potential targets for intervention.

A clear limitation of this study, as mentioned previously, is that this group of participants were not a clinical sample with moderate to severe symptoms of depression, but instead were drawn from a low risk community sample. As such, it was not possible to test for effects of severe depressive symptoms, and this may be a reason why no group differences were found in this study. Furthermore, depressive symptoms were characterised using a self-report measure rather than a structured clinical interview, which is important when considering the results for two reasons. Firstly, self-reported measures of depression may be unreliable due to the under- or misreporting of symptoms. Secondly, the use of a clinical interview to identify symptoms of depression may have identified a group of participants with more severe symptomatology; therefore comparing such a group with the control participants may have resulted in significant group differences. This approach has been taken previously in an investigation of depressive symptoms on diurnal cortisol levels in pregnancy, and it was found that a clinical diagnosis of depression more strongly predicted diurnal cortisol than did a self-report measure (O'Connor et al., 2013a).

A further limitation is that this study did not measure time of awakening or sleep duration, and these have been shown to be significant predictors of the cortisol awakening response in pregnancy (O'Connor et al., 2013a). Also, only three saliva samples were taken across each of two days in order to assess diurnal cortisol and alpha-amylase. A more accurate method to index diurnal measures, and indeed to

calculate the area under the curve, would have been to include more than three sampling times over the day.

Finally, the range for sAA concentrations in this study was extremely large; therefore there was a big within-group variation, and as a result this makes the detection of group differences difficult. One way to reduce variation in the measures would have been to analyse the samples in duplicate or triplicate, rather than in singlet, and to use a mean measure in the analysis. Future research should take this approach into consideration.

2.4.4 Conclusion

In conclusion, this study found no evidence to suggest that women with depressive symptoms during the second and third trimester have increased cortisol or alpha-amylase responses to an infant distress video compared with non-depressed controls. This is in contrast to a previous study, which found that women with symptoms of depression in the late first/early second trimester had increased salivary cortisol responses to an infant distress stimulus. Similarly, this study found no evidence that diurnal cortisol was raised in pregnant participants with symptoms of depression, however participants in late pregnancy with symptoms of depression had greater sAA levels. Therefore, if the effects of antenatal depression on fetal development are HPA-mediated, then early pregnancy may be a particularly vulnerable period. On the other hand, it could be that the HPA axis has a smaller role in mediating these effects than previously thought, and future research should pursue other potential mediating pathways, such as changes in sympathetic nervous system activity.

CHAPTER 3

INVESTIGATING THE INFLUENCE OF MATERNAL ANTENATAL DEPRESSIVE SYMPTOMS AND BIOLOGICAL MARKERS OF STRESS ON INFANT CORTISOL RESPONSES TO INOCULATION AND TEMPERAMENT

3 Overview of Chapter

This chapter investigates whether maternal antenatal symptoms of depression, as well as antenatal salivary cortisol and alpha-amylase, may predict infant cortisol responses to inoculation and temperament. Antenatal mood disturbance increases the risk for a number of adverse offspring outcomes, and a proposed mechanism by which risk is transferred is via changes in the maternal and infant HPA axis (Glover, 2011, Talge et al., 2007). It has also been suggested that maternal antenatal biomarkers of stress more strongly predict infant outcomes than self-reported measures of antenatal mood (Del Giudice et al., 2011). This chapter directly tests these ideas by assessing and comparing the predictive effects of both maternal self-reported depressive symptoms and salivary cortisol and alpha-amylase on infant salivary cortisol reactivity and temperament.

This study is an analysis of data collected as part of a short-term longitudinal study that took part in Oxford, UK, during 2013/14, described in Chapter 2. A subset of 88 mothers and their infants took part in this postnatal part of the study. Data on self-reported antenatal mood were available, as were 2 measures of maternal antenatal cortisol and alpha-amylase: mean change in response to a stressor and log AUC of diurnal measures. Participants were visited at home two months after birth. Mothers reported postnatal mood and infant behaviour, and also collected infant saliva samples on the day of their infant's first inoculation.

This chapter begins with an overview of studies that have investigated the relationship between antenatal mood disturbance, infant cortisol, and infant temperament. A description of the subsample of participants who took part in this study and the methods are presented, followed by the results of the data analyses. This chapter concludes with a discussion of the study findings in the context of the field, as well as the strengths and limitations, and the conclusions of this research.

This is the first study to use both self-reported measures of maternal antenatal mood as well as two biomarkers of antenatal stress (salivary cortisol and alpha-amylase) collected in two different contexts (in response to a stressor and diurnally) to predict infant cortisol reactivity and temperament.

3.1 Introduction

3.1.1 Antenatal mood disturbance and infant cortisol

A limited number of studies have investigated the relationship between antenatal mood disturbance and offspring cortisol release, and a number of these have particularly focused on diurnal measures of offspring cortisol as an index of HPA function. For example, Gutteling and colleagues (2005a) reported a significant association between increased levels of maternal cortisol in pregnancy and increased child cortisol levels on the first day of school (mean age 4.5 years). Interestingly, this study also found a significant association between maternal fear of bearing a handicapped child during pregnancy and increased levels of cortisol in the offspring. However, an important consideration is that this study is limited by a very small sample size (N=29). Thus, these findings should be interpreted with caution, especially in light of a recent study, with a much larger sample of 192 children aged 4.5 years, which identified a significant association between maternal symptoms of depression during pregnancy and *decreased* offspring cortisol levels (Laurent et al., 2013). However, in this larger study symptoms of maternal antenatal depression were measured retrospectively, which introduces potential problems of misreporting (Laurent et al., 2013).

Three studies have also considered the relationship between antenatal mood disturbance and offspring diurnal cortisol in later childhood/early adolescence. In a sample of 74 10-year olds, O'Connor *et al* (2005) reported that antenatal anxiety was associated with individual differences in diurnal cortisol. In particular, offspring of anxious mothers had an increased cortisol awakening response (CAR) and decreased

afternoon cortisol levels. However, two studies of cortisol in adolescence have reported conflicting findings. Antenatal anxiety was found to be significantly associated with a decreased CAR and a flatter diurnal profile in a group of 58 14-15 years olds (Van den Bergh et al., 2008a). Further, in females only, such changes to diurnal cortisol were significantly associated with depressive symptoms. Thus, this was the first study to provide direct evidence that changes in offspring HPA function may mediate the association between antenatal mood disturbance and offspring depression. The association between antenatal anxiety and a diminished CAR and flatter diurnal profile has been recently replicated in a much larger group of adolescents (N=889) (O'Donnell et al., 2013). Importantly, these effects were independent of obstetric factors, and there were also similar effects for antenatal depression (O'Donnell et al., 2013). Thus, the existing literature reports conflicting findings regarding antenatal maternal mood and diurnal measures of offspring cortisol in childhood and adolescence. One possible explanation is the varying methodologies employed in these studies (e.g. retrospective *vs.* prospective study designs; self-report measures and clinical diagnoses of maternal antenatal mood; different offspring ages; and sampling times/methods), therefore direct comparison of the existing findings is challenging.

When assessing HPA function in very young infants, there are a number of difficulties associated with collecting diurnal cortisol samples, such as varying feeding schedules and irregular sleep times. Further, evidence suggests that the diurnal cortisol pattern does not become established until approximately 3 months of age (Mantagos et al., 1998). Therefore, measuring cortisol reactivity to a stressor may be a more sensitive method for detecting changes in HPA function of very young infants. A few studies have

used this method to assess infant/child HPA function, and have utilized a number of different paradigms to induce a cortisol stress response, including the heel-stick procedure, inoculation and stressful laboratory tasks. A clear advantage of assessing cortisol reactivity to the heel stick procedure is that this is usually carried out within 24 hours of birth. Therefore, postnatal influences on HPA function are limited, and any effects of maternal mood are as a result of antenatal, rather than postnatal, exposure. Leung and colleagues (2010) demonstrated a significant association between perceived stress in pregnancy and increased neonatal cortisol responses to the heel-stick (N=84). In the same study, they further demonstrated that such increased responses to acute stress persisted into the postnatal period: there was also a significant association between maternal antenatal perceived stress and infant cortisol response to a toy removal task at 10 months of age (Leung et al., 2010). The finding of increased reactivity to the heel-stick has been replicated in a larger sample of 116 mother-infant dyads; Poggi Davis and colleagues (2011b) reported that increased maternal cortisol and psychological distress in pregnancy was related to increased neonatal cortisol responses to the heel-stick (Davis et al., 2011b). Thus, the results of these two studies suggest that antenatal mood disturbance is associated with a more reactive neonatal cortisol response.

Assessment of cortisol responses to inoculation is also a useful method for assessing HPA reactivity in infancy; although unlike the heel-stick procedure it is possible that the postnatal environment may influence cortisol reactivity at a later age. While this paradigm has been used frequently to assess infant behavioural reactivity to a stressor, assessments of cortisol reactivity are currently limited. A recent study has used this

paradigm in the developing world (Fernandes et al 2014), where the incidence of both antenatal and postnatal depression is significantly higher than in developed countries (Gavin et al., 2005). In this study, 58 mother-infant dyads, based in a very poor area of rural South India, were assessed and maternal antenatal depressive symptoms significantly predicted infant salivary cortisol responses to inoculation at 2 months (Fernandes et al., 2014b). Interestingly, this association was found to be U-shaped, such that those infants exposed to both high and low levels of antenatal depression demonstrated the largest cortisol responses to inoculation. Although somewhat unexpected, this intriguing U-shaped relationship supports the hypothesis that in stressful environments, moderate levels of maternal antenatal stress may be optimal for the development of fetal HPA stress responses. However, this finding has yet to be replicated in a developed country, where the types and levels of stress experienced during pregnancy may be quite different to those in rural south India. The only other study to employ this pre/post inoculation paradigm in the context of antenatal stress was conducted on a sample of preterm infants exposed to exogenous glucocorticoids during gestation (Glover et al., 2005), and it was reported that exogenous glucocorticoids significantly predicted the magnitude of the cortisol response to inoculation at 4 months. Thus, the use of this paradigm in healthy infants from a developed country has yet to be explored.

Another method for assessing HPA function in young infants is to measure cortisol reactivity to a stressful laboratory task, such as a strange situation or still-face paradigm. In 2008, Oberlander and colleagues used a stressful infant habituation information-processing task in an attempt to identify a potential mechanism by which maternal

antenatal mood disturbance may result in changes to offspring HPA function. Specifically, they investigated the moderating role of DNA methylation of a gene involved in the negative feedback loop of the HPA axis (the glucocorticoid receptor gene - NR3C1). Results from this study showed that maternal symptoms of depression in late pregnancy were associated with increased neonatal NR3C1 DNA methylation, and these changes in DNA methylation were significantly associated with increased cortisol reactivity at 3 months of age (N=82) (Oberlander et al., 2008). This is the first finding from human research to suggest that the long-term impact of maternal antenatal mood disturbance on changes to infant HPA function may be mediated by alterations of infant DNA methylation (discussed further in Chapter 4).

Thus, accumulating evidence suggests that antenatal mood disturbance has implications for offspring HPA function, such that those infants exposed to antenatal mood disturbance show increased cortisol reactivity, which can be detected during the neonatal period and into childhood (more details of these studies are presented below in Table 9). Therefore, a potential mechanism by which antenatal mood disturbance impacts on fetal and infant development may be via enduring changes in HPA reactivity. However, key aspects of this association remain unclear and require further investigation.

Table 9 Summary of studies that have investigated the relationship between antenatal mood disturbance and offspring cortisol during infancy, childhood and adolescence

Study	N	Infant age	Measure of prenatal stress	Measure of infant cortisol	Main finding
Poggi Davis et al (2011) *J Child Psychol Psychi*	116	24 hours	PSS, CES-D, STAI and plasma cortisol	Response to heel stick procedure	Elevated concentrations of maternal cortisol during the late second and third trimesters, and elevated levels psychological distress throughout pregnancy, were associated with increased infant cortisol response to the heel stick procedure ($p<0.05$).
Leung *et al* (2010) *Clinical Pediatrics*	84	24- 48 hours and 10 months	APS	Response to a heel stick procedure (24-48hrs). Response to toy removal task (10 months)	Maternal perceived stress during pregnancy significantly predicted infant cortisol reactivity at 2 days and 10 months after birth ($p's < 0.05$).
Fernandes *et al* (2014) *Child Care Health Dev*	58	2 months	EPDS	Response to inoculation	Exposure to antenatal maternal depression independently predicted cortisol responses to inoculation ($p=0.04$). This association was U-shaped, so that those infants exposed to both high and low levels of antenatal depression showed the greatest reactivity.
Oberalnder *et al* (2008) *Epigenetics*	82	3 months	HAM-D, HAM-A, EPDS	Infant-controlled habituation-information processing task	Prenatal exposure to increased third trimester maternal depressed/anxious mood was associated with increased methylation of NR3C1 at a predicted NGFI-A binding site. Increased NR3C1 methylation at this site was also associated with increased salivary cortisol stress responses at 3 months ($p=0.018$).
O'Connor *et al* (2012) *Dev psychobiol*	125	17 months	Amniotic fluid cortisol as an index of maternal stress	Cortisol response to separation-reunion task	Infants who were exposed to high levels of *in utero* cortisol had higher baseline cortisol measures, and a blunted cortisol response to the stressor ($p<0.05$).

De Bruijn *et al (2009) Dev Psychobiol*	103	3 years	EPDS, STAI	Response to a frustrating task	Girls exposed to antenatal depression and anxiety showed higher cortisol levels compared to non-exposed girls (p=0.02). No differences were found in boys.
Laurent *et al* (2013) *Dev Psychol*	192	4.5 years	Mothers retrospectively reported antenatal depressive symptoms	Morning and evening samples over 3 consecutive days	Prenatal exposure to maternal depression predicted lower child cortisol levels.
Gutteling *et al* (2004) *Stress*	24	4.9 years	PRAQ-R, PSS, Everyday Problem List	Response to inoculation	There was no significant salivary cortisol response to the inoculation. Higher maternal morning cortisol, fear of giving birth to a handicapped child, and more reports of daily hassles in early pregnancy were related to higher child cortisol levels.
Gutteling *et al* (2005) *Psychoneuroendocrinology*	29	5.31 years	PRAQ-R, PSS, Everyday Problem List and diurnal salivary cortisol.	Salivary cortisol collected over 3 days: weekend, 1st day at school, one week after back to school	Children of mothers who had higher levels of cortisol and fear of bearing a handicapped child had higher levels of cortisol on school days. Neither group showed habituation on the second school day.
O'Connor *et al* (2005) *Biol Psychiatry*	74	10 years	Crown-Crisp Anxiety Index	Diurnal salivary cortisol collected over 3 consecutive days	Prenatal anxiety, but not depression, was significantly associated with a larger cortisol awakening response ($p<0.05$).
Van den Bergh *et al* (2008) *Neuropsychopharm*	58	14-15 years	STAI	Diurnal salivary cortisol	Antenatal exposure to maternal anxiety was associated with a flatter diurnal profile (p=0.04), and in females only this was associated with depressive symptoms (p=0.007).

O'Donnel *et al* (2013) *Psychoneuroendocrinology*	889	15 years	Crown-Crisp Anxiety Index, STAI, EPDS	Diurnal salivary cortisol collected over 3 consecutive days	Maternal antenatal anxiety was associated with a modest alteration in adolescent cortisol, indexed by a decreased waking cortisol response and a flatter diurnal slope ($p<0.05$). These effects were independent of obstetric factors and postnatal mood. Similar effects were seen for maternal antenatal depression ($p<0.05$).

APS: Abbreviated Psychosocial Scale, BDI: Beck Depression Inventory, CES-D: The short form of the Centre for Epidemiological Studies Depression Inventory, EPDS: Edinburgh Postnatal Depression Scale, HAM-A: Hamilton Rating Scale for Anxiety, HAM-D: Hamilton Rating Scale for Depression, PRAQ-R: Pregnancy Related Anxiety Questionnaire revised version, PSS: Perceived Stress Scale, STAI: Speilberger Trait Anxiety Inventory.

3.1.2 Antenatal mood disturbance and infant temperament

A further potential mechanism by which antenatal depression may exert its influence on fetal and infant development is by inducing changes in infant temperament (Baibazarova et al., 2013, Blair et al., 2011a, Davis et al., 2007). Scientific interest in infant temperament stems from the notion that even very young infants show considerable variability in their reactions to the environment, and also because these early individual differences have implications for later neurocognitive development, behaviour and personality (De Pauw and Mervielde, 2010). Temperament has been defined as "individual differences in emotional, motor and attentional reactivity measured by latency, intensity and recovery of response, and self-regulation processed such as effortful control that modulate reactivity" (Rothbart, 2007). Theories suggest that such individual differences in temperament have a genetic basis (Whittle et al., 2006, Goldsmith et al., 1987), and this empirical question can be tested using behavioural genetics methods. Indeed, monozygotic twins are more similar in temperament than

dizygotic twins (Cyphers et al., 1990). However, findings from twin studies suggest that genetic differences between individuals account for approximately 20% to 60% of the variability in temperament characteristics, whereas the remaining 40% to 80% is attributable to environmental influences (Saudino, 2009).

Given that temperament characteristics appear to be vulnerable to environmental influences, it is possible that parental mood, which poses a large influence on the fetal and infant environment, may have effects on temperament. Existing evidence from observational studies suggests that maternal antenatal mood disturbance is associated with a more difficult or reactive offspring temperament (Davis et al., 2004b, Austin et al., 2005b, Werner et al., 2007, Davis et al., 2007, McGrath et al., 2008, Davis et al., 2004a, Huizink et al., 2002, Gutteling et al., 2005a, Blair et al., 2011b). A variety of aspects of mood disturbance have been associated with a reactive infant temperament, including antenatal depression and anxiety, as well as perceived stress, pregnancy-specific anxiety and stressful life events. Interestingly, the largest of the existing studies (N=247) not only found a significant association between symptoms of antenatal depression and anxiety and negative infant reactivity, but also reported that elevations in maternal cortisol in late pregnancy were significantly related to a more negative infant temperament (Davis et al., 2007). However, Davis et al. (2007) reported independent effects of antenatal depression and cortisol on infant behavioural outcomes, and were therefore unable to conduct a mediation analyses to assess whether maternal cortisol mediated the link between antenatal depression and infant outcomes. Further, such effects of antenatal mood on infant temperament appear to be independent of maternal

postnatal mood, as well as demographic and obstetric factors (Blair et al., 2011a, Davis et al., 2004a). More details of these studies are presented below in Table 10.

Further interest in temperament stems from repeatedly replicated findings that a reactive temperament is a precursor for later behaviours, such as attention problems, aggressive and antisocial behaviour (Campbell, 1995, Rothbart and Bates, 1998, Stolz and McCormick, 1998, Prior et al., 2008, Lahey et al., 2008). This supports the idea that changes in infant temperament may be one potential mechanism by which antenatal mood disturbance increases risk for adverse offspring outcomes.

Table 10 Summary of studies that have investigated the association between antenatal mood disturbance and offspring temperament

Study	N	Infant age	Measure of antenatal stress	Measure of infant temperament	Main finding
Poggi-Davis *et al* (2007) *JAACAP*	247	2 months	STAI, CES-D, maternal afternoon cortisol	Fear subscale of the ITQ	Elevated maternal cortisol in late pregnancy was significantly associated with greater maternal report of negative infant reactivity ($p<0.01$). Antenatal depression and anxiety also predicted infant temperament ($p<0.05$).
McGrath *et al* (2008) *Infant Behav Dev*	139	2 and 6 months	EPDS and CES-D	Maternal report of perceived temperament characteristics	Depressed mothers reported that their infants had more difficult temperaments at both time points than non-depressed mothers ($p<0.05$).
Huzink *et al* (2002) *JAACAP*	170	3 and 8 months	PRAQ-R, PSS, EPL	Bailey scales of infant development, ICQ	Pregnancy-specific anxiety explained 3.3% of the variance of attention regulation at 3 months. Perceived stress and pregnancy anxiety taken together explained 5% of the variance of attention regulation at 8 months. Perceived stress accounted for 8.2% of the variance of difficult behavior of the 3-month-old infant
Poggi-Davis *et al* (2004) *Infancy*	22	4 months	STAI, CES-D	Harvard Infant Behavioural Reactivity Protocol	Maternal anxiety and depression during the antenatal, but not the postnatal period, were significantly and positively related to infant negative behavioural reactivity to novelty ($p<0.05$).
Werner *et al* (2007) *Dev Psychobiol*	50	4 months	SCID	IBQ	Antenatal psychiatric diagnosis was associated with having a 'cry' reactivity classification.
Austin *et al* (2005) *Early Human Dev*	970	4 and 6 months	STAI, EPDS	SITQ	Maternal antenatal anxiety and depression scores were predictive of a more difficult infant temperament.

Gutteling *et al* (2005) *Eur Child Adoles Psychiatry*	103	2 years	PRAQ-R, PSS, GHQ-30, DHS, Daily life experiences questionnaire, EPS	ICQ, CBCL, Bailey scales of infant development	Perceived stress during pregnancy significantly predicted more behavioural difficulties (OR=1.12) and more externalising problems (OR=1.17). Fear of bearing a handicapped child predicted higher levels of restless/disruptive temperament (OR=1.39) and more attention problems (OR=1.46).
Blair *et al* (2011) *Stress*	120	2 years	STAI, Pregnancy-related anxiety scale	ECBQ	Pregnancy-specific anxiety between 13 and 17 weeks of gestation was associated with a negative infant temperament ($p<0.05$). This association was independent of postnatal anxiety, demographic and obstetric factors.

CBCL: Child Behaviour Checklist, CES-D: The short form of the Centre for Epidemiological Studies Depression Inventory, DHS: Daily Hassles Scale, ECBQ: Early Child Behaviour Questionnaire, EPDS: Edinburgh Postnatal Depression Scale, EPL: Everyday Problems List, EPS: Everyday Problems Scale, GHQ-30: 30-item General Health Questionnaire, IBQ: Infant Behaviour Questionnaire, ICQ: Infant Characteristics Questionnaire, ITQ: Infant Temperament Questionnaire, PRAQ-R: Pregnancy Related Anxiety questionnaire (revised version), PSS: Perceived Stress Scale, SCID: Structured Clinical Interview for DSM-IV, SITQ: Short Infant Temperament Questionnaire, STAI: Speilberger Trait Anxiety Inventory.

3.1.3 Rationale

There is evidence to suggest that there may be an association between antenatal mood disturbance and both infant temperament and cortisol reactivity to stress. However, there remain a number of outstanding questions regarding this association. For example, it has recently been suggested that maternal biological markers of stress in pregnancy may be better predictors of infant outcomes than self-reported measures of mood (Del Giudice et al., 2011). However, this hypothesis has yet to be directly tested in a human cohort. Also, the relationship between maternal antenatal stress reactivity and infant stress reactivity is currently unclear.

Further, antenatal mood disturbance has implications for both infant cortisol reactivity and temperament. However, there are a limited number of studies that have assessed the relationship between infant cortisol reactivity and temperament directly. Maternal reports of infant temperament, specifically distress to limitations, have been associated with infant cortisol responses to a separation task at 9 months of age (Gunnar et al., 1992). Further, 18 month olds with an inhibited temperament and insecure attachment showed exaggerated cortisol responses to a strange situation task (Nachmias et al., 1996), and also toddlers with a high fear temperament and insecure attachment had increased salivary cortisol responses to both the strange situation task and inoculation (Gunnar et al., 1996). However, the relationship between temperament and stress reactivity during young infancy is yet to be defined.

3.1.4 Aims and Hypotheses

3.1.4.1 Aim

The aim of this study is to investigate whether maternal self-reported antenatal depression and biological markers of antenatal stress predict infant cortisol reactivity to inoculation and infant temperament. A further aim is to assess the relationship between cortisol reactivity and temperament in early infancy.

3.1.4.2 Primary Objective

The primary aim of this study is to investigate the effects of maternal antenatal depressive symptoms and biological markers of stress (salivary cortisol and alpha-amylase reactivity to stress and diurnal measures) on infant cortisol responses to inoculation at two months of age.

3.1.4.3 Primary Hypothesis

1. Antenatal depression and maternal biological markers of stress during pregnancy will significantly predict infant cortisol reactivity. Those infants exposed to high levels of maternal antenatal depression and salivary cortisol/alpha-amylase will show the greatest cortisol reactivity to inoculation.
2. Maternal cortisol reactivity will be a stronger predictor of infant cortisol reactivity than self-reported depressive symptoms.

3.1.4.4 Secondary Objective

The secondary aim of this study is to investigate the effects of maternal antenatal depressive symptoms and biological markers of stress on infant temperament at two months of age.

3.1.4.5 Secondary Hypothesis

1. Antenatal depression and maternal biological markers of stress during pregnancy will significantly predict infant temperament at 2 months.
2. Maternal antenatal biological markers of stress will more strongly predict infant temperament than self-reported depression.

3.1.4.6 Tertiary Objective

The tertiary aim of this study is to investigate the relationship between infant cortisol reactivity and temperament in early infancy.

3.1.4.7 Tertiary hypothesis

There will be a significant and positive correlation between infant cortisol reactivity to inoculation and infant distress to limitations. Those infants who show the greatest distress to limitations will have the greatest salivary cortisol responses to inoculation.

3.2 Methods

3.2.1 Participants

Participants from this study were from the cohort described in Chapter 2, for more details see page 69. Of the 103 participants who took part in the antenatal study, 88 mother-infant pairs also participated in the postnatal part of the study (nine participants declined to take part in the postnatal part of the study, three participants delivered too late to be included in this sample, one participant moved abroad, one participant had a termination because of pregnancy complications, and one participant lost her baby in late pregnancy). Of the infants included in this study, 39 (44.3%) were male and 49 (55.7%) were female, and average infant age at the postnatal visit was 8 weeks and 4 days old (range = 5 weeks to 14 weeks 3 days). 44 (50%) infants were born via vaginal delivery, 29 (32.9%) by assisted delivery, and 15 (17%) by caesarean section.

This research study was reviewed and approved by the Research Ethics Committee South Central Oxford B (REF: 12/SC/0473), and all participants provided informed consent for their infants and themselves to take part in the study.

3.2.2 Procedure

Mothers were assessed during either the 2nd or 3rd trimester of pregnancy. Self-reported measures of depression, anxiety and perceived stress were recorded, as well as salivary cortisol and alpha-amylase reactivity to a stressor and diurnal salivary cortisol and alpha-amylase measures. More details of this procedure can be found in Chapter 2, page 70.

Participants were visited at home approximately 2 months after they had given birth, and reported postnatal mood symptoms and infant behaviour. Mothers were asked to collect four saliva samples from their infant on the day of their first inoculation at 8 weeks of age. Of the 88 participants who took part in the postnatal study, 74 obtained and returned infant saliva samples. 60 participants collected samples at the 8-week appointment. However, in some cases it was not possible for mothers to collect infant saliva at this appointment, and instead collected saliva from their infants at their 12 week (n=10) or 16 week inoculations (n=4). On average, infants were 68 days old (range = 50 to 177 days) when they received inoculations and saliva samples were collected.

Samples were collected using Infant Saliva Swabs and Swab Storage Tubes (Salimetrics, UK). Mothers were asked to hold one end of the swab and place the other end into the infant's mouth for 60-90 seconds, so that 2 thirds of the swab became saturated with the infant's saliva. Participants collected the first sample any time during the day before the inoculation, the second sample immediately after inoculation, and the third and fourth samples 20 and 40 minutes following inoculation respectfully. On average, the second sample was taken 4 minutes after inoculation; the third sample 25 minutes after

inoculation and fourth sample 45 minutes after inoculation. Participants were provided with four Infant Saliva Swabs and four Swab Storage Tubes, and a stamped-addressed envelope to return the samples. The samples were centrifuged on receipt, and stored at -20°C. Participants were also asked to record the time when each sample was taken, the time of inoculation, the time of the infants last feed before inoculation, and whether that feed was breast milk or formula.

3.2.3 Measures

3.2.3.1 Maternal mood

Maternal antenatal mood was assessed using self-report measures of depression, anxiety and stress (Edinburgh Postnatal Depression Scale, Speilberger Trait Anxiety Inventory, Perceived Stress Scale) during either the 2nd or 3rd trimester of pregnancy. More details of this can be found in Chapter 2 page 70. Mothers also reported mood using the same scales at the postnatal visit.

