NEUROPHYSIOLOGICAL AND NEUROENDOCRINE DEVELOPMENT IN ADOLESCENTS BORN TO WOMEN WITH GESTATIONAL DIABETES MELLITUS

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Abstract

GDM affects more than one in ten live births globally, and incidence rates are increasing alongside the obesity epidemic. There is a substantial body of evidence indicating that children born to women with GDM are at increased risk of neurodevelopmental abnormalities and of developing diabetes and metabolic disorder during childhood or later in life. However, very little is known about the underlying physiology.

This thesis describes research utilising transcranial magnetic stimulation (TMS), a form of non-invasive brain stimulation, to investigate candidate physiological processes possibly underlying GDM-related neurodevelopmental sequelae. In Chapter 2, research is presented that suggests that children of women with GDM have lower cortical excitability and unpredictable inhibitory neuroplastic responses to continuous theta burst stimulation (cTBS), a repetitive form of TMS designed to induce a long-term depression-like neuroplastic response.

Recent research suggests that a relatively simple technique—namely, the indirect measurement of the corticospinal volleys elicited by TMS (I-waves)—can strongly predict the response to cTBS and, further, that the variability in responses may reflect in part variability in the interneuron networks activated by TMS, rather than in neuroplastic mechanisms. Given the high variability in neuroplastic responses observed in Chapter 2, the aim of following chapters was to apply this technique to GDM-exposed adolescents. First, however, this technique was applied for the first time to adolescents to determine its validity. In Chapter 3, the association between I-waves and the neuroplastic response to cTBS was determined to be present in adolescents. Surprisingly, however, the association was not detected in males and appeared to weaken with advancing age.

In Chapter 4, this association was not found to differ between adolescents exposed to GDM and a control group. However, the altered neuroplastic response to cTBS observed in Chapter 2 was not replicated. The combination of the results presented in Chapters 2 and 4 suggest that differences in cortical excitability and synaptic plasticity induced by GDM may be subtle and difficult to replicate without

much larger samples and more sensitive techniques. Nevertheless, they may represent important avenues of research, especially when considering that globally (especially in the developing world) most cases of GDM and GDM-related developmental pathology are significantly more severe.

In Chapter 5, it is shown that GDM exposure is associated with decreased pre-awakening and morning cortisol secretion but a similar cortisol awakening response (CAR). Given that altered cortisol profiles are associated with metabolic pathology, this may represent an important pathway between GDM exposure and later metabolic dysfunction. Further, cortisol has important roles in the brain, including in synaptic plasticity, so altered cortisol secretion may prove important to the neurodevelopmental outcomes experienced by individuals born to women with GDM.

Millions of children are born each year having experienced GDM during gestation, and these numbers are climbing. The research in this thesis contributes to the important and growing body of research describing the pathophysiological features of GDM exposure with the goal of reducing neurodevelopmental morbidity and the substantial health and economic impacts of GDM.

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Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and, where applicable, any partner institution responsible for the joint award of this degree. The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

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Signature:

Date: _____

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General introduction

The ‘Developmental Origins of Health and Disease (DOHaD)’ hypothesis, initially termed the ‘Fetal Origins of Adult Disease’, underlies a conceptual framework and associated research field which postulates that environmental factors during the preconceptional, prenatal, and early postnatal periods play important roles in determining long-term health and the susceptibility to disease. Under this framework, the fetus, if exposed to an adverse intrauterine environment, responds by developing adaptations that foster both its immediate viability and its survival of similar environments later in life. For example, a fetus may downregulate its organ function and growth in response to a nutrient deficit, promoting its survival to term and potentially facilitating adaptations to subsequent malnutrition. Such adaptations, often referred to as ‘programming’, manifest as irreversible changes to tissue structure, hormonal secretion, organ function, and gene expression. Many lines of evidence, including epidemiological data and data from extensive clinical and experimental studies, support the DOHaD framework and indicate that the effects of early environmental exposures, some common forms of which are maternal stress, infection, obesity, metabolic dysfunction, smoking, and other teratogens, do indeed play important roles in influencing later susceptibility to many chronic diseases.

One important early influence on development is gestational diabetes, a metabolic disorder that occurs during pregnancy in women without a history of diabetes. Gestational diabetes is highly prevalent, affecting more than one in ten live births, and its consequences are most obvious in terms of neurodevelopment and metabolic health. Individuals born to women with gestational diabetes are at increased risk of neurodevelopmental disorders such as autism and attention-deficit hyperactivity disorder, may have lower average intelligence, and often experience motor difficulties. Further, exposed children are at much higher risk of developing type 2 diabetes during childhood or later in life, a risk that cannot be fully explained by genetic factors. Although these consequences have been well known since at least the early 1990s, there is a paucity of human physiological data regarding the possible mechanisms underlying both the altered neurodevelopment and metabolic dysfunction in these individuals. The research presented in this thesis aims to begin filling this gap

by measuring, in humans, candidate neurophysiological processes that might underlie neurodevelopmental differences induced by gestational diabetes, and by characterising their cortisol secretion profiles, a possible mechanism underlying increased risk of developing diabetes.

Following an introduction and review of the literature in Chapter 1, a study will be presented in which cortical excitability and a form of neuroplasticity are compared between adolescents exposed or not exposed to gestational diabetes. During and after this research it became increasingly clear that the technique used to record this data—a form of non-invasive brain stimulation—suffers from high variability, but that much of this may be attributable to another neurophysiological characteristic, namely the interneuron network activated by the stimulus, that is relatively easy to measure and which had not yet been investigated in adolescents. Thus, in subsequent chapters, this characteristic is further explored (Chapter 3) and then applied to the gestational diabetes cohort (Chapter 4) in an attempt to more comprehensively examine their neurophysiological profile. In Chapter 5, the focus will shift to cortisol, wherein this cohort's cortisol awakening response and morning cortisol secretion are characterised and compared with a control group. This thesis will close with a general discussion of the findings, with a focus on their implications and the potential directions that this field might take in the future.

Chapter 1: A review of the literature

1.1 GESTATIONAL DIABETES MELLITUS

Gestational diabetes mellitus (GDM) refers to hyperglycaemia first detected during pregnancy (World Health Organization et al., 2014). GDM is a common disorder, affecting approximately 14% of all live births globally (Cho et al., 2018). In Australia, GDM affects between 9.6–13.6% of pregnancies (Australian Institute of Health and Welfare, 2019, Moses et al., 2011), and the incidence is up to four times higher in certain high-risk populations, including some Asian, Aboriginal Australian, and Torres Strait Islander women (Moses et al., 2011). As I will discuss below, incidence rates vary based on the diagnostic criteria used; there is no global consensus, and the preferred criteria vary even at a local level. In any case, millions of women experience GDM globally each year, and millions of children are born having experienced, to some degree, an adverse intrauterine environment as a consequence. Thus, understanding the neurodevelopmental consequences of exposure to GDM is of great importance to public health.

In the following sections, I will describe the pathophysiology of GDM and outline the major diagnostic and therapeutic guidelines.

1.1.1 Glucose homeostasis

The pancreas, through the release of various hormones, functions to regulate blood glucose concentrations within a narrow range of 4-6 mM. This regulation is accomplished chiefly by the opposing action of glucagon and insulin and is referred to as glucose homeostasis. Glucagon increases, whereas insulin decreases, blood glucose concentration. When blood glucose is low, such as during sleep or between meals, glucagon is released by α-cells to promote hepatic glycogenolysis and gluconeogenesis, releasing glucose into the blood. Conversely, insulin is released by β-cells in response to elevated blood glucose concentrations, such as those occurring after a meal. Insulin binds to receptors on liver, muscle, and adipose cells and functions

to allow cellular uptake of glucose from the blood, thereby reducing blood glucose concentration (Roder et al., 2016).

Dysregulation or dysfunction of the insulin-signalling pathway results in metabolic dysfunction. For example, autoimmune destruction of pancreatic β-cells inhibits the ability of the body to produce insulin and therefore to regulate blood glucose, resulting in Type 1 diabetes mellitus (T1DM). Conversely, cellular sensitivity to insulin may decrease and, particularly when coupled with insufficient insulin production, result in a state of insulin resistance and consequent hyperglycaemia. When sufficiently severe, outside of pregnancy, this is defined as Type 2 diabetes mellitus (T2DM).

A similar process occurs normally during pregnancy and, as I will discuss below, can lead to a similar form of diabetes.

1.1.2 Pathophysiology of GDM

In normal pregnancy, the mother's body undergoes a complex series of physiological changes to support the demands of the growing fetus, including changes to cardiovascular, respiratory, haematologic, renal, and metabolic systems (Plows et al., 2018). In the latter category, one important change is in insulin sensitivity. During early gestation, insulin sensitivity increases to promote glucose uptake and storage by adipose tissue in preparation for later energy demands (Di Cianni et al., 2003). However, during mid-pregnancy there is a surge in placental secretion of diabetogenic hormones, including growth hormone, corticotropin-releasing hormone, placental lactogen, and progesterone. Together, these hormones promote a state of insulin resistance and, consequently, slight hyperglycaemia (Phelps et al., 1981). Such a state is preferred by the fetus; maternal blood glucose is transported readily across the placenta and acts to support fetal growth. Maternal adaptations to pregnancy-induced insulin resistance include stimulation of pancreatic β-cell hyperplasia by human placental lactogen and increased glucose-stimulated insulin secretion, resulting in greater insulin production (Parsons et al., 1992). The inability to overcome pregnancy-induced insulin resistance by producing adequate compensatory insulin leads to the development of GDM (Mack and Tomich, 2017).

1.1.3 Classification, diagnosis, and treatment

The exact threshold for diagnosis and classification of GDM depends on the criteria used. To date, there has been no consensus amongst health professionals and organisations. However, it is now recommended by the World Health Organization (WHO), the International Federation of Gynaecology and Obstetrics, the Endocrine Society, and the Australian Diabetes in Pregnancy Society (ADIPS) that the International Association of Diabetes and Pregnancy Study Group (IADPSG) criteria be used in the diagnosis of GDM. The IADPSG criteria were based on the results of the Hyperglycaemia and Adverse Pregnancy Outcomes (HAPO) study, a large, multinational, multicentre study of 23,000 pregnant women (HAPO et al., 2008). The criteria are more conservative than previously-used guidelines and have resulted in significant increases in GDM diagnoses and associated healthcare costs (Egan et al., 2017). A major finding of the HAPO study that prompted the development of more stringent diagnostic criteria was a continuous increase in maternal and fetal risk of adverse outcomes with increasing maternal glycaemia, even below the diagnostic threshold for GDM. The IADPSG therefore recommend that all pregnant women undergo a fasting plasma glucose (FPG) test at their first prenatal visit, wherein a reading equal to or greater than 92 mg/dL is indicative of GDM. Women not reaching this threshold are recommended to undergo a 2-hour 75 g oral glucose tolerance test (OGTT) between 24 and 28 weeks of gestation. Still, disagreements remain, and variations of screening protocols exist based, in part, on definitions of risk and on which groups should be screened for early GDM. In Australia, ADIPS recommend the IADPSG criteria with the caveat that early screening with an FPG, as described above, should be performed only with women at moderate to high risk of developing GDM. Risk factors include ethnicity (Asian, Indian subcontinent, Aboriginal, Torres Strait Islander, Pacific Islander, Maori, Middle Eastern, African), overweight (BMI greater than 25 kg/m^2), and previous GDM or diabetes.

Variations in diagnostic criteria complicate estimates of the prevalence of GDM. Using the IADPSG criteria, the International Diabetes Federation estimated that 18 million live births (14%) worldwide were affected by GDM in 2017 (Cho et al., 2018). Less stringent classification or diagnostic criteria may reduce this estimate by a few percentage points, but, regardless of the criteria used, GDM is a common disorder and has substantial economic and public health implications. Importantly, almost 90% of

cases of hyperglycaemia during pregnancy occur in low- and middle-income countries where maternal healthcare is limited; thus, many cases of GDM globally result in more severe maternal and fetal complications than are common in first-world countries such as Australia.

In the next section, I will describe the complications arising from GDM, with a focus on the neurodevelopmental consequences that may be experienced by a child exposed to GDM during gestation.

1.2 CONSEQUENCES OF GESTATIONAL DIABETES

Gestational diabetes is associated with a host of complications for both mother and child. GDM is a complicated and multifaceted disorder with a range of physiological processes that may be considered as either mechanistic features or comorbidities. Chief amongst these is overweight or obesity. GDM is twice as prevalent in overweight women, and up to 8 times more common in severely obese women, when compared with women of normal weight (Cho et al., 2018, Australian Institute of Health and Welfare, 2019). Both obesity and GDM are associated with increased insulin resistance, blood glucose, and chronic inflammation, and overweight and obesity contribute directly to the insulin resistance underlying GDM. Thus, when considering the causes or predictors of offspring outcomes following GDM, one must also consider overweight and obesity. These disorders, at least at the population level, are essentially inextricable. The processes and sequelae of GDM typically also apply to children born to overweight or obese mothers with GDM. As obesity has been more extensively investigated in the context of neurodevelopment, some of the literature reviewed in this chapter will span both GDM and obesity.

When considering the effects of some pathophysiological process, it is important to recognise the artificial dichotomies used to classify such processes as being present or not. The principal feature of GDM—hyperglycaemia—exists not in a binary state, but rather on a spectrum, as do the effects of hyperglycaemia and other features of GDM on the development of a child. The developmental consequences of GDM exposure described in this chapter and in later chapters therefore represent averages, and these averages, importantly, represent some of the best cases; most women enrolled in studies in English-speaking countries have received the best possible

medical care. The core findings of the HAPO study, described earlier, were continuous associations between maternal blood glucose concentrations, including those below the diagnostic criteria for GDM, with adverse pregnancy outcomes. Indeed, there were no obvious thresholds for blood glucose at which risks increased (HAPO et al., 2008). In other words, with respect to outcomes, a diagnosis of GDM does not represent a physiological threshold, but rather one of convenience.

With these caveats in mind, I will now discuss the common perinatal complications arising from GDM.

1.2.1 Pregnancy and perinatal complications

GDM increases the risk of several serious perinatal complications (HAPO et al., 2008, Billionnet et al., 2017, O'Sullivan et al., 2011, Wendland et al., 2012). A study of over 700,000 births in France in 2012 found that the risks of preterm birth (odds ratio [OR]: 1.3 [95% CI: 1.3, 1.4]), Caesarean section (OR: 1.4 [95% CI: 1.4, 1.4]), pre-eclampsia/eclampsia (OR: 1.7 [95% CI: 1.6, 1.7]), macrosomia (OR: 1.8 [95% CI: 1.7, 1.8]), respiratory distress (OR: 1.1 [95% CI: 1.0, 1.3]), birth trauma (OR: 1.3 [95% CI: 1.1, 1.5]), and cardiac malformations (OR: 1.3 [95% CI: 1.1, 1.4]) were increased in women with GDM compared with the non-diabetic population (Billionnet et al., 2017). Similar observations were made in a large systematic review of eight studies totalling over 44,000 women (Wendland et al., 2012).

Once delivered, babies of women with GDM are at increased risk of neonatal hypoglycaemia; exposure to maternal hyperglycaemia results in fetal hyperglycaemia and hyperinsulinaemia, and when access to the maternal glucose supply is suspended, neonatal hyperinsulinaemia and hypoglycaemia may ensue. If not properly managed, this can lead to brain injury (Esakoff et al., 2009).

With respect to the most severe outcome, despite several very large studies, it remains uncertain whether GDM is associated with an increased risk of perinatal death (Johns et al., 2018). The French study mentioned previously (Billionnet et al., 2017) found, amongst over 700,000 births, that the odds of perinatal mortality were increased by 30% in term-born infants of women with GDM compared with the non-diabetic population (OR: 1.3 [95% CI: 1.0, 1.6]). When excluding women to whom antidiabetic medication was dispensed in the following year—that is, women who may have had

pregestational diabetes—the increased risk of perinatal mortality persisted only in women treated for GDM with diet modification. Conversely, two large population-based cohort studies of over 1 million women in Canada (between 1996 and 2010) and Sweden (between 1991 and 2003) demonstrated that perinatal mortality in offspring of women with GDM was either lower (relative risk [RR]: 0.63 [95% CI: 0.43, 0.93]) or similar (OR: 0.8, non-significant) to the non-diabetic population (Fadl et al., 2010, Feig et al., 2014).

With respect to the basic pathophysiology underlying most of the perinatal sequelae of GDM, it can be summarised that the hyperglycaemic intra-uterine environment stimulates the fetal pancreas to hyperinsulinaemia and accelerated anabolism, resulting in fetal overgrowth and potential macrosomia. Such growth and metabolic patterns cause or contribute to various complications of pregnancy and birth, and hypoglycaemia following birth.

1.2.2 Metabolic dysfunction and diabetes risk

Women who are diagnosed with GDM are at significantly elevated risk of developing T2DM. In a recent meta-analysis of 43 studies including nearly 5 million women, the risk of developing T2DM for women with GDM was seven times that of women with normal glucose tolerance during pregnancy (Benhalima et al., 2019). It is estimated that 50% of women with GDM will develop T2DM in the 5–10 years following the index pregnancy (Kim et al., 2002, Bellamy et al., 2009, Benhalima et al., 2019). There is also evidence for an association between GDM and subsequent risk of cardiovascular disease which is only partially explained by BMI (Shostrom et al., 2017).

There is a significant body of evidence indicating that offspring of women with GDM are also at increased risk of diabetes and adverse metabolic phenotypes (e.g. Silverman et al., 1995, Boney et al., 2005, Lawlor et al., 2011, Nehring et al., 2013, Landon et al., 2018). The association between GDM and offspring metabolic profile and particularly the mechanisms by which the intrauterine environment may alter offspring metabolic phenotype have been studied most extensively in animals. Increased fetal exposure to glucose leads to hypertrophy of the endocrine pancreas and, potentially, to fetal hyperinsulinaemia, even when maternal hyperglycaemia is

mild (Aerts and Van Assche, 2006). Fetal hyperinsulinaemia has been shown to adversely affect hypothalamic development, resulting in hypoplasia of the ventro-medial nucleus, which is responsible for modulating insulin secretion (Plagemann et al., 1992). Thus, alterations in hypothalamic development, particularly in more severe cases of GDM, may compromise the development of the insulin-regulatory system, predisposing offspring to metabolic dysfunction and diabetes (Aerts and Van Assche, 2006, Aerts et al., 1988).

Indeed, in rats hypothalamic hypoplasia persists into adulthood (Plagemann et al., 1992). Although rats born of diabetic mothers have adequate pancreatic mass, normal islet-cell distribution, and normal basal glycaemia and insulinaemia, their response to glucose stimulation is impaired. Offspring of mildly diabetic mothers exhibit a decreased insulin response and impaired glucose tolerance, whilst in offspring of severely diabetic mothers the opposite is observed (Aerts et al., 1997). Impaired glucose tolerance is observed to a similar degree in various other rat and monkey models involving perinatal hyperinsulinaemia (Oh et al., 1988, Plagemann et al., 1992, Susa et al., 1992). Importantly, these studies have also demonstrated that normalisation of maternal glycaemia during pregnancy prevents adverse metabolic development in offspring (Damm et al., 2016).

Although human research is significantly more complex, involving myriad variables potentially confounding an association between maternal and offspring health, there are several studies supporting the findings in the animal research described above. For example, in a study of 597 adults, 21% of individuals born to women with GDM developed T2DM or pre-diabetes by 27 years of age, and this risk was eight times greater than in offspring of non-diabetic mothers (Clausen et al., 2008). Interestingly, the odds of developing diabetes in GDM-exposed individuals was also significantly higher than in those born to both women with T1DM and women genetically predisposed to T2DM. In a related study, children of both women with GDM and women with T1DM had lower insulin sensitivity and pancreatic β-cell function than offspring of non-diabetic and genetically-predisposed women (Kelstrup et al., 2013).

The role of genetics in the intergenerational transfer of adverse metabolic phenotype was explored further in a cleverly designed study of discordant sibships by Dabelea et al. (2000). In this study, BMI and diabetes prevalence were compared in

siblings born before or after their mother was diagnosed with diabetes. The risk of diabetes was significantly higher in siblings born after a maternal diabetes diagnosis than those born before (OR: 3.7). The mean BMI was also 2.6 kg/m^2 higher in offspring of diabetic compared with non-diabetic pregnancies. These outcomes were not influenced by a paternal diagnosis of diabetes. Although this study assessed the effect of maternal T2DM, rather than GDM, the results suggest that intrauterine exposure to hyperglycaemia conveys risk for diabetes and obesity in offspring beyond that attributable to genetic factors alone (Dabelea et al., 2000).

Some reviews of the topic have expressed concern that an association between maternal diabetes (especially GDM) might be confounded, and largely or wholly attributable to, maternal BMI (Kim et al., 2011, Philipps et al., 2011, Kawasaki et al., 2018). These concerns were based on the attenuation of the association between maternal diabetes and offspring metabolic health by maternal BMI in some studies, and the failure to correct for maternal BMI in others. However, many of the studies included in these reviews were conducted in populations in which, at least at the time of study, universal screening for GDM was not offered. Thus, bias may result from women of normal weight (and who are thus not considered high-risk) being misclassified as non-diabetic; adjustment for pre- or early-pregnancy BMI therefore may over-attenuate the association between maternal diabetes and offspring outcomes (Fraser and Lawlor, 2014).

More recently, a large, racially and ethnically diverse study of 4,160 children involved in the HAPO study provided robust evidence that maternal GDM (and, more generally, hyperglycaemia) results directly in impaired glucose tolerance in children. Importantly, the associations in this study were not attenuated by maternal or childhood BMI (Lowe et al., 2019). This study, in addition to the evidence from animal studies and studies of women with T2DM, as described above, provides a strong foundation for the hypothesis that the intrauterine diabetic environment programs the fetal metabolic system.

In the next section, I will discuss the outcomes of most relevance to later chapters of this thesis—the neurodevelopmental consequences of maternal hyperglycaemia. This section will contain a discussion of both cognitive and neuromotor sequelae and, as above, will contain relevant literature investigating the effects not only of GDM *per se*, but also of obesity and other sources of hyperglycaemia.

1.3 NEURODEVELOPMENTAL CONSEQUENCES OF GESTATIONAL DIABETES

The mechanisms described in previous sections, summarised broadly as altered fetal growth, are known to alter brain development in children born to women with hyperglycaemia, including GDM. Indeed, the fetal brain experiences periods of exquisite sensitivity to perturbations of the intra-uterine environment such as those produced by maternal dysglycaemia (Meredith, 2015).