3.2.3.2 Maternal biological markers of stress

Maternal salivary cortisol and alpha-amylase responses to acute stress and diurnal measures were taken during either the second or third trimester. More details of this procedure are available in Chapter 2 page 70. For analyses in this chapter, two measures of maternal salivary cortisol and alpha-amylase were used: mean change in response to stressor and log AUC of diurnal cortisol and alpha-amylase. Cortisol mean change in response to the stressor was calculated by subtracting the baseline cortisol from the 20 minutes post-stressor cortisol. Details of how log AUC cortisol and alpha-amylase were calculated are available in Chapter 2, page 75.

3.2.3.3 Infant temperament

Infant behavior was assessed via maternal report 2 months post birth using 5 subscales (57 items) of the Infant Behaviour Questionnaire (IBQ): activity, smiling, novelty, distress to limitations and soothability. (Gartstein and Rothbart, 2003). Mothers were

required to report how often their infant engaged in various behaviours during the past week using a 7-point Likert scale from 'Never' to 'Always'. These subscales have shown good internal consistency (Cronbach's alphas 0.7-0.9) and reliability (Gartstein and Rothbart, 2003). This scale is available in the appendices (page 298).

3.2.3.4 Infant salivary cortisol

74 mothers collected and returned the infant saliva samples. Of the 296 samples, 11 had insufficient saliva to quantify the cortisol concentration. Samples that were taken more than one hour after the inoculation were excluded (8 samples removed). Outliers that were more than three standard deviations outside of the mean were removed (7 outliers excluded). Descriptive characteristics of the cortisol measures taken at each time point are presented in Table 12.

Salivary cortisol concentrations were quantified using a direct double-antibody radioimmunoassay (RIA) with utilisation of 125I-cortisol as the ligand. More details of this process are available in Chapter 2, page 75.

3.2.4 Statistical analysis

3.2.4.1 Group comparisons

Infants born to participants of the 'depression-symptom group'[3] are referred to as 'depression-exposed' infants, and the infants born to women in the control group are referred to as 'control infants'.

Characteristics of the control infants and depression-exposed infants were compared using t-tests and chi squared tests. Correlations between infant characteristics, maternal mood, infant cortisol and temperament were then assessed using Pearson's bivariate correlations.

3.2.4.2 Assessing infant salivary cortisol responses to inoculation

For consistency with the previous chapter, the infant cortisol data was first analysed using a repeated measured ANOVA in order to assess changes in cortisol concentration over time. The four infant cortisol measures were entered as repeated measures; group (depression-exposed *vs.* control) and infant gender were entered as between-subjects factors. Maternal postnatal depression was used as a covariate in this analysis, as a continuous variable, as was infant age at the time of inoculation.

However, due to the smaller sample size of this study, using the measure of antenatal depression as a categorical variable reduces the power to detect significant effects.

[3] Details of the depression-symptom and control groups are available in Chapter 2. Mothers were defined as the depression-symptom group if they scored 10 or above on the antenatal EPDS measure. Those mothers who scored 9 or below comprised the control group.

Therefore, this data was re-analysed using antenatal depression as a continuous variable, entered as a predictor into a linear regression model. Mean cortisol change at 20 minutes post-inoculation was used as the outcomes variable. Infant age at the time of inoculation, gender and maternal postnatal depression were entered as covariates. Postnatal depression was entered at a later stage of the model so that the effects of antenatal depression could be examined independently of postnatal depression.

Linear regression models were also used to investigate whether maternal antenatal salivary cortisol and/or alpha-amylase predicted infant cortisol responses to inoculation. Two measures of each salivary biomarker were used as predictors: mean change in response to the stressor and log AUC of the diurnal measures. Regression models were constructed as described above, with salivary cortisol or alpha-amylase entered in the place of antenatal depression. Where maternal salivary cortisol was used as a main predictor, maternal days of gestation when salivary cortisol was measured was also entered into the model as a covariate.

3.2.4.3 Assessing infant temperament

Linear regression models were also used to analyse the infant temperament data, and four domains of temperament were used as outcome variables (activity, smiling, distress to limits and soothing). Birth weight was used as a covariate because it correlated with some domains of temperament. Also, postnatal depression was used as a covariate in all analyses, because of its known effects on infant development (Murray, 1992). Again, postnatal depression was entered at a later stage of the analyses so that the effects of

antenatal depression could be examined independently of postnatal depression. Gender was used as a predictor variable in all analyses, because there is evidence to suggest that antenatal depression may act on male and female infants differently (O'Connor et al., 2002b, O'Connor et al., 2003, Rodriguez and Bohlin, 2005, Van den Bergh and Marcoen, 2004b, Bergh et al., 2005). Maternal antenatal depression and maternal antenatal measures of salivary cortisol and alpha-amylase (mean change in response to stressor and log AUC) were used as main predictors in the regression models, and were entered as continuous variables.

3.3 Results

3.3.1 Demographic characteristics

For consistency with the previous chapter, infant demographic data is presented split by group (control vs. depression-symptom) and trimester of antenatal assessment (2nd vs. 3rd). Of the 88 infants included in this study, 40 were born to mothers assessed in the 2nd trimester (n=32 control and n=8 depression-exposed) and 48 were born to mothers assessed in the 3rd trimester (n=35 control and n=13 depression-exposed). Due to the young age of the infants, responses on the novelty items of the IBQ were very low (22-40%); therefore the novelty subscale was not included in these analyses. Demographic characteristics of the infants in the 4 groups were compared, and are presented below in Table 11. No significant differences were found for the following variables:

a) Gender; ($x^2_{(1)}$=0.639, p=0.424)
b) Birth weight; ($F_{(3)}$=0.490, p=0.690)
c) Gestational age at delivery; ($F_{(3)}$=2.171, p=0.098)
d) Method of delivery; ($x^2_{(4)}$=1.538, p=0.820)
e) Delivery complications; ($x^2_{(1)}$=0.548, p=0.459)
f) Age of infant at postnatal visit; ($F_{(3)}$=1.699, p=0.174)
g) Activity; ($F_{(3)}$=1.053, p=0.374)
h) Smiling; ($F_{(3)}$=1.221, p=0.306)
i) Distress; ($F_{(3)}$=1.432, p=0.239)
j) Soothing; ($F_{(3)}$=1.787, p=0.155)

Table11 Infant demographic characteristics and maternal mood, split by group and trimester of antenatal assessment

Maternal trimester at assessment	Second trimester		Third trimester	
Demographic Variables	Control Infants (N=32)	Depression-exposed infants (N=8)	Control Infants (N=35)	Depression-exposed infants (N=13)
Infant Characteristics				
Gender (n, %)				
Male	9 (28.1)	5 (62.5)	19 (54.3)	6 (46.2)
Female	23 (71.9)	3 (37.5)	16 (45.7)	7 (53.8)
Birth weight, Kg (m, SD)	4.32, 0,54	3.61, 0.45	3.40, 0.44	3.40, 0.57
Gestational age at birth, weeks (m, SD)	39.9, 1.34	41.0, 0.71	40.0, 0.92	40.29, 1.25
Delivery method (n, %)				
Vaginal	20 (62.5)	3 (37.5)	15 (42.9)	6 (46.2)
Forceps	5 (15.6)	2 (25)	11 (31.4)	2 (15.4)
Ventouse	4 (12.5)	1 (12.5)	2 (5.7)	2 (15.4)
Elective caesarean	-	1 (12.5)	2 (5.7)	-
Emergency caesarean	3 (9.4)	1 (12.5)	5 (14.3)	3 (23.1)
Delivery complications (n,%)	16 (50)	3 (37.5)	13 (37.1)	4 (30.8)
Age at postnatal assessment, weeks (n, %)	8.49, 1.82	10.0, 2.25	8.45, 1.75	8.78, 1.61
Infant behaviour (m, SD)				
Activity	3.49, 0.72	3.89, 0.76	3.41, 0.62	3.50, 0.80
Smiling	3.92, 1.69	4.65, 0.91	4.35, 1.27	3.77, 1.03
Distress	4.08, 0.89	4.25, 0.65	4.31, 0.57	3.83, 0.83
Soothing	4.68, 0.99	3.99, 0.52	4.21, 1.00	4.37, 1.07
Maternal mood				
Antenatal mood (mean, SD)				
EPDS (depression)	3.69, 2.74	12.75, 3.41	4.17, 3.12	13.84, 3.60
TAI (trait anxiety)	32.41, 7.27	44.50, 6.23	33.57, 6.73	46.69, 5.63
PSS (perceived stress)	8.31, 4.87	18.13, 8.06	9.14, 4.85	18.69, 5.63
Postnatal mood (mean, SD)				
EPDS (depression)	6.19, 4.17	5.75, 4.46	6.97, 3.17	10.00, 4.74
TAI (trait anxiety)	33.78, 9.32	37.29, 7.27	35.69, 8.98	40.85, 8.97
PSS (perceived stress)	11.28, 6.6.4	12.00, 4.75	12.74, 5.47	15.23, 5.70

*p<0.05, ***p<0.001. Infant behaviour was assessed using subscales of the Infant behaviour Questionnaire. EPDS: Edinburgh Postnatal Depression Scale, STAI: Spielberger Trait Anxiety Index, PSS: Perceived Stress Scale, SD: Standard Deviation.

For the measures of maternal antenatal mood, mothers of the depression-exposed infants who were assessed in the second trimester scored higher for antenatal depression, trait anxiety, and perceived stress than the mothers of the control infants (all p's<0.001). However there were no significant differences in the measures of postnatal depression, anxiety and perceived stress (all p's>0.05). Similarly, the mothers of the depression-exposed infants who were assessed in the second trimester scored significantly higher than control participants on measures of antenatal depression, anxiety and perceived stress (all p's<0.001). Mothers of the depression-exposed infants also scored significantly higher for postnatal depression than the control group ($t_{(46)}$=-2.559, p=0.014), however there were no group differences in measures of postnatal anxiety or perceived stress.

3.3.1.1 Infant cortisol

There were no significant associations between the last feed (breast milk or formula), the time difference between the last feed and inoculation, and any of the infant salivary cortisol measures. Descriptive characteristics of the infant cortisol samples are presented in Table 12**.**

Table 12 Descriptive characteristics of infant cortisol samples

Infant cortisol (nmol/L)	N	Minimum	Maximum	Mean	SD
1. Baseline	71	0.34	39.59	7.57	8.71
2. Immediately post-inoculation	70	0.34	50.07	8.33	10.89
3. 20 minutes post-inoculation	66	0.68	48.10	16.25	13.07
4. 40 minutes post-inoculation	63	0.38	38.38	8.96	9.22

On average, the second sample was taken 4 minutes after inoculation; the third sample 25 minutes after inoculation and fourth sample 45 minutes after inoculation.

3.3.2 Correlations between variables

3.3.2.1 Antenatal mood, biological markers of antenatal stress and birth outcomes

Measures of antenatal depression, anxiety and perceived stress were all highly and significantly correlated with each other (all p's<0.001). Perceived stress was significantly associated with gestational length (r=0.221, p=0.039), and also gestational length was associated with labour complications (r=0.221, p=0.003). However, these effects did not remain significant following a correction for multiple testing. There were no other significant correlations (all p's>0.05).

3.3.2.2 Infant cortisol and other variables

No measures of infant cortisol were significantly correlated with any infant variables, maternal antenatal mood or maternal biological markers of stress (all p's>0.05).

Baseline infant cortisol was negatively associated with cortisol mean change in response to inoculation (r=-0.524, p<0.001), suggesting that those infants with lower baseline cortisol showed the greatest cortisol change in response to inoculation. The three cortisol measures taken post-inoculation were significantly and positively correlated with each other (all p's<0.001).

3.3.2.3 Infant temperament and other variables

Birth weight was significantly and positively correlated with infant activity (r=0.232, p=0.030), smiling (r=0.233, p=0.030) and soothing (r=0.230, p=0.032). This suggests that those infants with the greatest birth weights show the greatest levels of activity, smiling and soothability. However, these significant effects did not survive a correction for multiple testing. Infant temperament variables were not significantly correlated with any other birth outcomes or maternal antenatal mood or salivary cortisol/alpha-amylase (all p's>0.05).

3.3.2.4 Correlations between infant cortisol and temperament

Infant smiling was negatively correlated with infant cortisol at 20 minutes (r=-0.295, p<0.01) and 40 minutes (r=-0.339, p<0.01) post-inoculation. Smiling was also negatively and significantly correlated with mean cortisol change in response to inoculation (r=-0.272, p<0.05). This indicates that those infants who show the greatest level of smiling have the smallest cortisol responses to inoculation. No other measures of infant temperament and cortisol were significantly correlated (all p's>0.05). These results are presented below in Table 13.

Table 13 Correlations between infant cortisol and temperament

		Infant cortisol (nmol/L)				
		Baseline	**T0**	**T20**	**T40**	**Mean change in response to stressor**
Infant Temperament	**Activity**	-0.186	-0.209	-0.127	-0.048	0.014
	Smiling	0.027	-0.206	**-0.295****	**-0.339****	**-0.272***
	Distress	-0.130	-0.138	-0.129	-0.048	-0.041
	Soothing	-0.012	-0.035	0.019	0.097	0.027

*p<0.05, **p<0.01. Temperament was assessed using 4 items from the Infant Behaviour Questionnaire. T0: sample taken immediately post-inoculation, T20: sample taken 20 minutes post-inoculation, T40: sample taken 40 minutes post-inoculation.

3.3.3 Infant salivary cortisol responses to inoculation

3.3.3.1 Maternal antenatal depression and infant cortisol

Repeated-measures ANOVA

A repeated measures ANOVA was used to assess the effects of maternal antenatal depression on infant cortisol responses to inoculation. There was a significant within-subjects effect of time ($F_{(1)}$=2.949, p=0.035), reflecting an increase in salivary cortisol from baseline to 20 minutes post-inoculation ($t_{(60)}$=-5.250, p<0.001), followed by a decrease in salivary cortisol from 20 to 40 minutes post-inoculation ($t_{(56)}$=6.415, p<0.001). However, there were no significant between-subject effects of antenatal depression, or infant gender, or a gender*antenatal depression interaction (all p's>0.05). There was also no significant effect of trimester in which the mother was assessed or postnatal depression on cortisol response to the inoculation, but there was a significant effect of infant age at time of inoculation ($F_{(1)}$=7.808, p=0.008). The negative correlation between infant age and mean cortisol change in response to the stressor (r=-0.260, p=0.043) suggests that younger infants have larger cortisol responses. These results are depicted graphically in Figure 14.

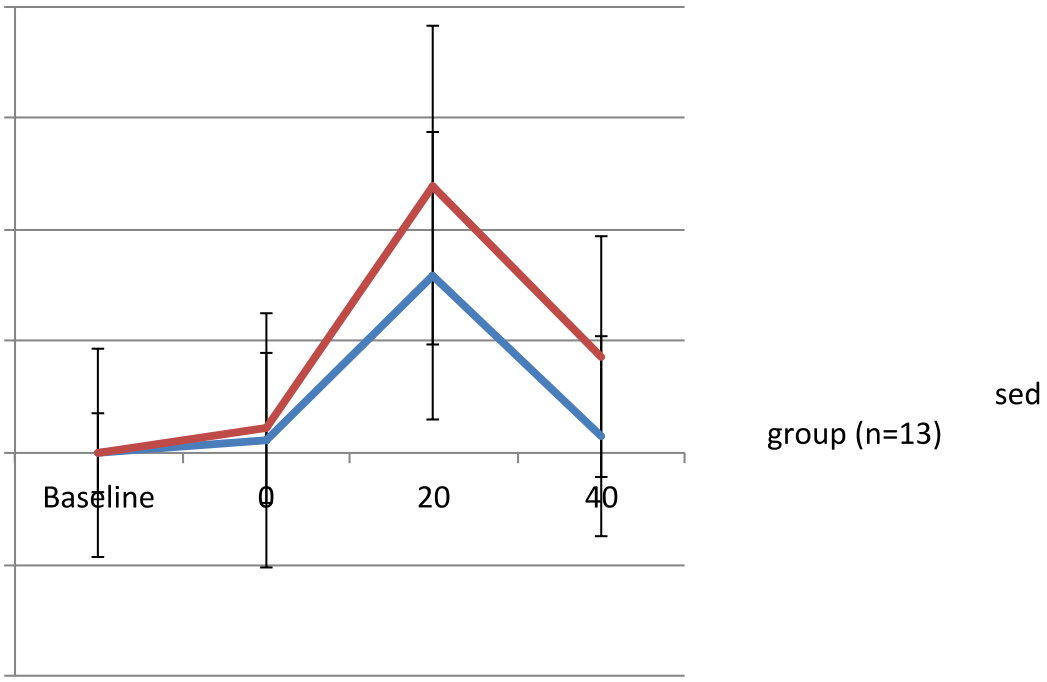


Figure 13 Infant salivary cortisol responses to inoculation.

Regression analyses

A regression model was used to assess whether antenatal depression, used as a continuous variable, predicted cortisol reactivity. Mean change in cortisol concentration 20 minutes post-inoculation was used as the outcome variable (because the repeated measures ANOVA identified a peak in concentrations at this time point). However, the results of the regression analyses parallel the ANOVA results: infant age significantly predicted mean change in cortisol concentrations (β=-0.295, p=0.026). However, no other variables significantly predicted cortisol mean change (all p's>0.05).

A previous study of maternal antenatal depression and infant salivary cortisol responses to inoculation reported a curvilinear association (Fernandes et al., 2014a). To investigate whether a similar relationship existed in the current data set, curve estimation techniques were applied. However, a quadratic model did not significantly explain this association (R^2=0.010, p=0.732).

3.3.3.2 Maternal antenatal biomarkers of stress and infant cortisol

Maternal Cortisol

Neither maternal mean cortisol change in response to the stressor, nor log AUC diurnal cortisol significantly predicted infant cortisol reactivity ((β=-0.082, p=0.535) and (β=0.058, p=0.679) respectively). No other variables significantly predicted infant cortisol mean change at 20 minutes (all p's>0.05), apart from infant age.

Maternal Alpha-Amylase

Maternal mean change in alpha-amylase in response to the infant distress film did not significantly predict infant cortisol reactivity. However, the mean change in alpha-amylase*infant gender interaction term was significant (β=-0.259, p=0.049). To investigate this interaction the data were split by gender, and the regression models repeated. Maternal alpha-amylase mean-change did not significantly predict male or female infant cortisol reactivity (males: β=0.221, p=0.264, females: β=-0.267, p=0.121). However, the differing direction of the Beta values explains the significant interaction terms in the previous analyses.

Neither log AUC alpha-amylase nor a log AUC alpha-amylase*gender interaction significantly predicted infant cortisol reactivity.

3.3.4 Infant temperament

3.3.4.1 Maternal antenatal depression and infant temperament

Maternal postnatal depression was a significant predictor of infant activity (β=0.401, p<0.001) and distress to limitations (β=0.328, p=0.004), suggesting that those infants exposed to high levels of maternal postnatal depression showed the greatest levels of activity and distress to limitation. Similarly, infant birth weight was a significant predictor of activity (β=0.225, p=0.025) and soothability (β=0.225, p=0.043), suggesting that those infants with the greatest birth weights showed the highest levels of activity and soothability.

However, antenatal depression alone did not significantly predict any of the domains of temperament, and neither did infant gender (all p's>0.05). There was a significant interaction between antenatal depression and infant gender to predict infant activity (β=-0.203, p=0.041). To investigate the interaction, the data were split by gender and the model repeated. Antenatal depression did not significantly predict activity in male (β=0.176, p=0.224) or female infants (β=-0.164, p=0.283), though the differing direction of the Beta values explains the significant interaction term. The results are presented in Table 14.

Table 14 Regression models using antenatal depression to predict infant temperament

Model		Activity		Smiling		Distress to limits		Soothability	
		B	**p**	***B***	**p**	***B***	**P**	***B***	**p**
1	Birth weight	**0.232**	**0.030**	**0.233**	**0.030**	-0.033	0.761	**0.230**	**0.032**
2	Birth weight	**0.240**	**0.028**	0.211	0.054	-0.053	0.634	**0.230**	**0.035**
	Antenatal depression	0.142	0.185	-0.049	0.646	-0.012	0.910	-0.100	0.354
	Gender	-0.022	0.835	-0.124	0.254	-0.124	0.265	0.053	0.624
3	Birth weight	**0.210**	**0.039**	**0.220**	**0.045**	-0.078	0.466	**0.223**	**0.043**
	Antenatal depression	0.028	0.784	-0.015	0.893	-0.107	0.332	-0.129	0.255
	Gender	-0.012	0.904	-0.127	0.242	-0.116	0.279	0.056	0.608
	Postnatal depression	**0.383**	**0.000**	-0.115	0.306	**0.319**	**0.005**	0.097	0.386
4	Birth weight	**0.225**	**0.025**	0.209	0.056	-0.070	0.511	**0.225**	**0.043**
	Antenatal depression	0.002	0.984	0.004	0.974	-0.120	0.283	-0.132	0.250
	Gender	-0.012	0.906	-0.127	0.239	-0.115	0.281	0.056	0.609
	Postnatal depression	**0.401**	**0.000**	-0.127	0.255	**0.328**	**0.004**	0.099	0.380
	Gender*Antenatal depression	**-0.203**	**0.041**	0.144	0.181	-0.098	0.359	-0.025	0.819

3.3.4.2 Maternal antenatal biomarkers of stress and infant temperament

Maternal cortisol

Maternal cortisol mean-change in response to a stressor did not significantly predict any of the domains of infant temperament (all p's>0.05). However, a significant gender*maternal cortisol interaction predicted infant distress to limitations (β=0.292, p=0.048). Further investigation revealed that for male infants, maternal mean cortisol change significantly predicted distress to limitations (β=0.384, p=0.016), such that those infants whose mothers had the greatest cortisol change in response to the stressor, show the greatest distress to limitations. However, maternal mean cortisol change did not significantly predict distress to limitations in female infants (β=-0.059, p=0.677).

Maternal log AUC cortisol did not significantly predict any of the infant temperament measures (all p's>0.05). However, the interaction between log AUC cortisol and infant gender was significant in predicting infant smiling (β=0.376, p=0.001). Further investigation revealed log AUC cortisol significantly predicted smiling in male (β=-0.449, p=0.011) and female infants (β=0.350, p=0.025). However, it appears that the direction of effect is opposite for male and female infants. In male infants, increased maternal cortisol is associated with decreased smiling, whereas for female infants, increased maternal cortisol is associated with increased infant smiling.

The interaction between maternal log AUC cortisol and infant gender also approached significance in predicting infant distress to limits (β=0.193, p=0.099). However, log AUC cortisol was not significant in predicting distress to limits in either male (β=-0.201,

p=0.286) or female infants (β=0.213, p=0.181), however the opposite direction of the Beta values explains the significant interaction term.

Maternal Alpha-Amylase

Maternal alpha-amylase mean-change in response to stressor did not significantly predict any of the domains of temperament, and neither did an alpha-amylase*gender interaction (all p's>0.05)

Similarly, log AUC alpha-amylase was not significant in predicting any domains of infant temperament. However an interaction between log AUC alpha-amylase and infant gender was significant in predicting distress to limitations. For male infants, the effect of log AUC alpha-amylase approached significance (β=-0.350, p=0.079), but was non-significant for females (β=0.154, p=0.253). This suggests that male infants exposed to the highest levels of maternal alpha-amylase show the least distress to limits.

3.3.5 Summary of findings

Infant cortisol reactivity to inoculation

- Maternal antenatal depression did not significantly predict infant cortisol reactivity to inoculation.
- Similarly, maternal antenatal measures of salivary cortisol and alpha amylase did not significantly predict infant cortisol reactivity to inoculation.

Infant temperament

- Maternal antenatal depression did not significantly predict any domains of temperament.
- For male infants, those whose mothers had the greatest change in salivary cortisol in response to the stressor showed the greatest distress to limitations.
- For male infants, those whose mothers had the greatest diurnal cortisol levels showed the lowest levels of smiling. Conversely, female infants whose mothers had the greatest diurnal cortisol levels showed the highest levels of smiling.
- For male infants, those whose mothers had the highest diurnal alpha-amylase levels showed the lowest levels of distress to limitation.

Relationship between infant cortisol reactivity and temperament

- Those infants who showed the greatest levels of smiling had the smallest salivary cortisol responses to inoculation.
- There were no other significant associations between infant cortisol reactivity and temperament.

3.4 Discussion

This study investigated the effects of maternal antenatal depressive symptoms and biological markers of stress on infant salivary cortisol responses to inoculation and temperament. The main finding from this study is that maternal antenatal depression did not significantly predict infant salivary cortisol responses to inoculation at two months of age. Similarly, there were no significant effects of maternal antenatal biomarkers of stress on infant cortisol reactivity. This finding does not support the hypothesis that maternal antenatal mood disturbance exerts its influence on infant development by inducing changes in the infant HPA axis.

A further finding from this study was that maternal antenatal depression did not significantly predict infant temperament. However, there was evidence to suggest that maternal antenatal biomarkers of stress predicted some aspects of temperament. These findings should be interpreted with caution given the exploratory nature of the analyses and the large number of statistical tests carried out.

This study also identified that those infants with the greatest levels of smiling had the smallest cortisol responses to inoculation. Thus, this is the first study to investigate the relationship between temperament and cortisol reactivity in very young infants, and is also the first study to use measures of antenatal mood disturbance, as well as antenatal biomarkers of stress to predict infant cortisol reactivity and temperament.

3.4.1 Findings in context

3.4.1.1 Infant cortisol responses to inoculation

Although the infants in this study showed a significant increase in salivary cortisol in response to inoculation, no association was found between maternal antenatal symptoms of depression and the magnitude of infant salivary cortisol change. This is in contrast to previous studies that have reported significant associations between maternal antenatal psychological distress and increased neonatal cortisol responses to the heel stick procedure (Davis et al., 2011b, Leung et al., 2010), increased cortisol reactivity to a stressful laboratory task at 3 years of age (de Bruijn et al., 2009), and increased cortisol on the first day of school in 5 year old children (Gutteling et al., 2005b). Further, a previous study based in rural south India reported a curvilinear association between maternal antenatal depression and infant cortisol reactivity to inoculation at 2 months of age. Those infants of mothers with both very high and very low levels of antenatal depression showed the greatest cortisol responses to inoculation, whereas infants of mothers with moderate levels of depression during pregnancy had the smallest cortisol responses to inoculation. Such a curvilinear association was tested for in the current study, however there was no evidence to support a quadratic model.

One possible explanation for the lack of association found here is that the participants were drawn from a low-risk community sample and levels of maternal antenatal depression were relatively low. Had the participants been recruited from a high-risk population with moderate to severe levels of depression, there may have been a significant association between antenatal depressive symptoms and infant cortisol

reactivity. That being said, previous studies have reported associations between maternal antenatal mood disturbance and increased infant cortisol in low-risk community samples (Davis et al., 2011a, Leung et al., 2010), as well as high-risk socio-economically disadvantaged samples (Fernandes et al., 2014a), and a clinical sample of depressed participants (Oberlander et al., 2008).

This study also did not find an association between maternal biological markers of stress in pregnancy and infant cortisol reactivity. It has been hypothesised that maternal biomarkers of antenatal stress are better predictors of infant outcomes than self-reported measures of antenatal mood (Del Giudice et al., 2011). This study was able to directly test this hypothesis, however no effects of either antenatal mood or biomarkers on infant cortisol reactivity were evident. Further, this is the first study to directly test the association between maternal cortisol reactivity during pregnancy and infant cortisol stress reactivity, and this study found no significant associations. A clear limitation however, is that the infant distress video administered to mothers during the antenatal session failed to induce a significant salivary cortisol response in the majority of participants. Had a more efficient probe of the HPA axis been administered to participants, such as the Trier Social Stress Test, then perhaps there may have been a stronger association between maternal and infant cortisol reactivity.

However, an alternative explanation as to why no significant effects on infant cortisol were found in this study is because perhaps the effects of antenatal mood disturbance on changes in infant HPA function are more subtle and variable than previously thought. Previous studies assessing infant cortisol are limited by small sample sizes (n's = 58-

116), and have used a range of infant stressors (heel-stick, inoculation, laboratory tasks), and sampling times. Further, previous studies may be confounded by the lack of controlling for infant sleep times, feeding schedules, and whether the infants are fed breast milk or formula. This may be an important factor because recent research with rhesus macaques has found that maternal cortisol is expressed in breast milk, and breast milk cortisol has implications for offspring growth and temperament (Hinde et al., 2014). Therefore replication of the association between maternal antenatal mood disturbance and increases in infant cortisol reactivity to stress in a large cohort is required before firm conclusions regarding this association can be made.

3.4.1.2 Infant temperament

This study also found no association between maternal antenatal symptoms of depression, and any domains of infant temperament. Again, this is in contrast to a number of studies that have reported significant associations between maternal antenatal psychological distress and a more reactive or difficult infant temperament (Davis et al., 2007, McGrath et al., 2008, Davis et al., 2004a, Austin et al., 2005a, Gutteling et al., 2005a, Blair et al., 2011a). A likely explanation is the low levels of maternal antenatal depression in this study, however a large proportion of previous studies to report on associations between antenatal mood disturbance and infant temperament have also used low-risk community samples of women, with low rates of mood disorder (Davis et al., 2007, McGrath et al., 2008, Davis et al., 2004a, Austin et al., 2005a, Gutteling et al., 2005a, Blair et al., 2011a). Further, it has been estimated that only 20% of infants

manifest adverse outcomes associated with antenatal depression exposure. As such, a sample size of just 88 mother infant dyads, with relatively low levels of antenatal depression exposure, may be insufficiently powered to detect such effects.

However, this study did identify an association between maternal antenatal cortisol and infant temperament. Male infants exposed to high levels of maternal antenatal cortisol showed the greatest distress to limitations and also the lowest levels of smiling. This finding is in line with a previous report of a significant association between raised maternal cortisol during pregnancy and a more negative infant temperament (Davis et al., 2007). Conversely, the current study also found that raised maternal cortisol was associated with increased smiling in female infants and raised maternal alpha-amylase was associated with lower distress to limitation in male infants. However, the associations between maternal antenatal biomarkers of stress and infant temperament characteristics should be interpreted with caution for two important reasons. Firstly, a number of linear regression models were used to analyse the data in this study, however findings were not corrected for multiple comparisons. Thus, it is possible that some significant effects may represent false-positives. Secondly, the analyses using antenatal biomarkers to predict infant temperament were largely exploratory, and therefore replication in a different cohort is required before firm conclusions can be drawn.

3.4.1.3 Infant cortisol and temperament

This study found a significant negative correlation between infant smiling and cortisol reactivity, suggesting that those infants who show the greatest levels of smiling also

show the least cortisol reactivity in response to inoculation. Such a finding is in line with previous reports of associations between cortisol reactivity and temperament in older infants in children. For example, increased cortisol reactivity has been associated with increased distress to limits at 9 months of age (Gunnar et al., 1992), an inhibited temperament at 18 months of age (Nachmias et al., 1996), and a high fear temperament in a group of toddlers (Gunnar et al., 1996). This is the first study, however, to identify that associations between infant temperament and cortisol reactivity can be detected in very young infants. Importantly, the correlation between maternally reported temperament and infant salivary cortisol reactivity lends strength and validity to the use of maternally reported measures of infant behaviour a very young age.

3.4.2 Strengths and Limitations

This study has a number of strengths, including a prospective longitudinal design, and the novel approach of using both antenatal self-reported measures of mood and biological markers of stress to predict both infant cortisol reactivity and temperament. This study also used validated and widely used measures of maternal antenatal mood and infant temperament.

However, a number of limitations should be considered. Although postnatal depression was controlled for, maternal postnatal cortisol was not. The moderate sample size of this study (n=88), in combination with low rates of antenatal depression, makes the detection of antenatal depression effects difficult. Maternal mood was self-reported rather than defined by clinical diagnostic criteria, which could lead to misreporting. Also,

the measure of infant temperament was based on maternal report, which may be subject to reporter-bias.

3.4.3 Conclusions

In conclusion, neither antenatal symptoms of depression nor antenatal cortisol predicted infant cortisol reactivity to inoculation at 2 months of age. These findings do not support the hypothesis that changes to both the maternal and infant HPA axis underlies the association between maternal antenatal depression and offspring outcomes. Thus, the role of the HPA axis in mediating associations between maternal antenatal mood disturbance and adverse offspring outcomes may be more subtle than previously thought.

CHAPTER 4

INVESTIGATING THE EFFECTS OF ANTENATAL DEPRESSIVE SYMPTOMS ON INFANT NR3C1 1F AND BDNF IV DNA METHYLATION

4 Overview of Chapter

This chapter investigates the effects of maternal antenatal depressive symptoms and cortisol on infant NR3C1 and BDNF DNA methylation. This chapter also investigates whether DNA methylation of these genes may predict infant cortisol responses to a stressor (inoculation) and infant behaviour (temperament). It has been suggested that the effects of antenatal depression on fetal and infant development are mediated by changes to the maternal and fetal HPA axis. Further, changes to fetal HPA function may result from alterations of DNA methylation at HPA-related genes. Indeed, there is evidence from animal and human studies that exposure to adverse environmental influences in early development result in increased DNA methylation of the NR3C1 1F region. A previous study has also shown that exposure to antenatal depressed mood resulted in increased infant NRC31 1F DNA methylation, which also significantly predicted infant cortisol responses to a stressor at 3 months, though this finding requires replication. Consideration of BDNF DNA methylation stems from the fact that exposure to antenatal depression increases risk for offspring psychopathology, and changes in BDNF DNA methylation has been implicated in a number of psychiatric disorders. However, it is currently unknown whether exposure to maternal antenatal depression may have implications for infant BDNF DNA methylation.

This study is an analysis of data collected as part of a short-term longitudinal study that took part in Oxford, UK, during 2013/14, described in Chapters 2 and 3. A subset of 57

mothers and their infants took part in this study; 20 mothers met criteria for antenatal depressive symptoms and 37 mothers were controls. Data on self-reported mood during pregnancy and 2 months after birth were available, as were 2 measures of maternal antenatal cortisol: mean change in response to a stressor, and the log AUC of diurnal cortisol. 2 months after birth two buccal swabs were taken from each infant, from which DNA was extracted and analysed for NR3C1 1F and BDNF IV DNA methylation. Maternal reports of infant temperament were available, as were infant cortisol responses to inoculation.

This chapter begins with a description of epigenetic processes with a focus on DNA methylation. The role of epigenetics in psychiatric disorders and changes in DNA methylation in response to early environmental adversity are discussed, with a focus on the NR3C1 and BDNF genes. A description of the subsample of participants who took part in this study and the methods are presented, and the results of this chapter follow. This chapter concludes with a discussion of the findings of this study in the context of the field, as well as the strengths and limitations, and the conclusions of this research.

This is the first study to investigate how both biological and psychological markers of stress in pregnancy may influence infant DNA methylation. This is also the first study to investigate whether changes in infant BDNF DNA methylation may be susceptible to antenatal mood disturbance.

4.1 Introduction

4.1.1 Epigenetic regulation of the genome

Phenotype is not only determined by the genetic make-up of an organism, but is a product of continuous interaction between genes and the environment. There are environmentally regulated intracellular components such as transcription factors that regulate gene expression. However, the presence and influence of such factors is dynamic, and cannot explain the long lasting, and in some cases inter-generational, effects of the environment on the phenotype.

As such, interest in epigenetic regulation of the genome, which may explain such phenomena, has grown substantially over the last decade. Epigenetics describes the process by which the expression of a gene is altered without a change in the DNA sequence. Although every cell within an organism carries the same genetic code, there is great diversity in the structure and function of groups of cells. Epigenetic processes support this diversity by controlling gene expression and silencing, which may be under both spatial and temporal control. The majority of patterns of epigenetic marks within the genome are inherited (Herman and Baylin, 2003, Jaenisch and Bird, 2003), however some are responsive to environmental influences such as stress, and are particularly vulnerable to change during development (Weaver et al., 2004, Oberlander et al., 2008, Roth et al., 2009, Waddington, 1953). There are a number of epigenetic processes, such as histone modifications and chromatin remodelling, however this chapter is focused on one epigenetic process which has been extensively studied: DNA methylation.

4.1.1.1 DNA methylation

Eukaryotic DNA is composed of four nucleotides: cytosine, guanine, thymine and adenine. DNA methylation occurs at cytosine bases of DNA, which are converted to 5-methylcytosine by the enzyme DNA methyltransferase (DNMT). The cytosine base is usually adjacent to a guanine nucleotide within the DNA sequence, resulting in two methylated cytosine bases sitting diagonal to one another on opposite DNA strands. These regions are known as CpG sites, and are demonstrated in Figure 15. There are two families of DNMT enzymes: *de novo* DNMTs, which are responsible for new methylation of cytosine nucleotides, and maintenance DNMTs, which are involved in copying the pattern of methylation from an existing DNA strand onto its new partner during replication (Bestor, 2000). The intracellular process responsible for guiding DNMTs to target CpG sites remains unclear, however current research suggests that DNMTs may be part of large chromatin-remodelling complexes (Jenuwein and Allis, 2001).

In mammals, DNA methylation is found sparsely but globally, and is concentrated at specific CpG sites (Unterberger et al., 2012). For many years it was thought that increased or hyper-methylation at the promoter region of a gene, i.e. the transcription start site, resulted in reduced gene transcription because the methyl group prevented the binding of transcription factors (Razin and Cedar, 1991). However, the current understanding of the role of DNA methylation in gene expression is more complex. Evidence suggests that DNA methylation can vary considerably between different cell

types, and in some cases DNA hypo-methylation of the promoter region corresponds with very little or no gene expression (Suzuki and Bird, 2008).

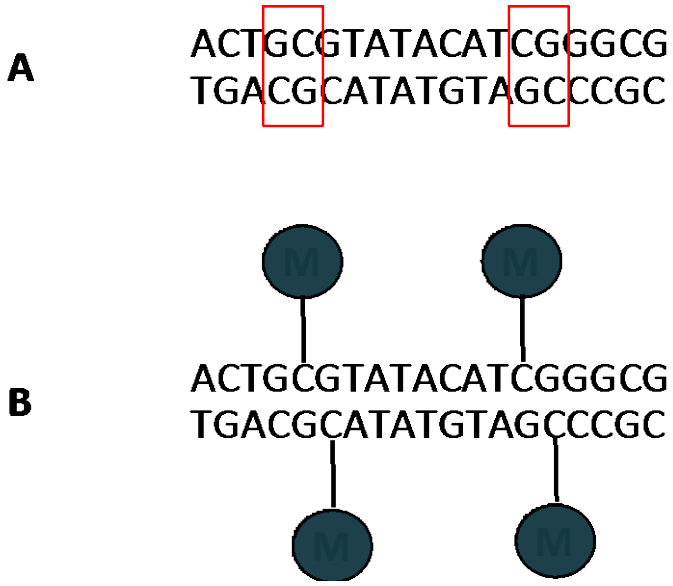


Figure 14 CpG sites. Part A. shows un-methylated double stranded DNA with two CpG sites (adjacent C and G nucleotides within a DNA strand) highlighted with a red box. Part B. shows methylated DNA, with four methyl groups attached to the C nucleotides of the CpG sites

Thus, DNA methylation is an intricate and tightly controlled intracellular process, which is essential for cell replication, differentiation, and normal growth and development. DNA methylation also has dynamic characteristics, and is mechanisms by which external environmental influences may regulate gene expression.

4.1.2 Epigenetics and psychiatric disorders

Although it held much promise, the sequencing of the human genome has not resulted in major breakthroughs in our understanding of the development of psychiatric disorders. As a result, attention has now been directed towards epigenetic alterations as a potential mechanism that could explain the aetiologies of such disorders. This is because many features of psychiatric disorders are consistent with an epigenetic dysregulation hypothesis: there is often discordance between monozygotic twins, a fluctuating disease course with periods of remission and relapse, generally a late age of onset, and parent of origin and sex effects.

In particular, much epigenetic psychiatric research has focused on schizophrenia and bipolar disorder. One candidate gene that has been consistently implicated in the development of neuropsychiatric disorders is Brain-derived Neurotropic Factor (BDNF). BDNF is part of the neurotrophin growth factor family and activates 2 distinct receptors in the brain (p75 neurotrophin receptor and tropomyosin related kinase B tyrosine kinase receptor - TrkB). The p75 neurotrophin receptor is activated by all members of the neurotrophin growth factor family, and its downstream action depends on the ligands bound to the receptor and what cell it is expressed in (Casaccia-Bonnefil et al., 1996, DeFreitas et al., 2001, Chao, 2003). BNDF also binds to TrkB: BDNF activation of this receptor is linked to at least 3 intracellular signalling pathways, and is essential for the normal development of the vertebrate nervous system (Kalcheim et al., 1987, Klein et al., 1993, Pezet and Malcangio, 2004). BDNF signalling is also needed for long-term potentiation and neurogenesis, and is therefore essential for learning and memory as well as reward-related processes (Pencea et al., 2001).

BDNF dysregulation has been implicated in the pathogenesis of schizophrenia (Nieto et al., 2013), and in particular is thought to be associated with the cognitive deficits of the disease (Nieto et al., 2013). However, BDNF has also been studied in a number of other common psychiatric disorders, notably depression. Depressed patients have decreased levels of circulating BDNF that normalise following antidepressant treatment (Sheline et al., 2003). Similarly, post mortem studies have demonstrated decreased BDNF expression in the brains of suicide victims and depressed patients (Sen et al., 2008, Molendijk et al., 2011). Studies providing direct evidence of changes in DNA methylation are also beginning to emerge (Chen et al., 2011, Mill et al., 2008, Keller et al., 2010, Toledo-Rodriguez et al., 2010). Using post-mortem tissue samples, Chen *et al.* (2011) identified decreased promoter BDNF methylation in the prefrontal cortex (PFC) of depressed patients, and, similarly, Mill and colleagues (2008) also found decreased BDNF DNA methylation in the PFC of people who have schizophrenia. Conversely, Keller *et al.* (2010) showed an increase in BDNF promoter methylation in the Wernicke area of suicide victims compared with non-suicide deaths, and Toledo-Rodriguez *et al.* (2010) found increased methylation of the BDNF promoter in blood cells of teenagers who had been exposed to maternal antenatal smoking. Thus, whilst this limited human evidence suggests that the direction of change in BDNF DNA methylation is inconsistent across different tissues and disorders, it highlights a potentially important role for BDNF methylation in such disorders.

Although this is a rapidly developing field of research, current evidence points to an inherent role of epigenetic processes in psychiatric disorders. Epigenetic mechanisms

could potentially explain how environmental influences, particularly in early development, could contribute to the development of psychiatric disease.

4.1.3 Epigenetics and early environmental influences

Much of the research regarding the influence of early life stress on epigenetic modifications has focused on the gene encoding the glucocorticoid receptor (GR), NR3C1. To date, compelling evidence from rodent studies suggests that the quality of parenting during early life has implications for the epigenetic regulation of NR3C1 in the offspring (Meaney, 2001). A pioneering model of parenting in rodents has identified stable individual differences in parenting behaviour of dams: some are classified as 'high licking/grooming' (high LG) animals, whereas others are classified as 'low licking/grooming' (low LG) (Meaney and Szyf, 2005, Szyf et al., 2005). A high frequency of licking and grooming, as well as arched-back nursing, is thought to be synonymous with a supportive and sensitive parenting style in humans, whereas a low frequency of grooming represents perhaps a more insensitive and unsupportive caregiving behaviour. These naturally occurring differences in early maternal behaviour are associated with individual differences in responses to stress and behaviour in the offspring. As adults, offspring of low LG dams are more fearful and show exaggerated responses to stress when compared with offspring of high LG dams (Stern, 1997, Liu et al., 1997, Caldji et al., 1998, Francis et al., 1999).

Biologically, low levels of maternal care in rodents are associated with increased methylation of exon 1F of the glucocorticoid receptor *NR3C1* gene in the offspring. Exon

1F lies within the promoter region, and is under the regulatory control of Nerve Growth Factor 1A (NGF1-A); a transcription factor involved in initiation of gene transcription, and subsequent expression of the GR protein (McGowan et al., 2009). Increased levels of methylation at exon 1F results in decreased binding of NGF1-A, and a subsequent decreased expression of the GR in the hippocampus (Weaver et al., 2004). These animals also have impaired regulation of the HPA stress response, and demonstrate more anxiety-like behaviours.

Interestingly, these effects persist into adulthood, but can be reversed by cross fostering at an early stage (Francis et al., 1999), further strengthening the idea that the epigenetic changes are the result of environmental exposure rather than inherited. As adults, central infusion of a histone deacetylase inhibitor also reverses these early effects of parenting, resulting in no differences between the low and high LG offspring in histone acetylation, DNA methylation, NGF1-A binding, GR expression and HPA responses to stress (Weaver et al., 2004, Weaver et al., 2005). These compelling findings suggest a causal relationship between maternal behaviour, epigenetic regulation of the NR3C1 gene and HPA stress reactivity. Thus, early environmental adversity may establish an epigenetic profile for offspring, which has implications for HPA reactivity and behaviour, but which has the potential to be reversed.

As this is a relatively new field of research, translational studies of humans are limited. None the less, findings from the few existing studies that have investigated exposure to early life stress and NR3C1 exon 1F methylation have been consistent with the animal findings (McGowan et al., 2009, Romens et al., 2014, van der Knaap et al., 2014). When

comparing suicide victims both with (n=12) and without (n=12) a history of childhood abuse, McGowan and colleagues (2009) showed that those with a history of childhood abuse had increased methylation of exon 1F in the hippocampus, and also found decreased GR mRNA transcripts. Romens *et al.* (2014) reported increased NR3C1 exon 1F methylation in DNA extracted from whole blood samples from a group of young adolescents exposed to physical abuse in childhood. Similarly, in a much larger sample of 468 adolescents, van der Knaap and colleagues (2014) used DNA from whole blood to detect increased NR3C1 1F methylation in adolescents who had been exposed to stressful life events and traumatic experiences.

Given that epigenetic changes are particularly susceptible to change during early development, it is possible that even earlier environmental challenges during the antenatal period may have implications for offspring DNA methylation. Indeed, this has been investigated using two different assessments of maternal antenatal psychological distress. Using DNA extracted from cord blood, Oberlander and colleagues (2008) identified that those infants who had been exposed to heightened levels of maternal anxiety and depressed mood during the third trimester of pregnancy had increased methylation of the NR3C1 1F promoter region at birth. Further, there was a significant association between methylation of this region and infant cortisol responses to a stressful stimulus at 3 months of age. The moderate sample size of 82 participants should be considered when interpreting the results, however this study highlights the possibility that NR3C1 1F methylation may, in part, mediate the association between antenatal depression and altered infant responses to stress. A similar study by Radtke and colleagues (2011), but with a smaller sample size of just 25 participants, questioned

whether antenatal exposure to intimate partner violence may also have similar effects on DNA methylation. Using whole blood samples from young adolescents, they found that those who had been exposed to maternal intimate partner violence during pregnancy did indeed have increased methylation of the NR3C1 1F region. Unfortunately, this study did not assess stress reactivity of these adolescents, and therefore whether such changes in stress reactivity persist into later life remains an important unanswered question.

Although much of the research has focused on epigenetic regulation of NR3C1 in the context of early environmental influences, interest in the effects of early life stress on BDNF methylation has begun to emerge. From animal studies, it has been well documented that exposure to adverse experiences during both the antenatal and postnatal period results in decreased expression of BDNF mRNA transcripts and BDNF proteins (Fumagalli et al., 2007, Boersma et al., 2014, Branchi et al., 2004, Lippmann et al., 2007). Such findings suggest that changes in BDNF expression may be attributable to changes in BDNF methylation. In 2009, Roth and colleagues (2009) found that postnatal maltreatment of pups by stressed dams resulted in increased methylation of BDNF exon IV, and an associated decrease of BDNF expression in the PFC. These DNA methylation effects persisted into adulthood, and those females with a history of abuse demonstrated similar abusive behaviours to their own offspring. Interestingly, treatment during the postnatal period with zebularine, a DNA methylation inhibitor, sufficiently decreased BDNF IV methylation and rescued the BDNF expression in the PFC to normal levels. As with the NR3C1 findings presented above, this suggests a causal effect of maternal behaviour on BDNF DNA methylation and expression.

Recently, the effects of maternal antenatal stress on offspring BDNF methylation have also been investigated. Boersma *et al.* (2014) demonstrated that pups exposed to maternal antenatal stress had increased BDNF exon IV methylation in the amygdala and hippocampus, and an associated decrease in BDNF protein expression. This finding suggests that, like the NR3C1 gene, BDNF is susceptible to changes in DNA methylation of the promoter region following exposure to maternal antenatal stress, and such changes may, in part, mediate the association between antenatal stress exposure and adverse offspring outcomes.

4.1.4 Outstanding questions

A number of outstanding questions remain regarding associations between antenatal environmental exposures and offspring DNA methylation. Clearly, we currently have little information to determine whether the type and intensity of fetal exposure to an adverse intra-uterine environment is important for DNA methylation changes. For example, it could be that exposure to maternal smoking or substance abuse during pregnancy may result in a different offspring epigenetic profile than exposure to maternal distress. Similarly, we could hypothesise that the intensity of exposure could correlate with the degree of change in DNA methylation.

In consideration of exposure to antenatal mood disturbance and infant NR3C1 methylation, some key outstanding questions are yet to be addressed. Firstly, the findings from Oberlander *et al.* (2008), that antenatal depression correlates with increased infant NR3C1 1F methylation, are yet to be replicated in a different cohort,

which is critical before firm conclusions regarding this association can be made. Further, it has been suggested that maternal antenatal biological markers of stress may be better predictors of infant outcomes than self-reported mood (Del Giudice et al., 2011), however it is currently unknown whether maternal antenatal cortisol may better predict infant DNA methylation than self-reported mood.

Also, because the Oberlander study used cord blood taken at birth, it is currently unknown whether epigenetic changes, which result from exposure to antenatal depressed mood, may persist into the postnatal period. Finally, the human studies described above have either used brain tissue or blood in order to assess DNA methylation. It is currently unknown, however, whether detection of changes in NR3C1 methylation as a result of exposure to antenatal depressed mood in other peripheral tissues, such as buccal cells, is possible. This is an important question when considering replication of these findings in larger cohorts, as the collection of buccal cell samples is quicker, easier and more economical than the collection of blood samples or post mortem brain tissue.

In terms of BDNF DNA methylation, the key outstanding question here is whether the animal findings are replicable in humans, as it is currently unknown whether exposure to antenatal mood disturbance results in changes in offspring BDNF IV methylation.

4.1.5 Aims and Hypotheses

1. **NR3C1**

Aim: The primary aim of this study is to investigate whether antenatal depression and/or maternal antenatal cortisol predicts increased infant NR3C1 1F DNA methylation. A further aim is to test whether changes in NR3C1 methylation are associated with infant stress responses (cortisol) and behaviour (temperament).

Hypothesis: This study will replicate previous findings: antenatal depression will be associated with increased infant NR3C1 DNA methylation. This increased NR3C1 methylation will predict larger infant cortisol responses to inoculation, and will also predict a more reactive infant temperament (greater levels of activity and distress to limitations).

2. **BDNF**

Aim: A secondary aim of this study is to investigate whether increases in maternal depression predict increased BDNF IV methylation, and also whether such changes in methylation are associated with changes in infant stress responses (cortisol) and behaviour (temperament).

Hypothesis: The animal findings will be replicated in a human cohort; those infants exposed to antenatal depression will have increased methylation of BDNF IV. However, there is no previous literature linking BDNF DNA methylation and infant cortisol

responses to stress or infant temperament. Therefore no clear hypotheses can be made regarding this association, and the analyses presented herein are exploratory.

4.2 Methods

4.2.1 Participants

Participants from this study were from the cohort described in Chapter 2, for more details see page 69. Of the original 103 participants who were recruited to the study, the first 57 mothers to be visited postnatally took part in this study. Demographic variables of the 57 participants who took part in this study, and of the whole cohort are shown in Table 15. The demographic characteristics of this subsample were compared to the whole Oxford Pregnancy Cohort; no significant differences were found for the following maternal measures:

a) Age; ($t_{(101)}$=-1.37, p=0.174)
b) Education; ($x^2_{(4)}$=5.81, p=0.214)
c) Ethnicity; ($x^2_{(6)}$=8.34, p=0.212)
d) Alcohol consumption; ($x^2_{(1)}$=1.92, p=0.166)
e) Trimester at antenatal assessment; ($x^2_{(1)}$=2.12, p=0.146)
f) Planned pregnancy; ($x^2_{(1)}$=1.67, p=0.196)
g) Previous history of mental health disorders; ($x^2_{(3)}$=2.96, p=0.399)
h) Symptoms of depression (EPDS); ($t_{(101)}$=0.93, p=0.170)

However, when compared to the whole cohort, the subsample of participants used in this study had significantly lower trait anxiety scores ($t_{(101)}$=0.10, p<0.001) and perceived stress scores ($t_{(101)}$=1.64, p<0.05).

Table 15 Maternal demographic characteristics of the Oxford Pregnancy Study and the subsample of participants used in this study

	Whole cohort (N=103)	**Epigenetic cohort (n=57)**
Age (m, SD)	31.48 (4.60)	32.04 (4.35)
Education (n,%)		
GCSE	1 (1)	0
A-level	3 (2.9)	1 (1.8)
Undergraduate degree	36 (35)	25 (43.9)
NVQ	7 (6.8)	3 (5.3)
Postgraduate degree	56 (54.5)	28 (49.1)
Ethnicity (n,%)		
Caucasian	94 (91)	51 (89.4)
Black	1 (1)	1 (1.8)
Asian	7 (6.8)	4 (7.0)
Mixed Race	1 (1)	1 (1.8)
Alcohol units/week (n, %)		
None	86 (83.5)	45 (78.9)
1-5	17 (16.5)	12 (21.1)
Cigarettes/week (n, %)		
None	86 (83.5)	51 (89.5)
Did not answer	17 (16.5)	6 (10.5)
Trimester at assessment (n, %)		
Second	50 (48.5)	24 (42.1)
Third	53 (51.5)	33 (57.9)
Planned Pregnancy (n, %)	88 (85.4)	51 (89.5)
Previous history of metal health problems (n, %)	29 (25.9)	17 (29.8)
Antenatal mood (mean, SD)		
EPDS (depression)	6.19 (5.13)	5.77 (4.52)
TAI (trait anxiety)	36.00 (9.50)	35.89 (2.74)***
PSS (perceived stress)	11.27 (6.90)	10.28 (5.67)*

*p<0.05, ***p<0.001. EPDS: Edinburgh Postnatal Depression Scale, STAI: Spielberger Trait Anxiety Index, PSS: Perceived Stress Scale.

This research study was reviewed and approved by the research Ethics Committee South Central Oxford B (REF: 12/SC/0473), and all participants provided informed consent for their infants and themselves to take part in the study.

4.2.2 Procedure

Participants were assessed during either the second or third trimester of pregnancy. Self-reported measures of antenatal depression, anxiety and perceived stress were recorded, as well as salivary cortisol and alpha-amylase responses to an infant distress video, and diurnal salivary cortisol and alpha-amylase. More details of this procedure are available in Chapter 2, page 69.

Participants were also assessed at home approximately 2 months after they had given birth. Self-report measures of postnatal depression, anxiety and perceived stress were obtained, as well as a maternal-report measure of infant behaviour. At the postnatal assessment two buccal swabs were obtained from the infant and following the postnatal visit, mothers collected a series of saliva samples from the infant on the day of their inoculation, to be assayed for the hormone cortisol. More details of these procedures are available in chapter 3, page 122.

4.2.3 Measures

4.2.3.1 Maternal mood

Maternal antenatal mood was assessed via self-report during either the 2nd or 3rd trimester of pregnancy, and at 2 months after birth. Mothers reported their mood using paper-based questionnaires to assess current symptoms of depression (EPDS), trait anxiety (STAI) and perceived stress (PSS). More details of these measures are available in Chapter 2, page 72.

4.2.3.2 Maternal antenatal cortisol

Maternal cortisol reactivity to an infant distress stimulus was measured during either the 2nd or 3rd trimester of pregnancy, and diurnal measures of salivary cortisol were also obtained during either the second or third trimester. More details are available in Chapter 2, page 70.

4.2.3.3 Infant behavior

Infant behavior was assessed via maternal-report 2 months post birth using 4 sub-scales of the Infant Behaviour Questionnaire (IBQ) (Gartstein and Rothbart, 2003). This measure identifies 5 domains of infant temperament: activity, smiling, novelty, distress and soothing. More details of this measure are available in Chapter 3, page 124.