Most of the evidence that will be presented here is derived from studies of women in developed countries who, therefore, have generally received adequate care and management of diabetes. Thus, the findings I will present should be taken in that context and, when extended to the offspring of many, if not most, cases worldwide, might be considered mild. The infants of women with well-treated diabetes during pregnancy are therefore typically asymptomatic at birth. However, there is now a substantial, and still-growing, body of evidence that presents a more concerning picture of neurodevelopment throughout childhood and adolescence, indicating that offspring of even mild, well-treated diabetic mothers have greater potential for experiencing intellectual, language, motor, and behavioural impairments (Perna et al., 2015).

1.3.1 Neurodevelopmental disorders

Autism spectrum disorder (ASD) refers to a group of complex neurodevelopmental disorders characterised by repetitive and characteristic patterns of behaviour and difficulties with communication and interaction (American Psychiatric Association, 2013). Global prevalence of ASD is estimated at 1 in 160 children (Elsabbagh et al., 2012), a figure that is known to be increasing with more sensitive and inclusive diagnostic criteria. A recent estimate places the incidence of ASD at 1 in 59 (Baio et al., 2018), with males about 4 times as likely to receive a diagnosis than females. Presentations of ASD vary widely in general type and severity, so it may be unsurprising that the etiopathology of ASD remains unclear. Indeed, different forms may be precipitated by different causes and mechanistic pathways. However, twin studies estimate heritability of ASD between 64 and 91% (Tick et al., 2016). ASD, therefore, is strongly influenced by genetics, but the lack of full

concordance in monozygotic twins indicates that their remains an important role of environmental factors in the etiopathogenesis of the disorder (Lehn et al., 2007, Newschaffer et al., 2007). A growing body of evidence implicates maternal diabetes and metabolic dysfunction, including GDM, in the development of ASD in offspring. A recent systematic review and meta-analysis of 12 studies found a consistent pattern of higher ASD incidence in children of diabetic mothers, with a pooled relative risk of 1.48 (CI: 1.25–1.75, $p < 0.001$) (Xu et al., 2014).

Several possible mechanisms have been proposed to explain the association between maternal hyperglycaemia and offspring ASD. Maternal hyperglycaemia can result in fetal hypoxia (Eidelman and Samueloff, 2002), and inadequate oxygen supply to the fetus may impair neurodevelopment and thereby infer greater risk of ASD (Burstyn et al., 2011, Kolevzon et al., 2007). Maternal hyperglycaemia is also associated with increased free-radical production and impaired antioxidant defence, resulting in oxidative stress in the cord blood and placenta (Biri et al., 2006, Chen and Scholl, 2005). Several studies have also reported higher oxidative stress in individuals with ASD (Chauhan et al., 2004, Ming et al., 2005, Yao et al., 2006).

Interestingly, there is evidence that postprandial hyperglycaemia, rather than diabetes *per se*, is an important maternal metabolism-related driver of ASD pathogenesis: pre-pregnancy obesity combined with GDM more than doubles the risk of ASD (Connolly et al., 2016, Hoirisch-Clapauch and Nardi, 2019). In this model, the underlying factor is insulin resistance. Although insulin resistance also underlies GDM, in the case of pre-pregnancy obesity instances of intrauterine hyperglycaemia are frequent and, crucially, occur throughout pregnancy, including during early pregnancy, when the fetal brain is most susceptible to insults that promote ASD. Structural and diffusion magnetic resonance imaging reveals that ASD brains exhibit disrupted neuronal connectivity due to disordered neuronal migration; frontal connectivity is often excessive and disorganised, while connectivity between the frontal lobe and other brain areas is reduced and disorganised (Di Martino et al., 2014, Ha et al., 2015). Neuronal migration starts very early in pregnancy, ending at around 26–29 weeks' gestation (Tau and Peterson, 2010), corresponding approximately to the time GDM is usually first recognised. Thus, although GDM likely contributes to ASD in other ways, and may contribute to the mechanisms described here earlier than its time of progression into and diagnosis as frank diabetes, obesity is likely the primary

maternal metabolic risk factor. As I have discussed earlier, however, obesity is integral to a complete understanding of GDM, and is comorbid with most cases of GDM. The two diseases therefore are likely to each contribute to ASD pathogenesis and, when comorbid, may confer risk greater than each would independently. In a large study of over 230,000 births, Sacks et al. (2016) found that individuals born to women with GDM were at increased risk of long-term neuropsychiatric disease, including ASD, independently of maternal BMI, maternal age, and gestational age at birth (OR: 1.83).

Another interesting possibility is that maternal metabolic conditions, including both obesity and GDM, interrupt maternal immune regulation, likely through inflammatory processes, leading to the production of autoantibodies reactive to the fetal brain. The fetal blood-brain barrier is permissive during fetal development, as the fetus relies on maternal immunoglobulin G (IgG) antibodies for protection against pathogens. However, this places the fetus at risk of potentially pathogenic antibodies (Krakowiak et al., 2017). Indeed, approximately 23% of mothers of children with ASD produce specific patterns of autoantibodies to fetal brain proteins that have been found in only 1% of mothers of typically developing children (Krakowiak et al., 2017, Braunschweig et al., 2008, Braunschweig et al., 2012, Brimberg et al., 2013, Singer et al., 2008, Zimmerman et al., 2007), and these antibodies have been associated with more severe cognitive and behavioural symptoms (Piras et al., 2014). In a recent study of mothers of children with severe ASD, these ASD-specific autoantibodies were more common in women with metabolic conditions during pregnancy, especially GDM; women with GDM were 3.2-fold more likely to have anti-fetal brain autoantibodies (Krakowiak et al., 2017).

GDM is also associated with the development of attention deficit and hyperactivity disorder (ADHD). ADHD is an early-onset neurodevelopmental disorder characterised by overactivity, inattention, and impulsivity (American Psychiatric Association, 2013). ADHD results in functional impairments in academic, family, and social settings, and affects approximately 7.2% of children worldwide (Thomas et al., 2015). As with ASD, the aetiology of ADHD is complex and not completely understood. There is a strong genetic component (Grimm et al., 2018), while environmental risk factors have also been identified (Polanska et al., 2012). Maternal hyperglycaemia creates an inflammatory environment by promoting oxidative stress via the production of reactive oxygen species within the embryo and fetus. It has been

suggested that ADHD may be due to central nervous system inflammatory response in the fetus caused by maternal inflammation and immune response. Maternal diabetes may also modulate maternal epigenetic mechanisms, leading to downstream effects on fetal neurodevelopment. Recently, Xiang et al. (2018) found, in a study of over 330,000 births, that children born to women with GDM requiring antidiabetic medication were more likely to have ADHD (HR: 1.26). A meta-analysis by Zhao et al. (2019) supports this finding, suggesting that maternal diabetes, especially GDM, is a probable risk factor for ADHD.

1.3.2 Neurocognitive development

In addition to frank neurodevelopmental disorders such as ASD and ADHD, GDM has been implicated as a risk factor for suboptimal development of cognitive function. There is a growing, albeit heterogeneous and incomplete, body of research suggesting that children born to women with GDM experience with greater frequency adverse cognitive development. Although some studies have not found an association between GDM and offspring cognitive development (Ornoy et al., 2001, Rizzo et al., 1991), most studies involving relatively large samples have produced consistent negative associations (Fraser et al., 2012, Bonilla et al., 2012, Dionne et al., 2008). Most studies have not controlled for potential confounders such as maternal BMI, age, tobacco and alcohol use, and socio-economic status. Further, the field is complicated by inconsistency between studies of young offspring, in which GDM is consistently associated with poorer cognitive and language development, and studies of older offspring, in which these associations generally are not found (Adane et al., 2016). However, this discrepancy may reflect a normalisation of cognitive function as children grow older; the effects of GDM may be at least partially reversible given that cognitive development is subject to myriad external influences following birth (Ronfani et al., 2015).

Indeed, there is evidence that most of the variation in cognitive function attributed to GDM exposure may in fact be explained by covariates largely relating to socioeconomic status and the child's environment (Clausen et al., 2013, Fraser et al., 2014). Two recent systematic reviews (Adane et al., 2016, Robles et al., 2015) have concluded that GDM does represent a likely risk factor for adverse cognitive

development, but that the evidence remains relatively weak given the heterogeneity in findings and overall quality of evidence. The independent effects of GDM are therefore likely to be small but may contribute synergistically to adverse cognitive outcomes when combined with poor maternal health, obesity, and an adverse developmental environment.

Nevertheless, for some domains there is relatively consistent, albeit not conclusive, evidence that GDM contributes to poorer cognitive development. As in other areas, there is more data on obese mothers, and the independent effects of GDM are often hard to extricate from those of maternal weight. Perhaps the most extensively investigated domain is language. Interestingly, a Spanish study (Torres-Espinola et al., 2015) found evidence for temporarily accelerated development of language in children of obese mothers, followed by a rapid deceleration until 18 months of age, suggesting that accelerated prenatal growth may advance cognitive development in early perinatal life but hinder subsequent development. Similar trends were observed in children of women with GDM. Another study in 2-year-old children found a trend for weaker language performance in offspring of mothers with GDM (Tertti et al., 2015).

Delays or impairments in motor skills are also commonly observed in offspring of mothers with metabolic disturbances during pregnancy (Ornoy, 2005, Ornoy et al., 2015). Early studies found lower gross and fine motor skills in 6–12-year-old children of women with either GDM or pregestational diabetes compared with controls (Ornoy et al., 1999, Ornoy et al., 2001, Ornoy et al., 1998). In these studies, negative correlations were found between maternal glycated haemoglobin (HbA1c), acetonuria, and total motor scores, suggesting that the severity and duration of maternal hyperglycaemia may influence the child's motor development. In a recent study of 2504 mother-child dyads, children of obese mothers were twice as likely to fail to reach typical developmental milestones (≤ 2 SD below the age norm) for fine and gross motor skills, in addition to communication, social skills, and problem solving (Girchenko et al., 2018).

Although the field is complex and still growing, it is clear that GDM contributes to adverse neurodevelopment. That is, individuals born to women with GDM exhibit differences in their behaviour and functional neurodevelopmental outcomes. However, the physiology underlying these relationships remains unclear. We know, for example, that GDM and obesity are associated with ASD and likely contribute mechanistically

to its pathophysiology, but what is actually occurring to the child's brain? What neurophysiological mechanisms are affected? These are the questions that underlie the research in this thesis, and to date little has been done in humans to answer them. In the next section, I will discuss some of candidate mechanisms and processes, including the macro- and micro-structure of the brain and the mechanisms of communication between brain cells.

1.4 POSSIBLE NEUROPHYSIOLOGICAL MECHANISMS

1.4.1 Brain macrostructure

Brain development is a highly complex but organised process. In humans, the cerebral cortex starts developing from a single layer of cells at approximately 33 days post-conception (Bystron et al., 2008). Amongst mammals, there is significant variation in the macroscopic structure of the brain with respect to the degree of folding. Some species, like mice, have cerebra that have almost completely smooth surfaces (*lissencephalic*). Others, like humans, have brains with complex folded structures characterised by 'hills' (gyri) and 'valleys' (sulci) (*gyrencephalic*) (DeFelipe, 2013). In humans, the process of gyrification (folding) begins *in utero*, peaks during adolescence, stabilises during adulthood, and eventually declines during the ageing process (Raznahan et al., 2011). Disrupted gyrification has been associated with a range of neurodevelopmental sequelae, including both autism (Yang et al., 2016, Wallace et al., 2013, Schaer et al., 2013, Schaer et al., 2015, Libero et al., 2019, Libero et al., 2014, Kohli et al., 2019b, Kohli et al., 2019a, Kates et al., 2009, Ecker et al., 2016, Duret et al., 2018, Bos et al., 2015) and cognitive deficits (Duret et al., 2018, Chung et al., 2017, Gregory et al., 2016, Gautam et al., 2015, Docherty et al., 2015).

Gyrification involves the differential expansion of the cerebral cortex, which initially is smooth, to produce an intricately folded structure. This process is dictated by genetic, biochemical, and environmental factors, and disruptions to this precisely orchestrated series of events can lead to altered brain structure and function (Quezada et al., 2018). In humans, gyrification starts during the second trimester of gestation and is mostly complete by term. The exact mechanisms underlying gyrification are still unknown, and the study of the process and those factors that might influence it is complicated by a lack of suitable animal models; rodents are lissencephalic, so many

of the most powerful and malleable models are of little use. Nevertheless, some data are available, and altered gyrification has been associated with several distinct intrauterine complications (Quezada et al., 2018), including intrauterine growth restriction (Dubois et al., 2008, Egana-Ugrinovic et al., 2013), preterm birth (Engelhardt et al., 2015, Kim et al., 2016, Moeskops et al., 2015), hypoxia (Bregant et al., 2013, Smith et al., 2015), and infection (Picone et al., 2014). To date, there has been no investigation of a possible link between GDM and gyrification. Although it may be expected that mild or well-managed GDM should have little effect on brain macrostructure, GDM is associated with mild hypoxia, increased risk of preterm birth, and chronic inflammation, which may produce similar effects to infection-induced inflammation, so there is a possibility that GDM could contribute to altered gyrification in humans, especially when severe or under-treated. Such an effect could represent one mechanistic link between maternal diabetes and both autism and cognitive deficits in offspring.

1.4.2 Brain microstructure

The brain is composed of two main types of tissue: grey matter (GM) and white matter (WM). GM contains the cell bodies, or somas, of neurons and glial cells, the neuronal dendrites, short-range intra-cortical axons, and the stems of long-range axons extending into the white matter. WM consists of densely packed, often myelinated, axons that emanate from the soma of neurons in GM and project to distal sites in the brain or elsewhere in the body. Glial cells are also present in WM (Alexander et al., 2019). Thus, the WM acts to allow GM in different areas of the brain to communicate by providing organised, highly efficient routes for information to travel from region to region.

Naturally, disturbances to microstructural development during gestation are associated with significant functional neurodevelopmental impairments. The most well understood perinatal insult that alters brain microstructure is preterm birth. More accurately, there is a linear association between gestational age at birth and brain microstructure, with even mildly preterm individuals exhibiting widespread microstructural alterations compared with their term-born peers (Kelly et al., 2016). These white matter abnormalities are associated with the common neurological and

behavioural delays experienced by preterm individuals, including compromised language and communication, motor control, and cognitive function. Although the field is still rapidly expanding, there is a great deal of research elucidating specific links between microstructural brain development and neurodevelopmental outcomes in children and adults born preterm (e.g. Thompson et al., 2014, Kelly et al., 2016, Vollmer et al., 2017, Collins et al., 2019, Duerden et al., 2019). A review of this literature is beyond the scope of this thesis. However, the similarities between the neurodevelopmental outcomes in preterm individuals and those born to diabetic women suggest that similar microstructural alterations may also be present in the latter group.

There is some limited evidence in this area; a study of newborns of obese mothers found altered white matter development, similar to that seen in preterm infants, compared with those born to women of normal weight (Ou et al., 2015). Another study examined three birth cohorts and found that maternal BMI was associated with altered white matter development in 10- and 26-year-old individuals born preterm, but not in a smaller cohort of 6-year-olds. The patterns of microstructural change were not consistent between the age groups, and differed from those seen in infants previously (Ou et al., 2015), so the effects of maternal obesity, or the intrauterine environment generally, may evolve over the age-span. To date, these are the only studies in this area, and an association between GDM and white matter development remains to be investigated.

1.4.3 Brain function

So far, I have described the structural features of the brain that could be affected by an adverse intrauterine environment such as that produced by GDM. However, the brain's structure only partly accounts for its function. The function of the brain ultimately depends on the communication between its neurons; the structure provides the substrate and organisation to allow and facilitate communication, but the transfer of information between neurons requires specific mechanisms. In this section, I will briefly describe the anatomy of the neuron, the connections between neurons, and the primary mechanism by which the brain is able to alter these connections—synaptic

plasticity. Subsequently, I will examine how synaptic plasticity might be affected by the intrauterine environment.

1.4.3.1 The neuron and the synapse

Neurons are distinct from other cell types due to their specialisation for rapid, intercellular electrical signalling. This specialisation for communication is evident in the morphology of the neuron, the most obvious characteristic of which is its extensive branching structure. Neurons consist of a body (soma), a major process (the axon), and an elaborate arborisation of smaller process, or dendrites, that branch out from the soma. Neurons communicate with one another through connections between their axons and dendrites. The number of inputs a neuron receives is determined by the complexity of its dendritic network and varies from one to about one hundred thousand. Neurons are specialised secretory cells; the connections made between one neuron and another—the synapse—represent an elaborate secretory apparatus found in most polarised epithelial cells. The axon terminal of the presynaptic neuron is immediately adjacent to a specialised region of receptors on the postsynaptic, target neuron. In most cases, a synapse involves no physical continuity between the communicating neurons; rather, communication is performed via secretion of molecules. These neurotransmitters are released by the presynaptic terminal, traverse the synaptic cleft (extracellular space), and bind to receptors on the postsynaptic neuron (Kandel and Schwartz, 1982).

The information received by a postsynaptic neuron travels down the receiving dendrite and is integrated and "read" at the origin of that neuron's axon. An axon, depending on the type of neuron, may span a few hundred micrometres or much farther, as in the axons running through the human spinal cord to the foot, which are about a metre long. The event that allows information to travel such distances is called an action potential, a self-propagating wave of electrical activity consisting of depolarisation of the axon's membrane. The action potential is initiated at the base of the axon (the hillock) and travels to the terminus where synaptic contacts are made, whereby synaptic vesicles—specialised secretory organelles—release their neurotransmitters. Neurotransmitters, when binding with receptors on the postsynaptic cell, modify the electrical properties of that cell, thereby transmitting information from one neuron to the other (Hammond, 2008).

The synaptic connections between neurons provide the basic "wiring" of the brain's circuitry. However, in contrast to the wiring of an electrical device, the wiring of the brain is dynamic and constantly changing. In addition to facilitating the transmission of information about the brain (and body), it is the function of the synapse to facilitate dynamic change in neural activity and connectivity. Such changes in synaptic activity arise from several forms of plasticity—collectively termed synaptic plasticity—that function on timescales ranging from milliseconds to years. More broadly, these processes are responsible for the phenomenon commonly known as neuroplasticity, the process by which neuronal networks reorganise the strength of their connections in response to external and internal stimuli. Neuroplasticity is believed to represent the main process by which the central nervous system stores and retrieves information, and therefore to underlie learning and memory (Hammond, 2008, Kandel et al., 2014).

1.4.3.2 Synaptic plasticity

Short-term forms of synaptic plasticity generally involve changes to the amount of neurotransmitter released by the presynaptic neuron. For example, facilitation, augmentation, and potentiation function by enhancing neurotransmitter release via the action of calcium ions within the presynaptic terminal. Conversely, synaptic depression involves a reduction in neurotransmitter release due to activity-dependent depletion of synaptic vesicles. On the other hand, long-term forms of synaptic plasticity, which act on timescales of tens of minutes or longer, arise from molecular mechanisms which themselves vary in timescale, from initial post-translational modifications of existing synaptic proteins to later changes to gene expression. This form of synaptic plasticity can produce long-term modifications including the growth of synapses, and ultimately produce lasting changes to brain function. Plasticity of this type resulting in an increase in synaptic transmission is referred to as long-term potentiation (LTP), whilst the opposite is termed long-term depression (LTD) (Purves et al., 2013).

Whilst several cellular processes may underlie LTP and LTD, the most extensively studied and well understood, and perhaps the most important, involve the ionotropic N-methyl-d-aspartate (NMDA) receptor, which is activated by glutamate. Glutamate is the most important neurotransmitter for normal brain function; nearly all excitatory neurons in the central nervous system are glutamatergic, and half of all

synapses release this neurotransmitter (Purves et al., 2013). When postsynaptic NMDA receptors are activated by glutamate, entry of calcium ions through the NDMA receptor cation channels is impeded by a voltage-dependent magnesium blockade (Nowak et al., 1984). However, when an NMDA receptor is activated concurrently with postsynaptic depolarisation, the magnesium blockade is released, allowing calcium to enter the cell (Cooke and Bliss, 2006). Large increases in intracellular calcium result in the activation of various kinases, ultimately increasing the conductance and insertion of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors into the postsynaptic membrane. In this way, NMDA receptors produce neuroplastic change; the postsynaptic cell is now more sensitive to glutamatergic synaptic transmission and the synapse is stronger. NMDA receptors thus act as coincidence detectors: the receptor detects the coincidence of presynaptic activation (glutamate release) and postsynaptic activation (concurrent depolarisation). As a result, cells that commonly fire together develop stronger synapses, facilitating future co-activation. This is the mechanistic foundation of LTP.

LTD, on the other hand, is produced by a variation of this mechanism. When the presynaptic and postsynaptic activity is mismatched, resulting in only a small amount of calcium influx through postsynaptic NMDA receptors, postsynaptic excitability and synaptic strength can decrease. Small postsynaptic increases in intracellular calcium preferentially activate protein phosphatase cascades, which dephosphorylate AMPA receptors and promote their removal from the postsynaptic membrane (Carroll et al., 1999, Lee et al., 1998). Thus, depending on the type of neuronal activity, NMDA receptors can either strengthen (LTP) or weaken (LTD) glutamatergic synapses (Kandel et al., 2014).

1.4.3.3 The involvement of LTP and LTD in learning and memory

The involvement of activity-dependent synaptic plasticity in learning and memory has been well documented and has driven a substantial body of research over the last five decades. An analysis of this field is far beyond the scope of this work (for review, see Takeuchi et al., 2014), but I will here provide a brief introduction to the main types of evidence available elucidating the neurophysiological mechanisms underlying learning and memory. Much of this research has focussed on LTP, especially in the mammalian hippocampus, a brain region thought to be central to memory. The identification of the role of NMDA receptors in LTP induction provided

a molecular explanation for the links between LTP and the theta rhythm, a prominent neural oscillation detected in the brains of animals engaged in exploratory behaviour (Vanderwolf, 1969), which has been known for decades to be associated with learning and memory (Klemm, 1976). Indeed, LTP exhibits many properties that are characteristic of learning and memory, including rapid formation, long duration, associativity, and cooperativity (Bliss and Collingridge, 1993). These findings led to a variety of methods to stimulate the brain electromagnetically using stimulation patterns based upon the theta rhythm (Larson and Lynch, 1986) to induce LTP and modulate learning and behaviour in animals. In Section 1.5 I will describe a form of non-invasive brain stimulation also based on the theta rhythm used to induce LTP and LTD in humans, which comprises a principal methodology of the research presented in later chapters.