4.2.3.4 Infant salivary cortisol

Infant salivary cortisol response to inoculation was measured at approximately 8 weeks post birth. Details of this protocol are available in Chapter 3, page 122.

4.2.3.5 Infant NR3C1 1F and BDNF IV DNA methylation

Buccal swab collection

Infants were on average 53.6 days old (SD=9.99, range=26-98 days) when the buccal swabs were collected. Two buccal samples were taken from each infant, one from the right and one from the left cheek. A third buccal sample was taken from the lower lip of 9 infants to be used as pilot samples. The buccal swabs were obtained using Catch-All soft sample swabs (Epicentre Ltd), which were firmly bushed across the inside of the infant's cheek/lip six times, and stored in sterilised 2ml tubes. Samples were stored at -20^{o}C at the Department of Psychiatry, Oxford, before being shipped to the Department of Psychology at Columbia University, New York, on dry ice (World Courier Ltd), where they were stored at -80^{o}C until analysis.

DNA isolation

Buccal cell DNA isolation was performed using a QIAamp DNA mini kit (Qiagen Ltd, USA) in accordance with the manufacturers protocol, and the buccal DNA was quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific Ltd, USA). The extracted DNA was in a range of 6-31ng/μl.

Bisulphite pyrosequencing

DNA methylation at specific CpG sites was analysed using the quantitative bisulphite-pyrosequencing method. The extracted buccal DNA (125ng) was bisulphite converted using an EpiTect Bisulphite kit (Qiagen Ltd, USA) according to the manufacturer's instructions, and stored at -20^{o}C until further analysis. A PyroMark PCR kit (Qiagen Ltd, USA) and PCR primers specific for NR3C1 and BDNF were used to obtain biotinylated PCR products and a specific sequencing primer was then used to determine CpG methylation in the regions of interest. Two separate assays were used to assess 10 CpG sites within NR3C1 exon 1F, and one assay was used to assess 5 CpG sites within BDNF exon IV, details of the primers used are available in Table 16. The PCR and pyrosequencing primers were designed using PyroMark Assay Design Software 2.0. The regions of interest within NR3C1 and BDNF were chosen based on previous research that has identified these areas as susceptible to epigenetic regulation following early exposure to adverse environmental influences (McGowan et al., 2009, Oberlander et al., 2008, Boulle et al., 2012, Kundakovic et al., 2014). Pyrosequencing was performed using a PyroMark Q24 pyrosequencer (Qiagen Ltd, USA) using specific pyrosequencing primers. Before sample analysis, each pyrosequencing assay was validated using standard curves by analysing 0, 20, 40, 60, 80, and 100% methylated human genomic DNA standards. Those standards were generated by mixing commercially available unmethylated and hypermethylated DNA standards (EpiTect PCR Control DNA Set, Qiagen) in the following ratios: 0:5; 1:4; 2:3; 3:2; 4:1; and 5:0 (10ng of total bisulfite-converted DNA). Following assay validation, 10ng of each bisulfite-converted sample

DNA was run in a 25-µL PCR reaction; 3µL of PCR product was run on a gel to confirm the band size and 20µL was run on a pyrosequencer. The average DNA methylation levels of specific CpG sites were quantified using PyroMark Q24 2.0.4 software (Qiagen Ltd, USA).

Table 16 Details of PCR and pyrosequencing primers, and their genomic coordinates

NR3C1 1F Assay 2* – CpG sites 5-1 - chr5:143,404,013-143,404,147	
PCR primer forward- Biotinylated	/5Biosg/ GTTGTTATTAGTAGGGGTATTGG
PCR primer reverse	AACCACCCAATTTCTCCAATTTCTTTTC
Pyrosequencing primer (reverse)	CAACTCCCCCACTCCAAACCC
NR3C1 1F Assay 1* – CpG sites 6-10 - chr5:143,404,011-143,404,097	
PCR primer forward	AGTTTTAGAGTGGGTTTGGAG
PCR primer reverse-Biotinylated	/5Biosg/ AAAACCACCCAATTTCTCCAATTTCTT
Pyrosequencing primer (forward)	GAGTGGGTTTGGAGT
BDNF IV* – CpG sites 1-5 - chr11:27,701,519-27,701,826	
PCR primer forward	GGGTTGGAAGTGAAAATATTTGTAAA
PCR primer reverse-Biotinylated	/5Biosg/CCCCATCAACCAAAAACTCCATTTAATCTC
Pyrosequencing primer (forward)	GGTAGAGGAGGTATTATATGATAG

*Genomic coordinates show genomic regions amplified by PCR and are based on the UCSC Genome Browser Human Dec. 2013 (GRCh38/hg38) Assembly.

4.2.4 Statistical analysis

4.2.4.1 Group comparisons

For analysis, participants were split into two groups based on their mother's antenatal EPDS score: those scoring 9 or below comprised the control group, where as those scoring 10 or above comprised the 'depression-exposed' group.

Independent samples t-tests and chi-squared tests were used to determine group differences in demographic characteristics, maternal mood and infant behaviour. Then, Pearson's correlations were used to assess the relationship between variables.

4.2.4.2 Regression analysis

Multiple regression models were used to address the aims of the chapter, because the outcome data was sampled at a single time point (DNA methylation of Buccal tissue collected at two months of age), and the aim of this chapter is to investigate whether antenatal depression and cortisol predict DNA methylation, and whether DNA methylation predicts infant cortisol and temperament. Thus, regression analyses were used to examine predictive models.

Firstly, linear regressions were used to assess whether maternal antenatal symptoms of depression, or cortisol, significantly predicted infant DNA methylation. Then, further regression models were used to examine whether infant NR3C1 and BDNF DNA methylation predicted infant cortisol reactivity to a stressor, or infant behaviour (temperament). Within each model, batch number of DNA methylation analysis was entered at step 1 as a covariate, as was postnatal depression because of its known effects on infant development (Murray, 1992). The main predictor variables were entered at

step 2, as was infant gender because there is evidence to suggest that maternal antenatal mood disturbance may impact on male and female infants differently (O'Connor et al., 2002b, O'Connor et al., 2003, Rodriguez and Bohlin, 2005, Van den Bergh and Marcoen, 2004b, Bergh et al., 2005). Where maternal cortisol was entered as a predictor variable, maternal days of gestation was also included in the analyses as a covariate. Finally, interaction variables were entered at step 3, which were created using standardised or centred variables.

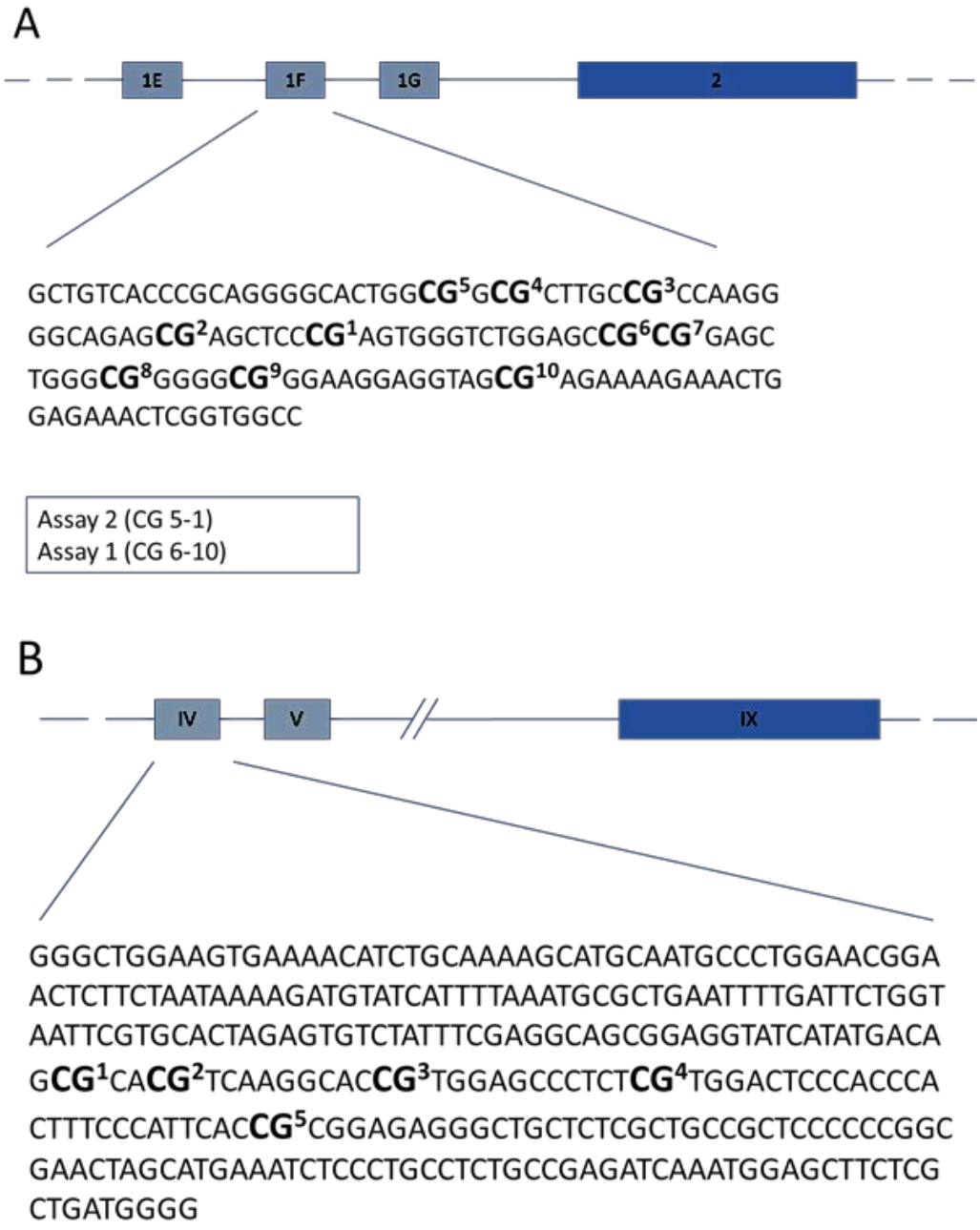


Figure 15 Part A and B are diagramatic representations of the DNA regions assessed in this study. Part A depicts three exons within the glucocorticoid receptior gene (NR3C1) adjacent to the major coding exon 2. This study assessed a 136 bp region encompassing exon IF. 10 CpG sites were examined using 2 assays designed with separate sequencing primers. Part B shows the 308 bp region of BDNF assessed in this study, which encompasses the untranslated exon IV. 5 CpG sites were assessed within this region using one assay.

4.3 Results

4.3.1 Demographic characteristics

The demographic characteristics of this sample are displayed in Table 17. All of the mothers in this study were primiparous, and their average age was 32.04 (SD=4.35). This primarily Caucasian (89.5%) group of women were highly educated, with 49.1% having a postgraduate degree. 21.1% reported consuming 1-5 units of alcohol per week, 89.5% of participants reported that their pregnancy had been planned, and 29.8% reported a previous history of mental health disorders.

For analysis, participants were divided into a control group (n=37) and a depressive symptom group (n=20). The demographic characteristics of these two groups were compared, and are presented in Table 17. No significant differences between control and depressive-symptom groups were found for the following variables:

a) Age; ($t_{(15)}$=0.25, p=0.816)
b) Education; ($x^2_{(3)}$=0.56, p=0.906)
c) Ethnicity; ($x^2_{(6)}$=7.90, p=0.246)
d) Alcohol consumption; ($x^2_{(1)}$=0.68, p=0.410)
e) Trimester at antenatal assessment; ($x^2_{(1)}$=1.85, p=0.174)
f) Planned pregnancy; ($x^2_{(1)}$=0.01, p=0.924)
g) Previous history of mental health disorders; ($x^2_{(3)}$=1.86, p=0.602)

However, as expected the depression-symptom group scored significantly higher on self-reported measures of antenatal depression ($t_{(55)}$=2.30, p<0.001), anxiety ($t_{(55)}$=0.49, p<0.001) and perceived stress ($t_{(55)}$=0.34, p<0.001).

Table 17 Demographic characteristics of the control group and depressive symptom group

Demographic Variables	Control Group (N=37)	Depressive symptom group (N=20)
Age (mean, SD)	32.14 (4.33)	31.85 (4.50)
Education (n, %)		
A-level	1 (2.7)	0
Undergraduate degree	16 (43.2)	9 (45)
NVQ	2 (5.4)	1 (5)
Postgraduate degree	18 (48.6)	10 (50)
Ethnicity (n, %)		
Caucasian	35 (94.6)	16 (80)
Black	0	1 (5)
Asian	1 (2.7)	3 (15)
Mixed Race	1 (2.7)	0
Alcohol units/week (n, %)		
None	28 (75.7)	17 (85)
1-5	9 (24.3)	3 (15)
Cigarettes/week (n, %)		
None	33 (89.2)	18 (90)
1-5	4 (10.8)	2 (10)
Days of gestation at antenatal assessment (mean, SD)	199.78 (86.82)	209.35 (46.78)
Planned Pregnancy (n, %)	33 (89.2)	18 (90)
Previous history of mental health problems (n, %)	10 (27)	7 (35)
Antenatal mood (mean, SD)		
EPDS (depression)	4.68 (2.82)	12.2 (2.12)***
STAI (trait anxiety)	23.19 (5.71)	42.75 (5.88)***
PSS (perceived stress)	7.81 (4.47)	14.85 (4.82)***

***p<0.001. EPDS: Edinburgh Postnatal Depression Scale, STAI: Spielberger Trait Anxiety Index, PSS: Perceived Stress Scale.

Of the 57 infants included in this study, 25 (43.9%) were male and 32 (56.1%) were female. Mean birth weight was 3.33Kg (SD=0.64), and 21 mothers (36.8%) reported delivery complications. Mean scores and standard deviations of the temperament domains were: activity (3.52, 0.71), smiling (4.02, 1.21), distress to limitations (4.25, 0.78) and soothing (4.31, 0.96).

In this chapter, the infants of mothers in the depression-symptom group are referred to as 'depression-exposed infants', and those born to the control participants are referred to as 'control infants'.

The characteristics of the depression-exposed and control infants were compared, and are presented below in Table 18. No significant group difference was found for the following variables:

k) Gender; ($x^2_{(1)}$=0.02, p=0.898)

l) Age when buccal swab was taken; ($t_{(55)}$=0.28, p=0.779)

m) Birth weight; ($t_{(54)}$=-0.15, p=0.881)

h) Method of delivery; ($x^2_{(4)}$=8.64, p=0.071)

i) Smiling; ($t_{(55)}$=-0.85, p=0.400)

j) Distress; ($t_{(55)}$=-1.50, p=0.140)

k) Soothing; ($t_{(55)}$=1.34, p=0.186)

However, the mothers with symptoms of depression reported significantly more delivery complications than control participants ($x^2_{(1)}$=4.07, p=0.04), and also reported that their infants had higher activity levels ($t_{(55)}$=-2.04, p=0.047).

Table 18 Demographic characteristics of the depression-exposed and control infants

Demographic Variables	Control Infants (N=37)	Depression-exposed Infants (N=20)
Gender (n, %)		
Male	16 (43.2)	9 (45)
Female	21 (56.8)	11(55)
Infant age in days (mean, SD)	53.8 (7.4)	53.1 (13.8)
Birth weight, Kg (mean, SD)	3.35 (0.44)	3.27 (1.02)
Method of delivery (n, %)		
Vaginal	22 (59.5)	5 (25)
Forceps	9 (24.3)	6 (30)
Ventouse	2 (5.4)	4 (20)
Elective caesarean	1 (2.7)	2 (10)
Emergency caesarean	2 (5.4)	3 (15)
Delivery complications (n, %)	10 (27)	11 (55)*
Infant behaviour (mean, SD)		
Activity	3.38 (0.66)	3.77 (0.73)*
Smiling	3.92 (1.22)	4.21 (1.19)
Distress	4.14 (0.85)	4.46 (0.80)
Soothing	4.43 (1.01)	4.08 (0.82)

*p<0.05. Infant behaviour was measured by maternal report at 2 months using the Infant Behaviour Questionnaire (IBQ)

4.3.2 Correlations

4.3.2.1 Correlations between maternal and infant characteristics

Pearson's correlations were used to investigate the relationship between maternal and infant characteristics. As expected, maternal depressive symptoms were significantly correlated with self-reported maternal anxiety ($r=0.742$, $p<0.001$) and perceived stress ($r=0.811$, $p<0.001$). However, measures of maternal mood were not significantly correlated with any of the demographic characteristics assessed, including: age, ethnicity, educational qualifications, living with partner, household income and alcohol consumption (all p's>0.05). There was also no significant correlation between any of the maternal and infant characteristics (all p's>0.05).

4.3.2.2 Correlations between CpG sites

Within each NR3C1 assay there was a high degree of correlation in the level of DNA methylation between sites; methylation at each CpG site was highly and significantly correlated with DNA methylation at every other CpG site within the same assay (all p's<0.001). This same high degree of correlation was not evident within the BDNF assay, as only the mean methylation of all 5 CpG sites was significantly correlated with CpG3 ($r=0.670$, $p<0.001$), 4 ($r=0.548$, $p<0.001$) and 5 ($r=0.769$, $p<0.001$). There were no significant between-assay correlations of CpG methylation (all p's>0.05).

4.3.2.3 Correlations between maternal and infant characteristics, and CpG methylation

The number of alcohol units consumed per week was correlated with NR3C1 1F DNA methylation at CpG1 ($r=0.289$, $p<0.05$), 4 ($r=0.287$, $p<0.05$) and 5 ($r=0.298$, $p<0.05$). Also, antenatal depression was correlated with BDNF IV CpG5 methylation ($r=-0.287$, $p<0.05$), antenatal anxiety was significantly correlated with BDNF CpG3 ($r=-0.318$, $p<0.05$), 4 ($r=-0.265$, $p<0.05$) and 5 methylation ($r=-0.280$, $p<0.05$), and perceived stress was significantly associated with BDNF CpG3 ($r=-0.290$, $p<0.05$) and 5 methylation ($r=-0.304$, $p<0.05$). However, none of these associations remained significant following a correction for multiple tests.

4.3.3 NR3C1

4.3.3.1 Maternal antenatal depression and infant NR3C1 methylation

For one of the control participants, there was not sufficient bisulphite-converted DNA available to run these analyses. Therefore, 36 participants are included in the control group, and 20 in the depression-exposed group.

Mean methylation of all 10 CpG sites was firstly used as the outcome variable in the regression model. An interaction between antenatal depression and infant gender significantly predicted mean NR3C1 methylation (β=-0.350, p=0.017), however there was no significant effect of antenatal depression (β=-0.019, p=0.896) or gender alone (β=0.089, p=0.524). These results are presented below in Table 19.

Table 19 Regression model using antenatal depression to predict mean NR3C1 methylation

Model		Unstandardized coefficients		Standardised coefficients *Beta*	t	Sig.
		B	Std. error			
1	Batch number	0.238	0.234	0.167	1.208	0.233
	Postnatal depression	0.338	0.400	0.117	0.845	0.402
2	Batch number	0.263	0.241	0.155	1.089	0.282
	Postnatal depression	0.352	0.432	0.122	0.815	0.419
	Antenatal depression	0.066	0.184	0.053	0.360	0.720
	Infant gender	0.069	0.185	0.054	0.372	0.711
3	Batch number	0.157	0.233	0.093	0.676	0.502
	Postnatal depression	0.427	0.412	0.148	1.037	0.305
	Antenatal depression	-0.024	0.179	-0.019	-0.132	0.896
	Infant gender	0.114	0.177	0.089	0.642	0.524
	Antenatal depression*infant gender	-0.435	0.176	**-0.350**	**-2.475**	**0.017**

To further investigate the significant interaction term, the regression model was reconstructed for males and females separately. For male infants, antenatal depression significantly predicted mean NR3C1 methylation (β=0.439, p=0.044); male infants exposed to maternal antenatal depression had greater mean CpG methylation than control male infants. For female infants, was no significant effect of antenatal depression (β=-0.329, p=0.099), but the strong Beta value (-0.329) and a p-value of 0.099 suggests a trend towards significance. However, the direction of effect for female infants appears to be opposite to that of males: those female infants exposed to antenatal depression appear to have *decreased* NR3C1 methylation across the 10 CpG sites compared to control females. These findings are presented graphically in Figure 17.

This regression model was reconstructed a number of times using % methylation at each individual CpG site as the outcome variable. At CpG sites 2 and 9, an antenatal depression*gender interaction also significantly predicted methylation (β=-0.285, $p<0.05$) and (β=-0.330, $p<0.05$) respectively).

The dataset was split by gender and this model was repeated for CpG2 and 9. At CpG2, the effect of antenatal depression in predicting methylation for the males approached significance (β=0.395, p=0.067), but was not significant in predicting female methylation (β=-0.239, p=0.231). Interestingly, at CpG9 the opposite is seen: the effect of antenatal depression in predicting methylation in the females approached significance (β=-0.360, p=0.066), however was non-significant in predicting methylation in the males (β=0.288, p=0.191). The opposite direction of the β values explains the significant interaction effects.

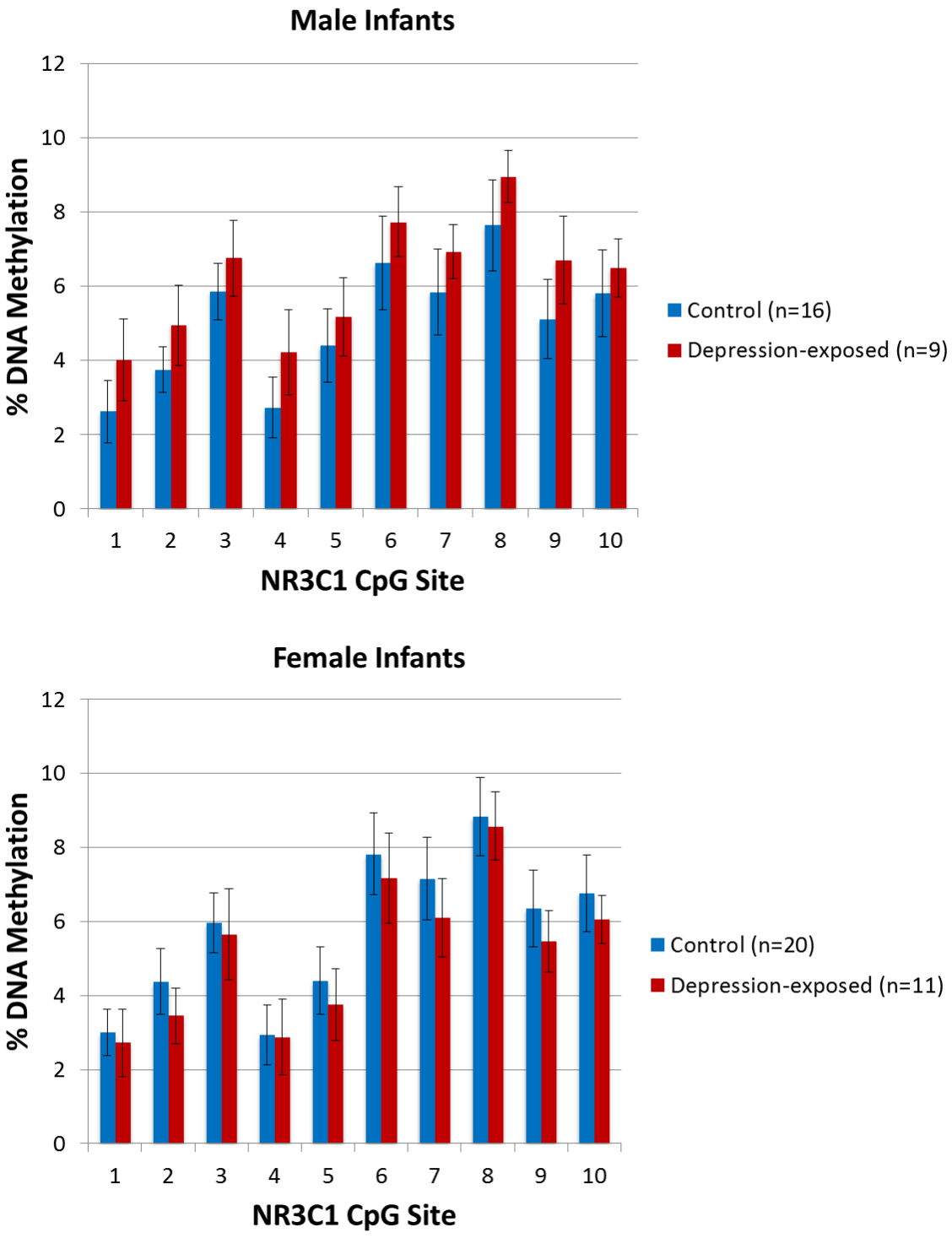


Figure 16 Percent methylation at each CpG site within the assessed NR3C1 region for the depression-exposed and control infants.

4.3.3.2 Maternal antenatal cortisol and infant NR3C1 methylation

A prominent theory to explain associations between antenatal mood disturbance and adverse infant outcomes suggests that increased circulating maternal glucocorticoids influence the development of the infant HPA axis, potentially via epigenetic modifications of HPA-related genes (Glover, 2011, Talge et al., 2007, Braithwaite et al., 2014). To test this hypothesis, maternal cortisol was used as a main predictor in regression analyses, in the place of antenatal depression, to predict infant NR3C1 methylation. Maternal days of gestation was also included in the analyses as a covariate. Two measures of maternal cortisol were used as main predictor variables in this analysis: log AUC diurnal cortisol[4] and mean change in response to a stressor[5]. These measures were entered into the regression model as continuous variables.

Both measures of maternal cortisol were used as predictors, however neither of these measures significantly predicted mean NR3C1 methylation, and neither did the interaction variables (all p's>0.05).

[4] A Kolmogorov-Smirnov test was performed and logAUC for maternal cortisol was found to be normally distributed ($D_{(56)}$=0.111, p=0.085).

[5] A Kolmogorov-Smirnov test was performed and mean change in maternal cortisol in response to the stressor was found to be normally distributed ($D_{(57)}$=0.128, p=0.201).

4.3.3.3 Infant NR3C1 1F DNA methylation and behaviour

Linear regression models were constructed to assess whether mean NR3C1 1F DNA methylation may predict infant behaviour, and the four domains of temperament (activity, smiling, distress to limitations and soothability) were used as the outcome variables.

Results of these analyses showed that mean NR3C1 1F DNA methylation[6] did not significantly predict any domains of temperament, and neither did infant gender or a gender*NR3C1 1F methylation interaction (all p's>0.05).

[6] A Kolmogorov-Smirnov test was performed and the NR3C1 methylation data were found to be normally distributed ($D_{(54)}$=0.091, p=0.200).

4.3.3.4 Infant NR3C1 1F DNA methylation and cortisol responses to inoculation

A full set of infant saliva samples was only available for 41 infants: 15 had been exposed to antenatal depression and 26 were controls.

A regression analysis was used to investigate whether infant NR3C1 1F DNA methylation may predict infant cortisol responses to inoculation, and mean cortisol change at 20 minutes post-inoculation was used as the outcome variable.

The results of these analyses showed that infant NR3C1 1F methylation did not significantly predict cortisol mean change in response to inoculation, neither did infant gender or the NR3C1 methylation*gender interaction (all p's>0.05).

4.3.4 BDNF

4.3.4.1 Maternal antenatal depression and infant BDNF IV DNA methylation

Linear regression models were used to assess whether antenatal depression may predict infant BDNF methylation, and mean % methylation of the 5 BDNF CpG sites was used as the outcome variable. Antenatal depression significantly predicted mean BDNF methylation (β=-0.343, p=0.013), however there was no significant effect of gender (β=-0.249, p=0.079) or an antenatal depression*gender interaction (β=0.620, p=0.538). The results of these analyses are available below in Table 20.

Table 20 Regression model using antenatal depression to predict infant BDNF IV mean DNA methylation

Model		Unstandardized coefficients		Standardised coefficients *beta*	t	Sig.
		B	Std. error			
1	Batch number	0.114	0.217	0.072	0.524	0.602
	Postnatal depression	-0.405	0.382	-0.145	-1.061	0.294
2	Batch number	0.253	0.205	0.160	1.232	0.224
	Postnatal depression	-0.402	0.376	-0.144	-1.069	0.290
	Antenatal depression	-0.419	0.164	**-0.336**	**-2.565**	**0.013**
	Infant gender	-0.322	0.158	-0.271	-2.034	0.047
3	Batch number	0.274	0.209	0.173	1.310	0.196
	Postnatal depression	-0.421	0.380	-0.151	-1.107	0.274
	Antenatal depression	-0.427	0.165	**-0.343**	**-2.590**	**0.013**
	Infant gender	-0.296	0.165	-0.249	-1.796	0.079
	Antenatal depression*infant gender	0.100	0.162	0.084	0.620	0.538

To further explore this finding, this model was reconstructed using % methylation at each individual CpG site as the outcome variable. At CpG3, antenatal depression significantly predicted methylation (β=-0.417, p=0.002), however there was no significant effect of gender (β=-0.170, p=0.212), or a gender*antenatal depression interaction (β=0.221, p=0.102). These findings are demonstrated graphically in Figure 18.

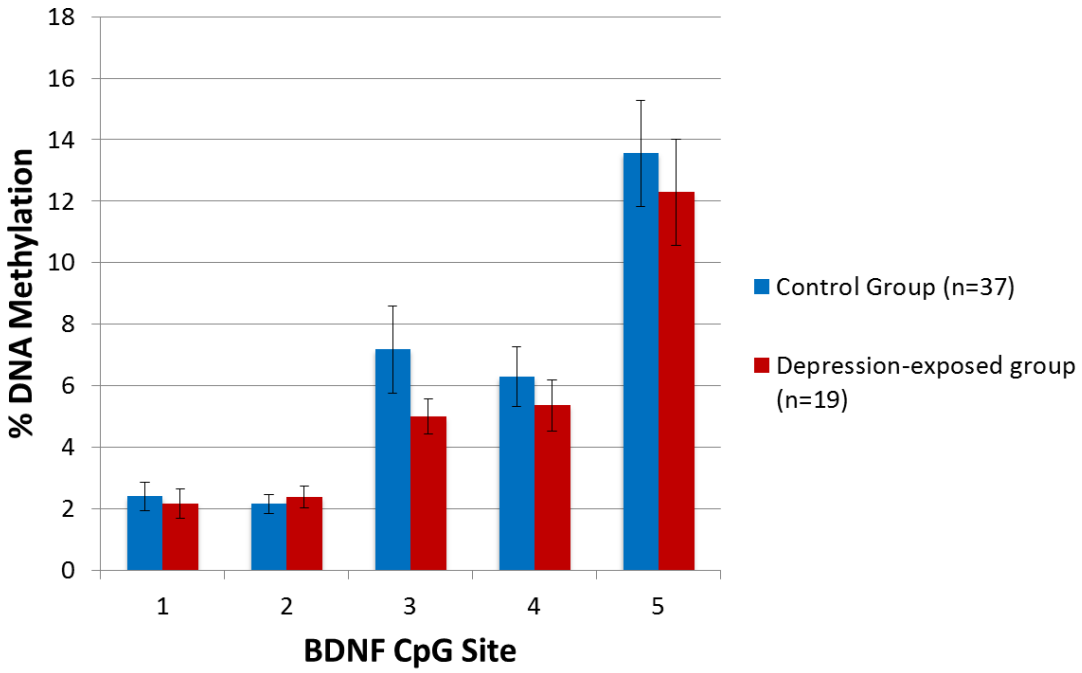


Figure 17 Infant BDNF IV DNA methylation (%) at each assessed CpG site, split by group.

There was one more significant finding: at CpG1 there was a significant effect of infant gender to predict methylation (β=-0.305, p=0.039). Further investigation revealed that at this site, male infants had significantly higher % DNA methylation than females (2.57%

vs. 2.10%). There were no other significant effects of antenatal depression, gender, or antenatal depression*gender interaction (all p's > 0.05) .

4.3.4.2 Maternal antenatal cortisol and infant BDNF IV DNA methylation

As before, regression models were used to assess whether measures of antenatal maternal cortisol predicted infant BDNF IV DNA methylation. The models were constructed as described in part 4.3.3.2, and mean BDNF methylation was used as the outcome variable.

However, in all models maternal cortisol did not significantly predict BDNF methylation. There were also no significant effects of gender or maternal cortisol*gender interactions (all p's>0.05).

4.3.4.3 Infant BDNF IV DNA methylation and behaviour

As for NR3C1, regression analyses were used to assess whether BDNF methylation[7] may predict infant behaviour. No significant effects were found for the domains of distress to limitation or soothing, however a significant BDNF methylation*gender interaction significantly predicted activity (β=-0.334, p=0.018). Further investigation revealed that for male infants only, BDNF methylation significantly predicted activity (β=-0.617, p=0.004), presented below in

Table 21. The negative beta value suggests that those male infants with the lowest levels of BDNF methylation show the highest activity levels.

Table 21 Regression model using mean BDNF methylation to predict infant activity, split by gender

Gender	Model		Unstandardized coefficients		Standardised coefficients *beta*	t	Sig.
			B	Std. error			
Male	1	Batch number	-0.131	0.209	-0.133	-0.628	0.537
		Postnatal depression	-0.064	0.342	-0.040	-0.187	0.853
	2	Batch number	-0.181	0.175	-0.183	-1.033	0.313
		Postnatal depression	-0.412	0.305	-0.255	-1.352	0.191
		Mean BDNF methylation	-0.491	0.150	**-0.617**	**-3.262**	**0.004**
Female	1	Batch number	0.237	0.170	0.254	1.397	0.174
		Postnatal depression	0.373	0.349	0.195	1.069	0.295
	2	Batch number	0.221	0.181	0.237	1.220	0.234
		Postnatal depression	0.279	0.356	0.197	1.064	0.297
		Mean BDNF methylation	0.038	0.133	0.056	0.287	0.776

[7] A Kolmogorov-smirnov test was performed and the BDNF methylation data were found to be normally distributed ($D_{(54)}$=0.074, p=0.200).

BDNF methylation also significantly predicted infant smiling (β=-0.206, p=0.034). The negative beta value indicates that those infants with the lowest levels of BDNF methylation show the greatest levels of smiling. There were no other significant effects of BDNF methylation, gender, or a BDNF methylation*gender interaction on infant behaviour (all p's>0.05).

4.3.4.4 Infant BDNF IV DNA methylation and cortisol responses to inoculation

A regression analysis was used to investigate whether BDNF methylation predicted infant cortisol responses to inoculation.

Mean BDNF methylation did not significantly predict mean change in infant cortisol at 20 minutes post-inoculation. Also, there were no significant effects of gender or a gender*BDNF methylation interaction (all p's>0.05).

4.3.5 Summary of findings

NR3C1

- Male infants exposed to antenatal depression had significantly increased DNA methylation of the assessed NR3C1 1F region compared with controls. The effect of antenatal depression was not significant for female infants, though a trend suggested that those female infants exposed to antenatal depression had decreased NR3C1 1F DNA methylation compared with control females.
- Maternal cortisol did not predict infant NR3C1 1F DNA methylation.
- Infant NR3C1 1F DNA methylation did not predict infant temperament or cortisol responses to inoculation.

BDNF

- Infants exposed to antenatal depression had significantly decreased methylation at BDNF CpG3 compared to controls.
- Maternal antenatal cortisol did not predict infant BDNF IV DNA methylation.
- In males, lower levels of BDNF IV DNA methylation predicted higher activity levels. In both male and female infants, lower levels of BDNF IV DNA methylation predicted increased levels of smiling.
- Infant BDNF IV DNA methylation did not predict cortisol responses to inoculation.

4.4 Discussion

This study was an analysis of the methylation of two key genes in samples taken from 57 infants, 20 of whom had been exposed to maternal depressive symptoms during pregnancy. The main finding of this study is that male infants who had been exposed to maternal depressive symptoms had increased methylation of the promoter region of the GR gene, NR3C1. This result is in line with findings from both the human (Oberlander et al., 2008) and animal literature (Weaver et al., 2004), but suggests potential sex effects. Although antenatal depression predicted methylation, there was no significant effect of maternal cortisol in predicting methylation, which is surprising given the theoretical model that describes changes of both the maternal and fetal HPA axis as a direct result of mood disturbance in pregnancy (Talge et al., 2007, Glover, 2011).

The secondary aim of this study was to investigate whether exposure to antenatal depression predicted infant BDNF methylation. It was expected that antenatal depression exposure would predict increased infant BDNF IV methylation. This hypothesis was primarily driven from animal research that has linked antenatal stress with increased offspring BDNF IV methylation and decreased BDNF protein expression in the amygdala and hippocampus. However the opposite direction of effect was seen in the current study: antenatal exposure to maternal depressive symptoms predicted decreased methylation of infant BDNF IV, specifically at CpG3, which is adjacent to a suspected CREB binding site. This is the first study to investigate this association in a human cohort.

4.4.1 Findings in context

4.4.1.1 NR3C1

This study replicated previous findings and showed that male infants exposed to maternal antenatal depressive symptoms had overall increased methylation of the NR3C1 1F region. However, this study differs from the previous findings in two significant ways. Firstly, this result highlights potential sex effects on changes in NR3C1 methylation, as significant effects of antenatal depression exposure were only found for male infants. Notably, all of the existing animal literature, which has linked exposure to early life stress with increased NR3C1 1F DNA methylation, has derived solely from male rodents. In the human literature, McGowan and colleagues (2009) only analysed DNA methylation in the brains of male suicide victims, and found significant increases in NR3C1 1F methylation following childhood abuse. Van der Knaap *et al* (2014) demonstrated increased NR3C1 1F methylation in DNA of blood extracted from teenagers exposed to significant life events or trauma, however found no significant effect of gender on methylation. Further, Romens and colleagues (2014) demonstrated increased NR3C1 1F methylation following childhood abuse, and Oberlander *et al.* (2008) report increased NR3C1 1F methylation following exposure to maternal antenatal depression. However, neither study report any effects of gender, or that such effects were tested for.

Thus, this is the first study to identify that male infants may be more susceptible to increased NR3C1 1F methylation following antenatal depression exposure than female infants. This finding compliments a body of literature which suggests that males may be

more susceptible to the effects of maternal antenatal distress than females (O'Connor et al., 2002b, O'Connor et al., 2003, Rodriguez and Bohlin, 2005, Van den Bergh and Marcoen, 2004b, Bergh et al., 2005). For example, following exposure to antenatal psychological distress males are more likely to have negative emotionality, hyperactivity and behavioural problems (O'Connor et al., 2002b, O'Connor et al., 2003), and may also be more likely to show symptoms of ADHD than females (Van den Bergh and Marcoen, 2004b, Rodriguez and Bohlin, 2005). Therefore, sex differences in NR3C1 1F methylation could, in part, explain the association between antenatal mood disturbance and more adverse outcomes seen in males than females. Of course, it is very unlikely that differential methylation of just one gene could explain complex behaviours and emotional difficulties in later life, and it is highly probable that a number of other genes as well as other antenatal and postnatal environmental factors play a role. Nonetheless, replication of this finding would certainly add weight to this hypothesis, and could potentially lead to further investigation of sex differences in genome-wide methylation following antenatal distress exposure.

The second aspect of this study that sets it apart from previous research is that the DNA used in this analysis was extracted from infant buccal tissue. Previous studies have shown that exposure to early life stress results in increased NR3C1 1F methylation in post mortem hippocampal brain tissue (McGowan et al., 2009), cord blood (Oberlander et al., 2008) and whole blood (van der Knaap et al., 2014, Romens et al., 2014). However, this is the first study to demonstrate the same association using buccal tissue. This has important implications for replication and further investigation of NR3C1 1F methylation

in larger cohorts, because collection of buccal cell samples is easier and more economical than extracting DNA from blood or post mortem brain tissue.

However, a recurring problem in this field is that epigenetic mechanisms are highly tissue specific, and tissue specific methylation has yet to be characterised at the genome-wide level. Thus, it is unclear whether methylation in one tissue type is representative of another. Studies of consistency of methylation profiles across peripheral tissues have produced conflicting results, although they are generally limited by the small sample sizes and candidate gene approach (Thompson et al., 2013, Armstrong et al., 2014). Just one recent study has used post mortem brain tissue to investigate the variability in DNA methylation across a number of brain regions (n=9), and for a subset of participants blood samples taken before death were also available (n=3). This study found that between-tissue variation in DNA methylation greatly exceeded between-individual differences, which highlights the importance of DNA methylation in regulating cell-specific gene expression (Davies et al., 2012). Unfortunately, NR3C1 methylation was not assessed in this study, although it could be assumed from these findings that perhaps the methylation profile of this gene in brain tissue may be very different to that seen in peripheral tissue, especially as the GR is expressed centrally. On the other hand, it could be that methylation of NR3C1 in peripheral and central tissue has a high degree of inter-individual correlation, and the results obtained from buccal swabs could be a reliable marker of central methylation. However, these ideas are speculative and clearly more research is needed as understanding this association is critical in driving further research.

A somewhat surprising finding from this study was that maternal cortisol did not predict infant NR3C1 methylation. It has been proposed that one possible mechanism by which antenatal depression impacts on fetal and infant development is via the programming of both the maternal and fetal HPA axis during pregnancy, and that this programming may be mediated by epigenetic modifications of the infant genes related to the HPA axis (Talge et al., 2007, Glover, 2011). As such, it was expected that maternal cortisol levels would significantly predict infant NR3C1 methylation. However, this study did not find any evidence to support this theory. This lack of association may be attributable to failures in accurately measuring maternal cortisol, though two different measures of maternal cortisol were assessed in this study and neither significantly predicted infant methylation. Also, maternal depressive symptoms significantly predicted infant NR3C1 1F DNA methylation in males. Thus, it may be that maternal cortisol does not mediate the association between antenatal depression and changes in infant NR3C1 1F DNA methylation.

Further, this study failed to replicate the previous finding that infant NR3C1 1F DNA methylation predicted infant cortisol responses to a stressor (Oberlander et al., 2008). However, there were a number of methodological differences between this study and the Oberlander (2008) study. For example, this study assessed infant cortisol responses to inoculation at 2 months of age, whereas the previous study assessed infant cortisol responses to a habituation-information processing task at 3 months. Also, the previous study assessed methylation in DNA extracted from cord blood, and it may be that DNA methylation in blood is more reliably correlated with stress responses than DNA methylation of DNA in buccal tissue.

4.4.1.2 BDNF

This is the first study in humans to investigate whether exposure to maternal antenatal depressive symptoms may have implications for offspring BDNF methylation. Previous animal research has demonstrated an increase in BDNF IV methylation, and a subsequent decreased BDNF expression, in the offspring of dams exposed to antenatal stress (Boersma et al., 2014). Human studies of adult psychopathology have also reported increased BDNF IV methylation (Toledo-Rodriguez et al., 2010, Keller et al., 2010). In contrast, the current study found that infants exposed to antenatal depression had decreased methylation of the BDNF IV region, specifically at CpG3, which is adjacent to a CREB binding site. Our knowledge of the relationship between BDNF IV methylation and BDNF protein expression, which derives solely from animal research, would suggest decreased methylation results in more active CREB binding and more expression of the BDNF protein in those infants exposed to antenatal depression (Ikegame et al., 2013).

However, there are a number of problems with this assumption. Firstly, although there is a high degree of homology between the human and rodent BDNF gene sequences (Boulle et al., 2012), our knowledge of the role of epigenetics in regulating gene expression is very limited. Therefore, it would be unwise to assume that the BDNF gene is regulated in the same manner across species, and that decreased methylation is associated with increased protein expression in humans. Indeed, the BDNF gene is composed of a complex assortment of un-translated regulatory elements, which are regions of the gene that are transcribed into mRNA but are not translated into the functional protein. Only one of these regions was evaluated in this study: exon IV. As such, methylation changes in the regulatory regions of this gene are very complex and difficult to interpret in

relation to gene expression. Secondly, there are limitations in translating findings from animal models of antenatal stress to human mood disorders. Therefore, the previous rodent study is not wholly comparable to this finding as different mechanisms may be driving changes in gene methylation. Finally, the issue of whether changes in methylation detected in a peripheral tissue are relevant to central methylation and expression also apply here.

On the other hand, given that BDNF is essential for neurogenesis, it is possible that the decreased BDNF methylation seen in infants exposed to antenatal depression in this study could relate to increased BDNF expression in order to support advanced neurodevelopment. Such an explanation would support the theory that stress during pregnancy increases risk for preterm delivery (Dole et al., 2003), and therefore fetuses of stressed mothers undergo advanced or rapid development (Pike, 2005, Worthman and Kuzara, 2005). Indeed, a very recent study has shown that foetuses of mothers with negative mood have an advanced neurobehavioral profile, when compared with control foetuses at the same gestational age (Doyle et al., under review). Further support comes from a functional MRI study of children and adolescents, some of whom had experienced maternal deprivation in early life. Children with a history of early adversity had mature connectivity between the amygdala and medial prefrontal cortex and resembled the adolescent phenotype (Gee et al., 2013), suggesting that they had undergone, or indeed were still undergoing, some form of rapid brain development. Whilst the association between antenatal depression and decreased BDNF methylation is certainly consistent with this idea of 'advanced development' in fetuses of prenatally stressed women, a wide range of genes are involved in neurodevelopment, and it is unlikely that changes in BDNF

expression alone could support such advanced or rapid brain development. Therefore further research is required to corroborate these findings, and investigate DNA methylation changes in other key neurodevelopment genes.

4.4.2 Strengths and Limitations

This study had a number of strengths, including a prospective longitudinal design, the testing of infant methylation of two key genes, as well as the novel approach of using both biological and psychological measures of maternal antenatal psychological distress as predictors of infant methylation. It is also the first study to replicate the previous NR3C1 finding in cord blood (Oberlander et al 2008) using buccal tissue, which has positive implications for replication in larger cohorts.

This study also has a number of limitations that should be addressed. Firstly, although comparable to previous studies, this study has a modest sample size of 57 participants. This makes the detection of moderate to small effects of antenatal depression on methylation very difficult. Nonetheless, the replication of the finding that early life stress results in increased NR3C1 1F DNA methylation adds weight to the reliability of the finding (Oberlander et al., 2008, McGowan et al., 2009, Romens et al., 2014, van der Knaap et al., 2014). Secondly, the participants who met criteria for depressive-symptoms in this study were not clinically diagnosed. Instead, they self-reported current levels of mood using a paper-based questionnaire, and a cut-off approach was used to identify a group of participants who reported depressive symptoms during pregnancy. The use of this cut-off is a standard approach with good sensitivity and specificity to identify

participants with depressive symptoms in a community sample. However, previous research on antenatal depression has shown that clinical diagnoses of depression correlate more reliably with biological markers of depression, such as diurnal cortisol, than do self-report measures (O'Connor et al., 2013a). Therefore, using clinical diagnostic criteria to define the depression-exposed group in this study may have yielded more significant and reliable findings. But, if this approach had been taken, the depression-exposed group would probably have been smaller, and as a result there would have been less power to detect significant results. A further limitation is that this study did not account for multiple cell types that may be present when taking the buccal swab. For example, as well as buccal cells it is likely that the samples from which DNA was extracted contained other cell types, such as immune cells. As different cell types have different methylation patterns, the composition of cells within a sample will therefore affect the methylation results. However, this problem is unavoidable when using buccal swabs, and the method of cell sorting is generally only applied to blood samples. Finally, maternal-reported measures antenatal of mood and infant behaviour may be subject to misreporting and reporter-bias.

4.4.3 Conclusion

In conclusion, this study found that male infants exposed to antenatal depression had increased methylation of NR3C1 1F compared with non-exposed controls. This study adds to a body of research that shows that exposure to early environmental adversity results in increased NR3C1 1F methylation (Oberlander et al., 2008, Romens et al., 2014,

van der Knaap et al., 2014), and also replicates a previous finding that antenatal depression predicts increased infant NR3C1 1F methylation (Oberlander et al., 2008). However, this is the first study to identify potential sex effects in changes in DNA methylation as a result of exposure to antenatal depressed mood. A surprising result from this study is that maternal cortisol did not significantly predict infant NR3C1 1F methylation, and this study failed to replicate the previous finding that infant NR3C1 1F methylation predicts infant stress responses. Thus, these findings suggest that perhaps the effects of antenatal depression on infant development may not be medicated by changes to both the maternal and fetal HPA axis.

This study also found that those infants exposed to antenatal depression had decreased methylation of BDNF exon IV, specifically adjacent to a CREB binding site, suggesting that these infants had increased BDNF expression. This finding is contrary to previous animal research which found associations between antenatal stress and increased BDNF IV methylation (Boersma et al., 2014). Maternal antenatal cortisol did not significantly predict infant BDNF methylation, again suggesting that the effects of antenatal depression on changes in infant DNA methylation may not be HPA-driven.

CHAPTER 5

WHY ARE SOME INFANTS MORE SUSCEPTIBLE TO THE EFFECTS OF ANTENATAL MOOD DISTURBANCE THAN OTHERS?

INVESTIGATING THE MODERATING ROLE OF 5-HTTLPR

5 Overview of Chapter

This chapter investigates the role of the serotonin transporter polymorphism (5-HTTLPR) in moderating the association between antenatal anxiety/depression and infant temperament and childhood behavioural difficulties. It is clear that infants are not uniformly affected by antenatal mood disturbance. Thus, investigating why some infants may be more susceptible to antenatal mood disturbance is necessary to a) understand the mechanisms by which depression during pregnancy impacts on fetal and infant development, and b) effectively inform on intervention designs in order to target 'at risk' individuals.

This study is an analysis of data collected as part of a large UK-based cohort study: the Avon Longitudinal Study of parents and Children (ALSPAC). Serotonin transporter (5-HTTLPR) genotype data was available for n=5,084 individuals, who comprised the sub-sample used for analysis in this study. Second and third trimester antenatal mood data was also available, as well as data on infant temperament at 6 months and child behavioural difficulties at 42 and 81 months.

This chapter begins with an overview of the existing literature, which suggests that the 5-HTTLPR genotype moderates the association between antenatal mood disturbance and infant development. A description of the ALSPAC cohort and the methods employed in this analysis follow, and the results of hierarchal linear regression models, which were used to address the aims of this chapter, are presented. This chapter concludes with a discussion of the findings.

5.1 Introduction[8]

There is a growing awareness that environmental exposure to stress can shape developmental trajectories as early as the fetal period. This has been described in terms of the fetal programming hypothesis (Gluckman and Hanson, 2005, Barker, 1998) that stipulates that the phenotype of a fetus may be altered during the antenatal period in accordance with maternal cues. Maternal depression and anxiety during pregnancy are associated with an increased risk of behavioural and emotional disturbances in offspring (O'Connor et al., 2002b, Van den Bergh et al., 2005, Talge et al., 2007). An important challenge in this field is to disentangle the extent to which intergenerational transmission of mood disturbances is attributable to genetic or environmental factors, or both. The recent use of genetically sensitive study designs has confirmed that stressful insults that influence the antenatal environment, such as high levels of maternal anxiety, do have long-term effects on offspring outcomes which are, in part, independent of genetic factors (Rice et al., 2010).

It is clear, however, that there are individual differences in the extent to which developing fetuses are susceptible to maternal antenatal anxiety, and developmental programming more broadly. Early investigations in this area were interpreted largely in light of the classic 'diathesis-stress model' (Monroe and Simons, 1991a) (see Chapter 2 for more details), which proposed that some children are more susceptible to develop behavioural and emotional problems in unfavourable environments than others.

[8] The data presented in this chapter has been published in the Journal of the American Academy of Child and Adolescent Psychiatry. Braithwaite EC, Ramchandani PG, O'Connor TG, van IJzendoorn MH, Bakermans-Kranenburg MJ, Glover V, Netsi E, Evans J, Meaney MJ, Murphy SE (2013). No moderating effect of 5-HTTLPR on associations between antenatal anxiety and infant behavior. *JAACAP*; 52:5 (519-526).

Supporting evidence indicates, for example, that psychosocial stress (one type of unfavourable environment) predicts behavioural and emotional problems in children depending on individual differences in phenotypic factors, such as temperament (Van Zeijl et al., 2007, van Aken et al., 2007a, Bradley and Corwyn, 2008) and impulsivity (Lengua et al., 2000). Genetic factors also define susceptibility to environmental influences. For example, the serotonin transporter polymorphism, 5-HTTLPR, has been identified as a moderator of the impact of stressful life events on depressive symptoms (Caspi et al., 2003), whereby the short allele is thought to confer susceptibility to depressive symptoms (see Chapter 1, page 45). However, there is considerable debate about the validity of these findings given two negative meta-analyses of the gene by environment (GxE) interactions (Risch et al., 2009, Munafò et al., 2009), one positive meta-analysis, and two relevant papers that suggest that the initial finding may be a false positive (Duncan and Keller, 2011, Fergusson et al., 2011).

This chapter extends this line of research by testing the hypothesis that 5-HTTLPR moderates the effect of maternal antenatal anxiety on infant temperament and behavioural difficulties. Two previous investigations have addressed this question (Pluess et al., 2011, Oberlander et al., 2010). In a small-scale study of 75 mothers and their children, Oberlander *et al* (2010) reported that high levels of maternal antenatal anxiety predicted anxious and depressive symptoms in children, but only those with two copies of the short allele. In contrast, increased aggression and externalizing behaviours were predicted by high third trimester anxiety only in children with two copies of the long allele. These findings suggest that 5-HTTLPR does moderate behavioural outcomes of maternal antenatal anxiety; however, these results must be interpreted with caution given the small sample size. In a larger cohort study (N=1513),

Pluess and colleagues (2011) reported that the 5-HTTLPR polymorphism significantly moderated the effect of second trimester maternal antenatal anxiety on infant negative emotionality. They found that maternal reports of emotional difficulties at 6 months were predicted by maternal self-reported antenatal anxiety only in infants who carried at least one copy of the short allele, whereas there was no association in those infants homozygous for the long allele. Such evidence is consistent with the idea that 5-HTTLPR may moderate the effect of maternal antenatal anxiety on the developing fetus, with short allele carriers showing increased susceptibility to such an environmental insult.

5.1.1 Rationale

This chapter investigates the role of 5-HTTLPR in moderating the association between antenatal anxiety/depression and temperament in infancy, and behavioural difficulties in childhood in a larger sample than studied to date. Identifying individuals who may be susceptible to the effects of antenatal mood disturbance is essential in order to understand the mechanisms by which antenatal mood disturbance impacts on fetal and infant development, and also to inform future targeted interventions.

5.1.2 Aims and Hypotheses

5.1.2.1 Aim

The aim of the current study is to provide a substantive replication of previous findings by assessing the moderating effect of 5-HTTLPR on the association between maternal antenatal anxiety and temperament in infants aged 6 months, in a large population cohort, and to extend them by examining whether these effects persist beyond infancy.

5.1.2.2 Objectives

i. Replicate the previous study, which found that 5-HTTLPR moderates the association between antenatal anxiety and infant behaviour at 6 months, in a large population cohort.
ii. Extend previous findings beyond 6 months and investigate whether 5-HTTLPR moderates the association between antenatal anxiety and behavioural difficulties in later childhood.

iii. Determine whether 5-HTTLPR also moderates the association between antenatal depression and infant behaviour at 6 months and in later childhood.

5.1.2.3 Hypotheses

i. Those infants with two copies of the short allele will have a more reactive temperament when exposed to high levels of antenatal anxiety than those infants with two copies of the long allele.

ii. The 5-HTTLPR genotype will moderate the association between antenatal anxiety and later childhood behavioural difficulties up to 81 months.

iii. The 5-HTTLPR genotype will also moderate the association between antenatal depression and infant reactivity at 6 months and behavioural difficulties up to 81 months.

5.2 Methods

5.2.1 Participants

The present study is an analysis of data collected as part of the Avon Longitudinal Study of Parents and Children (ALSPAC) (Boyd et al., 2012). ALSPAC is large population based longitudinal study in which pregnant women from the Avon area of the UK (the former county surrounding the city of Bristol) were recruited with delivery dates between April 1991 and December 1992. Initially, 14,551 pregnant women were enrolled during the early stages of the study, and 13,801 mothers remained within the study. There were a total of 14,062 live births, and 13,985 surviving offspring at 12 months. Questionnaires were sent to parents at regular time intervals during pregnancy and following birth. DNA samples were obtained from over 10,000 children and 5-HTTLPR genotypes were available for 5631 individuals. In the current study, analyses were carried out only using data from infants of white Caucasian ethnicity, because 5-HTTLPR allele frequencies are inconsistent between different ethnic groups (Gelernter et al., 1997). Thus, a subsample of n=5084 infants was used for the current analysis. Ethical approval for the study was obtained from the ALSPAC law and ethics committee and from local research and ethics committees. All participants provided informed consent.