There is also a substantial body of pharmacological evidence linking LTP with learning and memory (Maren and Baudry, 1995). Much of this research describes the blockade of NMDA receptors using pharmacological antagonists and a resulting impairment in learning (e.g. Sakimura et al., 1995, Davis et al., 1992, Butelman, 1989, Mondadori et al., 1989, Venable and Kelly, 1990).

Given the fundamental link between synaptic plasticity, learning and memory, and, by extension, cognitive function and general neurodevelopment, an important question is raised: do individuals who experience an adverse intrauterine environment have impaired or altered synaptic plasticity? The answer to this question could inform an understanding of the mechanisms linking intrauterine development and subsequent brain development. To date, there has been no research in humans directly relating synaptic plasticity to the intrauterine environment, but there is some indirect evidence of possible causal pathways in addition to more direct, experimental evidence from animal models. Additionally, there is evidence linking dysregulated synaptic plasticity with neurodevelopmental disorders such as autism. This I will describe in the next section.

1.4.4 Synaptic plasticity is influenced by the intrauterine environment

As described earlier, fetuses of obese and diabetic women are likely to be chronically exposed to insulin resistance and excess glucose. Consequently,

inflammatory changes in fetal skeletal and adipose tissue increase peripheral insulin resistance and insulin production, which may have significant effects on the central nervous system (Cordner and Tamashiro, 2015). The insulin receptor is highly expressed in the hippocampus, and growing evidence implicates insulin signalling in learning and memory (Zhao et al., 1999, Zhao and Alkon, 2001) by modulating synaptic plasticity through, for example, NMDA receptor-dependent mechanisms (Liu et al., 1995, Christie et al., 1999). In rats, maternal high-fat diet (HFD; a common obesity model) has been associated with decreased hippocampal expression of the insulin receptor and glucose transporter in offspring persisting into adulthood (Cordner and Tamashiro, 2015). A similar mechanism may involve leptin; the leptin receptor is also highly expressed in the brain, and maternal obesity has been shown in rats to decrease hippocampal expression of the leptin receptor in offspring. Like insulin, leptin signalling is thought to play a critical role in learning by regulating synaptic plasticity and neurotransmitter trafficking (Cordner and Tamashiro, 2015). Leptin is also a neurotrophic factor, and abnormalities in leptin signalling during fetal development have been associated with decreased stem cell differentiation and growth (Desai et al., 2011).

There is growing evidence that these chemical and functional changes are associated with structural alterations in the brains of offspring exposed to maternal obesity or adverse metabolic phenotypes. For example, rats born to dams fed a HFD throughout gestation and lactation exhibit atrophy of pyramidal neuron dendrites in both the hippocampus and amygdala. These changes to neuronal morphology may partially explain the alterations in synaptic plasticity and learning in these animals (Janthakhin et al., 2017). In a study of mice, HFD-exposed offspring displayed increased body weight, circulating insulin, and strongly increased hypothalamic insulin signalling. Additionally, HFD offspring showed a global gene expression profile that differed significantly from control mice, with alterations in a number of key pathways involved in neurogenesis, axogenesis, and regulation of synaptic plasticity (Bae-Gartz et al., 2019). Similarly, HFD-exposed mice have lower brain-derived neurotrophic factor (BDNF) in the hippocampus compared with control mice, and this is associated with impaired dendrite arborisation and spatial learning (Tozuka et al., 2010).

1.4.4.1 Synaptic plasticity in neurodevelopmental impairment

Long-term depression deregulation is believed to be an important mechanism in the pathophysiology of ASD. LTD is crucial during early development for the regulation of synaptic weighting, functioning to modulate and regulate the changing wiring of the brain during maturation. In the human cortex, beginning in the earliest embryonic stage and lasting until approximately 2 years of age, synapses are formed at an amazing rate, at times reaching 40,000 synapses per second (Scott and Beauvais, 2010). Following this process, the brain contains far more synapses than it requires. Synaptic pruning, a competitive and activity-dependent process, takes place until approximately 10 years of age, by which time around 50% of synapses have been lost (Huttenlocher, 1990). Synaptic pruning is crucial to proper development of brain circuits and cognitive functions. In ASD, pruning is impaired (Hutsler and Zhang, 2010); thus, dysregulation of the mechanisms underlying pruning, which also underlie LTD, may contribute both to the development of ASD and to later symptomatology (Hansel, 2019). Indeed, genetic studies of ASD have identified several risk genes that are key regulators of synaptic plasticity, including genes involved in synaptic scaffolding proteins, chromatin remodelling, and those encoding receptors (Bourgeron, 2015).

Synaptic plasticity is also implicated heavily in ADHD. ADHD is believed to result from gene–environment interaction, and the heritable components are largely associated with variability in genes that encode proteins involved in dopamine and noradrenaline signalling and directed neurite growth (Poelmans et al., 2011, Kasparek et al., 2015). As described in the previous section, adverse perinatal development leads to altered brain morphological development, including altered dendritic development and reduced synaptic density (Murmu et al., 2006). Taken together, the data on genetic and environmental factors influencing ADHD imply that changes in synaptic plasticity, especially resulting from altered dendritic development, which result from altered dopaminergic function, may be important factors underlying the neurobiology of ADHD (Kasparek et al., 2015).

1.4.4.2 Synaptic plasticity and cortical excitability in humans born preterm

It is clear that changes to mechanisms underlying synaptic plasticity are important features of common neurodevelopmental disorders, including those associated with GDM and other intrauterine exposures. However, to date direct

neurophysiological evidence in humans who have experienced developmental disturbances is sparse. To my knowledge, the only such studies have been performed in preterm-born adolescents. Pitcher et al. (2012a) found that adolescents born preterm had a significantly lower LTD-like neuroplastic response to non-invasive brain stimulation. Adolescents born early preterm (≤ 32 weeks completed gestation) did not exhibit a significant neuroplastic response to stimulation, while those born late preterm (33–37 weeks) had weaker responses than those born at term. This pattern of increasingly adverse response with greater prematurity is consistent with the neurodevelopmental outcomes, discussed earlier, experienced by individuals born preterm. In this study, preterm adolescents also had lower salivary cortisol concentrations. Cortisol is a hormone with important roles in neuroplasticity, as I will discuss in a later section of this chapter.

In the next section, I will describe the techniques used both in the studies mentioned above and in the research presented later in this thesis. Specifically, I will describe several forms of non-invasive brain stimulation used to measure cortical excitability and synaptic plasticity in humans. Subsequently, I will discuss the hormone cortisol and its roles in synaptic plasticity.

1.5 NON-INVASIVE STIMULATION OF THE HUMAN BRAIN

During the late nineteenth and early twentieth centuries, the electrical stimulation of exposed cortical tissue contributed greatly to the understanding of the human cortex. However, naturally these techniques were of limited applicability, especially to the healthy human brain. In 1954, Gualtierotti and Paterson (1954) tried to stimulate the unexposed human cerebral cortex by applying an electric current through the intact scalp. Although they obtained motor responses, the procedure was extremely painful and inefficient, since most of the current flowed through the scalp rather than into the brain. This technique was improved in 1980 by Merton and Morton (1980) when they used a single high-voltage capacitive discharge, allowing deeper penetration of the current into the brain. Although this was an improvement, the pain associated with the procedure was strong enough to prevent its widespread clinical or research use.

In parallel with the development of methods of electrical stimulation of the brain, the concept of non-invasive neurostimulation using a time-varying magnetic field was gradually evolving following the discoveries of magnetic induction by Faraday (1831) and eddy currents by Foucault in 1885. The first known attempt to induce magnetic brain stimulation was by d'Arsonval (1896), who induced phosphenes, vertigo, and syncope by applying an alternating current to a coil surrounding the head. Several replications and variations of this work were performed in the following years, and similar techniques were used to stimulate peripheral nerves (Bickford and Freming, 1965).

Finally, in 1985 Barker and Jalinous (1985) introduced transcranial magnetic stimulation (TMS), achieving non-invasive and painless stimulation of the human motor cortex using a stimulator consisting of a capacitor discharging into a stimulating coil placed on the scalp. TMS introduced a novel research tool for studying the functionality, morphology, and connectivity of various regions of the intact human brain.

1.5.1 Transcranial magnetic stimulation

TMS induces electric currents in the brain via Faraday's principle of electromagnetic induction. Briefly, Faraday discovered that an alternating—but not a constant—electric current in a conductor induces a current in the opposite direction in a nearby conductor (Faraday, 1831). This was formalised as the law of induction, or Faraday's law, and generalised as one of the four foundational equations of classical electromagnetism, the Maxwell equations. Basically, this law states that a changing magnetic field produces an electric field. Any electric current induces a magnetic field around it; with TMS, an electric current, which grows to a peak and diminishes back to zero rapidly (<1 ms), is passed through a coil placed over the head. This current produces a magnetic field perpendicular to the plane of the coil with a corresponding time-course, rising to about 2.5 Tesla and falling rapidly. This rapidly fluctuating magnetic field passes unimpeded through the scalp and skull and induces a current in the brain tissue (a conductor) that flows parallel to the plane of the coil but in the opposite direction to the original current (Rotenberg et al., 2014).

In the commonly used figure-of-eight coil, which consists effectively of two overlapping circular coils, the induced current is maximal under the intersection of the two coils. This is a type of 'focal' coil, designed to concentrate current at the junction of the coils in order to better target specific brain regions. However, even with focal coils, the current induced in the brain reaches only superficial brain structures—about 2cm deep—because electromagnetic induction follows the inverse cube law. That is, the power of the magnetic field decreases exponentially with distance. The basic mechanism of neural activation by TMS relies on forcing free charges in intra- and extra-cellular volumes to move coherently by an electric field. Depolarisation or hyperpolarisation is induced in cell membranes that interrupt current progress, resulting in initiation of an action potential in affected neurons (Roth and Zangen, 2012).

1.5.2 Measuring cortical excitability with single-pulse TMS

TMS is commonly applied to the primary motor cortex (M1). Whereas there are no simple, immediate methods for recording the activity of other cortices, stimulation of the motor cortex offers the significant benefit of direct, non-invasive recording of the brain's output by simple electromyographic recording at a target muscle. Often, stimulation is applied to the M1 representation of the hand, which is conveniently located superficially along the precentral gyrus. The effect of stimulation of this manner can be characterised by examining the properties of the compound muscle action potential, which, in this context, is referred to as a motor evoked potential (MEP).

Several distinct features of the MEP, including latency, magnitude, and area, can be used to derive unique information about the activity of corticospinal pathways recruited by a single TMS pulse. Latency, or the time between the stimulus (pulse) and the motor response (MEP onset), is reflective of the number of synapses between the stimulation site and the target muscle (mostly within the cortical region stimulated) as well as the integrity of the white matter fibres involved. MEP magnitude is by far the most commonly utilised measure of single-pulse TMS of the motor cortex and is generally measured as the peak-to-peak amplitude (trough-to-peak) in millivolts (mV).

By recording the stimulation intensity required to elicit an MEP of a given amplitude, one may obtain a measure of cortical excitability. This is most commonly operationalised as the motor threshold (MT). The MT is defined as the minimum stimulus strength (usually in percentage of maximum stimulator output [MSO]) that produces a small MEP (usually > 50 μV) in the target muscle in at least half of 10 or more consecutive trials. Put simply, it is the minimum stimulation intensity required to reliably induce a motor response. The MT can be measured at rest (resting motor threshold, RMT) or during slight isometric activation of the target muscle (active motor threshold, AMT). The MT reflects the excitability and the local density of a central core of excitatory interneurons and corticospinal neurons in the muscle representation in the M1, as well as the excitability of the target spinal (or brainstem) motorneurons (Hallett, 2000, Tassinari et al., 2003). At the cellular level, the MT reflects the membrane excitability of axons as mediated by the permeability of voltage-gated sodium channels (Cincotta et al., 2012). The MT is not influenced by pharmacological modulation of γ-aminobutyric acid (GABA)-mediated inhibition or NMDA receptor-mediated excitation (Cincotta et al., 2012, Ziemann et al., 2015), but does seem to involve fast excitatory neurotransmission through AMPA receptors, suggesting that the strength of excitatory synaptic connections in the stimulated network influences the MT (Di Lazzaro et al., 2003). Structurally, the MT is thought to reflect the integrity of white matter. Specifically, greater excitability, as measured with both RMT and AMT, is associated with greater regional fractional anisotropy (FA) in white matter tracts underlying the primary motor, premotor, and posterior prefrontal cortices (Kloppel et al., 2008). FA provides a measure of directional organisation of white matter and depends on such factors as the degree of myelination, axon diameter, axon density, fibre geometry, and general maturation of white matter (Le Bihan et al., 2001, Le Bihan, 2003, Beaulieu, 2002) and is associated with a host of cognitive tasks and abilities (e.g. Wolbers et al., 2006, Bengtsson et al., 2005, Tuch et al., 2005, Klingberg et al., 2000). Thus, the MT represents a relatively simple technique that can provide information about white matter properties and potentially elucidate mechanisms linking adverse neurodevelopment with functional outcomes.

Indeed, Pitcher et al. (2012b) reported that, in a group of 151 children aged 10–13, those born preterm had higher RMT (indicating lower cortical excitability). Specifically, there was a linear association between gestational age at birth and RMT,

such that even mildly or moderately preterm children experienced reduced excitability, and these changes were associated with poorer motor skill development, especially manual dexterity. Thus, reduced excitability—likely reflecting, to some degree, altered white matter integrity and functional connectivity—appears to provide a mechanistic pathway between prematurity and adverse functional neurodevelopment. Given the neurodevelopmental similarities between preterm-born individuals and individuals born to women with metabolic disorders, an aim of the research presented in this thesis was to apply similar methods to children and adolescents born to women with GDM to determine whether similar mechanisms might contribute to adverse neurodevelopment in this group.

1.5.3 Inducing synaptic plasticity using repetitive stimulation

TMS offers several techniques to non-invasively induce and detect neuroplastic changes in the excitability of neuronal networks in the human brain. This is achieved by applying repetitive trains of magnetic pulses with specific patterns and frequencies of delivery and is referred to generally as repetitive TMS (rTMS). As with other forms of brain stimulation, due to the ease of quantifying changes in the motor cortex by measuring cortical excitability with single-pulse TMS, most of the work investigating rTMS-induced neuroplasticity has focussed on the primary motor cortex, but these protocols have been applied to a range of cortical regions (Boroojerdi et al., 2000, George et al., 1996, Nyffeler et al., 2006) and such investigations have become more common with the advancement of neuroimaging techniques such as electroencephalography (EEG) and functional magnetic resonance imaging (fMRI) (Guller and Giacino, 2014, Kimiskidis, 2016, Bergmann et al., 2016, Tremblay et al., 2019).

Early work with rTMS in humans involved the application of simple protocols employing evenly spaced magnetic pulses. Generally, low frequency (~1 Hz) stimulation reduces excitability of the stimulated region, while higher frequencies (> 5 Hz) tend to increase excitability (Chen et al., 1997, Gangitano et al., 2002, Maeda et al., 2000b, Muellbacher et al., 2000, Berardelli et al., 1998). These changes are generally observed as decreases and increases, respectively, in the size of MEPs evoked by single TMS pulses and reflect various forms of inhibitory and facilitatory

neuroplastic responses. However, even these relatively simple paradigms are complicated by a range of factors, and the magnitude and direction of excitability changes are influenced by factors other than stimulation frequency. For example, short trains of high frequency stimulation may produce suppression or facilitation of MEPs depending on whether stimulation is delivered at low or high intensities (Modugno et al., 2001). Similarly, the duration of stimulation may determine the type of response elicited (Maeda et al., 2000a).

1.5.3.1 Theta burst stimulation

As mentioned earlier (Section 1.4.3.3), animals engaging in exploratory behaviour exhibit bursts of high frequency neuronal activity in the hippocampus in phase with the 4–7 Hz theta frequency rhythm (Okeefe and Nadel, 1979), and this neuronal activity is associated with the induction of LTP, learning, and memory. Based on the link between theta oscillations and learning and memory, a number of early stimulation protocols were developed for application in animals and neuronal preparations that involved delivering bursts of high frequency (i.e. 100 Hz) stimulation at the theta frequency of 5 Hz (Larson and Munkacsy, 2015).

In 2005, Huang et al. (2005) introduced theta burst stimulation (TBS), an rTMS paradigm based on the hippocampal theta rhythm that is safe for use in humans and that, depending on the pattern of bursts, can induce LTP-like or LTD-like neuroplastic responses. TBS involves the delivery of bursts of three sub-motor-threshold pulses at 50 Hz with an interburst frequency of 5 Hz. When delivered in a continuous 40 second train (i.e. continuous TBS; cTBS), a lasting suppression of MEP amplitudes may be observed. Conversely, when bursts are delivered in intermittent trains of 2 seconds with 10 second intervals (intermittent TBS; iTBS) MEP amplitudes are facilitated.

1.5.3.2 Mechanisms of TBS

At the cellular level, cTBS and iTBS, like other plasticity-induction protocols (Stefan et al., 2002, Nitsche et al., 2003, Vlachos et al., 2012, Ciampi de Andrade et al., 2014), are NMDA receptor-dependent; the effects of TBS are blocked by NMDA receptor antagonists such as memantine and dextromethorphan (Huang et al., 2007, Wankerl et al., 2010). Additionally, TBS is also dependent on activation of Ca^{2+} channels (Wankerl et al., 2010). Because both NMDA receptors and Ca^{2+} channels are key agents at the postsynaptic membrane for the induction of plasticity (Tan et al.,

2013, Lenz et al., 2015), TBS is believed to induce LTP- and LTD-like phenomena (Suppa et al., 2016).

It may seem curious that stimulation involving the same frequencies (50 Hz at 5 Hz) and volume (600 pulses) can produce opposing effects due only to the introduction of breaks between bursts. To explain the opposing effects of cTBS and iTBS, Huang et al. (2011) developed a theoretical model based on a simplified postsynaptic model of plasticity. Assuming that LTP and LTD are triggered by the influx of calcium to the postsynaptic cell (see Section 1.4.3.2), the basic result of the model is that TBS produces a mix of excitatory and inhibitory effects which, depending on the pattern of stimulation, summate to yield either suppression or facilitation of cortical excitability. A short burst at 50 Hz produces a brief facilitation followed by later but weaker inhibition (Huang and Rothwell, 2004). Hence, iTBS, which involves short trains of TBS, allows the facilitatory effect to remain dominant, resulting in LTP. Conversely, cTBS maintains a continuous application of TBS such that the inhibitory effect is allowed to overcome the facilitation, resulting in LTD. This is supported by evidence indicating that halving the duration and total number of pulses delivered with cTBS (20 seconds, 300 pulses) results in facilitation rather than inhibition (Gentner et al., 2008). Mechanistically, the bias towards synaptic weakening or strengthening is controlled by the amount and rate of postsynaptic calcium influx which is a function of the rate and duration of stimulation (Huang et al., 2011, Suppa et al., 2016).

This model is supported by a range of experimental evidence. For example, dysfunction of inositol 1,4,5-trisphoshate receptors (InsP3Rs) required for LTP results in a conversion of LTD to LTP, while partial blockade of NMDA receptors to reduce the rate of calcium influx results in a conversion of LTP to LTD (Nishiyama et al., 2000). Further, increasing the number of TBS bursts appears to reduce the LTP-like effect (Larson and Lynch, 1986, Abraham and Huggett, 1997).

1.5.3.3 Variability of TBS after-effects

Like all current methods of non-invasively inducing neuroplasticity in humans, the response to TBS can be highly variable from one subject to another. In practice, group-level effects are often weak or, in some cases, insignificant (Pellegrini et al., 2018, Vallence et al., 2015, Jannati et al., 2019, Jannati et al., 2017). It is not necessarily the case that TBS protocols do not produce a neuroplastic response in most individuals, but rather that the direction of the response can vary. That is, cTBS may

produce after-effects consistent with LTP (facilitation), while iTBS will sometimes produce LTD-like suppression of excitability. A recent systematic review and meta-analysis (Chung et al., 2016) confirmed that both TBS protocols do indeed produce systematic changes as intended; amongst 87 studies, iTBS yielded moderately large MEP increases lasting up to 30 minutes (standardised mean difference (SMD) = 0.71, $p < 0.00001$), while cTBS yielded similarly reduced MEPs lasting up to 60 minutes (SMD = −0.9 , $p < 0.00001$). Nevertheless, at the level of a single study, particularly when relatively small, as most studies have been, the variability in TBS after-effects can be problematic. Thus, several studies have attempted to identify and quantify those factors responsible for or associated with response variability. In this section I will briefly describe these factors (for review, see Ridding and Ziemann, 2010).

Neurotrophins are key molecules in activity-dependent modulation of synapse structure and function. Genetic polymorphism in such molecules has been a key area of interest in NIBS-induced plasticity variability. Most of the research has focussed on a common single-nucleotide polymorphism (SNP) in the brain-derived neurotrophic factor (BDNF) gene. Several studies have shown that subjects with an amino acid substitution (valine to methionine) at codon 66 (val66met) have a reduced or absent response to either or both cTBS and iTBS (Cheeran et al., 2008, Antal et al., 2010). However, these results were not replicated by two more recent studies (Voti et al., 2011, Mastroeni et al., 2013), so the importance of BDNF polymorphisms in influencing TBS response remains unclear. There is also limited evidence for roles of polymorphisms in NDMA receptor subunits (Mori et al., 2011) and the catechol-O-methyltransferase (COMT) gene (Lee et al., 2014), but further research is required.

The plasticity response is influenced strongly by the circadian system and, in particular, the hormone cortisol. The cellular mechanisms underlying cortisol's effects on synaptic plasticity are well known and will be the topic of Section 1.6.2. Although most of the research in this area concerns animals and brain slices, there is increasing evidence for cortisol's role in TBS and other forms of non-invasive plasticity induction in humans (Sale et al., 2008, Sale et al., 2010, Sale et al., 2007). For example, Clow et al. (2014) reported that the magnitude of the cortisol awakening response, the initial burst of cortisol secretion upon awakening, was positively associated with the strength of the LTD-like response to cTBS. As I will discuss in detail later, cortisol, in simple

terms, promotes facilitation of synaptic strength in small concentrations but favours inhibition at higher and more prolonged concentrations.

Other state factors have important effects on neuroplasticity. Perhaps the most extensively studied is the influence of the history of synaptic activity within a stimulated cortical region (Ridding and Ziemann, 2010), a phenomenon known as metaplasticity. Metaplasticity is defined as modification in the magnitude or direction of plasticity by previous activity in the same postsynaptic neuron or network (Abraham, 2008, Hulme et al., 2013), and may manifest as a result of endogenous neural activity, voluntary muscle contraction (when stimulating the motor cortex), or by priming the stimulated region with various forms of stimulation. Metaplasticity theory, for which there is considerable supporting evidence, implies simply that the threshold for LTP/LTD induction is flexible and dependent on the history of activity at a synapse.

For example, Todd et al. (2009) showed that when iTBS is delivered 10 minutes prior to cTBS, the after-effects (of cTBS alone) switch from inhibition to facilitation. Murakami et al. (2012) extended these findings by testing all possible pairs of iTBS and cTBS separated by an interval of 15 minutes. After application of identical protocols (iTBS before iTBS, cTBS before cTBS), the expected after-effects were suppressed. Conversely, pairs of different protocols (iTBS before cTBS, cTBS before iTBS) resulted in an enhancement of the non-primed effects. That is, when primed by iTBS, cTBS produced greater suppression than cTBS alone, and *vice versa*. Gamboa et al. (2011) extended the investigation of priming both cTBS and iTBS by identical protocols by priming at 2, 5, and 20 minutes. Consistent with the findings reported by Todd et al. (2009), in most cases the after-effects were suppressed. Two exceptions were noted: cTBS primed by cTBS at 20 minutes produced suppression similar to cTBS alone, and iTBS primed by iTBS at 2 minutes produced similar facilitation to iTBS alone. Thus, there is some evidence for an interval-dependent priming effect. Indeed, Goldsworthy et al. (2012) showed that when cTBS was primed with cTBS using a 10-minute interval, the resulting LTD-like suppression was significantly increased in magnitude and duration. This effect was robust and resistant to reversal (de-depression) by voluntary contraction or short-duration iTBS (Goldsworthy et al., 2015). Importantly, it is possible to prime the motor cortex and influence the response to TBS behaviourally, namely by contracting the target muscle. This may occur

unintentionally and represents an important potential confounder in TBS studies; EMG should always be monitored throughout an experiment and subjects should be instructed to avoid any contraction of the target muscle. However, some protocols require voluntary contraction, such as when the active motor threshold is being assessed. Thus, several studies have attempted to elucidate the mechanisms controlling behavioural metaplasticity. In the context of TBS, Gentner et al. (2008) showed that performing a brief tonic voluntary contraction prior to a short period of cTBS (20 seconds, half of the normal protocol), which normally produces facilitation, results instead in suppression of excitability. Similarly, Iezzi et al. (2008) demonstrated that priming with phasic voluntary finger movements can reverse the effects of both iTBS and cTBS. Behavioural engagement during plasticity-inducing stimulation can also alter the after-effects. For example, the effects of both iTBS and cTBS are reduced when applied during voluntary contraction of the target muscle (Huang et al., 2008). Nevertheless, the specific relationships between motor and cortical activity and subsequent synaptic plasticity remain unclear. That is, the effects of different magnitudes, intervals, and time courses of prior muscle activity are unknown.

The factors known to influence the response to TBS that I have discussed thus far all relate to intrinsic differences in the mechanisms underlying synaptic plasticity, whether they are trait factors, such as genetics, or transient variations in synaptic mechanisms such as those produced by cortisol exposure or previous synaptic activity. However, recent data suggest that some of the variation in neuroplastic response to TBS may be due instead to differences in the populations of cortical neurons that are stimulated by TMS pulses, rather than to differences in synaptic plasticity *per se* (Hamada et al., 2013). In the next section, I will describe this phenomenon and how it can be quantified. This will necessitate a brief discussion of the corticospinal mechanisms of TMS.

1.5.4 Origin of descending activity elicited by TMS

Cervical epidural recordings reveal that a single TMS pulse to the motor cortex evokes a series of descending waves of corticospinal activity (Di Lazzaro and Rothwell, 2014). The earliest of these discharges is termed the D-wave because it is a result of direct activation of corticospinal axons in the subcortical white matter. The

later waves are termed I-waves because they result from indirect, trans-synaptic activation of corticospinal cells. I-waves are numbered in order of appearance and are thought to result from increasingly complex inputs to corticospinal cells (Suppa et al., 2016).

The descending corticospinal activity evoked by focal TMS delivered using a figure-of-eight coil differs depending on the orientation of the coil (and thus the current flow in the cortex). Whereas TMS with a posterior–anterior (PA) induced current preferentially evokes an I1-wave (at threshold intensities), TMS with anterior–posterior (AP) current preferentially evokes later I3-waves in some subjects and D- or I1-waves in others (Di Lazzaro et al., 2001a). On the other hand, TMS that induces latero–medial (LM) current recruits both D- and I1-waves even at low intensities (Di Lazzaro et al., 1998). These findings suggest that TMS with different current directions may activate different neuronal elements and/or different sites of the same neuronal elements within the primary motor cortex (Di Lazzaro et al., 2008). This is important to consider when applying different forms of TMS. For example, in contrast to the monophasic pulses typically applied with single-pulse TMS, such as those used when measuring excitability, TBS is applied with a biphasic waveform, which induces current in two opposing directions in the brain. Although neuronal activation can occur in either phase of a biphasic stimulus, stimulation occurs preferentially in the reverse cycle of current flow because this is the time at which there is maximum charge transfer (Groiss et al., 2012). Thus, TBS preferentially activates the brain during anterior–posterior current flow, in contrast to monophasic single-pulse TMS frequently used to measure TBS after-effects, which generally stimulates with posterior–anterior current and potentially activates different neuronal elements.

1.5.4.1 Assessing interneuron recruitment

The measurement of I-wave recruitment, in the context of a TBS experiment, is a straightforward and reasonably quick process. Simply, MEP onset latencies are recorded by stimulating using PA, AP, and LM currents (by adjusting the orientation of the coil) delivered using single-pulse TMS during mild contraction (~10% of maximum voluntary contraction) of the target muscle. Mildly suprathreshold MEPs evoked by PA (early I-waves) or AP (potential late I-waves) currents are compared with relatively high intensity stimulation by LM current (D-wave). The rationale is that, in individuals in whom late I-wave generating circuitry is more excitable, AP

MEPs will have longer latencies than PA and LM MEPs, because more complex circuits are involved in stimulating output cells (with AP current) (Hamada et al., 2013). Indeed, AP MEP latencies are often observed to be 1-2ms longer than PA MEPs, and 4-5ms longer than LM MEPs (Day et al., 1989). The difference between LM and AP latencies (AP-LM) is used as the primary index of late I-wave recruitment because LM latencies are highly consistent, allowing reliable estimation of I-wave recruitment (larger AP-LM values indicating later I-waves).

1.5.4.2 Variable interneuron recruitment influences the response to TBS

Epidural recordings have shown that, whereas iTBS preferentially enhances later I-waves while leaving the I1 wave unaffected, cTBS supresses the earliest I-wave (I1) with relatively little effect on the D-wave and later I-waves (Di Lazzaro and Rothwell, 2014). Based on these data, Hamada et al. (2013) hypothesised that individuals in whom early I-waves were more easily recruited would respond strongly to cTBS, whereas those in whom later I-waves were preferentially recruited would respond better to iTBS. However, Hamada et al. (2013) found that individuals in whom late I-waves are more easily recruited exhibit both stronger inhibition following cTBS and facilitation following iTBS, whereas those in whom early I-waves are recruited typically exhibit the opposite response to one or both of the protocols. In this study, I-wave latency explained about 50% of the variation in TBS response. This effect was replicated in subsequent studies, in which I-wave recruitment explained smaller yet substantial proportions of the of the variation in cTBS response ranging from around 13% to 26% (Huang and Mouraux, 2015, Hordacre et al., 2017). In all these studies, subjects who recruited late I-waves were more likely to show the "expected" inhibition following cTBS and/or facilitation following iTBS, in contrast to the unpredictable responses observed in subjects who recruited earlier I-waves.

Given the effects of TBS on corticospinal activity measured by epidural recording, these results are somewhat curious. Indeed, there remains uncertainty about the mechanisms underlying the association between I-wave recruitment and TBS response, but it has been suggested it may be related to different cellular locations of I-wave inputs to corticospinal neurons (CSNs) and whether the rules for synaptic plasticity are the same at different sites. It has also been suggested (Amassian et al., 1987) that early I-wave inputs are located on proximal parts of the CSNs, whereas later I-wave inputs are located on more distal dendrites. Thus, the results observed by

Hamada et al. (2013) and others may reflect differential behaviour of oligosynaptic, dendritic inputs and monosynaptic, proximal inputs.

The reliability of the association between I-wave recruitment and TBS response is not yet entirely certain. In a recent study by Rocchi et al. (2018), I-wave latency was not associated with the response to cTBS. However, this was a small study (n = 13), and the sample appeared to consist largely of "non-responders" to cTBS and may therefore be underpowered to detect the effect in question. Nevertheless, I-wave measurement appears to be a promising technique to better predict the response to NIBS techniques and potentially to optimise these techniques in therapeutic settings. The results described above, however, also raise the possibility that the measurement of plasticity mechanisms in previous studies is influenced by variable interneuron recruitment, a factor that might vary with other characteristics of interest and thus represent a potential confounder.

1.6 CORTISOL

In the previous section, I discussed the factors that contribute to variability in the neuroplastic response to non-invasive brain stimulation techniques, including variable interneuron recruitment. This section will focus on another such factor—cortisol. Here I will describe the basic physiology of cortisol, the role of cortisol in modulating synaptic plasticity and, finally, the role of cortisol in metabolism and its possible relevance to the neurodevelopment and metabolic function of individuals born to women with GDM.

1.6.1 The hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis is a major endocrine system that plays key roles in basal homeostasis and the body's response to stress (DeMorrow, 2018, Fries et al., 2009). The glucocorticoid hormone cortisol is the primary end-product of the HPA axis and binds to receptors that are present in almost every tissue in the body. The HPA axis functions as a complex series of feedback interactions between three components: the hypothalamus, the pituitary gland, and the adrenal glands. The hypothalamus responds to basal, circadian-regulated neural input and

stress-induced input by increasing secretion of corticotropin-releasing hormone (CRH). CRH is released by the paraventricular nucleus (PVN) into the portal blood and stimulates the anterior pituitary gland to secrete adrenocorticotropic hormone (ACTH), which in turn circulates to the adrenal cortex where it stimulates the release of cortisol into the bloodstream. Cortisol achieves negative feedback by limiting the secretion of both CRH and ACTH, whilst ACTH also limits CRH secretion (DeMorrow, 2018).

The importance of the HPA axis is illustrated by the wide range of functions that cortisol is involved in, including metabolism, energy homeostasis, immune function, and neural function. Cortisol is perhaps best known for its role in regulating the response to stress. Indeed, a dynamic cortisol response, marked by a rapid rise and decline in response to stress, is thought to be crucial to facilitate adequate coping with perceived threats in the environment, and dysregulation of the cortisol response to stress is associated with neuropsychiatric disorders including major depression and anxiety (Zorn et al., 2017).

1.6.2 Cortisol modulates neuroplasticity

Upon binding to their receptors, corticosteroids become potent modulators of intracellular calcium concentrations (Krugers et al., 2005), thereby regulating the electrical properties of neurons. In this way, corticosteroids can have strong modulatory roles in synaptic plasticity, and these effects can be classified as either rapid, non-genomic effects, or slower, genomic effects. These effects are mediated by two different type of receptor: mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs). The rapid effects are confirmed, through experiments with specific agonists, antagonists, and inactivation of MRs, to be a product mainly of low-affinity membrane-bound MRs (mMRs) and involve both an increase in presynaptic glutamate release and altered postsynaptic glutamate receptor and calcium channel properties. The slower, genomic effects are generally attributed to the activation of GRs resulting from prolonged exposure to high circulating corticosteroids, for example following a stressful event. However, there are likely some GR-mediated presynaptic effects (Prager and Johnson, 2009). Work by Olijslagers et al. (2008) and others indicates corticosterone (the primary glucocorticoid in rats) binds to presynaptic mMRs leading

to activation of the extracellular signal-regulated kinases (ERK1/2) signalling pathway, resulting in increased glutamate release and thus increased excitatory neurotransmission and LTP.

Corticosteroids affect various aspects of postsynaptic AMPA receptor (AMPAR) trafficking. The nature of these effects indicates that they include both rapid, non-genomic effects and slow, protein-synthesis-dependent (i.e. genomic) effects, resulting again from the activation of mMRs and mGRs, respectively (Krugers et al., 2010). Corticosteroids rapidly increase the lateral diffusion of GluA1 and GluA2 subunits and promote the synaptic increase in GluA2-containing AMPA receptors following LTP-induction. However, mGR activation following high corticosteroid exposure reduces the ability to induce LTP and prevents the activity-dependent increase in synaptic GluA2-containing AMPARs that is promoted by mMR activation (Krugers et al., 2010).

In addition to their effects on AMPARs, corticosteroids modulate the activity of NMDA receptors (NMDARs). Indeed, this may be a major mechanism by which their effects are exerted on AMPARs; as discussed earlier (Section 1.4.3.2), NMDARs are major contributors to synaptic plasticity through their influence on AMPAR function. Research indicates that brief exposure to cortisol/corticosterone rapidly enhances NMDAR function, thereby facilitating bidirectional synaptic plasticity, likely via mMR activation. However, high or prolonged corticosteroid exposure leads to increased synaptic expression of GluN1 and GluN2A subunits, inhibiting NMDAR activity and leading to a loss of synaptic facilitation via mGR activation (Prager and Johnson, 2009). These data support NMDAR modulation as a major mechanism underlying the effects of corticosteroids on glutamatergic transmission, including modulation of AMPAR function, and consequently on bidirectional synaptic plasticity in a manner which depends on the degree and time-course of corticosteroid exposure. Indeed, electrophysiological data further suggest that corticosterone targets NMDAR-dependent plasticity via GR activation (Krugers et al., 2006). Wiegert et al. (2005) induced synaptic plasticity in hippocampal slices by 10 Hz stimulation or theta burst potentiation, which both require NMDAR activity. The authors observed that the suppressive effects of corticosterone on 10 Hz stimulation were prevented by treating the slices with the GR antagonist mifepristone. Theta burst potentiation, which also involves voltage-gated calcium channels and possibly other mechanisms, was altered

by corticosterone only when isolating the NMDAR-dependent component using nifedipine, suggesting that corticosterone selectively hampers NMDAR-dependent forms of plasticity, while leaving some other forms unaffected.

In addition to NMDARs, mGRs appear to directly modulate $GABA_A$ receptors, leading to a reduction in neuronal excitability by causing hyperpolarisation of the postsynaptic neuron. This mechanism likely adds to mGR-mediated suppression of LTP and a concomitant facilitation of LTD by increased endocytosis of GluA2 AMPAR subunits.

Thus, glucocorticoids can accentuate synaptic function (Krugers et al., 2010), driving both the strengthening and weakening of synapses, and can thereby promote apparently opposing mechanisms depending on the dynamics of MR and GR activation and synaptic input. It should be noted that these dynamic effects are believed to be important for the processing and storage of memories of stressful or emotional events. Briefly, the rapid promotion of synaptic transmission and LTP is important for the storage of salient information related to the event, while later inhibition of LTP and promotion of LTD apparently serve to increase the storage threshold for information reaching the network later (Joels et al., 2006), thereby preventing relatively non-salient information after the event from interfering with or reconfiguring the network, resulting in a greater likelihood of the original information being consolidated.

It is important to note that the mechanisms described here largely pertain to membrane-bound corticosteroid receptors. However, the inverted U-shape dose response curve is believed to apply similarly to corticosteroid interactions with genomic MRs and GRs, and likely reflect in part a function of the different affinities of gMRs and gGRs for cortisol/corticosterone leading to opposing effects at the transcriptional level (Prager and Johnson, 2009).

Importantly, individual differences in the MR:GR ratio, particularly at synapses, could influence phenotypical differences in the initial response to stress, the interaction between stress and synaptic plasticity, and to the formation and recall of stress-related or traumatic memories. Similarly, differences in hypothalamic-pituitary-adrenal (HPA) function, such as basal cortisol production or the cortisol awakening response, may alter an individual's stress response and synaptic function. Indeed, animal research indicates that healthy circadian glucocorticoid patterns are important for

learning-dependent synaptic formation and maintenance (Liston et al., 2013), and disrupted patterns of glucocorticoid secretion are associated with cognitive deficits (e.g. Gibson et al., 2010, Evans et al., 2011) and a range of neuropsychiatric disorders (e.g. Jagannath et al., 2013, Wulff et al., 2010).

Thus, it is important to further our understanding of the interactions between corticosteroids and synaptic function, in addition to factors which may alter the function of the hypothalamic-pituitary-adrenal (HPA) axis and its downstream targets. As mentioned above, cortisol may be an important measure during human plasticity-inducing brain stimulation experiments. For example, Pitcher et al. (2012a) found in adolescents lower salivary cortisol concentrations with decreasing gestational age at birth, and these concentrations were associated with a corresponding decrease in LTD-like neuroplastic response following cTBS. Conversely, cortisol has been shown to inhibit LTP induced by paired associative stimulation (PAS) (Sale et al., 2008). PAS-induced LTP was lower in the morning (following the CAR) and in the evening following hydrocortisone administration relative to when endogenous plasma cortisol concentrations were low. Thus, there is evidence in humans for the importance of cortisol as a modulator of LTP and LTD-like synaptic plasticity by non-invasive brain stimulation which corresponds with the known mechanisms observed in cell and animal models. For this reason, cortisol may be considered an important covariate in research in which synaptic plasticity is induced using brain stimulation protocols like TBS. Thus, in the neurophysiological research presented in later chapters of this thesis, a measurement of cortisol is taken. Such measurement comprises a single saliva sample collected proximally to the induction of plasticity in order to capture possible influences of circulating cortisol on the synaptic response to TBS.

1.6.3 The cortisol awakening response

Basal cortisol secretion follows a distinct diurnal rhythm. Normally, the highest cortisol production occurs during the second half of the night, with a peak in the early morning hours (during sleep). Cortisol concentrations then drop gradually throughout the day and into the first half of the night (Fries et al., 2009). Superimposed on this basal rhythm is a brief spike in cortisol secretion that occurs immediately after awakening in the morning, lasting 30–45 minutes. This phenomenon is termed the

cortisol awakening response (CAR). The CAR has become a topic of intense investigation following the introduction of salivary cortisol sampling, which allows non-invasive, naturalistic, and accurate measurement of cortisol. There are several reasons why the CAR has become an important research topic. Firstly, the CAR appears to be under somewhat independent control from basal and stress-induced cortisol secretion (Schmidt-Reinwald et al., 1999). Secondly, twin studies indicate that there is genetic influence over the CAR that is distinct from the heritability of the basal cortisol profile (Wust et al., 2000). Thirdly, the CAR is independently associated with stress and health in important ways, and has been suggested to be a distinct and useful index of HPA axis function (Steptoe and Serwinski, 2016).

As described above, cortisol release is under control of a hormonal cascade initiated by the PVN of the hypothalamus. The PVN itself is under strong regulation by higher brain structures, including the hippocampus, amygdala, and prefrontal cortex (Fries et al., 2009). Additionally, there are connections between the PVN and the suprachiasmatic nucleus, another hypothalamic structure that is sensitive to light (through the visual system) and is involved in circadian regulation. Indeed, the CAR is sensitive to fluctuations in light in the environment and can be augmented by light exposure towards the end of the sleep period. Noise, in contrast, has little effect on the CAR (Steptoe and Serwinski, 2016). As the name suggests, the CAR is not simply a steep section of the diurnal cortisol profile, but rather a response to the process of waking from sleep. The CAR does not occur when waking from daytime naps, however, nor does it occur following repeated awakening during the night. Interestingly, when wake time is expected, there is an increase in ACTH during the hour before wake time, but this does not occur if no wake time is expected. Indeed, although the exact functions of the CAR remain unclear, a prominent theory is that the CAR plays a preparatory role in "priming" the individual for the anticipated demands of the day (Fries et al., 2009). This is supported by research indicating that larger CARs are exhibited when people are anticipating a demanding task that day (Stalder et al., 2010a, Stalder et al., 2010b). Further, studies involving repeated measurements over time suggest that up to 70% of the variation in the CAR is attributable to day-to-day fluctuations rather than stable characteristics (Ross et al., 2014).

1.6.3.1 Measuring the CAR

Due to the ease of collecting salivary cortisol samples, most participants in CAR studies collect samples at home by passive drool or by chewing on special cotton swabs. Given that the CAR is dynamic, precise timing of the samples is critical, complicating ambulatory studies with adherence and accuracy problems. The first sample should be collected immediately after waking, and if delayed by even a minute or two the resulting measurement of the CAR may be misleading. If a delayed waking sample is collected, the CAR is likely to have already begun, leading to smaller recorded increase from "baseline" to the subsequent peak and a flatter CAR slope. The exact timing and frequency of subsequent samples varies in the literature, with the choice often reflecting a cost/accuracy trade-off; more samples produce more accurate results but increase study costs. Thus, smaller studies often employ protocols calling for 4-5 samples, whilst larger epidemiological studies often use only two sampling times (typically waking and at 30 or 45 minutes post-awakening). Although two-sample protocols such as this provide an approximation of the underlying CAR, they cannot be sure to capture the peak, which may fall between 30 and 45 minutes post-awakening (PA) (Clow et al., 2004, Smyth et al., 2015, Wust et al., 2000). Importantly, the assumption in such studies that the CAR peak will occur at around 30 minutes PA, with some random variation amongst individuals, is likely not valid. There is evidence for systematic differences in the timing of the peak related to cognitive function (Evans et al., 2012), adolescent development (Oskis et al., 2009), sex (Pruessner et al., 1997, Schlotz et al., 2004, Wust et al., 2000), and menstrual phase (Wolfram et al., 2011). Therefore, expert consensus guidelines (Stalder et al., 2016) stipulate that a minimum of three samples should be collected to measure the CAR: on awakening, 30 minutes PA, and 45 minutes PA. Studies aimed at elucidating more fundamental CAR mechanisms or that may be more sensitive to temporal dynamics should include additional samples at 15 and 60 minutes PA.