5.2.2 Measures

5.2.2.1 Maternal antenatal anxiety

At 18 and 32 weeks of pregnancy maternal anxiety was assessed using the anxiety items from the Crown Crisp Index, a validated self-rating inventory (Birtchnell et al., 1988,

Sutherland and Cooper, 1992). The Crown Crisp Index is a 24-item questionnaire with 4 sub-scales each composed of 8 items. Participants are asked to rate their responses to questions related to feelings and behaviours on a 4-point scale from "very often" to "never". Studies using this measure have previously demonstrated a robust association between maternal antenatal anxiety and adverse child behaviour outcomes (O'Connor et al., 2002b).

5.2.2.2 Maternal antenatal depression

Maternal antenatal depression was assessed at 18 and 32 weeks of pregnancy, and also 8 weeks after birth, using the Edinburgh Postnatal Depression Scale (EPDS). A description of this measure can be found in Chapter 2, page 72.

5.2.2.3 Infant temperament at 6 months

The Infant Temperament Questionnaire (Carey and McDevitt, 1978) was used to assess child temperament at 6 months of age. The questionnaire identifies 9 domains of temperament: activity, adaptability, approach, distractibility, intensity, mood, persistence, rhythmicity and threshold. Parents completed the questionnaire using a 6-point scale response from "almost never" to "almost always". This questionnaire has previously been shown to have good test reliability and internal consistency (Carey and McDevitt, 1978). One temperament characteristic (infant reactivity) was chosen *a priori* for the present study. Infant reactivity was chosen for assessment as it has been shown to be an important characteristic in terms of infant plasticity and is influenced by maternal antenatal anxiety (Davis et al., 2004a). Individual items from the Infant

Temperament Questionnaire corresponding to reactivity were identified independently by three raters (Paul G. Ramchandani, Marinus van IJzendoorn, Marian Bakermans-Kranenburg). The identified items fell almost exclusively within the adaptability, approach, intensity and threshold subscales. Therefore, a scale to assess infant reactivity was established by calculating the sum of the z scores for the adaptability, approach, intensity and threshold domains, and this showed good internal consistency (α=0.768). A similar method has been used previously to calculate an infant reactivity measure (Ramchandani et al., 2010). The questions in these scales show significant overlap with those used in the study by Pluess *et al.* to asses infant negative emotionality (Pluess et al., 2011). For example there are similar questions on the infant's reaction to loud noises, the degree of protest when being dressed, and how quickly the infant calms down after an unpleasant stimulus.

5.2.2.4 Child behavioural problems

Child emotional and behavioural problems were measured at 42 months (3.5 years) using the Rutter Revised Scale and at 81 months (6.9 years) using the Strengths and Difficulties Questionnaire.

The Rutter Revised Scale is a parent-report measure commonly used to assess child psychopathology and prosocial behavior (Rutter, 1967, Hogg et al., 1997). This measure has three problem behavior subscales: conduct problems, emotional problems and hyperactivity. Scores on the three subscales are summed to create a 'total difficulties' scale. Parents are required to rate child behaviours on a 3-point scale from 'does not apply' to 'certainly applies'.

The Strengths and Difficulties Questionnaire (SDQ) is a well-validated parent-report assessment of child emotional and behavioural problems (Goodman et al., 1998). The SDQ assesses specific child symptoms related to attention/hyperactivity, conduct and emotional problems, and a 'total behavioural problems' score encompasses these three subscales. The total SDQ score shows good predictive validity of clinician rated mental health disorders (Goodman and Goodman, 2011).

5.2.3 Genotyping

Child DNA was extracted from peripheral blood samples and genotyped as described previously by Araya and colleagues (Araya et al., 2009). For analysis, the six genotypes were grouped by expression level: low expression: SS, SL_G, L_GL_G; medium expression: SL_A, L_GL_A; and high expression: L_AL_A. The genotypes were in Hardy-Weinberg equilibrium (X^2=13.56, p=0.42), and the distribution of the 5-HTTLPR triallelic genotypes are shown in Table 22. The triallelic 5-HTTLPR variable was treated as a polytomous variable, and entered into the statistical models (as described below) using 'low', 'medium' and 'high' group terms.

Table 22 Distribution of the 5-HTTLPR genotypes in the ALSPAC sample

	5-HTT expression level		
	Low	*Medium*	*High*
5-HTTLPR Genotypes (n, %)	SS (16.6%) SL_G (6%) L_GL_G (8%)	SL_A (44.1%) L_AL_G (7.1%)	L_AL_A (25.4%)

5.2.4 Covariates

A number of variables were included as covariates in this analysis in order to control for possible confounding effects. Covariates were identified and added to the analysis if they were either associated with the outcome or predictor variable, or there was empirical evidence to suggest that they may act as cofounders. The following variables were included as covariates in this analysis: maternal age, smoking during pregnancy, alcohol consumption during pregnancy, maternal education, household crowding, infant gender, and postnatal depression (assessed 8 weeks after birth).

5.2.5 Statistical Analysis

First, descriptive characteristics for antenatal depression and anxiety, 5-HTTLPR, infant reactivity and behaviour measures at 42 and 81 months were examined. Second, associations of behavioural outcomes (infant reactivity and total behavioural difficulties at 42 and 81 months) with antenatal anxiety and depression, and 5-HTTLPR were examined using Pearson (two-tailed) correlations. Due to the large number of Pearson correlations, a conservative approach was used and Bonferroni corrections applied.

Third, hierarchal regression models were used to predict the outcome of interest (either infant reactivity or total behavioural difficulties at 42 or 81 months). In the first step of the regression model, all covariates were entered, including infant gender. Infant gender was entered because there is evidence to suggest that males and females are affected differently as a result of exposure to antenatal depression (Murray, 1992). At step 2, the predictor variable (either antenatal anxiety or depression) and the moderator variable (5-HTTLPR) were entered. Three two-way interaction variables

were entered at step 3 (predictor*moderator, predictor*gender and moderator*gender), and finally a three-way interaction was entered at step 4 (predictor*moderator*gender)[9]. All interaction terms were created using centred and standardised variables in order to avoid problems of multicolinearity (Aiken and West, 1991).

[9] The predictor*moderator interaction is carried by the predictor*moderator*gender interaction but only represents the interaction when all lower order terms have been partialled. Therefore, including the three two-way interactions is an imperative step.

5.3 Results

5.3.1 Descriptive Statistics

Demographic variables of the subsample used in this study and the whole ALSPAC cohort are shown in Table 23. The demographic characteristics of the subsample were compared to the remaining sample, and significant differences were found for a number of variables:

a) Maternal age; participants in the subsample were slightly older than the remaining sample ($t_{(14080)}$=20.029, p<0.001).
b) Smoking; participants in the subsample smoked on average fewer cigarettes per day than the remaining sample ($x^2_{(3)}$=84.25, p<0.001).
c) Alcohol; the subsample with genetic data consumed significantly fewer units of alcohol per week than the remaining sample ($x^2_{(32)}$=49.81, p<0.05).
d) Maternal educational qualifications; participants of the subsample had, on average, higher educational qualifications than the remaining sample ($x^2_{(4)}$=207.92, p<0.001).
e) Household Crowding; there was no significant difference between the participants with genetic data and the remaining ALSPAC sample on the crowding index ($t_{(113230)}$=-11.986, p=0.818).
f) Infant gender; there was a higher proportion of boys in the subsample compared to the remaining sample ($x^2_{(1)}$=4.63, p<0.05).
g) Living with partner; a higher proportion of participants in the subsample lived with their partner compared to the remaining sample ($x^2_{(2)}$=53.36, p<0.001).

h) Postnatal depression; the subsample of participants used in this analysis had significantly lower postnatal depression scores than the remaining sample ($t_{(11708)}$=-3.091, p<0.001).

Table 23 Descriptive characteristics for the whole ALSPAC sample, and the subsample of white Caucasians with genetic data.

Variables	Whole ALSPAC sample (N=13,801)	Subsample with genetic data (n=5,084)
Maternal Measures		
Maternal age (mean, SD)	28.0, 4.97	29.14, 4.60
Smoking during pregnancy (n, %)	2436 (20)	741 (16.9)
Alcohol during pregnancy (n, %)	2200 (31.8)	1052 (20.7)
Mother's highest educational qualification (n, %)		
CSE	2522 (20.2)	671 (13.2)
Vocational	1228 (9.8)	441 (8.7)
O level	4323 (34.6)	1800 (35.5)
A level	2803 (22.5)	1324 (26.1)
Degree	1607 (11.0)	833 (16.4)
Household crowding (mean, SD)	1.67, 0.66	1.57, 0.66
Living situation (during pregnancy) (n, %)		
Living with partner	12426 (84.7)	4766 (94.8)
Living without partner	1160 (8.5)	262 (5.2)
Maternal postnatal depression (8 weeks) (mean, SD)	6.06, 4.80	5.85, 4.59
Infant Measures		
Infant Gender (n, %)		
Male	7318 (51.7)	2685 (52.8)
Female	6825 (48.3)	2399 (47.2)
Infant reactivity 6 months (mean, SD)	0.00 (2.73)	-0.07 (2.74)
Rutter Revised Scales at 42 months		
Emotional Difficulties Score (mean, SD)	2.55 (1.75)	2.46 (1.71)

Conduct Difficulties Score (mean, SD)	3.63 (2.37)	3.62 (2.32)
Hyperactivity Score (mean, SD)	2.63 (1.81)	2.64 (1.81)
Total behavioural difficulties Score (mean, SD)	12.54 (5.74)	12.40 (5.54)
Strengths and Difficulties Questionnaire at 81 months		
Emotional Difficulties Score (mean, SD)	1.49 (1.66)	1.43 (1.61)
Conduct Difficulties Score (mean, SD)	1.57 (1.44)	1.54 (1.42)
Hyperactivity Score (mean, SD)	3.38 (2.37)	3.35 (2.36)
Total behavioural difficulties Score (mean, SD)	7.29 (4.72)	7.13 (4.58)

The infant behavioural characteristics of the subsample were also compared to the remaining ALSPAC sample, see Table 23. Infants of the subsample had significantly fewer behavioural difficulties at 42 months ($t_{(10022)}$=-2.31, p<0.01) and at 81 months ($t_{(8399)}$=-3.71, p<0.01) and scored lower on emotional difficulties score at 81 months ($t_{(8418)}$=-3.46, p<0.001) than the remaining ALSPAC sample. However, there were no significant differences between the samples on measures of reactivity at 6 months ($t_{(10472)}$=-2.50, p=0.742), emotional difficulties ($t_{(10022)}$=-4.91, p=0.098), conduct difficulties ($t_{(10022)}$=-0.45, p=0.072) and hyperactivity ($t_{(10022)}$=0.35, p=0.547) at 42 months, and conduct difficulties ($t_{(8428)}$=1-2.51, p=0.184) and hyperactivity ($t_{(8403)}$=-1.51, p=0.714) at 81 months.

5.3.1.1 Antenatal mood data

Antenatal mood data was available for between 4653 and 4965 participants, depending the measure, see Table 24. A paired samples T-test showed that levels of antenatal

anxiety and depression were significantly higher at 32 weeks than 18 weeks of pregnancy (anxiety: ($t_{(4605)}$=-4.87, p<0.001), depression: ($t_{(4696)}$=-3.19, p<0.01)).

Table 24 Details of antenatal anxiety and depression scores at 18 and 32 weeks gestation

	Mean	SD	Range	N
Antenatal anxiety 18 weeks	4.64	3.36	0 – 16	4653
Antenatal anxiety 32 weeks	4.87	3.45	0 – 16	4913
Antenatal depression 18 weeks	6.45	4.56	0 – 28	4707
Antenatal depression 32 weeks	6.71	4.86	0 – 29	4965

5.3.1.2 Infant reactivity and behavioural difficulties

Descriptive statistics for infant reactivity at 6 months, and behavioural difficulties at 42 and 81 months are presented in Table 25. There was no significant difference in reactivity score between boys and girls ($t_{(4483)}$=-34.13, p=0.473).

At 42 months, boys had significantly more conduct difficulties than girls ($t_{(4570)}$=5.34,p<0.001). However, there were no significant differences between boys and girls on measures of emotional difficulties ($t_{(4570)}$=-3.57,p=0.642), hyperactivity ($t_{(4570)}$=-5.07,p=0.175) and total behavioural difficulties ($t_{(4570)}$=3.55, p=0.124).

At 81 months, boys scored higher than girls on the hyperactivity score ($t_{(4292)}$=10.88, p<0.001) and boys also scored higher on the total behavioural difficulties score ($t_{(4286)}$=-7.32,p<0.001). There were no significant differences between boys and girls on the

measure of emotional difficulties ($t_{(4293)}$=-1.71,p=0.705) and conduct difficulties ($t_{(4297)}$=4.49,p=0.249).

Table 25 Descriptive characteristics for infant reactivity at 6 months, and behavioural difficulties at 42 and 81 months.

	Boys				Girls				Total			
	Mean	**SD**	**Range**	**N**	**Mean**	**SD**	**Range**	**N**	**Mean**	**SD**	**Range**	**N**
Infant reactivity at 6 months	-0.28	2.68	-9.23 - 8.43	2376	0.01	2.78	-9.69 - 10.67	2109	-0.15	2.73	-9.69 - 10.67	4485
Rutter Revised Scales at 42 months												
Emotional Difficulties Score	2.38	1.73	0 - 10	2443	2.53	1.68	0 - 10	2129	2.45	1.71	0 - 10	4572
Conduct Difficulties Score	3.78	2.38	0 - 14	2443	3.39	2.19	0 - 16	2129	3.60	2.30	0 - 16	4572
Hyperactivity Score	2.78	1.84	0 - 8	2443	2.49	1.77	0 - 8	2129	2.64	1.81	0 - 8	4572
Total behavioural difficulties Score	12.65	5.54	0 - 35	2443	11.97	5.41	0 - 36	2129	12.34	5.49	0 - 36	4572
Strengths and Difficulties Questionnaire at 81 months												
Emotional Difficulties Score	1.40	1.64	0 - 9	2291	1.48	1.58	0 - 9	2004	1.43	1.61	0 - 9	4295
Conduct Difficulties Score	1.67	1.49	0 - 9	2292	1.47	1.41	0 - 8	2007	1.58	1.46	0 - 9	4299
Hyperactivity Score	3.70	2.43	0 - 10	2291	2.94	2.21	0 - 10	2003	3.35	2.36	0 - 10	4294
Total behavioural difficulties Score	7.76	4.84	0 - 31	2289	6.73	4.37	0 - 28	1999	7.29	4.66	0 - 31	4288

5.3.2 Correlations

5.3.2.1 Infant reactivity and behaviour variables

Correlation analyses were conducted to explore the bivariate associations between infant reactivity at 6 months and behavioural difficulties at 42 and 81 months[10]. This data is presented in Table 26. All variables were significantly and positively correlated with each other, indicating that the assessments of infant behaviour using different measures were stable over time. Further, because the behavioural difficulties subscales (emotional difficulties, conduct difficulties and hyperactivity) were highly correlated with the total behavioural difficulties score, all further analysis in this study was carried out using only the total behavioural difficulties subscale at 42 and 81 months.

5.3.2.2 Infant reactivity, behaviour variables, antenatal anxiety, antenatal depression, 5-HTTLPR genotype and covariates

Bivariate associations for infant reactivity, total behaviour difficulties at 42 and 81 months, antenatal anxiety and depression, 5-HTTLPR genotype and covariates were examined and are presented in Table 27[11]. Importantly, 5-HTTLPR was not correlated with infant reactivity (r=0.025, p=0.099), total difficulties at 42 months (r=0.014, p=0.333) or total difficulties at 81 months (r=0.022, p=0.153). On the other hand, second and third trimester anxiety and depression was significantly and positively associated with infant reactivity and total behavioural problems at 42 and 81 months. This indicates that, as expected, higher levels of anxiety and depression during

[10] Bonferroni correction p = α/no of correlations = 0.05/36 = 0.0014
[11] Bonferroni correction p = α/no of correlations = 0.05/42 = 0.0012

pregnancy are associated with a more reactive temperament at 6 months, and more behavioural difficulties at 42 and 81 months.

Table 26 Unadjusted associations for infant reactivity and behavioural difficulties at 42 and 81 months of age (n=4295)

		6 months	42 months				81 months			
		Reactivity	Emotional difficulties	Conduct difficulties	Hyperactivity	Total difficulties	Emotional difficulties	Conduct difficulties	Hyperactivity	Total difficulties
6 months	Reactivity	-								
42 months	Emotional difficulties	0.083**	-							
	Conduct difficulties	0.082**	0.202**	-						
	Hyperactivity	0.087**	0.206**	0.381**	-					
	Total difficulties	0.107**	0.620**	0.749**	0.669**	-				
81 months	Emotional difficulties	0.067**	0.360**	0.119**	0.069**	0.285**	-			
	Conduct difficulties	0.063**	0.148**	0.383**	0.263**	0.390**	0.262**	-		
	Hyperactivity	0.070**	0.129**	0.313**	0.477**	0.417**	0.198**	0.449**	-	
	Total difficulties	0.099**	0.297**	0.368**	0.397**	0.505**	0.619**	0.703**	0.778**	-

Table 27 Correlations between infant reactivity at 6 months, total behavioural difficulties at 42 and 81 months, 5-HTTLPR, antenatal depression, antenatal anxiety and the covariates

		Reactivity 6 months	Total difficulties 42 months	Total difficulties 81 months
Moderator	5-HTTLPR	0.025	0.014	0.022
Predictors	Antenatal anxiety 18 weeks	0.140**	0.222**	0.213**
	Antenatal anxiety 32 weeks	0.116**	0.232**	0.204**
	Antenatal depression 18 weeks	0.136**	0.203**	0.213**
	Antenatal depression 32 weeks	0.117**	0.213**	0.203**
Covariates	Maternal age	-0.320	-0.061**	-0.068**
	Smoking	0.016	0.081**	0.109**
	Alcohol	-0.005	0.048	0.036
	Maternal education	-0.020	-0.045	-0.105**
	Crowding	0.057**	0.010	0.052
	Infant gender	0.062**	-0.052**	-0.111**
	Living situation	-0.015	0.038	0.066**
	Postnatal depression (8 weeks)	0.134**	0.224**	0.214

**p<0.01

5.3.3 Moderator analysis

5.3.3.1 Infant reactivity at 6 months

i. Does 5-HTTPR moderate the association between antenatal anxiety and infant reactivity at 6 months?

Two hierarchical linear regressions were constructed to assess whether 5-HTTLPR moderated the association between infant reactivity and a) antenatal anxiety at 18 weeks b) antenatal anxiety at 32 weeks. The results of this analysis are presented in Table 28.

Second and third trimester maternal antenatal anxiety predicted infant reactivity at 6 months of age (β=0.174, p<0.001 and β=0.164, p<0.001 respectfully). That is, infants of mothers who had high levels of antenatal anxiety during pregnancy had a more reactive temperament than those infants of mothers who had low anxiety levels.

In line with previous findings there was a significant effect of infant gender on temperament (β=0.238, p<0.001), such that boys had a less reactive temperament than girls. There was not, however, a significant effect of the 5-HTTLPR genotype (β=-0.300, p=0.126), or an antenatal anxiety*5-HTTLPR interaction to predict infant reactivity at 6 months (antenatal anxiety at 18 weeks: β=-0.050, p=0.408; antenatal anxiety at 32 weeks and: β=0.028, p=0.624). Similarly, the three-way antenatal anxiety*5-HTTLPR*gender interaction did not significantly predict reactivity (all p's>0.05).

The gender*5-HTTLPR interaction term significantly predicted infant reactivity at 6 months (β=0.262, p<0.05). Further exploration of this interaction revealed that for female infants only, the 5-HTTLPR genotype predicted reactivity: those females with a high expression of the serotonin transporter had a significantly more reactive

temperament at 6 months than females with a low serotonin transporter expression level.

ii. Does 5-HTTLPR moderate the association between antenatal depression and infant reactivity at 6 months?

Two hierarchical linear regression models were constructed to investigate whether 5-HTTLPR moderated the association between antenatal depression and infant reactivity. The results of this analysis are presented in Table 29.

Consistent with the previous analysis, antenatal depression alone predicted infant reactivity (antenatal depression at 18 weeks: $\beta=0.128$, $p<0.001$; antenatal depression at 32 weeks: $\beta=0.070$, $p<0.05$), however the 5-HTTLPR genotype did not ($\beta=-0.264$, $p=0.179$ and $\beta=-0.328$, $p=0.087$).

The antenatal depression*5-HTTLPR term did not significantly predict infant reactivity (antenatal depression at 18 weeks: $\beta=0.024$, $p=0.591$; antenatal depression at 32 weeks: $\beta=0.034$, $p=0.393$). And further, the three-way interaction did not predict infant reactivity at 6 months (antenatal depression at 18 weeks: $\beta=-0.012$, $p=0.671$; antenatal depression at 32 weeks: $\beta=-0.018$, $p=0.467$).

Table 28 Linear regression models using 5-HTTLPR and maternal antenatal anxiety to predict infant reactivity at 6 months

	Antenatal anxiety at 18 weeks				Antenatal anxiety at 32 weeks			
Predictor variables	**B**	***β***	***t***	***p***	**B**	***β***	***t***	***p***
Step 1								
Maternal age	-0.014	-0.023	-1.376	0.172	-0.019	-0.031	-1.847	0.065
Smoking during pregnancy	-0.048	-0.011	-0.635	0.526	-0.013	-0.003	-0.178	0.859
Alcohol during pregnancy	-0.008	-0.009	-0.553	0.581	-0.002	-0.002	-0.120	0.904
Maternal education	0.004	0.002	0.098	0.922	0.008	0.004	0.215	0.830
Crowding	**0.211**	**0.045**	**2.716**	**0.007**	**0.197**	**0.043**	**2.625**	**0.009**
Gender	**0.328**	**0.060**	**3.719**	**0.000**	**0.312**	**0.057**	**3.610**	**0.000**
Living with partner	-0.366	-0.028	-1.720	0.086	**-0.460**	**-0.036**	**-2.275**	**0.023**
Postnatal depression	**0.051**	**0.084**	**4.246**	**0.000**	**0.059**	**0.098**	**5.272**	**0.000**
Step 2								
Antenatal anxiety	**0.174**	**0.211**	**4.151**	**0.000**	**0.164**	**0.202**	**4.091**	**0.000**
5-HTTLPR	-0.300	-0.077	-1.532	0.126	-0.335	-0.086	-1.747	0.081
Step 3								
Antenatal anxiety*5-HTTLPR	-0.050	-0.042	-0.828	0.408	0.028	0.024	0.491	0.624
5-HTTLPR*gender	**0.262**	**0.105**	**2.076**	**0.038**	**0.293**	**0.117**	**2.374**	**0.018**
Gender*antenatal anxiety	**-0.056**	**-0.106**	**-2.103**	**0.036**	**-0.073**	**-0.141**	**-2.885**	**0.004**
Step 4								
Antenatal anxiety*5-HTTLPR *Gender	0.030	0.040	0.787	0.431	0.003	0.004	0.077	0.939
	R^2=0.014	**F(2599)=3.233, P<0.001**			**R^2=0.011**	**F(2727)=2.738, P<0.01**		

Table 29 Linear regression models using 5-HTTLPR and antenatal depression to predict infant reactivity at 6 months.

	Antenatal depression at 18 weeks				Antenatal depression at 32 weeks			
Predictor variables	**B**	***β***	***t***	***p***	**B**	***β***	***t***	***p***
Step 1								
Maternal age	-0.017	-0.027	-1.621	0.105	-0.020	-0.032	-1.912	0.056
Smoking during pregnancy	-0.036	-0.008	-0.480	0.631	-0.007	-0.002	-0.099	0.921
Alcohol during pregnancy	-0.006	-0.006	-0.392	0.695	-0.002	-0.002	-0.108	0.914
Maternal education	0.012	0.005	0.314	0.753	0.014	0.006	0.351	0.726
Crowding	**0.170**	**0.036**	**2.194**	**0.028**	**0.190**	**0.041**	**2.523**	**0.012**
Gender	**0.329**	**0.060**	**3.716**	**0.000**	**0.341**	**0.062**	**3.950**	**0.000**
Living with partner	-0.377	-0.029	-1.767	0.077	**-0.455**	**-0.036**	**-2.245**	**0.025**
Postnatal depression	**0.048**	**0.079**	**4.239**	**0.000**	**0.056**	**0.091**	**4.765**	**0.000**
Step 2								
Antenatal depression	**0.128**	**0.210**	**4.121**	**0.000**	**0.070**	**0.121**	**2.427**	**0.015**
5-HTTLPR	-0.264	-0.068	-1.343	0.179	-0.328	-0.084	-1.710	0.087
Step 3								
Antenatal depression*5-HTTLPR	0.024	0.027	0.537	0.591	0.034	0.042	0.854	0.393
5-HTTLPR *gender	0.239	0.095	1.890	0.059	**0.276**	**0.111**	**2.239**	**0.025**
gender X antenatal depression	-0.044	-0.112	-2.232	0.029	-0.018	-0.049	-1.013	0.311
Step 4								
Antenatal depression*5-HTTLPR *Gender	-0.012	-0.022	-0.425	0.671	-0.018	-0.033	-0.680	0.497
	R^2=0.010	**F(2641)=2.457, P<0.01**			**R^2=0.011**	**F(2743)=2.872, P<0.01**		

5.3.3.2 Behavioural difficulties at 42 months

iii. Does 5-HTTLPR moderate the association between antenatal anxiety and total behavioural difficulties at 42 months?

As with the previous analysis, gender significantly predicted total behavioural difficulties at 42 months (β=-0.218, p<0.001); boys had more behavioural difficulties than girls at this age. Antenatal anxiety alone predicted total behavioural difficulties (antenatal anxiety at 18 weeks: β=0.279, p<0.001; antenatal anxiety at 32 weeks: β=0.286, p<0.001), however 5-HTTLPR genotype did not (β=-0.003, p=0.993 and β=0.268, p=0.998).

An antenatal anxiety*5-HTTLPR genotype interaction did not significantly predict total behavioural difficulties (antenatal anxiety at 18 weeks: β=0.024, p=0.840; antenatal anxiety at 32 weeks: β=0.053, p=0.636), and the three-way interaction was also non-significant (antenatal anxiety at 18 weeks: β=-0.016, p=0.831; antenatal anxiety at 32 weeks: β=-0.042, p=0.557).

iv. Does 5-HTTLPR moderate the association between antenatal depression and total behavioural difficulties at 42 months?

Antenatal depression significantly predicted total behavioural difficulties (antenatal depression at 18 weeks β=0.223, p<0.001; antenatal depression at 32 weeks: β=0.167, p<0.01), however there was no significant effect of 5-HTTLPR (β=-0.044, p=0.878 and β=-0.027,p=0.943). There was no significant effect of an antenatal depression*genotype interaction (antenatal depression at 18 weeks: β=-0.040, p=0.645; antenatal depression

at 32 weeks: β=0.091, p=0.249) or a three-way antenatal depression*genotype*gender interaction (antenatal depression at 18 weeks: β=0.024, p=0.669; antenatal depression at 32 weeks and β=-0.052, p=0.303).

5.3.3.3 Total behavioural difficulties at 81 months

v. Does 5-HTTLPR moderate the association between antenatal anxiety and total behavioural difficulties at 81 months?

Antenatal anxiety significantly predicted total behavioural difficulties at 81 months (antenatal anxiety at 18 weeks: β=0.237, p=0<0.001; antenatal anxiety at 32 weeks: β=0.236, p<0.001), however 5-HTTLPR did not (β=0.070, p=0.832 and β=0.148, p=0.647). There was no significant effect of an antenatal anxiety*genotype interaction (antenatal anxiety at 18 weeks: β=0.008, p=0.939; antenatal anxiety at 32 weeks: β=0.029, p=0.767) or a three way antenatal anxiety*genotype*gender interaction (antenatal anxiety at 18 weeks: β=0.000, p=0.999; antenatal anxiety at 32 weeks: β=-0.003, p=0.960).

vi. Does 5-HTTLPR moderate the association between antenatal depression and total behavioural difficulties at 81 months?