Although early studies suggested high test-retest stability of the CAR (Wust et al., 2000, Edwards et al., 2001), evidence has accumulated over the past two decades suggesting that the CAR is in fact subject to high intra-individual variability. For example, Hellhammer et al. (2007) employed structural equation modelling to CAR data collected over six consecutive days and found that the CAR on a single day is determined largely by situational, or state, factors (61–82%) rather than long term, trait

factors (15–37%). These findings were confirmed subsequently by studies providing state-specificity estimates of 78% (Almeida et al., 2009) and 64% (Stalder et al., 2010b). The number of sampling days required therefore depends largely on the type of study and whether state or trait characteristics are under investigation. To avoid state-related confounding, cross-sectional research requires crucially that state-related influences (e.g. study setting) are equal between groups such that state variability contributes in a random manner. In such cases, increasing sample size may be the most practical avenue for robust data. If trait characteristics are of interest, multiple sampling days will provide more accurate representations of the trait CAR. This, of course, leads rapidly into feasibility issues. Consensus guidelines (Stalder et al., 2016) recommend that CAR data are measured over two or more days, and this is advisable regardless of study type. The study days should also be considered carefully, however; the number of weekday and weekend measurements should be considered and balanced appropriately given that weekend and weekday demands likely differ.

Once the data have been collected, there are important considerations to be made when analysing and reporting it. When the purpose of a study is to investigate group differences in the CAR, and straightforward approach to analysis is the use of repeated-measure analysis of variance, wherein a main effect of time may be considered evidence for the presence of the CAR, and time-by-group interactions to represent group differences in CAR profile. However, summary indices of the CAR may also be computed, and these provide the benefits of a single continuous variable, including the potential to assess associations with other variables (e.g. by addition to a regression model). Perhaps the most common of these are area-under-the-curve (AUC)-based measures. Several variations of these measures exist, but they tend to be highly intercorrelated and have been shown to represent two main underlying components: 'total cortisol production' and 'change in cortisol levels'. The most common of the former measures is the AUC with respect to ground (AUCg), a measure that is calculated with reference to zero. Importantly, measures of this type should not be considered true measures of the CAR, but rather of total hormonal output (Clow et al., 2010). Nevertheless, AUCg is commonly reported as a measure of the CAR, and indeed is often the sole index used. On the other hand, the AUC with respect to increase (AUCi) is an index of pattern or rate of change that takes into account both sensitivity and intensity (Fekedulegn et al., 2007). Therefore, when assessing the CAR—a

dynamic process—AUCi or a similar measure should be employed (Stalder et al., 2016, Khoury et al., 2015). AUCg or a similar measure may be used as an index of hormonal output, which is a separate, but possibly equally interesting, phenomenon.

Importantly, the awakening sample (S1) should also be reported (Stalder et al., 2016). Clow et al. (2010) reviewed neurophysiological evidence of morning cortisol secretion and concluded that different processes are likely responsible for pre-awakening cortisol increase and the CAR. Therefore, in addition to its importance to the validity of CAR estimation, S1 provides novel information about a separate mechanism of the HPA axis.

1.6.4 Role of cortisol in metabolic dysfunction

In addition to its roles in the brain, cortisol features prominently in metabolic regulation, and dysregulation of the HPA axis has been associated with metabolic dysfunction and the development of T2DM. Thus, cortisol regulation represents a possible mechanism underlying the increased risk for metabolic dysfunction in individuals born to women with GDM. In this section, I will briefly describe cortisol's role in the metabolic system, followed by a discussion of the link between HPA axis dysregulation and metabolic dysfunction.

Cortisol is a prominent metabolic hormone and functions overall to promote catabolic processes by increasing the availability of all fuel substrates by mobilising glucose, free fatty acids, and amino acids from endogenous stores. Indeed, glucocorticoids, the class of hormone to which cortisol belongs, derived their name from their profound effects on plasma glucose levels. In this section, I will refer specifically to cortisol, because that is the hormone of interest here, but note that this is fundamentally a general discussion that applies similarly to other glucocorticoids; thus, the term 'cortisol' may more accurately be substituted for 'glucocorticoid'.

Cortisol regulates multiple aspects of glucose homeostasis. First, cortisol stimulates gluconeogenesis (the production of glucose from non-carbohydrate substrate) in the liver (Kraus-Friedmann, 1984). Second, cortisol exerts tissue-specific effects on glycogen metabolism (Kuo et al., 2015). In the liver, cortisol increases glycogen storage, whereas in skeletal muscle it promotes glycogenolysis and inhibits insulin-induced glycogen synthesis (Stalmans and Laloux, 1979, Exton et al., 1972,

Ruzzin et al., 2005). Cortisol also modulates glucagon and insulin secretion from the pancreas. Finally, cortisol inhibits glucose utilisation by reducing both glucose uptake and glucose oxidation in skeletal muscle and adipose tissue (Di Dalmazi et al., 2012, Kuo et al., 2013). Thus, cortisol is a diabetogenic factor and acts to oppose the effects of insulin, resulting in insulin resistance. These functions serve to increase blood glucose, a process related to cortisol's primary purpose of regulating the response to stress; metabolic adaptation during stress requires plasma glucose to be preserved for use by the brain (Charmandari et al., 2005). This process, under normal conditions, is transient and integral to normal functioning. However, chronic exposure to high or dysregulated cortisol concentrations contributes to pathologic hyperglycaemia, insulin resistance, diabetes, and obesity.

Dysregulation of the HPA axis, including the diurnal cortisol curve and the CAR, is associated with metabolic dysfunction. For example, Kuehl et al. (2015) found that lower total morning cortisol (AUCg) was associated with higher risk of metabolic syndrome, especially higher blood pressure. A flatter CAR (smaller AUCi) has been associated with metabolic disturbance (Lasikiewicz et al., 2008) and T2DM (Bruehl et al., 2009). Similarly, Hackett et al. (2014) reported that flatter diurnal cortisol slopes were associated with T2DM and, in a related study, found flatter diurnal slopes to predict the development of T2DM around 10 years later (Hackett et al., 2016). These findings are of particular importance to the research presented in this thesis; because individuals born to women with GDM are at significantly increased risk of developing metabolic disturbance and T2DM, HPA axis dysregulation represents a possible mechanistic link between maternal and child metabolic health. Nevertheless, such a mechanism has yet to be explored.

1.7 SUMMARY

GDM is a common disease of pregnancy that affects millions of women and their offspring each year. Even when well controlled, GDM may produce an adverse intrauterine environment characterised by hyperglycaemia and inflammation. A large body of evidence indicates that this environment, even when mild, can influence the neurodevelopment of the fetus and child, conferring increased risk of neurodevelopmental, cognitive, and motor difficulties. Further, children of mothers

with GDM are significantly more likely to develop diabetes during their lives, a risk that is not entirely explained by genetics. To date, there is no direct neurophysiological data to suggest what neural processes might be affected by exposure to GDM, and the physiological link between maternal and offspring metabolic dysfunction in humans is similarly unexplored. The experiments described in this thesis aim to begin exploring these questions, focusing on determining whether synaptic plasticity and neuronal excitability might underlie the neurodevelopmental differences produced by GDM, and whether an altered HPA axis could provide a mechanistic link between maternal and offspring diabetes.

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Chapter 2: Reduced cortical excitability, neuroplasticity, and salivary cortisol in children born to women with gestational diabetes mellitus

2.1 ABSTRACT

Background

Children exposed to gestational diabetes mellitus (GDM) *in utero* are at increased risk of neurodevelopmental difficulties including autism and impaired motor control. However, the underlying neurophysiology is unknown.

Methods

Using transcranial magnetic stimulation, we assessed cortical excitability and long-term depression (LTD)-like neuroplasticity in 45 GDM-exposed and 12 control children aged 11–13 years. Data were analysed against salivary cortisol and maternal diabetes severity and treatment (insulin [N = 22] or metformin [N = 23]) during pregnancy.

Findings

GDM-exposed children had reduced cortical excitability ($p = 0.003$), LTD-like neuroplasticity ($p = 0.005$), and salivary cortisol ($p < 0.001$) when compared with control children. Higher maternal insulin resistance (IR) before and during GDM treatment was associated with a blunted neuroplastic response in children ($p = 0.014$) and this was not accounted for by maternal BMI. Additional maternal and neonatal measures, including fasting plasma glucose and inflammatory markers, predicted neurophysiological outcomes. The metformin and insulin treatment groups had similar outcomes.

Interpretation

These results suggest that GDM can contribute to subtle differences in child neurophysiology, and possibly cortisol secretion, persisting into early adolescence.

Importantly, these effects appear to occur during second trimester, before pharmacologic treatment typically commences, and can be predicted by maternal insulin resistance. Therefore, earlier detection and treatment of GDM may be warranted. Metformin appears to be safe for these aspects of neurodevelopment.

2.2 INTRODUCTION

Gestational diabetes mellitus (GDM) affects 5–10% of pregnancies, with a higher prevalence in obese women (WHO, 2014). Emerging evidence indicates that children exposed *in utero* to GDM are at higher risk of neurodevelopmental difficulties, including attention deficit hyperactivity disorder (Nomura et al., 2012), autism spectrum disorders (Xu et al., 2014), and impaired motor development (Ornoy et al., 1999). Additionally, maternal obesity has independently been associated with a range of neurodevelopmental and psychiatric disorders in the offspring (Edlow, 2016). Animal research underpins the hypothesis that oxidative stress and inflammation associated with maternal hyperglycaemia are major drivers of altered neurodevelopment in GDM-affected fetuses (Sullivan et al., 2014), while obesity is associated with a chronic, low-grade, metabolically-induced inflammatory state (Pantham et al., 2015). Placental inflammation is observed in obesity- and GDM-affected pregnancies (Saloman et al., 2016), and intrauterine inflammation can evoke fetal brain injury (Elovitz et al., 2011). Further, maternal hyperglycaemia can retard dendritic development in the fetal brain (Jing et al., 2014). Taken together, these findings suggest that GDM-exposed fetuses experience an adverse environment *in utero* that contributes to abnormal neurodevelopment. However, there are currently no neurophysiological data in children exposed to GDM (or maternal obesity), so the mechanisms underlying these neurodevelopmental disturbances are unknown.

Evidence for a link between inflammation and suboptimal neurodevelopment also comes from studies of children born preterm. While the aetiology of preterm birth is multifactorial, the only established pathological, causal factor is infection and/or inflammation (Romero et al., 2007), the exposure to which significantly increases the risk of alterations in cortical microstructure and functional connectivity (Counsell et al., 2008), as well as poor neurodevelopmental outcomes postnatally (Leviton et al., 2013). These subtle but significant changes are believed to underlie the high incidence

of low severity neurodevelopmental impairments commonly reported in children born preterm, including cognitive, motor and behavioural impairments (Mwaniki et al., 2012) and altered neuroplasticity (i.e. the brain's ability to alter the strength of its synaptic connections) (Pitcher et al., 2012a). While GDM-exposed children born at term exhibit some of the same neurodevelopmental impairments as children born preterm, it is currently unknown if they exhibit similar abnormalities in neuroplasticity. Since neuroplasticity is widely accepted as a key mechanism underlying learning and memory, abnormalities in neuroplasticity may help to explain the physiological processes responsible for adverse neurodevelopmental outcomes in children exposed to GDM.

Here we used transcranial magnetic stimulation (TMS) techniques to investigate cortical excitability and the capacity for long-term depression (LTD)-like neuroplasticity in children born to women enrolled in the Metformin in Gestational diabetes (MiG) randomised controlled trial (Rowan et al., 2008) to explore potential associations between maternal hyperglycaemia and cortical function in the offspring. The MiG trial examined the safety and efficacy of the oral anti-hyperglycaemic agent metformin versus insulin to treat GDM, and demonstrated that metformin is equally effective and safe as insulin for both mother and child (Barrett et al., 2013, Battin et al., 2015, Rowan et al., 2011, Wouldes et al., 2016). A secondary aim of the present study was to compare the neurophysiologic outcomes of metformin- versus insulin-exposed children, as there remains a lack of long-term and neurophysiological data assessing the potential impact of metformin. This is important because, unlike exogenous insulin, metformin crosses the placenta (Charles et al., 2006) and interacts with the fetus in largely unknown ways. Reassuringly, the available evidence suggests that the likely effects of metformin in the fetus are anti-inflammatory (Scheen et al., 2015) and neuroprotective (Chung et al., 2015a). Thus, metformin may benefit the fetal brain in GDM-exposed pregnancies beyond its role in maternal glycaemic control.

2.3 METHODS

2.3.1 Ethical Approval

All procedures were approved by the Women's and Children's Health Network and University of Adelaide human research ethics committees and conducted in accordance with the Declaration of Helsinki (2008 revision). Participants were pre-screened for contraindications to TMS (Rossi et al., 2009b). Parents provided written, informed consent and accompanied their children to the experimental session.

2.3.2 Subjects

45 GDM-exposed (aged 11.8 ± 0.7 years [mean ± SD], 20 females) were recruited from the Adelaide arm of the MiG trial (Rowan et al., 2008). Their mothers had been treated for GDM not responding to lifestyle alteration, with a 1:1 random allocation at study entry to receive either insulin or metformin treatment. The mean (± SD) gestational age at study entry was 30 ± 2.6 weeks. Eight of the metformin-treated women in the current study had required supplemental insulin to achieve euglycaemia, but there was no difference in demographic or clinical characteristics of these women compared with the metformin-only treatment women, and these subjects are included in the metformin group. Twelve control children not exposed to GDM (aged 12.8 ± 0.8 years, 8 females) were recruited from labour ward records and matched as closely as possible for gestational age at birth (GA). Mothers in the GDM group had higher body mass indices (BMIs) (34.1 ± 6.8) than control group mothers (23.7 ± 4.6; $p < 0.001$).

2.3.3 Maternal measures

Insulin resistance was measured in mothers in the MiG trial using the homeostatic model assessment of insulin resistance at trial entry (HOMA-IR-Trial; 30 ± 2.6 weeks gestation) and at 36 weeks gestation (HOMA-IR-36). Additional perinatal records were obtained.

2.3.4 Recording procedures

Children were seated with their hands and forearms supported. Adhesive Ag/AgCl bipolar surface electrodes were applied over the right first dorsal interosseous (FDI) hand muscle to obtain surface electromyography (EMG) recordings. EMG signals were amplified (x1000; 1902 amplifier; CED), bandpass filtered (20 Hz–1 kHz) and digitized at 5 kHz (1401 interface; CED), and were stored offline for later analysis. Researchers were blinded to the treatment status of the participant's mother when collecting and analysing data.

2.3.5 Transcranial magnetic stimulation (TMS)

TMS is a non-invasive brain stimulation technique in which the motor cortex is electromagnetically stimulated to produce a motor evoked potential (MEP) recorded in a contralateral muscle using EMG (Di Lazzaro and Rothwell, 2014). Motor cortical excitability was assessed with single-pulse TMS applied to the left primary motor cortex (M1) representation of the right FDI muscle using a 70mm figure-of-eight coil connected to a monophasic Magstim 200^2 stimulator (Magstim Co, Whitland, UK). The coil was oriented with the handle pointing postero-laterally at a 45° angle to the sagittal plane, producing a posterior-anterior current flow across M1. The optimal site for consistently evoking MEPs in the FDI was determined and marked on the scalp. Resting motor threshold (RMT) was determined as the lowest TMS intensity required to evoke MEPs of at least 50 µV peak-to-peak amplitude in the resting FDI in at least five of ten consecutive trials. Active motor threshold (AMT) was assessed while the subject maintained approximately 10% of their maximum voluntary contraction and determined as the lowest TMS intensity required to evoke MEPs of at least 200 µV peak-to-peak amplitude in at least five of ten consecutive trials. The TMS intensity that evoked MEPs of ~1 mV peak-to-peak amplitude ($SI_{1\,mV}$) was also determined and used throughout the experiment for evoking test MEPs (Pitcher et al., 2015).

2.3.6 LTD-like neuroplasticity induction with cTBS

Continuous theta burst stimulation (cTBS; a repetitive TMS protocol) over M1 was used to induce LTD-like suppression of MEP amplitudes. Pharmacological studies

indicate cTBS-induced MEP suppression is NMDA-receptor-dependent and similar mechanistically to LTD (Huang et al., 2007). An air-cooled figure-of-eight coil connected to a Magstim Super Rapid stimulator (Magstim, Whitland, UK) was used to apply repetitive TMS to the optimal site for stimulating the right FDI. The cTBS protocol consists of 600 pulses applied in bursts of three pulses at 50 Hz, repeated at 5 Hz for a total of 40 s (Huang et al., 2005). Stimulation intensity was set to 80% of AMT. MEPs were recorded in blocks of 15 trials prior to cTBS (i.e. baseline) and at 0, 5, 10, 20 and 30 minutes following cTBS. The peak-to-peak amplitudes of the 15 MEPs in each block were measured and a mean amplitude calculated. Changes in MEP amplitude relative to baseline MEP amplitude were used as an index of LTD-like plasticity (Pitcher et al., 2012a). All MEPs were recorded at high gain and any with obvious EMG activity in the 200 ms before the TMS stimulus were discarded. Mean variability (coefficient of variation, COV) of MEP amplitudes was also calculated to determine correlation with plasticity induction (Hordacre et al., 2017).

2.3.7 Salivary cortisol

Saliva samples were obtained from each child immediately before TMS baseline measures (13:22 ± 0.25 hr) using a Salivette (Sarstedt) and standardized collection procedures. Salivettes were centrifuged to obtain saliva, which was stored at -20°C until assayed. Twenty-five microliter aliquots of saliva were assayed in duplicate for cortisol by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (HS-Cortisol; Salimetrics).

2.3.8 Statistical analysis

Data were analysed using SPSS (Version 24; IBM). Repeated-measures ANCOVAs (rmANCOVAs) with polynomial contrasts were performed with Bonferroni's correction for multiple comparisons. Between-subject factors were GDM group (GDM or Control) and Treatment Group (Control, Insulin, or Metformin). The within-subject factor was Time (0, 5, 10, 20, or 30 minutes post-cTBS). All potential covariates were assessed for statistical significance. Gestational age at birth (days) was found significant and remained as a covariate. Separate rmANOVAs for each treatment and GDM group were conducted on the raw MEP data with the factor Time

(baseline, 0, 5, 10, 20, and 30 minutes post-cTBS). *Post-hoc* comparisons were conducted where indicated to compare the post-cTBS and baseline MEP amplitudes. Linear regression analyses were used to examine the associations between cortisol, maternal characteristics, and mean and peak response to cTBS (Table 2.1). Mediation analyses, using the PROCESSING macro for SPSS (see Hayes and Rockwood, 2017), were performed using cortisol as a potential mediator. All associations were checked statistically and visually for the presence of potential outliers and to check for normality of data.

2.4 RESULTS

2.4.1 Baseline characteristics and cortical excitability

RMT (% maximum stimulator output) was higher in the GDM group (57.9 ± 12.3, $N = 42$) than in the Control group (44.9 ± 5.7, $N = 12$) when corrected for age ($F_{(1,51)} = 6.41$, $p = 0.003$, $\eta_p^2 = 0.199$) (Figure 2.1), but did not differ between the Metformin (58.8 ± 12.7, $N = 22$) and Insulin (56.9 ± 12.1, $N = 21$) groups ($p = 0.743$). Similarly, the stimulator intensity (% maximum stimulator output) required to evoke test MEPs (SI_{1mV}), AMT and cTBS intensity was higher in the GDM group (SI_{1mV}, 70.0 ± 14.3; AMT, 52.6 ± 7.9; cTBSi, 42.2 ± 5.8; all $p < 0.001$) compared with controls (SI_{1mV}, 57.3 ± 13.8; AMT, 43.9 ± 7.1; cTBSi, 35.2 ± 5.5), but did not differ between the Metformin and Insulin groups (all $p > 0.364$). Children in the Control group were older than those in the Metformin and Insulin groups, but there was no effect of age on any primary analyses when correcting for salivary cortisol concentration or GA. There were no effects of child sex or level of maternal education on any of the primary outcomes.

Ten of the GDM-exposed participants (22%) were unsuitable for the neuroplasticity analyses; 3 had RMTs which exceeded the maximum stimulator output, and 7 had assessable RMTs ($M = 75.9$) but were not able to produce supra-threshold test MEPs approaching 1mV (SI_{1mV}) required for the neuroplasticity induction protocol. The 12 Control participants were all able to be assessed (difference, $\chi^2 = 3.23$, $\varphi = 0.238$, $p = 0.072$).

2.4.2 LTD-like neuroplasticity is reduced in children exposed to GDM

The neuroplastic response to cTBS was blunted or absent in the GDM group compared with the Control group after correcting for GA ($F_{(1,42)} = 8.63$, $p = 0.005$, $\eta_p^2 = 0.170$) (Figure 2.2). There was a main effect of Time for the Control participants ($p = 0.008$), with reductions in MEP amplitudes from baseline at all post-cTBS time-points (all $p \leq 0.038$). There was no effect of Time for GDM-exposed participants ($p = 0.092$), and no change in MEP amplitudes from baseline at any time point (all $p \geq 0.163$). There was no effect of time in the Insulin ($F_{(5)} = 1.94$, $p = 0.097$) or Metformin ($F_{(5)} = 1.02$, $p = 0.369$) groups. When correcting for GA, mean ($F_{(1,45)} = 8.86$, $p = 0.005$, $\eta_p^2 = 0.171$) and peak ($F_{(1,45)} = 4.96$, $p = 0.031$, $\eta_p^2 = 0.106$) post-cTBS MEP suppression were greater in Control children than in GDM-exposed children. This was not accounted for by maternal BMI. *Post-hoc* analysis revealed that Control children demonstrated a greater LTD-like response to cTBS than insulin-exposed children ($p = 0.047$, 95% CI [-55.01 – -0.31]). Control and Metformin ($p = 0.153$, 95% CI [-50.02 – 5.38]) and Metformin and Insulin groups ($p = 1.00$, 95% CI [-30.61 – 19.92]) did not differ in response to cTBS.

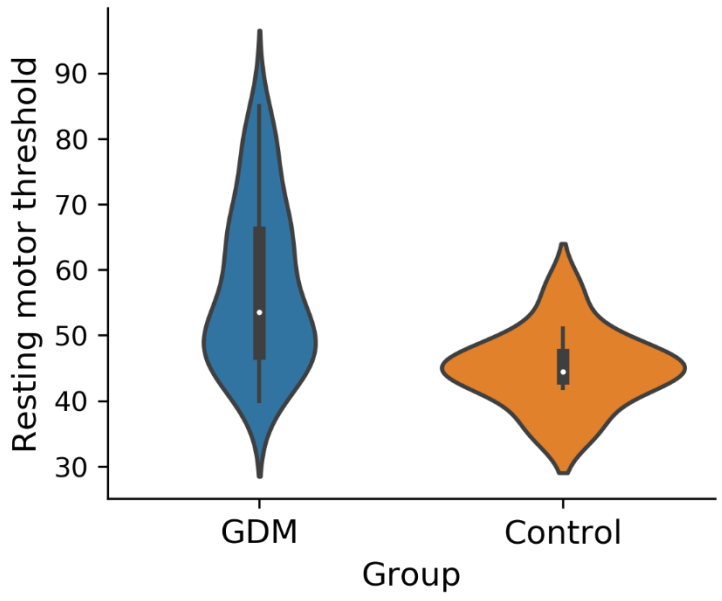


Figure 2.1: Resting motor threshold*. RMT was higher ($p < 0.001$) in the GDM group, indicating lower cortical excitability. RMT values represent % of maximum stimulator output.*

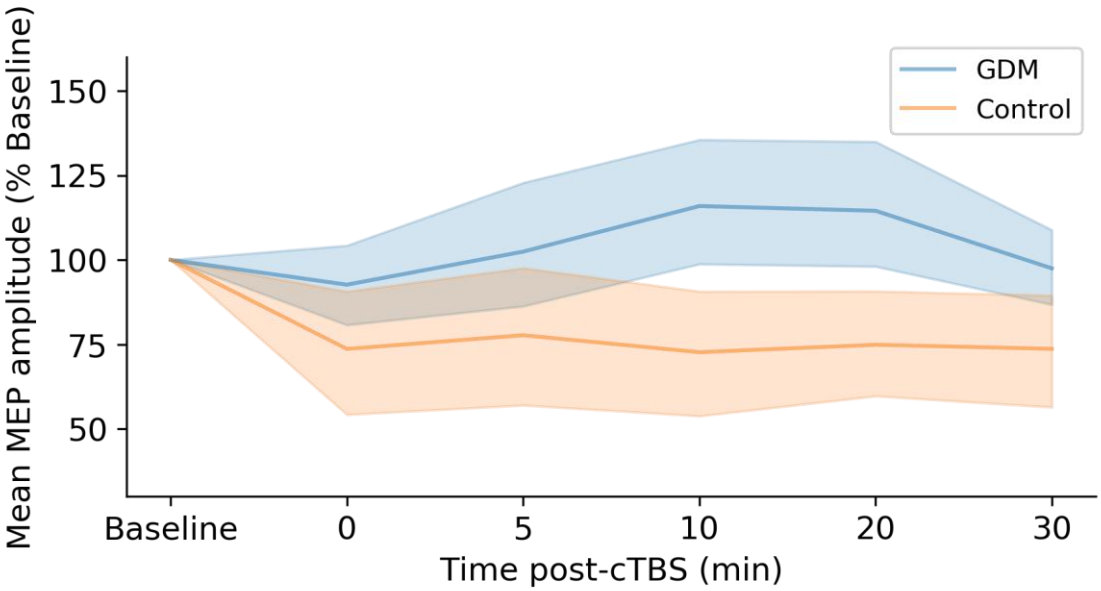


***Figure 2.2*: *Comparison of cTBS response between groups*.** *The control group exhibited significantly greater suppression of MEP amplitudes following cTBS, indicating a greater LTD-like neuroplastic response. Shaded areas represent 95% confidence intervals.*

2.4.3 Maternal insulin resistance and metabolic markers are associated with neurophysiological outcomes

Higher maternal insulin resistance at 36 weeks gestation (HOMA-IR-36) was associated with reduced mean ($F_{(1,25)}$ = 6.97, p = 0.014, R^2 = 0.225; Figure 2.3) and peak ($F_{(1,25)}$ = 4.20, p = 0.052, R^2 = 0.149) MEP suppression following cTBS in GDM-exposed children. HOMA-IR at trial entry also explained a near-significant proportion of variation in mean MEP suppression ($F_{(1,25)}$ = 3.91, p = 0.061, R^2 = 0.157). Further analysis of HOMA-IR-36 revealed an association with mean MEP amplitude variability throughout the experiments (coefficient of variation, COV), whereby higher maternal HOMA-IR-36 was associated with lower MEP COV (p = 0.045, R^2 = 0.160) when corrected for child age. Table 2.1 shows additional associations that were observed between child neurophysiology and maternal and neonatal metabolic and inflammatory markers.

Within the GDM group, a comparison was made to determine whether a previous GDM diagnosis is associated with different outcomes in children, as a previous

diagnosis would place a woman in 'high risk' status and potentially lead to earlier detection and treatment of GDM. Indeed, women with a previous diagnosis of GDM were assigned to treatment earlier (N = 8, 28.7 ± 3.2 weeks gestation) than both women in their first pregnancy (N = 13, 31.5 ± 1.1 weeks gestation, p = 0.007) and primiparous women without a prior GDM diagnosis (N = 11, 30.9 ± 2.7 weeks gestation, p = 0.025). Children of women with a previous diagnosis of GDM (51.7 ± 26.8) had stronger peak LTD-like responses to cTBS than did first-born children (87.9 ± 32.3, p = 0.004), while first-born children had stronger responses than children of women with a previous pregnancy not complicated by GDM (64.4 ± 14.1, p = 0.035).

In the GDM group, children who were heavier at birth had lower cortical excitability (p = 0.016, R^2= 0.168) and salivary cortisol (p = 0.049, R^2= 0.106) than did children who were lighter, and this was associated with greater cord C-peptide, glucose, and triglycerides. Similar observations were made with birth weight centiles.

2.4.4 Low salivary cortisol is associated with a reduced LTD-like neuroplastic response

Diurnal variations in human salivary cortisol concentrations are well characterised and affect neuroplasticity induction depending on time of day of the test (Sale et al., 2008, Pitcher et al., 2012a). Here, we found no group difference in the time of day (p = 0.953; mean = 13:22 h ± 1.9 hrs) at which the cTBS experiments were performed and no effect of time of day on any of the analyses. Immediately before cTBS, cortisol concentration ranged between 2.3–6.4 nmol/L in the Control group, 0.8–4.2 nmol/L in the Insulin group, and 0.9–4.9 nmol/L in the Metformin group. Mean (± SD) concentrations differed between the Control group (4.1 ± 1.6 nmol/L) and both the Metformin (2.1 ± 1.2 nmol/L; p < 0.001) and Insulin (1.9 ± 1.1 nmol/L; p < 0.001) groups. The Metformin and Insulin groups did not differ from each other (p = 0.690). Normal salivary cortisol concentrations in children are reported to be approximately 4 nmol/L at the corresponding time of day (McCarthy et al., 2009), suggesting that the values in our GDM-exposed subjects were low.

Cortisol explained over 20% of the variation in the mean response to cTBS ($F_{(1,42)}$ = 10.91, p = 0.002, R^2 = 0.210) (Figure 2.4). Cortisol was negatively associated with both RMT ($F_{(1,46)}$ = 4.92, p = 0.032, R^2 = 0.099) and SI_{1mV} ($F_{(1,46)}$ = 5.48, p =

0.024, R^2= 0.115), indicating that higher cortisol concentrations were associated with greater cortical excitability. Mediation analysis did not detect a mediation effect of cortisol on the relationship between GDM group and cortical excitability (indirect effect; SE = 2.70, 95% CI: -7.23, 3.52), suggesting GDM influences excitability directly or through other mechanisms. Conversely, cortisol was found to mediate the relationship between GDM group and mean neuroplastic response (indirect effect; SE = 9.06, 95% CI: -40.02, -4.37) with a strong direct effect on plasticity (B = -9.67, SE = 4.12, p = 0.024).

Higher mean MEP COV was also associated with stronger peak MEP suppression following cTBS (p = 0.011, R^2 = 0.142) and with higher cortisol concentration (p = 0.010, R^2 = 0.149). Group analysis revealed lower mean MEP COV in the Insulin group (M = 0.45) compared with the Control group (M = 0.60; p = 0.015), while the Metformin group (M = 0.54) had values similar to the Control (p = 0.258) and Insulin (p = 0.060) groups.

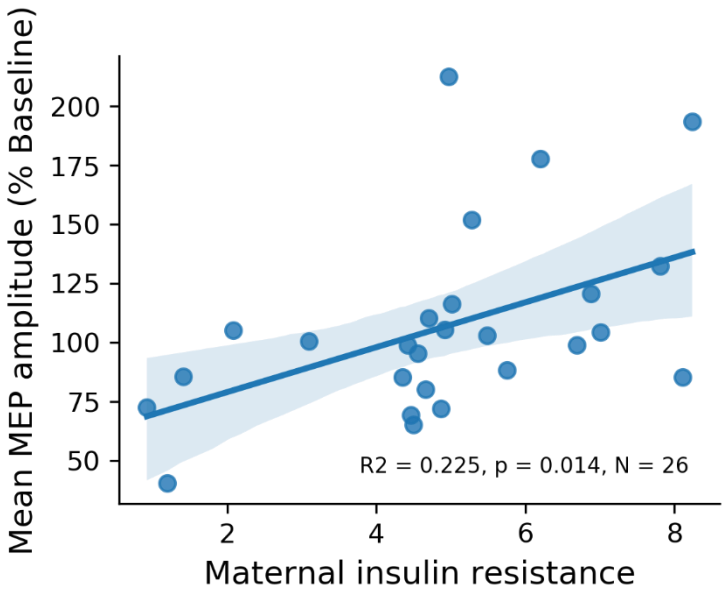


Figure 2.3: Association between cTBS response and maternal insulin resistance. *Greater maternal insulin resistance (HOMA-IR) at 36 weeks gestation was associated with responses to cTBS that were not consistent with LTD-like plasticity.*

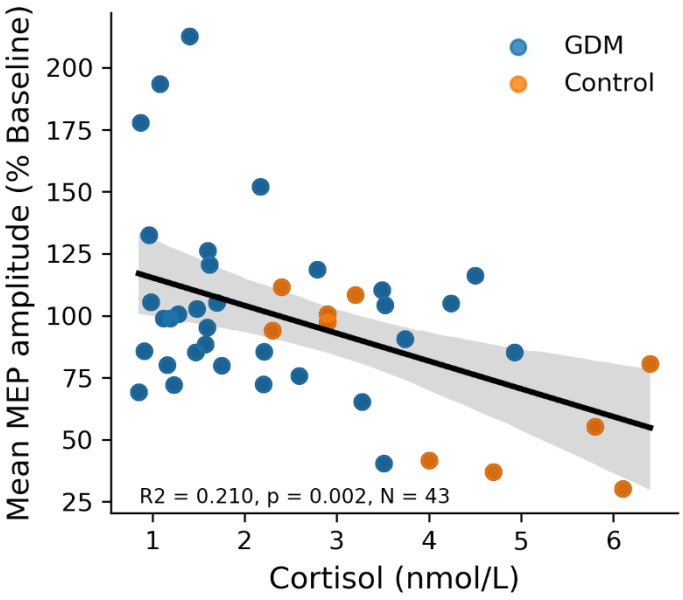


Figure 2.4: Association between cTBS response and salivary cortisol. *Higher salivary cortisol concentrations were associated with stronger LTD-like responses to cTBS (*p *= 0.002). Cortisol concentrations were higher in the control group (*p *< 0.001).*

Table 2.1: Associations between maternal and neonatal markers and child neurophysiological outcomes in GDM-exposed children*. Significant correlations and their direction are indicated with arrows (↑, positive).* * $p < 0.05$, ** $p < 0.01$. *'Entry', measurements at trial entry; '36 wks', measurements at 36 weeks gestation; IR, insulin resistance; FPG, fasting plasma glucose; #, correlation where* $0.05 < p < 0.08$.

	RMT	SI_{1mV}	Mean LTD	Response Direction	MEP COV	Cortisol
Maternal and neonatal characteristics						
IR – entry			# ↓			
IR – 36 wks			↓ * *n* = 26	↑ ** *n* = 26		
FPG – rand					↓ * *n* = 28	
FPG – 36 wks					↓ ** *n* = 28	
Insulin – 36 wks			↓ ** *n* = 26	↑ ** *n* = 26		
C-peptide – rand			#			
Cholesterol – 36 wks			↓ * *n* = 27			
Maternal BMI – rand						
Previous GDM diagnosis	↑ * *n* = 41		# ↑		# ↑	
Cord C-peptide					↓ * *n* = 14	
Cord C-reactive protein				↑ ** *n* = 29		
Cord triglycerides			↓ * *n* = 25	↑ ** *n* = 25		
Neonatal hypoglycaemia			↓ * *n* = 33	↑ * *n* = 33		↓ * *n* = 38
Apgar-5		↓ * *n* = 35				
Baby weight (g)		↑ * *n* = 34				↓ * *n* = 37

2.5 DISCUSSION

To our knowledge, this is the first study to provide neurophysiological evidence that exposure to GDM *in utero* is associated with reductions in cortical excitability and neuroplasticity—and an associated reduction in basal salivary cortisol—at 11–13 years of age in the offspring. Child neurophysiological responses were associated with several maternal perinatal factors—most prominently maternal insulin resistance and inflammation. Importantly, these effects were present in children whose mothers were diagnosed and treated per the Australian clinical guidelines current at the time of the trial, and who were monitored closely and treated within a randomised controlled trial. Neurophysiological outcomes in children exposed to metformin were comparable with those whose mothers were treated with insulin.

Children in our GDM-exposed sample exhibited reduced cortical excitability, possibly reflecting suboptimal or delayed neurodevelopment of underlying brain structures. Pharmacological studies show that the RMT reflects the properties of neuronal membrane voltage-gated sodium channels. Additionally, imaging studies show that the RMT reflects white matter maturation, myelination, and structural integrity, and individuals with lower RMTs have better white matter development and neurodevelopmental outcomes (Ziemann et al., 2015). For example, children born preterm have been shown to have compromised development of both white and grey matter, microstructural alterations, and altered neural connectivity remaining into adolescence (Counsell et al., 2008), which are believed to underlie the decreased cortical excitability and LTD-like plasticity observed in adolescents born preterm (Pitcher et al., 2012b, Pitcher et al., 2012a). While RMT is high in very young children and gradually reduces to near adult values by 12–14 years of age (Garvey and Mall, 2008), the relatively high RMTs seen in the GDM-exposed children were not explained by age at assessment, GA at birth, or cortisol independently. Additionally, RMTs could not be acquired in three GDM-exposed children, and their data are not included in the RMT comparison, so the effect size is likely to be underestimated. Given the association between cortical excitability and motor skills—particularly manual dexterity (Pitcher et al., 2012b)—these results suggest that alterations to cortical excitability may partially explain the motor function deficits experienced by some individuals exposed to GDM.

Overall, in children exposed to GDM the neuroplastic response to cTBS was absent or reduced when compared with their non-GDM-exposed peers, suggesting that GDM contributes to altered development of the neural mechanisms underlying LTD-like synaptic plasticity. Altered neurophysiological responses in children were most strongly predicted by maternal IR measured both before commencement of pharmacologic treatment in late second to early third trimester, as well as at 36 weeks gestation following 1-2 months of metformin or insulin treatment. Interestingly, maternal plasma glucose was not found to be associated directly with neuroplasticity, although this finding was based on two single measures of fasting plasma glucose, which may not reflect overall maternal glycaemia. However, several other maternal and neonatal metabolic markers were related. Of note, higher cord blood triglyceride and C-reactive protein concentrations were associated with poorer neuroplastic responses. Thus, the relationship between GDM and cortical development may be complex and involve, primarily, inflammatory mechanisms. IR measurements may act as composite measures of the sequelae of GDM, possibly by capturing inflammation, altered lipid metabolism, and altered fetal growth. Indeed, maternal IR was associated with maternal glucose, BMI, inflammatory markers, and cord triglycerides. Similar observations have been made in animal models, whereby maternal inflammation and hyperglycaemia alter fetal insulin signalling and, consequently, neurodevelopment by, for example, retarding dendritic development (Jing et al., 2014).

Neonatal hypoglycaemia also strongly predicted both plasticity and salivary cortisol in children, and this was not associated with the maternal factors mentioned above. Thus, alteration to the response to TMS in children is likely complicated further by neonatal metabolic factors and the immediate post-natal environment. Although GDM increases the risk of neonatal hypoglycaemia, we had limited access to neonatal records for the control group, so we cannot determine if neonatal hypoglycaemia similarly affects children not exposed to GDM.

Conversely, prior maternal diagnosis of GDM appeared to result in better outcomes in children. Children whose mothers had a previous pregnancy complicated by GDM had stronger neuroplastic responses than those whose mothers had not previously experienced GDM. Interestingly, the only systematic, observable difference between these groups was the time at which mothers were assigned a treatment; those with a previous GDM diagnosis were treated around two weeks

earlier, likely due to their increased risk status. Thus, earlier treatment may be responsible for better outcomes in children. However, this too is likely more complex, and may involve metabolic differences, as time of treatment allocation (weeks gestation) did not predict neuroplasticity independently.

Although the importance of the neuroplastic response to TMS to functional outcomes is unclear, it is known to involve mechanisms which underlie synaptic plasticity, learning, and memory, including activation and modulation of the NMDA receptor at excitatory synapses (Ziemann et al., 2015). Additionally, theta burst stimulation is known to influence motor learning (Teo et al., 2011). Thus, the response to plasticity induction is assumed to reflect the activity of the underlying synaptic machinery and therefore to be of importance to the associated functional domains. The extent to which the outcomes here relate to, for example, cognition and motor skills, should be explored further.

Low afternoon salivary cortisol observed in the GDM group represents one possible explanation for the altered neuroplastic responses exhibited by GDM-exposed children. Indeed, cortisol was found to be a mediator between GDM group and mean cTBS response. However, cortisol cannot explain entirely the variation in plasticity and did not influence or mediate the relationship between IR and cTBS response. Thus, it may be most likely that reduced neuroplastic response results from both a direct neurophysiological influence of GDM on cortical development and an indirect influence of sub-normal circulating cortisol, as corticosteroids alter both the function and subunit composition of NMDA-type glutamate receptors and hence synaptic plasticity (Tse et al., 2012), including the response to cTBS (Pitcher et al., 2012a).

It is important to note that we collected only a single measurement of cortisol. Given the importance of circadian patterns of cortisol release to cognitive (Gibson et al., 2010) and neuropsychiatric functioning (Wulff et al., 2010), we plan to explore further this relationship by assessing the cortisol awakening response in our sample to determine whether circadian patterns of cortisol secretion are altered.

To conclude, we found that gestational diabetes is associated with altered cortical and neuroendocrine development in offspring, but the extent to which these effects are causally linked, or are separate manifestations of an altered perinatal environment, is unclear. However, the severity of maternal insulin resistance prior to and during treatment, in addition to maternal and cord metabolic markers, appears to

predict these outcomes in offspring. Given that hyperglycaemia in these women was strictly controlled as per clinical guidelines for the remainder of their pregnancy, the data presented here suggest that the fetal brain is susceptible to even mild to moderate maternal insulin resistance and inflammation earlier in pregnancy, regardless of subsequent treatment, and that this represents a risk for impaired cortical development and HPA function into childhood and adolescence. While some 'high-risk' patients are diagnosed earlier, GDM is usually not diagnosed before the 24^{th}–28^{th} weeks of gestation. However, our findings (and others) suggest that by this time the neural pathology of fetuses exposed to GDM may already be established, and that prevention or reversal of associated adverse perinatal outcomes may be limited (Georgiou et al., 2011). Although our study is relatively small, the convergence of several characteristics of both mother and child on the primary outcomes strengthens our conclusions. Nevertheless, these data should be replicated and extended, and we plan to conduct more comprehensive neurodevelopmental and neuroendocrine assessments of this cohort in the future. Given our results, we suggest that methods for early prediction of such outcomes, such as HOMA-IR, metabolic and proteomic markers, and modelling approaches, may be an important step in developing more sensitive indices of risk (Ozgu-Erdinc et al., 2015). Early pharmacologic interventions, particularly in pregnancies at high risk or in which lifestyle advice is unlikely to suffice, may help to prevent the potential abnormalities in cortical physiology and neuroendocrine function identified in this study. Reassuringly, metformin appears safe with respect to those aspects of cortical development assessed here and may be an appropriate treatment for early GDM.

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Chapter 3: The association between I-wave recruitment and the response to cTBS is age- and sex-dependent

3.1 FOREWORD

In the previous chapter, I presented evidence suggesting that maternal GDM is associated with an altered neuroplastic response to cTBS. This response was not simply 'smaller', but also more variable. As I will explain below, cTBS is inherently variable, but the results described above suggested that the GDM group may exhibit more than the 'normal' amount of variability in response to stimulation. Shortly after completing this study, I learned of a TMS technique that had the potential to explain some of the variability in cTBS response. Therefore, I planned a study, which is presented in Chapter 4, to investigate whether this marker differed in the GDM group and therefore whether there was indeed some systematic difference in the variability of cTBS responses in the GDM group. Importantly, such an effect would likely be independent of synaptic plasticity, so insights here would further inform our understanding of the neurophysiological consequences of GDM exposure. However, the technique in question had to date only been used in adults. Therefore, I thought it prudent to validate the technique in younger participants while examining whether other characteristics, namely age and sex, altered the underlying phenomenon. Due to the time constraints of a PhD, the study, presented in this chapter, was conducted concurrently with the GDM group study in Chapter 4; however, as will become clear below, the surprising results of this study somewhat complicate the data presented in Chapter 4.

3.2 ABSTRACT

The neuroplastic response to continuous theta burst stimulation (cTBS) is inherently variable. The measurement of I-wave latencies has been shown to strongly predict the magnitude and direction of the response to cTBS, whereby longer latencies are associated with stronger LTD-like responses. However, potential differences in

this association relating to age and sex have not been explored. We performed cTBS and measured I-wave recruitment (via MEP latencies) in 60 participants (26 female) ranging in age from 11 to 78 years. In contrast to previous studies, there was not a significant association between I-wave latencies and cTBS response at the group level ($p = 0.272$, $R^2 = 0.021$). However, there were interactions between I-waves and both age and sex when predicting cTBS response. Subgroup analysis revealed that preferential late I-wave recruitment predicted cTBS response in adolescent females (< 18 yrs; $p = 0.0008$, $R^2 = 0.623$), but not in adolescent ($p = 0.376$, $R^2 = 0.039$) or adult males ($p = 0.572$, $R^2 = 0.033$). A trend was observed visually in adult females ($p = 0.414$, $R^2 = 0.068$). These data suggest that the generalisability of I-wave measurement in predicting the response to cTBS may be lower than initially believed and that the use and interpretation of this technique may require caution.