Antenatal depression at 18 weeks significantly predicted total behavioural difficulties at 81 months (β=0.133, p<0.01) whereas depression at 32 weeks did not (β=0.101, p=0.104). The antenatal depression*genotype interaction did not predict total difficulties at 81 months (antenatal depression at 18 weeks: β=0.010, p=0.912; antenatal depression

at 32 weeks: β=-0.004, p=0.964), and neither did a three-way antenatal depression*genotype*gender interaction (antenatal depression at 18 weeks: β=-0.023, p=0.692; antenatal depression at 32 weeks and β-0.033, p=0.953).

5.3.4 Summary of Findings

- This study did not replicate the findings of Pluess *et al.* (2011), that 5-HTTLPR moderates the association between antenatal anxiety and infant behaviour at 6 months.
- 5-HTTLPR did not moderate the association between antenatal anxiety and infant behavioural difficulties at 42 and 81 months.
- Also, 5-HTTLPR did not moderate the association between antenatal depression and infant behaviour at 6 months, or up to the age of 81 months.

5.4 Discussion

This study was an analysis of a large community sample of children from whom data from the antenatal period to age 81 months was available. The main finding of this study is that the effect of antenatal anxiety and depression during the second and third trimester on infant reactivity at 6 months and later behavioural problems is not moderated by the 5-HTTLPR genotype. Although the results show that antenatal anxiety and depression predict infant temperament and later behavioural problems, consistent with previous findings (O'Connor et al., 2002a, Pluess et al., 2010a), this study found no GxE interaction between antenatal anxiety/depression and 5-HTTLPR to predict infant temperament or later behavioural difficulties. These results remained robust when a three-way interaction to include child gender was entered into the model.

5.4.1 Findings in context

There were no moderating effects of 5-HTTLPR on infant outcomes, however one novel finding was obtained from this study: in girls only, the serotonin transporter genotype predicted reactive temperament at 6 months. Specifically, girls with a high expression level of the serotonin transporter had a more reactive temperament than those with a low expression level. Although there was no interaction with antenatal mood, it is an intriguing notion that a single polymorphism may contribute to a complex behaviour such as infant temperament. However, interpretation of this finding requires caution as, given the number of statistical tests carried out in this study, it may represent a false

positive. Thus, this finding requires replication in another large cohort before firm conclusions regarding this association can be drawn.

On the other hand, this study yielded many null findings, despite the large sample size, long-term period of the study, and detailed symptom measures. The results of this study are not consistent with those of Pluess *et al* (2011) which indicate that a combination of two short alleles and high levels of maternal antenatal anxiety present a cumulative risk factor for infant emotional difficulties at 6 months of age, in line with the diathesis-stress model. In this study however, there was no evidence of any type of interaction that resembled either a diathesis-stress or differential susceptibility model. In an attempt to comprehensively replicate the Pluess *et al* (2011) methodology, biallelic re-coding of the 5-HTTLPR genotype did not significantly change the outcome of the regression model[12]; there was no significant interaction between antenatal anxiety and the biallelic genotype (SS, SL, LL) to predict infant reactivity.

The variation in findings between this study and the Pluess *et al* (2011) study may be attributable to a number of factors, including the use of different questionnaire measures. As a measure of infant temperament, this study used reactivity as assessed by the Infant Temperament Questionnaire, whereas Pluess *et al* (2011) used a revised version of the Infant Behaviour Questionnaire, with the negative emotionality subscale for analysis. A different measure of maternal antenatal anxiety was also used: the anxiety scale of the Crown Crisp Index and the Brief Symptom Inventory. However, both are

[12] Results of the moderator analysis using the biallelic genotype are not presented here.

validated self-report measures of anxiety, and both have been used extensively, and are therefore unlikely to be the source of discrepancy.

It is possible that other methodological variations, or differences in the demographic characteristics of the two samples underlie the differences in the results. Although, it is important to note that the demographic characteristics of the Pluess et al (2011) are comparable to the subset of ALSPAC participants used in this study. For example, both samples have a generally high socio-economic status, indexed by high rates of education and income. The two samples were also similar in tobacco and alcohol consumption during pregnancy. Nonetheless, that two large cohort studies resulted in very different outcomes suggests that the role of the serotonin transporter polymorphism in moderating antenatal environmental influences on offspring behavioural outcomes may not be straightforward and consistent in differing circumstances. There is some evidence from studies of adults that the serotonin transporter polymorphism moderates the response to stressful events during the childhood years. However, even here the findings are not consistent (Risch et al., 2009, Karg et al., 2011, Munafo et al., 2009). Findings relating to childhood outcomes, such as positive and negative affect, are even less consistent (Araya et al., 2009, Hankin et al., 2011), and the results presented here suggest that 5-HTTLPR does not moderate the association between antenatal mood disturbance and childhood behavioural difficulties.

5.4.2 Strengths and limitations

There are a number of strengths to this study. First, the participants were drawn from a large population-based cohort, thus this study is relatively free from selection biases, which are usually associated with experimental and clinical trials. Second, the measures used in this study, namely the Crown Crisp Index, EPDS, Infant Temperament Questionnaire and the SDQ, have been extensively used and are well validated. Third, the sample size is very large: indeed the largest to date to investigate the moderating role of 5-HTTLPR on the effects of antenatal mood disturbance. Therefore, this study has sufficient statistical power to detect all but very small effects. Pluess *et al* (2011) found an interaction effect size of f2=0.004, and a power analysis revealed that to find this effect size with 80% power, a sample size of 2412 participants is required. Therefore, with a sample of 1513 participants their study was underpowered, whereas the current study was sufficiently powered to find such an effect. As highlighted by Duncan and Keller (2011), many GxE studies are significantly underpowered, resulting in a situation where positive findings from such studies may actually represent type 1 errors. Furthermore, given that multiple candidate gene by environment studies are carried out within and across many laboratories, it is inevitable that p values of less than 0.05 are found. Preferential publication of positive results means that false-positives enter the literature, and are thus subsequently difficult to replicate. Notably, there is strong precedent for this in the candidate gene literature.

This study also has limitations, which should be considered. First, measures of infant behaviour were based solely on maternal reports. Although this is perhaps inevitable in a large cohort study, it nonetheless raises the potential for the ratings of infant behaviour

to be subject to reporter bias. That is, anxious and depressed mothers may be more likely to over- or misreport behavioural difficulties of the infant, which may have led to greater associations between the variables. Second, there is evidence that the subsample of the ALSPAC cohort used in this study differed from the remaining sample of participants. For example, the participants of the subsample had higher levels of educational qualifications and income, consumed less alcohol and smoked less during pregnancy, and reported lower levels of psychological distress during pregnancy compared with the remaining ALSPAC sample. Furthermore, the infants of the subsample had less variability in the maternally-reported behavioural problems. Therefore, because the subsample were an advantaged sample whose children had fewer behavioural problems, this reduces the statistical power to detect moderating effects of the 5-HTTLPR genotype on associations between antenatal mood disturbance and adverse offspring outcomes.

5.4.3 Conclusion

In conclusion, this study has shown that within a large population-based cohort, the effects of maternal antenatal anxiety and depression on infant behavioural outcomes from 6 to 81 months are not moderated by the serotonin transporter genotype. Therefore it is likely that the role of 5-HTTLPR in moderating the effects of antenatal mood disturbance, if any, are more subtle and variable than previously thought.

CHAPTER 6

DISCUSSION

6 Overview of Chapter

Over the past decade is has been well established that antenatal mood disturbance increases risk for a number of adverse offspring outcomes, such as: low birth weight or premature birth, behavioural difficulties in childhood and psychiatric disease in adolescence and adult life. A striking aspect of these associations is that the effects appear to be, in part, independent from postnatal mood and shared risk genes between mother and infant. However, the remaining *in utero* biological mechanisms, which may mediate these associations, are yet to be elucidated.

Using a combination of data collected as part of a short-term longitudinal study based in Oxfordshire (The Oxford Pregnancy Study), and a large birth cohort based in western England (The ALSPAC Study), this thesis set out to address two specific questions:

3. Is maternal antenatal depression associated with alterations in maternal and infant HPA function?
4. Does the serotonin transporter polymorphism (5-HTTLPR) confer infant susceptibility to the adverse effects of maternal antenatal depression?

The three key findings from this thesis are:

i. Maternal antenatal symptoms of depression are not associated with increases in maternal antenatal or infant HPA reactivity. However maternal depression is associated with raised levels of alpha amylase across the day.
ii. Maternal antenatal depression is associated with significant increases in male infant NR3C1 1F DNA methylation, and decreases in infant BDNF IV DNA methylation.
iii. The serotonin transporter polymorphism (5-HTTLPR) does not moderate the association between antenatal depression and infant behavioural difficulties.

Taken together these findings do not support the hypothesis that the effects of antenatal depression on infant development are mediated via changes in maternal and infant HPA function. Also, infant 5-HTTLPR genotype may not confer susceptibility to the effects of maternal antenatal depression. However, changes in infant DNA methylation may be one potential mechanism that mediates associations between antenatal mood disturbance and offspring outcomes.

In this chapter, the findings of this thesis are summarised and discussed in the context of the pre-existing literature. Methodological considerations, as well as strengths and limitations of the thesis are discussed. Then, three thematic topics are considered: 1. Are alterations of the maternal and infant HPA axis a key mechanism by which antenatal mood disturbance impacts on infant outcomes? 2. The usefulness of epigenetic approaches to understanding intergenerational transmission of risk 3. Taking a candidate gene approach to understanding the effects of antenatal mood disturbance.

This chapter concludes with a consideration of clinical implications and scope for future research.

6.1 Summary of Findings

The aim of this thesis was to further elucidate the mechanisms by which antenatal mood disturbance may impact on fetal and infant development. Specifically, this thesis set out to address whether maternal antenatal depression is associated with increased maternal and infant HPA reactivity to acute stress, and whether antenatal depression may also have implications for infant DNA methylation and temperament. A further line of enquiry in this thesis was to attempt to understand why some infants may be more susceptible to the effects of maternal antenatal depression than others. There is evidence to suggest that approximately 20% of infants manifest the adverse outcomes associated with antenatal depression exposure. Therefore the secondary aim of this thesis was to investigate whether the infant 5-HTTLPR genotype, a proposed genetic susceptibility factor, may confer susceptibility to antenatal mood disturbance.

The aim of the study presented in Chapter 2 was to investigate the impact of maternal antenatal depressive symptoms on salivary cortisol and alpha-amylase reactivity to an infant distress stimulus. A further aim was to investigate effects of depressive symptoms on diurnal levels of salivary cortisol and alpha-amylase. Contrary to the study hypotheses, there were no significant effects of antenatal depression on psychological or biological responses to the infant distress film. Similarly, there were no significant effects of antenatal depression on diurnal salivary cortisol. However previous findings that the diurnal profile of salivary cortisol and alpha-amylase are maintained during pregnancy

were replicated in this study. A further finding was that participants with symptoms of depression in late pregnancy had significantly greater salivary alpha-amylase on awakening and across the day than control participants. These results suggest that mood disturbance in late pregnancy may be associated with increased sympathetic nervous system activity; resulting in increased vasoconstriction and reduced blood flow to the fetus. Thus, this finding could potentially explain associations between antenatal mood disturbance and low birth weight or premature babies.

The study presented in Chapter 3 was a follow-up of the infants born to the mothers assessed in Chapter 2. The aim of this study was to assess whether maternal antenatal symptoms of depression, or biological markers of antenatal stress, significantly predicted infant cortisol reactivity to inoculation and infant temperament at 2 months of age. Again contrary to expectation, neither antenatal depressive symptoms nor salivary biomarkers of antenatal stress significantly predicted infant cortisol responses to inoculation. Similarly, symptoms of antenatal depression did not predict infant temperament. Interestingly, there was evidence to suggest that antenatal salivary cortisol and alpha-amylase predicted some aspects of infant temperament. These results are the first to identify that biological markers of stress in pregnancy may be stronger predictors of infant behavioural outcomes than self-reported measures of antenatal mood.

The study presented in Chapter 4 aimed to investigate whether exposure to antenatal depression had implications for infant DNA methylation, using DNA samples taken from the same infants described in Chapter 3. This study identified that male, but not female, infants exposed to antenatal depression had increased methylation of the promoter

region of the glucocorticoid receptor gene, NR3C1. Also, both male and female infants exposed to antenatal depression had decreased DNA methylation of the promoter region of the BDNF gene. These findings suggest that exposure to maternal antenatal mood disturbance has implications for changes in infant DNA methylation, and this could be one potential biological mechanism by which antenatal mood disturbance impacts on fetal and infant development.

The study presented in Chapter 5 was an analysis of data collected as part of a longitudinal birth cohort study. The aim of this chapter was to investigate whether the effects of maternal antenatal depression and anxiety on infant behavioural difficulties were moderated a genotype proposed to confer susceptibility to environmental influences, the serotonin transporter polymorphism, 5-HTTLPR. However, this large, well-powered, direct replication of a previous study failed to find any evidence to suggest that those infants with two copies of the short allele were more at risk of developing behavioural difficulties when exposed to high levels of maternal antenatal depression and anxiety than infants with two copies of the long allele, or homozygous infants. Thus, the results of this study suggest that the previous report that 5-HTTLPR moderates the association between antenatal anxiety and infant behaviour may have been a false-positive.

6.2 Methodological considerations

The findings presented in this thesis should be interpreted in light of a number of methodological considerations, which are outlined in this section.

Recruitment strategy. Recruitment of participants is an important consideration when designing studies such as those presented in this thesis. The ALSPAC study used a population approach to recruiting participants; all pregnant women in a given area and time frame were approached and asked to take part in the study. As a result, the population of participants that were recruited to the ALSPAC study were a good representation of the general population (although not perfect as some participants will have declined to take part). However, a drawback to this approach is that a very large sample size is required. An alternative approach is to recruit participants from a clinical setting, and therefore a smaller sample size is required to investigate the effects, for example, of antenatal depression. However, findings generated from such a cohort of participants may not be generalizable to the wider population with any confidence.

The recruitment strategy for The Oxford Pregnancy Study lies in between the two approaches outlined above; participants self-selected to take part and were recruited from a number of community, as well as clinical settings. A strength of this recruitment strategy is that a variety of methods were used to recruit participants from the general population. However, it could be that systematic biases occurred. For example, some participants were recruited from the National Childbirth Trust (NCT), however women who attend NCT classes have a more affluent demographic than the general population,

given that there is a charge for attending this course. Further, because all participants self-selected to take part in The Oxford Pregnancy Study, then this group of participants are likely to be more motivated than the general population.

The majority of participants who took part in The Oxford Pregnancy Study responded to online adverts or posters (72 participants (70%)), which contained details about the study. The main strategy was to recruit any participants who met the inclusion criteria for the study, with the aim of assessing antenatal mood and splitting the sample into groups for analysis based on their EPDS score. As a result, 103 participants were recruited, of which 24 met this study's criteria for depressive symptoms. This method for recruitment was chosen because in order to assess biological measures, such as salivary cortisol and alpha-amylase, it was calculated that a moderate sample size upwards of 80 participants was needed. Further, previous studies of antenatal depression in Oxfordshire had experienced difficulties with recruiting participants. Therefore, given the limited time available to recruit a sufficient sample size (14 months), and known challenges with recruiting pregnant participants, it was decided that recruitment via community methods was the best approach to take. With a longer period of time available to recruit participants, an alternative method would have been to specifically recruit depressed participants via medical professionals. This may have resulted in more power to detect effects of antenatal depression on salivary biomarkers of stress and infant outcomes. However, this approach was not possible within the recruitment time frame, due to the time taken to approach and enlist medical professionals to help with recruitment, the time taken to address ethical constraints associated with recruiting participants in this way, and more potential participants would have not met inclusion

criteria due to using antidepressants during pregnancy. However, as noted above, if this approach had been taken then the findings would have been less generalizable to the population, and it is possible that the control and 'depressive-symptoms' groups may have been less comparable.

Assessment of antenatal depression. In The Oxford Pregnancy Study, symptoms of antenatal depression were self-reported using a paper-based questionnaire. A clear limitation of this approach is that participants may under or misreport their symptoms. An alternative method for assessing symptoms of depression is via the use of the Structured Clinical Interview for Depression (SCID). However, administration of the SCID is very timely and was not feasible within the timescale of the current project. However, despite the limitations of a paper-based questionnaire, the EPDS is a widely used and well-validated measure of depressive symptoms during the perinatal period, and has been shown to accurately predict clinical depression (Toreki et al., 2014, Cox et al., 1996, Matijasevich et al., 2014)

Treatment of the antenatal depression data. In this thesis, antenatal depression data has been used as both a categorical and continuous variable. The decision as to how the antenatal depression data was used was based on the statistical approach taken in each chapter. Where data were sampled at numerous time points, such as salivary cortisol and alpha-amylase responses to acute stress, data was analysed using a repeated-measures ANOVA. Where an ANOVA was used, antenatal depression was treated as a categorical

variable (i.e, control *vs.* depressive-symptom), using the pre-defined criteria of a score of 10 on the EPDS. However, where data were sampled at just one time point, such as maternal reports of infant temperament, regression models were used to analyse the data. In this case, antenatal depression was entered into the regression model as a continuous variable. The exception to this rule is in Chapter 4, where antenatal depression was treated as a categorical variable in predicting infant DNA methylation, for ease of interpreting the results.

Antenatal depression vs. anxiety. It has been suggested that the effects of antenatal mood disturbance on infant development may be specific to maternal anxiety. This idea is based on a body of work that suggests that symptoms of anxiety more likely reflect environmental stress rather than symptoms of depression, and in evolutionary terms, environmental stress may be very important for fetal programming or adaptation (Talge et al., 2007, Glover, 2011). This idea was tested using the current dataset, however the effects of antenatal anxiety on both maternal salivary cortisol/alpha-amylase, and infant cortisol/temperament, were consistent with the effects of maternal antenatal depression. A likely explanation is that symptoms of anxiety and depression are highly comorbid, are highly correlated in this sample of participants, and thus there was a high degree of overlap between the participants of the depression-symptom and anxiety-symptom group.

Small short-term longitudinal study vs. very large birth cohort. This thesis presents analyses of data collected from two different cohorts. The Oxford Pregnancy Study is a short-term longitudinal study conducted in Oxfordshire with a moderate sample size of 103 participants. In contrast the ALPAC study is a very large birth cohort study, with data on over 14000 participants (although genetic data was only available for a subset of approx. 5000 infants). Both study designs have strengths and weaknesses. The short-term longitudinal design has statistical limitations due to the relatively small sample size. On the other hand, because of the small sample size it is possible to administer a comprehensive assessment of participants, including: maternal antenatal and postnatal measures of mood and infant temperament, as well as maternal and infant biological markers of stress. On the other hand, the large cohort study is sufficiently powered to detect small effect sizes. This particularly important in candidate gene studies, because very large samples are required to detect interactions between genes and the environment. However, in such studies, detailed assessments of participants are often not possible, because of time and cost constraints.

6.3 Limitations

The limitations of this thesis are considered below:

6.3.1 Limitations of the Oxford Pregnancy Study

i. This study was an observational and not an experimental design. Although it addressed associations between antenatal depression, salivary biomarkers of stress and infant stress reactivity and temperament, it is not able to determine causal relationships.

ii. The number of women who met criteria for depressive symptoms (n=24) was small; therefore power is an issue when testing for complex effects of antenatal depression.

iii. Questionnaire measures were used to assess maternal antenatal and postnatal mood. Therefore these measures are subject to misreporting. Similarly, the measure of infant temperament was based on maternal report, and therefore potentially subject to reporter-bias.

iv. The study was limited to a single antenatal and postnatal assessment. Multiple antenatal and postnatal assessments would have provided useful information regarding timing effects and sensitive periods of gestation where antenatal depression is important for programming effects on the fetus.

v. Due to the small sample size and the high degree of co-morbidity, the differential effects of antenatal depression and anxiety on infant outcomes could not be explored in this study.

Limitations of the antenatal assessment

i. The infant distress stimulus was not an efficient probe of the HPA axis. Had a more potent stressor been used, such as the Trier Social Stress Test, a significant biological response may have been elicited.

ii. When the diurnal saliva samples were taken, time of awakening and sleep duration was not recorded, and these have been shown to affect awakening cortisol concentrations in pregnant women (O'Connor et al., 2013a).

Limitations of the postnatal assessment

i. The postnatal assessment was conducted when the infants were a very young age (2 months). Therefore it is possible that the infants were too young at this point to detect alterations in infant temperament associated with antenatal depression.

ii. There was a moderate variation in the ages of the infants at the postnatal assessment (5-14.5 weeks), which may have affected the results of the postnatal study.

iii. Infant daytime sleeping was not controlled for when measuring infant samples.

6.3.2 Limitations of the ALSPAC study

i. This study used a candidate gene approach to investigate moderating effects on associations between antenatal anxiety/depression and infant behaviour. Given the likelihood that antenatal mood impacts on fetal and infant development via multiple mechanisms involving multiple genes, and also has implications for

changes in DNA methylation, the candidate-gene approach may be an inappropriate method.

ii. All measures of maternal antenatal mood and infant behaviour were based on maternal reports, and are therefore subject to potential misreporting and reporter-bias.

iii. The subsample used in this study (for whom the genotype information was available) differed from the whole ALSPAC sample, and therefore the findings may not be applicable to the general population.

6.4 Strengths

There are a number of important strengths to this thesis which are considered below:

6.4.1 Strengths of The Oxford Pregnancy Study

i. This study was prospective in design, and used a combination of measures, both psychological and biological, to assess the effects of antenatal depression on both mothers and infants.

ii. The measures of maternal mood and infant behaviour used in this study have been extensively used and validated.

iii. This is the first study to assess the effects of antenatal depression on both salivary cortisol and alpha-amylase reactivity to a stressor as well as diurnal release.

iv. This is also the first study to use biological and psychological markers of antenatal stress to predict infant cortisol reactivity, DNA methylation and temperament.

v. Infant cortisol was measured before and after immunisation in order to study infant cortisol reactivity to a common stressor, and this approach proved an effective probe of the HPA axis.

vi. This is the first study to assess the effects of antenatal depression on infant BDNF IV DNA methylation.

6.4.2 Strengths of the ALSPAC study

i. This is large population-based cohort, and therefore is relatively free from selection biases often associated with experimental studies and trials.

ii. The large sample size gives the study good statistical power to detect small effect sizes.

iii. The measures of maternal mood and infant behaviour used in this study have been extensively used and validated.

iv. This study included multiple assessments of maternal antenatal and postnatal mood and infant behaviour.

6.5 Are alterations of the maternal and infant HPA axis a key mechanism by which antenatal mood disturbance impacts upon infant outcomes?

A prominent theory in the field of perinatal psychiatry is that antenatal mood disturbance impacts on fetal and infant development by inducing changes in both the maternal and infant HPA axes (Talge et al., 2007, Glover, 2011). This theory suggests that antenatal mood disturbance is associated with increased maternal glucocorticoids, namely cortisol, which crosses the placental barrier, so that fetal cortisol levels become raised. Increases in fetal cortisol concentrations disrupt the development of the fetal HPA axis, and as a result, offspring have a permanently over-reactive HPA axis. Such an over-reactive HPA axis then predisposes the offspring to develop the adverse outcomes associated with exposure to antenatal mood disturbance, such as behavioural difficulties in childhood and psychiatric disease in adulthood.

This theory clearly and succinctly explains how exposure to maternal antenatal mood disturbance may result in adverse offspring outcomes. Over the past few years, the majority of the research assessing effects of antenatal mood disturbance on infant development has focused on investigating this theory as a potential mediating mechanism, although there is a general consensus that this mechanism alone is rather simplistic and other systems are likely to also be involved in this association (Field, 2011, Glover, 2011, Monk et al., 2013). However, it is possible that the reason for the focus on the HPA axis is because this system is readily accessible and easy to measure, due to the availability of salivary cortisol as a reliable biomarker of HPA function.

There is currently some evidence to support the idea that antenatal mood disturbance may result in changes to both the maternal and fetal HPA axis. For example, there is evidence to suggest that maternal and fetal cortisol levels are correlated, and indeed are moderated by maternal anxiety (Glover et al., 2009a, O'Donnell et al., 2012). Antenatal anxiety has been associated with the down-regulation of the placental enzyme, 11-βHSD2, which usually metabolises cortisol into inactive cortisone. Thus, there appears to be a linear relationship between increases in maternal antenatal anxiety and increased exposure of the fetus to maternal cortisol. Further evidence in support of this theory is that some infants born to mothers with antenatal mood disturbance have increased cortisol responses to acute stress (Davis et al., 2011a, Brennan et al., 2008). There is also evidence to suggest that children with behavioural problems and adults with psychiatric disease have increased activity of the HPA axis (Bagner et al., 2010, Van den Bergh et al., 2008a, Mullen et al., 1986, Cowen, 2002).

However, there are clearly gaps in the theory for which there is currently insufficient supporting evidence. For example, evidence linking antenatal mood disturbance with increases in cortisol is mixed, and is generally limited to diurnal measures (Obel et al., 2005, de Weerth and Buitelaar, 2005, Buss et al., 2009, Entringer et al., 2011, Pluess et al., 2010b, Murphy et al., 2014). Further, there is no direct evidence linking increased maternal antenatal cortisol and increased infant cortisol in the same cohort of participants. Additionally, the underlying biological mechanisms that link increased circulating fetal cortisol with changes in offspring HPA function are yet to be elucidated.

The research presented in this thesis address two key components of this model for which there is currently insufficient supporting evidence: the link between antenatal mood disturbance and maternal cortisol, and the relationship between maternal antenatal and infant cortisol reactivity. However, the studies presented in this thesis found no evidence that maternal antenatal depressive symptoms were associated with increases in maternal salivary cortisol, measured both in response to a stressor and diurnally. Also, there was no evidence to suggest that maternal antenatal cortisol, or mood, was associated with infant salivary cortisol. Of course these findings should be interpreted in light of the limitations of this study, highlighted above. However, a recurrent finding and striking theme of this thesis is the lack of association between maternal antenatal depression and changes in both maternal and infant HPA function. Thus, a clear conclusion which can be drawn from these findings is that the role of maternal and infant HPA function in mediating associations between antenatal mood disturbance and adverse infant outcomes is more subtle and variable than previously thought. Further, this thesis identified that antenatal depressive symptoms are associated with increased maternal alpha-amylase levels, and also changes in infant BDNF DNA methylation. These two novel findings highlight that maternal antenatal mood disturbance has implications for alterations of a number of systems and pathways, and draws attention to the fact that future studies should focus on other potential underlying biological mechanisms.

6.6 The usefulness of epigenetic approaches to understanding intergenerational transmission of risk

Although the focus of this field has been directed towards maternal and infant HPA function, recent interest in epigenetic mechanisms as a method by which risk can be transferred from mother to infant, have begun to emerge. The concept that changes in gene expression can occur without a change to the DNA sequence is relatively new, and has initiated a paradigm shift in the way that genetic mechanisms are considered. Further, emerging evidence that epigenetic markers are susceptible to environmental influences has sparked great excitement in a number of fields, including Psychiatry.

In perinatal psychiatry, pioneering animal research from Michael Meaney's lab has demonstrated that maternal behaviour during the early postnatal period can have long-term impacts on offspring behaviour; those rodents exposed to low rates of maternal postnatal care show increased anxiety-like behaviours, and increased stress responses (Weaver et al., 2004, Weaver et al., 2001, Meaney, 2001). Further, these animals have increased methylation of the *nr3c1* promoter region, which corresponds with decreased glucocorticoid receptor (GR) expression in the hippocampus (Weaver et al., 2004). As the GR has an important role in the negative feedback-loop of the HPA axis, decreased expression of the GR explains the increased and maintained HPA stress responses demonstrated by these animals. Thus, this initial and very exciting research highlighted that adverse environmental exposures during the postnatal period can have long term implications for offspring DNA methylation-mediated behaviours and stress responses. Further, the application of cross-fostering study designs and DNA-methylation inhibitors

has convincingly demonstrated that this association is causal (Weaver et al., 2005, Francis et al., 1999).

However, the translation of animal findings to human research often poses a number of difficulties. When using rodents for research, particularly in understanding environmentally mediated effects, a huge advantage is that environmental and genetic variability between animals is limited. Therefore, the influence of one environmental factor, such as changes in maternal postnatal care, can often be studied independently of other varying factors. However, similar associations in humans are more difficult to understand for a number of reasons. Firstly, human genetic and environmental variation greatly exceeds that of rodents. Thus, it is near impossible to investigate a single genetic or environmental factor independently of others. To counter this problem, large sample sizes are needed so that variation can be controlled for. Secondly, in human studies it is not possible to assess DNA methylation in brain tissue, so peripheral tissue or post-mortem brain tissues are used instead. However, this poses problems because DNA methylation at the genomic level is yet to be fully characterised in individual tissues, therefore it is currently unknown whether DNA methylation profiles of peripheral tissue are reflective of central brain tissue.