3.3 INTRODUCTION

Transcranial magnetic stimulation (TMS) is a form of non-invasive brain stimulation used to stimulate the brain by delivering magnetic pulses through the intact scalp. Repetitive TMS (rTMS) involves delivering repeated pulses and, depending on the stimulation parameters, can produce changes in excitability of the stimulated area outlasting the stimulation period. Evidence suggests that these changes involve activity of N-Methyl-D-Aspartate (NMDA) receptors and therefore represent the early stages of synaptic plasticity in the human brain (Ziemann et al., 2008). rTMS techniques are used commonly in neurophysiological research and in the clinical management of several neurological and psychiatric diseases, including major depression (Guo et al., 2017, McClintock et al., 2018, Brunoni et al., 2019). One of the most common forms of rTMS is continuous theta burst stimulation (cTBS), which has been shown to decrease cortical excitability for up to 60 minutes (Huang et al., 2005).

A major issue with rTMS, including cTBS, is that there is significant intra- and inter-subject variability in responses (Pellegrini et al., 2018, Vallence et al., 2015, Jannati et al., 2019, Jannati et al., 2017). Importantly, responses vary not only in magnitude but in the direction of excitability change. cTBS, which is designed to induce inhibition-like changes in excitability, often induces changes consistent with

facilitation of excitability; as a result, studies often find weak or no group-wide effect of cTBS.

Factors that are known to influence the response to rTMS include age, gender, cortisol, attention, genetics, and muscle activity (when assessing the motor cortex) (Ridding and Ziemann, 2010). However, even when correcting for these factors, it is not entirely clear whether the variability in responses is due to intrinsic differences in the mechanisms underlying synaptic plasticity, or rather to differences in the populations of cortical neurons that are stimulated by TMS pulses. Different populations of neurons are more excitable in different people at different times, and different cortical networks can be more strongly influenced by rTMS, leading to variation in either the magnitude or direction of the response (Hamada et al., 2013). The descending volley evoked by a TMS pulse consists of a series of components: the early direct wave (D-wave) is produced by direct activation of corticospinal output cells, while the later components, known as indirect waves (I-waves), result from either monosynaptic input to corticospinal neurons from layer II/III interneurons (early I-waves) or more complex, oligosynaptic circuits including inhibitory neurons (late I-waves) (Di Lazzaro et al., 2012, Hamada et al., 2013). Recent research (Hamada et al., 2013, Huang and Mouraux, 2015, Hordacre et al., 2017) indicates that individuals in whom TMS is more likely to recruit late I-waves respond more strongly to TBS. The reason for this is not entirely clear, but is probably related to greater sensitivity of late I-wave-generating circuits to the effects of TBS (and other forms of NIBS) (Hamada et al., 2013). These researchers found a measure of I-wave recruitment to account for a substantial proportion of the variation in the response to TBS; therefore, I-wave measurement appears to be a promising technique to better predict and explain variation in the response to NIBS techniques and to optimise these techniques in therapeutic settings.

However, previous studies have not examined whether there are age- or sex-related differences in this relationship. Therefore, we aimed to test whether late I-wave recruitment could predict the response to cTBS similarly in males and females, whether the effect was present in adolescents, and whether the association changes in strength with increasing age.

3.4 METHODS

3.4.1 Ethical approval

All procedures were approved by the University of Adelaide Human Research Ethics Committee and conducted in accordance with the Declaration of Helsinki (2008 revision). Participants were pre-screened for contraindications to TMS (Rossi et al., 2009a) and provided written, informed consent.

3.4.2 Subjects

60 participants were recruited to the study (26 female). Participants ranged in age from 11 to 78 years (mean = 31.9, SD = 22.4, median = 23.0).

3.4.3 Electromyography

Participants were seated with their hands and forearms supported. Adhesive Ag/AgCl bipolar surface electrodes were applied over the right first dorsal interosseous (FDI) hand muscle to obtain surface electromyography (EMG) recordings. EMG signals were amplified (x1000; 1902 amplifier; CED), bandpass filtered (20 Hz–1 kHz), and digitized at 5 kHz (1401 interface; CED) and were stored offline for later analysis

3.4.4 Transcranial magnetic stimulation (TMS)

Motor cortical excitability was assessed with single-pulse TMS applied to the left primary motor cortex (M1) representation of the right FDI muscle using a 70mm figure-of-eight coil connected to a monophasic Magstim 200^2 stimulator (Magstim Co, Whitland, UK). Previous research has shown clearly that different descending volleys are elicited by single-pulse TMS depending on the direction of current flow across the motor cortex. Posterior-anterior (PA) current preferentially elicits early I-waves, whereas anterior-posterior (AP) current can recruit late I-waves. High-intensity latero-medial (LM) current evokes D-waves (Di Lazzaro et al., 2012, Hamada et al., 2013).

The protocol used here is equivalent to that described by Hamada et al. (2013). 3 different coil orientations were used to evoke motor-evoked potentials (MEPs): (1) PA-directed currents were produced by the coil held posterolaterally at an angle of about 45° to the midline; (2) AP-directed currents were elicited by placing the coil 180° to the PA current position; and (3) LM-directed currents were produced with the coil placed leftwards (90° from midsagittal line).

Using PA currents, the optimal site for consistently evoking MEPs in the FDI was determined and marked on the scalp, and resting motor threshold (RMT) was determined as the lowest TMS intensity required to evoke MEPs of at least 50 μV peak-to-peak amplitude in the resting FDI in at least five of ten consecutive trials. The active motor threshold (AMT) was defined as the lowest intensity to evoke an MEP of 200 μV, or visibly distinct from background EMG activity, in more than 5 of 10 consecutive trials while subjects maintained approximately 10% of maximum voluntary contraction of the target muscle, measured and monitored using a digital oscilloscope. We measured AMT with PA, AP, and LM currents (AMTpa, AMTap, and AMTlm, respectively). The TMS intensity that evoked MEPs of ~1 mV peak-to-peak amplitude (SI_{1mV}) was also determined, using PA current, and used throughout each experiment for evoking test MEPs (Pitcher et al., 2015). SI1mV was recorded 15 minutes after AMT measurements, following a period of muscle relaxation. Intensities are expressed as % of maximum stimulator output (MSO). All measurements were performed at the hotspot determined by PA currents, as previous research has shown that the direction of current does not significantly influence the position of the hotspot (Sakai et al., 1997, Arai et al., 2005).

3.4.5 Measurement of MEP onset latency to assess I-wave recruitment

The level of late I-wave recruitment was quantified by measuring MEP onset latencies using AP current in pre-contracted muscle at near-threshold stimulus intensities. AP MEP latencies were compared with MEP latencies using PA and LM currents. The rationale is that, in individuals in whom late I-wave generating circuitry is more excitable, AP MEPs will have longer latencies than PA and LM MEPs, because more complex circuits are involved in stimulating output cells (with AP current). Indeed, AP MEP latencies are often observed to be 1–2 ms longer than PA MEPs, and

4–5 ms longer than LM MEPs (Day et al., 1989). The difference between LM and AP latencies (AP-LM) was used as the primary index of late I-wave recruitment as LM latencies are highly consistent, allowing reliable estimation of I-wave recruitment (larger AP-LM values indicating later I-waves).

We measured onset latencies of MEPs using PA, AP, and LM currents with stimulus intensities of 110% AMTpa, 110% AMTap, and 150% AMTlm (or 50% MSO in subjects whose 150% AMTlm did not reach 50% MSO) (Hamada et al., 2013). Relatively high stimulus intensities were used with LM current to ensure a D-wave was evoked (Werhahn et al., 1994). 20 MEPs each with PA and AP currents, and 10 MEPs with LM currents, were recorded. After every tenth trial, subjects were asked to relax their hand to minimise fatigue. Signal (Version 6; Cambridge Electronic Design) was used to record, process and analyse EMG data. A custom-made script was used to measure MEP-onset latency, defined as the time-point where rectified EMG signals exceed a mean ± 2 SD of the pre-stimulus EMG level, expressed as milliseconds after the stimulus. For each subject and coil orientation, mean latencies were calculated and inspected directly to ensure validity.

3.4.6 LTD-like neuroplasticity induction using cTBS

cTBS was used to induce LTD-like suppression of MEP amplitudes. Pharmacological studies indicate that cTBS-induced MEP suppression is NMDA-receptor-dependent and similar mechanistically to LTD (Huang et al., 2007). An air-cooled figure-of-eight coil (rTMS coil) connected to a Magstim Super Rapid stimulator (Magstim, Whitland, UK) was used to apply repetitive TMS to the optimal site for stimulating the right FDI. The cTBS protocol consisted of 600 pulses applied in bursts of three pulses at 50 Hz, repeated at 5 Hz for a total of 40 s (Huang et al., 2005). Stimulation intensity was set to 70% of RMT (measured using the rTMS coil). MEPs were recorded in blocks of 15 trials prior to cTBS (i.e. baseline) and at 0, 5, 10, 20 and 30 minutes following cTBS with PA current. In each block, the mean of the peak-to-peak amplitudes of the 15 MEPs in each block was calculated. Changes in MEP amplitude relative to baseline MEP amplitude were used as an index neuroplasticity (cTBS response) (Pitcher et al., 2012a). All MEPs were recorded at high gain and any with obvious EMG activity in the 200 ms before the TMS stimulus

were discarded. cTBS was performed following a 15-minute rest period after baseline MEP recording to avoid metaplastic effects of prior cortical and muscle activity on neuroplastic response (Gentner et al., 2008, Iezzi et al., 2008, Goldsworthy et al., 2014).

3.4.7 Statistical analysis

Data were analysed using Python 3.7. The coefficient of variation (COV) was calculated on each participant's raw baseline MEPs (MEP COV). To test for differences in means of neurophysiological parameters between age groups and sexes, analyses of variance (ANOVA) were performed. Relationships between continuous variables, including the prediction of cTBS response by AP-LM, were tested using linear regression models. Interaction terms were added to models to test for age-dependent associations, including between age and AP-LM when predicting cTBS response. The sample was not sufficiently large to test for 3-way interactions, so regression models were constructed for each sex to assess sex-related differences in the interactions between age and physiological measures. Further, the sample was split into 4 groups: adolescent females, adult females, adolescent males, and adult males. In this way the I-waves–cTBS response association could be examined separately in, and compared between, each subgroup. Adolescent groups included participants aged between 11 and 15 years, while adult groups included participants aged between 19 and 78 years.

Type 3 sums of squares were used when calculating interaction effects. Normality of residuals was tested using normal quantile plots and the Jarque-Bera test. The assumption of linearity was tested by observing scatter plots and by plotting residuals against predicted values.

3.5 RESULTS

3.5.1 Baseline neurophysiological characteristics

Neurophysiological characteristics are presented in Table 3.1. Across all participants, there was not a net neuroplastic response to cTBS (mean = 108, SD = 33 % baseline; Greenhous-Geisser corrected p = 0.067). Individual cTBS responses

included both LTD- and LTP-like changes in MEP amplitudes (Figure 3.1). cTBS response was not predicted by Age (p = 0.782, R^2 = 0.001) or Sex (p = 0.773, R^2 = 0.001). Mean AP-LM latency difference across all participants was 3.59 ms (SD = 1.23, median = 3.56 ms), and was not associated with Age (p = 0.358, R^2 = 0.015) or Sex (p = 0.497, R^2 = 0.008).

Mean resting motor threshold (RMT) was 44.5% maximum stimulator output (SD = 7.4, median = 44.5), and was not associated with Sex (p = 0.626). Cortical excitability decreased (i.e. RMT increased) with increasing age (β = -0.100, p = 0.019, R^2 = 0.092). There were no group differences in baseline neurophysiological characteristics (all p > 0.264).

3.5.2 The association between I-wave recruitment and cTBS response is sex- and age-dependent

Amongst all participants, there was not a significant association between AP-LM and post-cTBS MEP amplitudes (p = 0.272, R^2 = 0.021). However, in a multiple regression model (p = 0.009, Adj. R^2 = 0.142) there was a significant Age*AP-LM interaction effect (β = 0.440, p = 0.001, ω^2 = 0.069) and main effects of AP-LM (β = -23.343, p = 0.001, ω^2 = 0.077) and Age (β = -1.612, p = 0.004, ω^2 = 0.054) (Figure 3.1, left).

Similarly, in a separate model, when predicting cTBS response there was a strong Sex*AP-LM interaction (β = -20.1, p = 0.005, ω^2 = 0.097) and main effects of Sex (β = 67.5, p = 0.011, ω^2 = 0.076) and AP-LM (β = 24.4, p = 0.024, ω^2 = 0.057; Model p = 0.022, Adj. R^2 = 0.114). These data suggest that the association between AP-LM and cTBS response is stronger in females (Figure 3.1, right), and might weaken with increasing age (Figure 3.1, left).

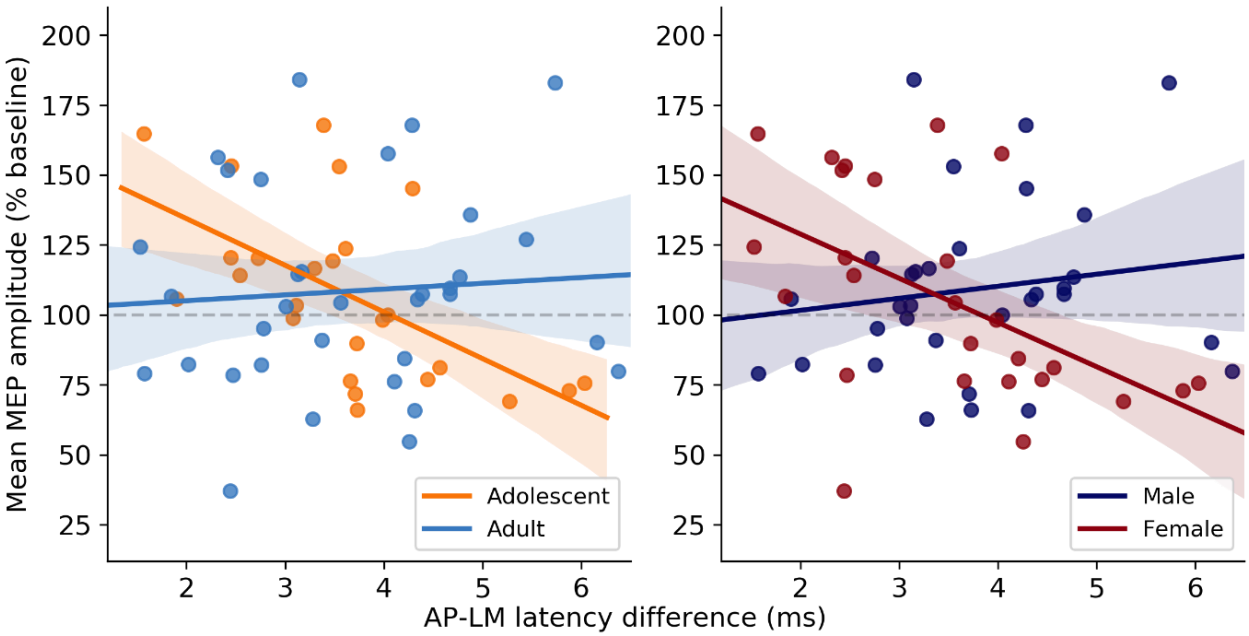


Figure 3.1: Interactions between I-wave recruitment, age, and sex when predicting cTBS response. *Later I-waves (larger AP-LM difference) were associated with stronger, LTD-like responses (lower MEP amplitudes) in adolescents and female participants. Shaded areas represent 95% confidence intervals for the regression lines.*

3.5.3 AP-LM predicts cTBS response in young females

In females greater AP-LM was associated with stronger, inhibition-like responses to cTBS (p = 0.006, R^2 = 0.272), and in a multiple regression model (p = 0.007, R^2 = 0.413) there was an Age*AP-LM interaction effect (β = 0.829, p = 0.043, ω^2 = 0.041) and main effects of Age (β = -2.7, p = 0.031, ω^2 = 0.048) and AP-LM (β = -34.6, p = 0.005, ω^2 = 0.097). Conversely, in males there were no associations or interactions between cTBS and AP-LM or Age (all $p > 0.345$, $\omega^2 < 0.0$).

Closer analysis revealed that AP-LM strongly predicted cTBS response in adolescent females (β = -20.8, p = 0.0008, R^2 = 0.623) but not in adult females (β = -11.0, p = 0.414, R^2 = 0.068), adolescent males (β = 4.7, p = 0.376, R^2 = 0.039), or adult males (β = 5.4, p = 0.572, R^2 = 0.033) (Figure 3.2). Thus, in our sample, the association

between I-wave recruitment (AP-LM) and cTBS response was present only in adolescent females.

Table 3.1: Neurophysiological characteristics by sex and age group*. Values represent means and standard deviations. AP-LM, latency difference between AP and LM MEPs (ms); cTBS response expressed as % of mean baseline MEP amplitude; MEP COV, coefficient of variation of baseline MEPs; RMT, resting motor threshold (% maximum stimulator output), AMT measures provided for PA, AP, and LM currents.*

Group (*n*)	Age	AP-LM	cTBS response	MEP COV	RMT	AMT-PA	AMT-AP	AMT-LM
Whole sample (*60*)	31.9 (22.4)	3.59 (1.23)	107 (33)	0.51 (0.20)	44.5 (7.4)	36.3 (6.3)	48.2 (9.3)	39.3 (6.4)
Adolescent female (*14*)	13.1 (1.1)	3.82 (1.33)	105 (35)	0.52 (0.18)	45.8 (6.5)	38.6 (5.8)	49.3 (7.2)	40.4 (5.2)
Adult female (*12*)	37.2 (17.4)	3.00 (0.98)	106 (41)	0.51 (0.17)	43.9 (6.1)	35.4 (5.8)	48.6 (9.8)	38.3 (5.6)
Adolescent male (*11*)	12.9 (1.2)	3.37 (0.67)	109 (27)	0.43 (0.10)	47.5 (7.8)	35.3 (6.4)	49.4 (11.9)	38.1 (3.8)
Adult male (*23*)	49.7 (21.0)	3.88 (1.42)	109 (33)	0.54 (0.26)	42.7 (8.1)	35.8 (6.8)	46.8 (9.2)	39.8 (8.3)

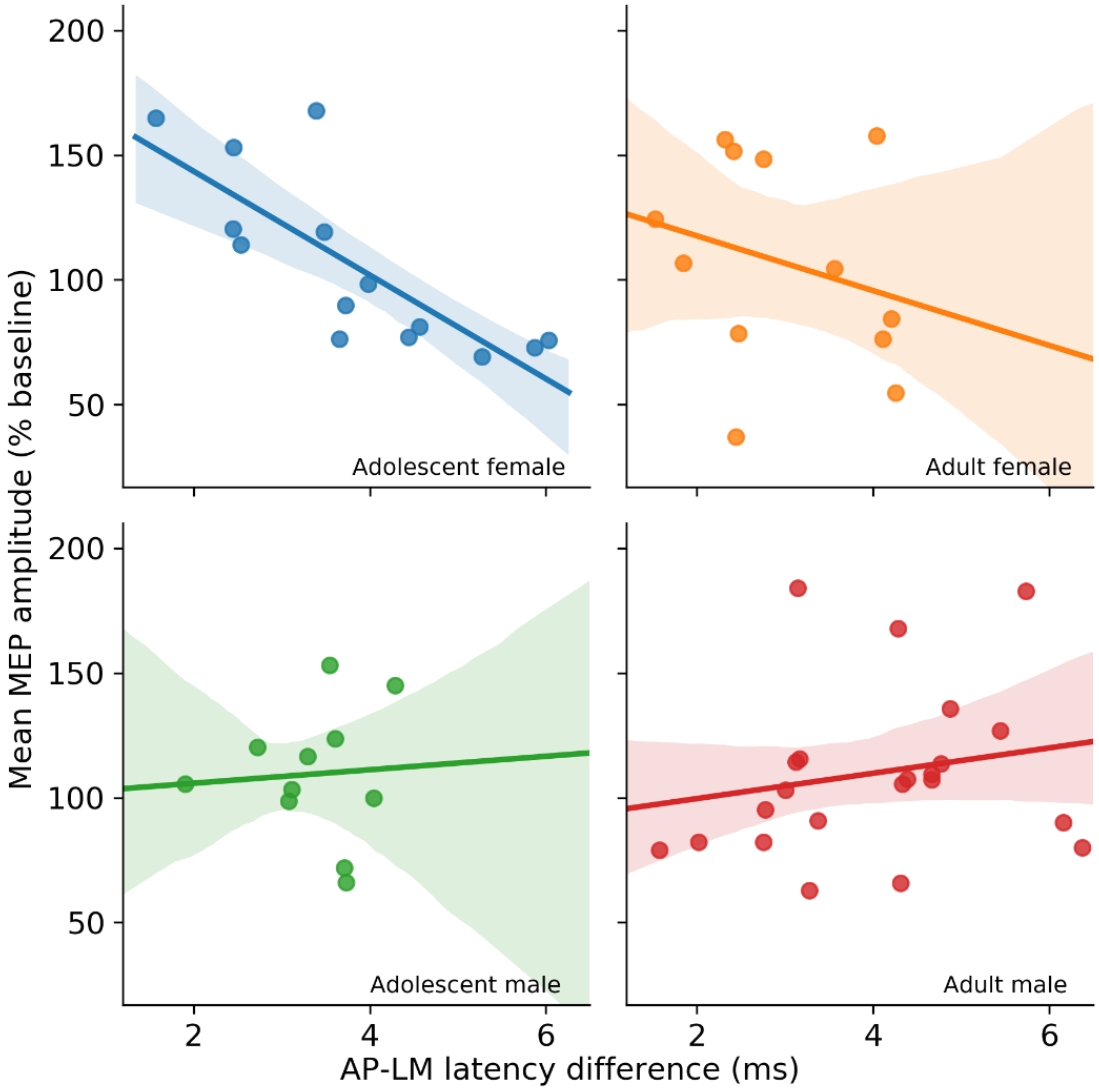


Figure 3.2: The I-waves-plasticity association differed by sex and age group. *Later I-waves (greater AP-LM latency difference) were associated with stronger, LTD-like cTBS response (lower MEP amplitude) in adolescent females, but not in other groups. Shaded areas represent 95% confidence intervals for the regression lines.*

3.6 DISCUSSION

We present evidence that the relationship between interneuron recruitment by TMS and the response to cTBS, whereby the recruitment of late I-waves is associated with stronger, LTD-like neuroplastic responses, is present only in young females.