However, despite the numerous difficulties in translating animal DNA methylation research to humans, the finding of exposure to early environmental adversities and increases in NR3C1 DNA methylation have been repeatedly reproduced in human cohorts. Increased NR3C1 exon 1F methylation and decreased GR mRNA have been detected in the hippocampus of suicide victims with a history of childhood abuse

(compared to control subjects and suicide victims without a history of childhood adversity) (McGowan et al., 2009). Increased NR3C1 1F DNA methylation has also been detected in DNA extracted from whole blood samples from young adolescents exposed to physical abuse in childhood (Romens et al., 2014), and adolescents exposed to stressful life events or trauma (van der Knaap et al., 2014).

Alterations in NR3C1 DNA methylation have also been associated with antenatal distress. For example, increased NR3C1 1F DNA methylation has been observed in whole blood of young adolescents whose mothers had experienced intimate partner violence during pregnancy (Radtke et al., 2011). Increased NR3C1 1F DNA methylation has also been found in cord blood of infants exposed to maternal depressed mood during pregnancy and this altered epigenetic state was predictive of stress responses in these infants at three months of age (Oberlander et al., 2008).

The findings presented in this thesis both replicate and extend the Oberlander et al. (2008) finding, by demonstrating that the effects of antenatal mood on NR3C1 1F methylation extend into the postnatal period, and are particularly pronounced in male infants. A further novel finding from this thesis is that exposure to antenatal depressive symptoms resulted in decreased infant *BDNF* IV methylation at CpG3. This CpG site is adjacent to the binding site of the transcription factor CREB, which controls *BDNF* transcription via a DNA methylation-dependent mechanism (Zheng et al., 2012).

A limitation of this research is the small sample size (n=57). Nonetheless there was a significant effect of antenatal depression on both genes that were investigated. The previous human studies have also been limited by small to moderate sample sizes

(n's=36-468), however an effect of early environmental adversity on NR3C1 DNA methylation is consistently reported. Repeated replications in different brain and peripheral tissues, and as a result of a number of different adverse environmental exposures, certainly adds weight to the idea that NR3C1 DNA methylation could be a marker of early environmental adversity.

Further, this strengthens the hypothesis that one potential mechanism by which antenatal mood disturbance may act on fetal and infant development is via enduring changes to offspring DNA methylation. Since this is a relatively new avenue of research, current studies, including the data presented in this thesis, are limited to just two genes (NR3C1 and BDNF). However, this initial evidence that antenatal depression can impact on infant DNA methylation is both very exciting and promising. Thus, there is much scope for future research in this area. In particular, large prospective cohort studies to assess changes in genome-wide DNA methylation are required to fully understand the extent to which antenatal mood disturbance may impact on offspring DNA methylation.

6.7 Taking a candidate gene approach to understanding the effects of maternal antenatal mood disturbance

As only a subset of infants manifest the adverse effects associated with exposure to antenatal mood disturbance, the identification of susceptibility and protective factors is a crucial step towards understanding underlying biological mechanisms. One commonly used technique to identify genetic risk factors for complex disorders, such as behavioural disorders and depression, is the candidate gene approach. This method directly tests the effects of genetic variants of a potentially contributing gene in an association study.

In 2003, Caspi and colleagues identified that the 5-HTTLPR polymorphism may confer susceptibility to environmental stress in the development of depressive symptoms. Since the initial publication of this finding, multiple studies have used this genotype to investigate susceptibility to environmental influences, both positive and negative. As a result, there is currently a plethora of research relating to the role of 5-HTTLPR as a susceptibility factor, although non-replications in the literature are evident (Munafo et al., 2009, Risch et al., 2009).

The role of this genotype in susceptibility to antenatal mood disturbance has previously been reported in prospective birth cohort based in The Netherlands. Pluess et al. (2011) reported that infants with two copies of the short allele were more likely to have negative emotionality at 6 months of age when exposed to antenatal anxiety that those infants with two copies of the long allele. One of the studies presented in this thesis was a large, well-powered direct replication, with the aim of firstly replicating the Pluess findings in another large cohort, and secondly to extend the findings past infancy and

into childhood. However, the study presented in this thesis found no moderating effect of 5-HTTLPR on associations between antenatal depression and anxiety and infant behavioural outcomes. Further, Pluess *et al.* (2011) reported an interaction effect size of f2=0.004. A power analyses revealed that to find this effect size with 80% power, a sample size of 2412 is required. Therefore with a sample of 1513 participants, their study was insufficiently powered to find such effect. However, the study presented in this thesis had a sample size of over 4000, and was therefore sufficiently powered to find an effect of this size. The fact that there was no moderation effect of 5-HTTLPR suggests that the original finding may have been a false-positive.

As a result of these findings, and a number of high profile failures to replicate key findings in this field, questions remain as to whether the candidate gene approach is useful in attempting to understand the effects of antenatal depression. Results presented in this thesis suggest perhaps not. Given the complexity of the potential mechanisms that are likely to be involved in this process, including heritable susceptibility genes, epigenetic processes, and hormonal changes, perhaps the candidate gene approach is too focused, and inappropriate to address the question of susceptibility. Further, as the role of epigenetic factors in moderating these effects has been highlighted as a potential mediating mechanism, such candidate-gene studies may be invalid if DNA methylation of the gene is not also simultaneously considered. On the other hand, it may be that previous research has been focused on the wrong candidate genes, and this approach may be appropriate if valid susceptibility genes are identified.

6.8 Scope for further investigation

There are many ways in which further investigation in this area may be undertaken. Currently, there is a large focus on alterations of the maternal and infant HPA axis as a potential mechanism by which antenatal mood disturbance impacts on fetal and infant development. However, one direction for future research would be to move beyond the HPA theory, and to pursue other potential mediating mechanisms such as changes in maternal sympathetic nervous system activity and immune function.

Further research into the effects of antenatal mood disturbance on infant DNA methylation is required. Although existing findings pertaining to the effects of antenatal mood disturbance on offspring DNA methylation, including those presented in this thesis, are intriguing and appear very promising, replication in much larger samples is required. Further, large scale whole epigenome studies are needed to comprehensively document the effects of antenatal mood on offspring DNA methylation changes, and detailed animal research, alongside studies of post-mortem brain tissue, are required to fully understand tissue-specific DNA methylation.

Alongside investigations into the role of epigenetics, there is also a lack of understanding of the role of genetics in fetal programming and the impact of antenatal mood disturbance on offspring development. It is clear that not all infants are affected uniformly by exposure to antenatal mood disturbance, therefore future twin, adoption and IVF studies are necessary to identify vulnerability and protective factors, and to elucidate the mechanisms of susceptibility and resilience.

6.9 Implications for clinical practice

There is a requirement for the development of psychological interventions to treat antenatal depression, and randomised-controlled trials. Firstly, these are necessary to further the development of treatments of antenatal depression, which are evidently necessary given the high prevalence rate, clear impact on infant development, and unclear effects of currently used pharmacological therapies. A more complete understanding of the mechanisms by which antenatal depression impacts on infant development is required before interventions can be confidently targeted at individuals. Thus, before that point is reached, all women with symptoms of antenatal depression should have access to non-pharmacological therapies. Further, randomised-controlled trials are required to provide the critically needed experimental evidence to establish a causal association between antenatal mood disturbance and offspring development.

6.10 Conclusions

In conclusion, the findings from this thesis suggest that the intergenerational transfer of risk from antenatal mood disturbance to infant development is not mediated by alterations of the maternal and infant HPA axis. However, changes in maternal sympathetic nervous system activity and infant DNA methylation may be two potential mediating mechanisms, and future research should continue to investigate these two pathways. A further finding from this thesis suggests that the adverse effects of antenatal depression on infant development are not related to the infant 5-HTTLPR genotype. This highlights that, given the complexity of the effects of antenatal depression on infant development, the candidate-gene approach may be inappropriate in attempting to identify susceptibility and protective factors.

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Appendices

The Oxford Pregnancy Study Measures

Edinburgh Postnatal Depression Scale

Instructions: We would like to know how you are feeling. Please **UNDERLINE** the answer which comes closest to how you have felt **IN THE PAST 7 DAYS**, not just how you feel today.

1. I have been able to laugh and see the funny side of things –
a) As much as I always could
b) Not quite so much now
c) Definitely not so much now
d) Not at all

2. I have looked forward with enjoyment to things -
a) As much as I ever did
b) Rather less than I used to
c) Definitely less than I used to
d) Hardly at all

3. I have blamed myself unnecessarily when things went wrong -
a) Yes, most of the time
b) Yes, some of the time
c) Not very often
d) No, never

4. I have been anxious or worried for no good reason -
a) No, not at all
b) Hardly ever
c) Yes, sometimes
d) Yes, very often

5. I have felt scared or panicky for no good reason -
a) Yes, quite a lot
b) Yes, sometimes
c) No, not much
d) No, not at all

6. Things have been getting on top of me -
a) Yes, most of the time I haven't been able to cope at all
b) Yes, sometimes I haven't been coping as well as usual
c) No, most of the time I have coped quite well
d) No, I have been coping as well as ever

7. I have been so unhappy that I have had difficulty sleeping -
a) Yes, most of the time
b) Yes, sometimes
c) Not very often
d) No, not at all

8. I have felt sad or miserable -
a) Yes, most of the time
b) Yes, some of the time
c) Not very often
d) No, not at all

9. I have been so unhappy that I have been crying -
a) Yes, most of the time
b) Yes, quite often
c) Only occasionally
d) No, never

10. The thought of harming myself has occurred to me -
a) Yes, quite often
b) Sometimes
c) Hardly ever
d) Never

Spielberger Trait Anxiety Index

Instructions: A number of statements which people have used to describe themselves are given below. Read each statement then circle a number to indicate how you **generally** feel. There are no right or wrong answers. Do not spend too much time on any one statement, but give the answer which seems to describe how you generally feel.

		Almost Never	Sometimes	Often	Almost Always
1.	I feel pleasant	0	1	2	3
2.	I feel nervous and restless	0	1	2	3
3.	I feel satisfied with myself	0	1	2	3
4.	I wish I could be as happy as others seem to be	0	1	2	3
5.	I feel like a failure	0	1	2	3
6.	I feel rested	0	1	2	3
7.	I am "calm, cool, and collected"	0	1	2	3
8.	I feel that difficulties are piling up so that I cannot overcome them	0	1	2	3
9.	I worry too much over something that really doesn't matter	0	1	2	3
10.	I am happy	0	1	2	3
11.	I have disturbing thoughts	0	1	2	3
12.	I lack self-confidence	0	1	2	3
13.	I feel secure	0	1	2	3
14.	I make decisions easily	0	1	2	3
15.	I feel inadequate	0	1	2	3
16.	I am content	0	1	2	3
17.	Some unimportant thought runs through my mind and bothers me	0	1	2	3
18.	I take disappointments so keenly that I can't put them out of my mind	0	1	2	3
19.	I am a steady person	0	1	2	3
20.	I get in a state of tension or turmoil as I think about my recent concerns and interests	0	1	2	3

Spielberger State Anxiety Index

Instructions: A number of statements which people have used to describe themselves are given below. Read each statement and then circle the appropriate number to the right of the statement to indicate **how you feel right now**, that is, at this moment in time, There are no right and wrong answers. Do not spend too much time on each statement but give the answer which seems to describe your present feelings best.

	Not at all	Somewhat	Moderately	Very much
1. I feel calm	1	2	3	4
2. I feel secure	1	2	3	4
3. I am tense	1	2	3	4
4. I feel strained	1	2	3	4
5. I feel at ease	1	2	3	4
6. I feel upset	1	2	3	4
7. I am presently worrying over possible misfortunes	1	2	3	4
8. I feel satisfied	1	2	3	4
9. I feel frightened	1	2	3	4
10. I feel comfortable	1	2	3	4
11. I feel self-confident	1	2	3	4
12. I feel nervous	1	2	3	4
13. I am jittery	1	2	3	4
14. I feel indecisive	1	2	3	4
15. I am relaxed	1	2	3	4
16. I feel content	1	2	3	4
17. I am worried	1	2	3	4
18. I feel confused	1	2	3	4
19. I feel steady	1	2	3	4
20. I feel pleasant	1	2	3	4

The Perceived Stress Scale

Instructions: The questions in this scale ask you about your feelings and thoughts **during the last week**. In each case, please indicate by circling *how often* you felt or thought a certain way.

0 = Never 1 = Almost Never 2 = Sometimes 3 = Fairly Often 4 = Very Often

1. In the last week, how often have you been upset because of something that happened unexpectedly? **0 1 2 3 4**
2. In the last week, how often have you felt that you were unable to control the important things in your life? **0 1 2 3 4**
3. In the last week, how often have you felt nervous and "stressed"? **0 1 2 3 4**
4. In the last week, how often have you felt confident about your ability to handle your personal problems? **0 1 2 3 4**
5. In the last week, how often have you felt that things were going your way? **0 1 2 3 4**
6. In the last week, how often have you found that you could not cope with all the things that you had to do? **0 1 2 3 4**
7. In the last week, how often have you been able to control irritations in your life? **0 1 2 3 4**
8. In the last week, how often have you felt that you were on top of things? **0 1 2 3 4**
9. In the last week, how often have you been angered because of things that were outside of your control? **0 1 2 3 4**
10. In the last week, how often have you felt difficulties were piling up so high that you could not overcome them? **0 1 2 3 4**

Positive and Negative Affect Scale

Instructions: This scale consists of a number of words that describe different feelings and emotions. Please read each item and mark the appropriate answer in the space next to the word. Indicate to what extent you feel this way now.

1. Very slightly or not at all
2. A little
3. Moderately
4. Quite a bit
5. Extremely

Interested	_____	Irritable	_____
Distressed	_____	Alert	_____
Excited	_____	Ashamed	_____
Upset	_____	Inspired	_____
Strong	_____	Nervous	_____
Guilty	_____	Determined	_____
Scared	_____	Attentive	_____
Hostile	_____	Jittery	_____
Enthusiastic	_____	Active	_____
Proud	_____	Afraid	_____

Visual Analogue Scales

Instructions: The following questions ask you about your experiences and feelings about the film. Please indicate your answer to the question by placing an X on the accompanying scale.

1) During the film, how much did you want to comfort the baby?

Did not want to comfort baby _____ Very much wanted to comfort baby

2) How upsetting did you find the film?

Not at all upsetting _____ Extremely upsetting

3) How good do you think you would be at comforting the baby?

Not at all good at comforting baby _____ Extremely good at comforting baby

Infant Behaviour Questionnaire

Instructions: We are interested to see how your baby has been behaving in certain situations in the **LAST WEEK**. Please read each question and indicate how often the baby did this during the **LAST WEEK** by circling one of the numbers.

1 Never	2 Very rarely	3 Less than half the time	4 About half the time	5 More than half the time	6 Almost always	7 Always	X Does not apply

The "does not apply" column is used when you did not see your baby in that situation. "Never" should be circled if you did see your baby in that situation but the baby never engaged in the behaviour.

Please circle a number for every item.

Feeding

When having to wait for food or liquids during the last week, how often did your baby:

1.Seem not bothered 1 2 3 4 5 6 7 X

2.Show mild fussing 1 2 3 4 5 6 7 X

3.Cry loudly 1 2 3 4 5 6 7 X

During feeding, how often did your baby:

4.Lie or sit quietly? 1 2 3 4 5 6 7 X

5.Squirm or kick? 1 2 3 4 5 6 7 X

6.Wave arms? 1 2 3 4 5 6 7 X

7.Fuss or cry? 1 2 3 4 5 6 7 X

When given a new food or liquid, how often did your baby:

8.Accept it immediately? 1 2 3 4 5 6 7 X

9.Reject it by spitting it out, closing mouth etc.? 1 2 3 4 5 6 7 X

10.Not accept it no matter how many times it was offered?

1 2 3 4 5 6 7 X

Sleeping

Before falling asleep at night during the last week, how often did your baby:

11.Show no fussing or crying? 1 2 3 4 5 6 7 X

During sleep how often did your baby:

12.Toss about? 1 2 3 4 5 6 7 X

13.Move from the middle of the cot to the end of the cot? 1 2 3 4 5 6 7 X

14.Sleep in one position only? 1 2 3 4 5 6 7 X

On waking, how often did your baby:

15.Fuss or cry immediately? 1 2 3 4 5 6 7 X

16.Lie quietly in his/her cot? 1 2 3 4 5 6 7 X

17.Coo or "talk"? 1 2 3 4 5 6 7 X

18.Cry within a few minutes? 1 2 3 4 5 6 7 X

How often did you baby:

19.Seem angry if left in his/her cot? 1 2 3 4 5 6 7 X

20.Seem happy if left in his/her cot? 1 2 3 4 5 6 7 X

21.Cry or fuss before going to sleep? 1 2 3 4 5 6 7 X

Bathing and Dressing

When being dressed or undressed, during the last week, how often did your baby:

22.Wave his/her arms and kick? 1 2 3 4 5 6 7 X

23.Squirm? 1 2 3 4 5 6 7 X

24.Smile or laugh? 1 2 3 4 5 6 7 X

When put into the bath, how often did your baby:

25.Startle? (gasp, throw out arms)	1	2	3	4	5	6	7	X
26.Smile?	1	2	3	4	5	6	7	X
27.Splash or kick?	1	2	3	4	5	6	7	X
28.Look surprised?	1	2	3	4	5	6	7	X

When placed in an infant seat or car seat, how often did your baby:

29.Wave arms and kick?	1	2	3	4	5	6	7	X
30.Squirm and turn body?	1	2	3	4	5	6	7	X
31.Lie or sit quietly?	1	2	3	4	5	6	7	X
32.Show distress at first then quiet down?	1	2	3	4	5	6	7	X

When you returned from having been away, and your baby was awake, how often did he/she:

33.Smile or laugh?	1	2	3	4	5	6	7	X

When introduced to a strange person, how often did you baby:

34.Refuse to go to the stranger?	1	2	3	4	5	6	7	X
35.Never “warm up” to the stranger?	1	2	3	4	5	6	7	X
36.Smile?	1	2	3	4	5	6	7	X

Soothing Techniques

Have you tried any of the soothing techniques in the last two weeks?
If so, how often did the method soothe your baby?
Circle X if you did not try this technique in the LAST TWO WEEKS.

37.Rocking	1	2	3	4	5	6	7	X
38.Holding	1	2	3	4	5	6	7	X
39.Singing or talking	1	2	3	4	5	6	7	X
40.Walking with your baby	1	2	3	4	5	6	7	X

41.Giving the baby a toy	1	2	3	4	5	6	7	X
42.Showing the baby something to look at	1	2	3	4	5	6	7	X
43.Patting or gently rubbing some part of your baby's body	1	2	3	4	5	6	7	X
44.Offering food or liquid	1	2	3	4	5	6	7	X
45.Offering the baby his/her dummy or security object	1	2	3	4	5	6	7	X
46.Changing your baby's position	1	2	3	4	5	6	7	X
47.Other	1	2	3	4	5	6	7	X

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The Oxford Pregnancy Study

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Participant Information Sheet

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. Please take time to read the following information and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like any more information. Part one tells you about the purpose of this study and what will happen if you take part. Part two gives you more detailed information about the conduct of the study. Thank you for reading this.

PART ONE

What is the purpose of the study?

We want to assess how mums respond to stimuli during pregnancy, and whether this may be altered in women who suffer from depression. We are also interested how changes in the mood mothers during pregnancy may influence the behaviour of the baby. This study is part of a D.Phil project, and we are aiming to include 100 pregnant women in total.

Why have I been invited?

You have been invited to take part in this study because you expressed an interest in being involved in our research.

Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part, you will be given a copy of this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are free to withdraw at any time and without giving a reason.

What will happen to me if I take part?

There are 3 parts to this study, which are explained below.

1. Part 1: Screening.
   If you would like to take part in this study, we will first ask you to complete a screening questionnaire, and based on your responses we may invite you to take part in parts 2 and 3 of the study.

2. Part 2: Prenatal assessment.
   This session will take place either at the Department of Psychiatry, Warneford Hospital, or if you would prefer, a researcher will visit you at home for this assessment. This session will take about an hour, and you are welcome to bring a friend/partner along to this session if you wish to do so. During this session you will be asked to:

   - Sign a consent form.
   - Fill in some questionnaires.
   - Watch a short film (5 minutes), which shows crying infants. Before and after you watch the film, we will ask you to give us some saliva samples (5 in total) and complete a questionnaire. This allows us to measure levels of the hormone cortisol and the enzyme α-amylase, which gives us an indication of how your body responds to stress.

   We would also like you to produce a series of saliva samples over 2 days (3 per day) following the prenatal assessment. The researcher will explain this procedure to you, and you will be provided with a pack of saliva collection tubes and a stamped, addressed envelope to return the samples.

During the prenatal assessment you will also be provided with a postcard in a stamped addressed envelope. If, after your baby is born, you wish to continue in the study, we will ask you to return to postcard to us. On receipt of the postcard, a researcher will contact you to arrange the postnatal visit (part 3). However, as the research team have no access to your medical records, they will be unaware of the outcome of the birth. Therefore, if the research team do not receive the card in the post by 4 weeks following the baby's due date, a member of the research team will contact you by email or telephone to see whether you want to continue in the study, and to arrange the postnatal visit. You will be asked by the researcher during the antenatal assessment whether you would prefer to be contacted by email or telephone, or not at all.

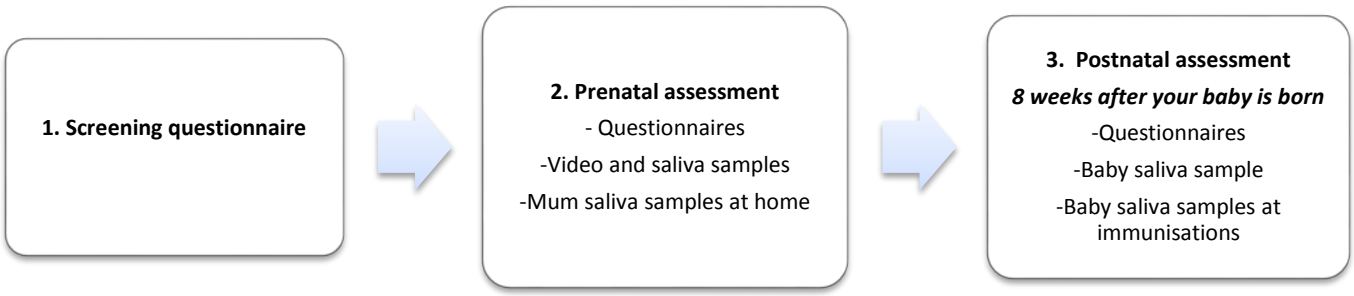
3. Part 3: Postnatal assessment.
   Approximately 2 months after you baby is born, a researcher will visit you at home or at another location if you would prefer. You will be asked to fill in some more questionnaires about you and your baby. The researcher will collect two samples of your baby's saliva. To do this the researcher will brush a swab across the inside of your baby's cheek for a few seconds. This will cause no harm or discomfort to your baby.

   We would like you to collect further saliva samples from your infant before and after their first immunisation at approximately 8 weeks of age. During the postnatal visit, the researcher will explain this procedure to you and you will be provided with a pack of infant saliva swabs and a stamped addressed envelope to return the samples. If you would like help collecting the saliva samples, a researcher will attend the immunisation appointment with you and take the saliva sample. The saliva samples will allow us to measure the levels of the hormone cortisol and enzyme α-amylase, which gives us an indication of how your baby responds to stress.

You will receive £20 as reimbursement for your participation in the study, and we will also reimburse travel expenses associated with attending the session at the Warneford Hospital.

Please note that:

- You can decide to withdraw from the study at any time.
- You do not need to answer any of the questions that you do not wish to.
- You and your baby's names will be removed from the information gathered in the study and it will not be possible to identify anyone from our reports on the study.



What are the possible disadvantages and risks of taking part?

The experiment involves watching a film of a crying baby, which may be mildly distressing to you. You are free to terminate the experiment at any point you wish to, without being required to give an explanation.

What are the possible benefits of taking part?

By taking part you will have an opportunity to make a contribution to our scientific understanding of how women and their babies may be influenced by changes in maternal mood during pregnancy.

Will my taking part be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in part 2.

This completes Part one. If the information in Part one has interested you and you are considering taking part, please read additional information in Part two before making a decision.

PART TWO

What will happen if I don't want to carry on with the study?

Even if you agree to take part, you are free withdraw from the study at any point, without giving us a reason. If you do decide to withdraw from the study, we may still use some of the data that you have already provided us, but this will be anonymised and will not be

linked to any of your personal details (e.g. name, address etc.).

What if there is a problem?

If you wish to complain about any aspect of the way in which you have been approached or treated during the course of this study, you should contact Dr Susannah Murphy on 01865 223601 or email Susannah.murphy@psych.ox.ac.uk, or you may contact the University of Oxford Clinical Trials and Research Governance (CTRG) office on 01865 572224 or via email to the head of CTRG: heather.house@admin.ox.ac.uk. The University has arrangements in place to provide compensation for any harm arising from participation in the Study, and for which the University is legally liable as the Sponsor.

Will my taking part in the study be kept confidential?

All information that is collected about you during the course of the research would be kept strictly confidential and would be accessible only to the research team. Responsible members of the University of Oxford may be given access to data for monitoring and/or audit of the study to ensure that we are complying with regulations.

Any information about you would be assigned a code and will not have your name on it. We are obliged to keep all research data until the youngest child in the study has turned 18; after this time it will be destroyed.

The only circumstances in which confidentiality would be breached would be in the rare situation in which it was judged that you or someone else was at risk of serious harm or if a court applied for the information. In these circumstances we would endeavour to discuss the matter with you and would disclose only information of immediate relevance.

What will happen to any samples I give?

During parts 2 and 3 of this study you will be asked to give samples of your saliva before and after the film, and your baby's saliva before and after their immunisation. We will analyse these samples for levels of the hormone cortisol and the enzyme α-amylase. These samples will not have your name on them and will be labelled only with your study code. They will be kept in the laboratory freezer until they have been analysed for cortisol and α-amylase levels, at which point they will be destroyed by incineration. We will not be able to give you any feedback on your individual results. No genetic tests will be carried out on these saliva samples.

During part 3 of this study (Postnatal assessment) a researcher will take 2 samples of

saliva from your baby. This saliva sample will be kept in the laboratory freezer until we analyse it. DNA will be extracted from the saliva and will be analysed to assess the activity of one particular gene (NR3C1), which is involved in how the body responds to mild stress, i.e. the 'fight or flight' response. We will not carry out a whole genetic analysis on this sample, and the results of the analysis will have no implication for the health of your baby. We will also ask for your permission to store your infant's DNA. This is so that if more genes are identified in the future as being susceptible to being more or less active in the same way, we may carry out a similar analysis. If you would not like us to store your infant's DNA, just let us know and we will note on your consent form that you will not be participating in this part of the study. We work closely with a group of researchers at Columbia University in the USA who are experts in this type of analysis, and it is possible that we may do part of this analysis in the USA. So, we would also like your permission to take your infant's DNA sample and any information which arises as a result of the analysis outside of the UK. Again, the sample and information would be fully anonymised and would not have any of your or your baby's personal information attached to it.

What will happen to the results of the study?

We may publish the results of this study in a scientific journal. Any research publication would not identify you individually. If you wish to obtain a copy of the published results, please inform the researcher. We would be delighted to send them to you when they are available.

Who is organising and funding the research?

The research is organised by the University of Oxford and funded by the Medical Research Council.

Who has reviewed the study?

This research has received ethical approval from the Oxfordshire Research Ethics Committee (Ethics number: 12/SC/0473)

Further information and contact details

Further independent information and advice about pregnancy-related matters can be obtained from Tommy's charity website (www.tommys.org). This charity also has an "Ask a Midwife" service that you can contact with any questions or concerns. You can call them on 0207 3983483 or email them at: info@tommys.org.

If you have any further questions about this particular study, please contact Elizabeth Braithwaite on 01865 613109, or email Elizabeth.braithwaite@psych.ox.ac.uk.

Thank you for taking the time to read this information sheet and considering whether to take part in this research.