Contrary to previous studies (Hamada et al., 2013, Huang and Mouraux, 2015, Hordacre et al., 2017) we did not find a significant association between I-wave recruitment and the response to cTBS when assessing the full sample. However, we did observe significant interactions between I-wave recruitment and both age and sex, indicating that the association was stronger in females and younger participants. These findings could, of course, be confounding each other, and because 3-way interactions would require a much larger sample (and involve difficulty in interpretation), we separated the sample into the four groups presented in the results: adolescent females, adult females, adolescent males, and adult males. Interestingly, the association between I-waves and cTBS response was not detectable to any degree in males, regardless of age. Conversely, there was a strong association between late I-wave recruitment and cTBS response in adolescent females. While the association was not significant in adult females, a trend was observed in the expected direction, and in females of all ages the effect remained, so it may be premature to conclude that the association is not present in adult females. In any case, these results were surprising, and we are not able to explain them fully. Therefore, we aim simply to report these data—while providing some possible explanations—so that they may be of use to other researchers.

The postulated marker of I-wave recruitment used here reflects the complexity of the circuits recruited by the TMS pulses (Hamada et al., 2013). More complex circuits, giving rise to later I-waves, are believed to be more sensitive to cTBS, such that individuals in whom late I-waves are recruited are also more sensitive to cTBS. The tendency for late I-waves to be recruited is presumed to reflect higher excitability of these more complex circuits, but structural differences have not been ruled out. In any case, I-wave recruitment is highly consistent in the short term (Hamada et al., 2013, Huang and Mouraux, 2015). The differences we have observed here must therefore reflect a difference in either the relationship between MEP latencies and I-wave recruitment (‘lateness’), or in the relationship between I-wave recruitment and

cTBS response. Given that the former is essentially an objective physiological marker (albeit with some error), the latter is more likely. Thus, in males, more complex cortical circuits may not have as large a sensitivity bias to cTBS; males with relatively excitable, simple circuits, in whom earlier I-waves are recruited, appear to respond to cTBS similarly to those in whom late I-waves are recruited.

We are not aware of any similar previous research examining sex differences with respect to I-waves, and we do not have the ability to explain these findings. However, sex differences are known to exist in other neurophysiological responses to brain stimulation. For example, females often respond somewhat more strongly to a variety of facilitatory and inhibitory plasticity-induction protocols (Kuo et al., 2008, Chaieb et al., 2008, Ridding and Ziemann, 2010), and there is evidence for specific roles of female sex hormones in this effect. For example, females in the high-estrogen phase of the menstrual cycle respond more strongly to transcranial direct-current stimulation and rTMS than males or females in the low-estrogen phase (Inghilleri et al., 2004, Lee et al., 2018, Chung et al., 2019).

Thus, it is possible that hormonal differences contributed to the present results, whereby the association between the complexity of circuits stimulated by TMS and their response to cTBS is a product of the female hormonal profile. Future studies should capture information about menstrual phase to determine its effect on plasticity and I-wave recruitment.

Although most of the apparent effect of age on the I-wave-cTBS response association was explained by sex, there remained an effect of age in females, whereby the association decreased with increasing age. This, too, was largely unexpected, but general speculation may be made. As age increases, it is possible that physiological determinants of the response to cTBS increase in number and complexity, thus reducing the relative importance of I-wave recruitment in predicting cTBS response. Such an effect is likely highly complex and without further research is difficult to explain. However, in females, hormonal changes over the lifespan, including a loss of estrogen following menopause, may contribute to changes in these functions, as they do with other neurological processes (Hara et al., 2015).

To summarise, we have presented evidence suggesting that the relationship between late I-wave recruitment and the neuroplastic response to cTBS is present only in young females. Deeper understanding of these findings will require both replication

in larger samples and further research into the possible mechanisms relating sex and age to the relationship between differential interneuron network recruitment and the response to cTBS. Measurement of late I-wave recruitment represents a promising technique to allow more accurate interpretation of TBS-induced neuroplasticity data and for better predicting therapeutic responses to TBS and other NIBS procedures. However, the data presented here suggest that greater caution may be warranted and that this technique may be less generalizable than originally believed (Hamada et al., 2013, Hordacre et al., 2017).

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Chapter 4: Cortical plasticity and interneuron recruitment in adolescents born to women with gestational diabetes mellitus

4.1 FOREWORD

As I mentioned at the beginning of the previous chapter, that study and the one presented here were conducted concurrently. Although the results of Chapter 3 raise questions about the technique used here, namely I-wave latency measurement, I should note that there can be little doubt about the presence of an association between I-wave recruitment and the response to cTBS. Rather, the extent to which this association is manifest in males and, potentially, older females, is unclear. The study in Chapter 3 suggests that the association may only be present, or at least useful, in young females. Nevertheless, the research presented in the present chapter remains useful; even if a comparison of I-waves and cTBS response can only reliably be performed in females, the data allow such an analysis, and additionally act as a replication of the study presented in Chapter 2. Note that the analyses in this chapter include all participants (including males) for the sake of simplicity, but the conclusions remain unchanged if the data are separated by sex.

4.2 ABSTRACT

Objective

Exposure to gestational diabetes mellitus (GDM) *in utero* is associated with a range of adverse cognitive and neurological outcomes. Previously, we reported altered neuroplastic responses to continuous theta burst stimulation (cTBS) in GDM-exposed adolescents. Recent research suggests that the relative excitability of complex oligosynaptic circuits (late I-wave circuits) can predict these responses. We aimed to determine if altered I-wave recruitment was associated with neuroplastic responses in adolescents born to women with GDM.

Methods

20 GDM-exposed adolescents and 10 controls (aged 13.1 ± 1.0 years) participated. cTBS was used to induce neuroplasticity. I-wave recruitment was assessed by comparing motor-evoked potential latencies using different TMS coil directions.

Results

Recruitment of late I-waves was associated with stronger LTD-like neuroplastic responses to cTBS (p = < 0.001, R^2 = 0.36). There were no differences between groups in mean neuroplasticity (p = 0.37), I-wave recruitment (p = 0.87), or the association between these variables (p = 0.41).

Conclusions

The relationship between I-wave recruitment and the response to cTBS previously observed in adults is also present in adolescents and does not appear to be altered significantly by *in utero* GDM exposure. Exposure to GDM does not appear to significantly impair LTD-like synaptic plasticity or interneuron recruitment.

4.3 INTRODUCTION

Gestational diabetes mellitus (GDM) affects approximately 10% of pregnancies, with higher prevalence in overweight and obese women (WHO, 2014). Evidence indicates that children exposed *in utero* to GDM are at higher risk of neurodevelopmental difficulties, including attention deficit hyperactivity disorder (Nomura et al., 2012), autism spectrum disorders (Xu et al., 2014), and impaired motor development (Ornoy et al., 1999). Recently, we reported (Van Dam et al., 2018) that children born to women with GDM had highly variable long-term depression (LTD)-like neuroplastic responses to magnetic brain stimulation. In our study, children's cortical plasticity was associated with the severity of the mother's GDM, particularly with the degree of maternal insulin resistance both before and during GDM treatment. These results were consistent with animal research suggesting that oxidative stress and inflammation associated with maternal hyperglycaemia (resulting from insulin resistance) are major drivers of altered neurodevelopment in GDM-affected fetuses (Sullivan et al., 2014). Both human and animal research suggests that GDM-exposed

fetuses experience an adverse environment *in utero* that contributes to abnormal neurodevelopment, possibly including altered synaptic plasticity and excitability. However, to our knowledge, ours was the first study to examine neurophysiological processes in humans exposed to GDM.

We previously used a form of repetitive transcranial magnetic stimulation (TMS), continuous theta burst stimulation (cTBS), to induce and measure LTD-like neuroplasticity. cTBS, along with its counterpart intermittent TBS (iTBS), induces after-effects on excitability in the stimulated area that outlast the period of stimulation by minutes to hours (Huang et al., 2005). Evidence suggests that these effects depend on the activity of N-Methyl-D-Aspartate (NMDA) receptors and hence it is thought that they represent an analogue of early stages of synaptic plasticity in the human brain (Huang et al., 2007, Ziemann et al., 2015). However, a principal issue with neuroplasticity induction by brain stimulation is that the responses are often highly variable both within and between individuals, with a number of known contributing factors, including age, cortisol, genetics, and prior muscle activity (Ridding and Ziemann, 2010). Recent research (Hamada et al., 2013, Huang and Mouraux, 2015, Hordacre et al., 2017) suggests that inter-individual differences in the cortical network activated by TMS pulses can also influence the response. Because different populations of cortical neurons are stimulated more easily or are more excitable in different people at different times, and because these populations can contribute differently to the neuroplastic response, a substantial proportion of the variability in neuroplastic responses may be explained by variable interneuron recruitment, rather than by variability in synaptic function *per se*.

The descending volley evoked by a single TMS pulse consists of several components. The earliest of these is believed to reflect the direct activation of the corticospinal output cells and is known as the direct (D)-wave. The later components result from indirect activation of output cells and are known as indirect (I)-waves. Early I-waves likely result from monosynaptic input to corticospinal neurons from layer II/III interneurons, whereas later I-waves reflect the activity of more complex, oligosynaptic circuits (Di Lazzaro et al., 2012, Hordacre et al., 2017).

Hamada et al. (2013) found that, in their sample, there was no net facilitatory or inhibitory neuroplastic response to iTBS or cTBS, respectively, due to high response variability. As is common in such experiments, there was variability not only in the

magnitude of neuroplastic responses, but also in the direction of responses. That is, some individuals exhibited inhibitory responses to iTBS, or facilitatory responses to cTBS—the opposite to what is expected. However, individuals in whom TMS recruited later I-waves had stronger neuroplasticity and, when treated as a group, displayed significant neuroplastic responses to both iTBS and cTBS in the expected directions. Similar results were observed by Hordacre et al. (2017). Although the mechanisms are not entirely clear, Hamada et al. (2013) suggest that the late I-wave-generating circuitry may be more sensitive to TBS than the early I-wave-generating circuitry.

Given that we previously observed both weaker and more variable neuroplastic responses to cTBS (including more LTP-like responses) in children exposed to GDM when compared with a control group, we aimed in the present study to determine whether I-wave recruitment differed in this group and whether this could explain the increased variability in neuroplastic responses we observed. Given that the association between I-waves and neuroplasticity has only been studied in adults, our results also provide novel evidence for the presence of this effect in adolescents.

4.4 METHODS

4.4.1 Ethical approval

All procedures were approved by the Women's and Children's Health Network and University of Adelaide human research ethics committees and conducted in accordance with the Declaration of Helsinki (2008 revision). Participants were pre-screened for contraindications to TMS (Rossi et al., 2009a). Parents and participants provided written, informed consent. Parents accompanied their children to the experimental session.

4.4.2 Subjects

20 GDM-exposed subjects (aged 12.7 ± 0.8 years [mean ± SD], 10 female), all of whom participated in our previous study (Van Dam et al., 2018), were recruited from the Adelaide arm of the Metformin in Gestational diabetes (MiG) trial (Rowan et al., 2008). Their mothers had been treated for GDM not responding to lifestyle

alteration, with a 1:1 random allocation at study entry to receive either insulin (n = 12) or metformin treatment (n = 8). Gestational age at trial entry varied from 20 weeks to 33 weeks (mean 30 ± 3.4 weeks). 3 of the metformin group received supplementary insulin to achieve maternal euglycaemia. 10 control participants (aged 13.6 ± 1.3 years, 7 females) whose mothers had normal glucose tolerance on routine testing at the end of the second trimester and no other recorded major pregnancy complications were recruited from labour ward records and matched as closely as possible for age, sex, gestational age at birth, and mode of delivery.

4.4.3 Electromyography

Participants were seated with their hands and forearms supported. Adhesive Ag/AgCl bipolar surface electrodes were applied over the right first dorsal interosseous (FDI) hand muscle to obtain surface electromyography (EMG) recordings. EMG signals were amplified (x1000; 1902 amplifier; CED), bandpass filtered (20 Hz–1 kHz), and digitized at 5 kHz (1401 interface; CED) and were stored offline for later analysis.

4.4.4 Transcranial magnetic stimulation (TMS)

TMS is a non-invasive brain stimulation technique in which the motor cortex is electromagnetically stimulated to produce a motor evoked potential (MEP), recorded in a contralateral muscle using EMG (Di Lazzaro and Rothwell, 2014). Motor cortical excitability was assessed with single-pulse TMS applied to the left primary motor cortex (M1) representation of the right FDI muscle using a 70mm figure-of-eight coil connected to a monophasic Magstim 200^2 stimulator (Magstim Co, Whitland, UK). Previous research has shown clearly that different descending volleys are elicited by single-pulse TMS depending on the direction of current flow across the motor cortex. Posterior-anterior (PA) current preferentially elicits early I-waves, whereas anterior-posterior (AP) current can recruit late I-waves. High-intensity latero-medial (LM) current evokes D-waves (Di Lazzaro et al., 2012, Hamada et al., 2013).

The protocol used here is equivalent to that described by Hamada et al. (2013). 3 different coil orientations were used to evoke MEPs: (1) PA-directed currents were

produced by the coil held posterolaterally at an angle of about 45° to the midline; (2) AP-directed currents were elicited by placing the coil 180° to the PA current position; and (3) LM-directed currents were produced with the coil placed leftwards (90° from midsagittal line).

Using PA currents, the optimal site for consistently evoking MEPs in the FDI was determined and marked on the scalp, and resting motor threshold (RMT) was determined as the lowest TMS intensity required to evoke MEPs of at least 50 μV peak-to-peak amplitude in the resting FDI in at least five of ten consecutive trials. The active motor threshold (AMT) was defined as the lowest intensity to evoke an MEP of 200 μV, or visibly distinct from background EMG activity, in more than 5 of 10 consecutive trials while subjects maintained approximately 10% of maximum voluntary contraction of the target muscle, measured and monitored using a digital oscilloscope. We measured AMT with PA, AP, and LM currents (AMTpa, AMTap, and AMTlm, respectively). The TMS intensity that evoked MEPs of ~1 mV peak-to-peak amplitude (SI_{1mV}) was also determined, using PA current, and used throughout each experiment for evoking test MEPs (Pitcher et al., 2015). SI1mV was recorded 15 minutes after AMT measurements, following a period of muscle relaxation. Intensities are expressed as % of maximum stimulator output (MSO). All measurements were performed at the hotspot determined by PA currents, as previous research has shown that the direction of current does not influence the position of the hotspot (Sakai et al., 1997, Arai et al., 2005).

4.4.5 Measurement of MEP onset latency to assess I-wave recruitment

The level of late I-wave recruitment was quantified by measuring MEP onset latencies using AP current in pre-contracted muscle at near-threshold stimulus intensities. AP MEP latencies were compared with MEP latencies using PA and LM currents. The rationale is that, in individuals in whom late I-wave generating circuitry is more excitable, AP MEPs will have longer latencies than PA and LM MEPs, because more complex circuits are involved in stimulating output cells (with AP current) (Hamada et al., 2013, Di Lazzaro et al., 2012). Indeed, AP MEP latencies are typically observed to be 1–2 ms longer than PA MEPs, and 4–5 ms longer than LM MEPs (Day et al., 1989). As TBS preferentially activates the brain during the second depolarising

phase of the current, that is, with an anterior-posterior current (Di Lazzaro et al., 2001b), those individuals who exhibit longer AP MEP latencies will be more strongly affected by TBS and show stronger neuroplastic responses (Hamada et al., 2013). The difference between LM and AP latencies (AP-LM) was used as the primary index of late I-wave recruitment because LM latencies are highly consistent, allowing reliable estimation of I-wave recruitment (larger AP-LM values indicating later I-waves).

We measured onset latencies of MEPs using PA, AP, and LM currents with stimulus intensities of 110% AMTpa, 110% AMTap, and 150% AMTlm (or 50% MSO in subjects whose 150% AMTlm did not reach 50% MSO) (Hamada et al., 2013). Relatively high stimulus intensities were used with LM current to ensure a D-wave was evoked (Werhahn et al., 1994). 20 MEPs each with PA and AP currents, and 10 MEPs with LM currents, were recorded. After every tenth trial, subjects were asked to relax their hand to minimise fatigue. Signal (Version 6; Cambridge Electronic Design) was used to record, process, and analyse EMG data. A custom-made script was used to measure MEP-onset latency, defined as the time-point where rectified EMG signals exceed a mean ± 2 SD of the pre-stimulus EMG level, expressed as milliseconds after the stimulus. For each subject and coil orientation, mean latencies were calculated and inspected directly to ensure validity.

4.4.6 LTD-like neuroplasticity induction with cTBS

cTBS was used to induce LTD-like suppression of MEP amplitudes. Pharmacological studies indicate that cTBS-induced MEP suppression is NMDA-receptor-dependent and similar mechanistically to LTD (Huang et al., 2007). An air-cooled figure-of-eight coil (rTMS coil), connected to a Magstim Super Rapid stimulator (Magstim, Whitland, UK), was used to apply repetitive TMS to the optimal site for stimulating the right FDI. The cTBS protocol consisted of 600 pulses applied in bursts of three pulses at 50 Hz, repeated at 5 Hz for a total of 40 s (Huang et al., 2005). Stimulation intensity was set to 70% of RMT (measured using the rTMS coil). MEPs were recorded in blocks of 15 trials prior to cTBS (i.e. baseline) and at 0, 5, 10, 20 and 30 minutes following cTBS with PA current. In each block, the mean of the peak-to-peak amplitudes of the 15 MEPs was calculated. Changes in MEP amplitude relative to baseline MEP amplitude were used as an index of neuroplasticity (Pitcher

et al., 2012a). All MEPs were recorded at high gain and any with obvious EMG activity in the 200 ms before the TMS stimulus were discarded. cTBS was performed following a 15-minute rest period after baseline MEP recording to avoid metaplastic effects of prior cortical and muscle activity on neuroplastic response (Gentner et al., 2008, Iezzi et al., 2008, Goldsworthy et al., 2014).

The coefficient of variation (COV) of baseline MEP amplitudes was also calculated to determine correlation with plasticity induction (Hordacre et al., 2017).

4.4.7 Salivary cortisol

Saliva samples were obtained from each child immediately before TMS baseline measures (at 13:22 ± 0.25 hr) using a Salivette (Sarstedt) designed for cortisol analysis. Salivettes were centrifuged to obtain saliva, which was stored at -20°C until assayed. Twenty-five microlitre aliquots of saliva were assayed in duplicate for cortisol concentrations by enzyme-linked immunosorbent assay (ELISA) according to manufacturer instructions (HS-Cortisol; Salimetrics).

4.4.8 Statistical analysis

Data were analysed using Python 3.7. Data were checked for normality using the Shapiro-Wilk test and were tested for equal sphericity and variance where appropriate using Bartlett's and Levene's tests, respectively. The associations between continuous variables (e.g. AP-LM latency and mean neuroplastic response) were tested using linear regression models. Interaction terms were added to regression models to test for group differences in the effect of AP-LM latencies (Group*AP-LM interaction) when predicting neuroplasticity. Group means were compared using an independent samples *t*-test (or a Welch's *t*-test where appropriate). To further examine the influence of I-wave recruitment on post-cTBS MEP amplitudes, data were analysed based on the presence or absence of late I-waves by median split on AP-LM latency, where AP-LM latency differences greater than the group median were considered to represent late I-waves, and those lower to represent early I-waves (Hordacre et al., 2017). The analysis was also performed based on a split at 4 ms AP-LM difference (Hamada et al., 2013). Results were comparable, so only the median split is reported below. Data were also

analysed by the direction of neuroplastic response (inhibitory, < 100% baseline; or facilitatory, > 100% baseline) to examine the difference in I-wave recruitment.

4.5 RESULTS

4.5.1 Baseline characteristics and cortical excitability

The mean (± SD) resting motor threshold (RMT) of all participants was 47.4 ± 7.3 (% MSO). RMT did not differ significantly between the GDM (46.6 ± 6.0) and control (49.1 ± 9.6) groups ($p = 0.379$). Within the GDM group, individual RMTs in the present study correlated strongly with RMTs assessed previously (reported in Van Dam et al., 2018) ($p < 0.001$, $R^2 = 0.574$). RMT was not influenced by age ($p = 0.251$), sex ($p = 0.563$), gestational age at birth ($p = 0.967$), or maternal GDM treatment type ($p = 0.738$). Gestational age at birth did not differ between the GDM (38.8 ± 1.5 weeks) and control (39.3 ± 0.86 weeks) groups ($p = 0.358$; grand mean 39.0 ± 1.3 weeks).

Neuroplastic responses measured in the previous study were not correlated to those measured in the present study ($R^2 = 0.069$, $p = 0.279$). In contrast to the previous, maternal insulin resistance was not associated with the neuroplastic response ($R^2 = 0.116$, $p = 0.233$). However, lower insulin ($R^2 = 0.286$, $p = 0.033$) and maternal C-peptide concentrations ($R^2 = 0.681$, $p < 0.001$) at trial entry were strongly correlated with stronger LTD-like neuroplastic responses

4.5.2 I-wave recruitment predicts the neuroplastic response to cTBS

Among all participants there was not a net neuroplastic response to cTBS; in a repeated measures ANOVA on raw MEP amplitudes there was no main effect of Time ($p = 0.126$). This was due to the large variability in responses, with both LTD- and LTP-like effects observed in different participants (Figure 4.1, left). The mean post-cTBS MEP amplitude (% baseline) at the group level was 107 ± 29.

Mean (± SD) AP-LM latency difference among all participants was 3.55 ± 1.04 ms. AP-LM was strongly associated with mean neuroplastic response to cTBS, explaining 36% of the variation in post-cTBS MEP amplitudes ($\beta = -16.817$, $R^2 = 0.36$, $p = < 0.001$) (Figure 4.1, right). Individuals in whom late I-waves were recruited

(longer AP-LM difference) were more likely to show inhibitory responses to cTBS, while individuals with shorter AP-LM differences tended to exhibit facilitatory responses. Similarly, participants in the early I-waves group, as determined by a median split on AP-LM difference, had significantly larger MEP amplitudes (123 ± 21 % baseline) following cTBS than did individuals in the late I-waves group (91 ± 27) (p = 0.002) (Figure 4.2). The late I-waves group did not exhibit a significant neuroplastic response to cTBS (compared with baseline) (p = 0.458). However, the early I-waves group exhibited a response to cTBS consistent with significant facilitation of MEPs (p = 0.006). Individuals who exhibited facilitation-like responses to cTBS had significantly shorter AP-LM latency differences (2.94 ± 0.67ms) than those who exhibited inhibition-like responses (4.36 ± 0.89 ms) (p < 0.001).

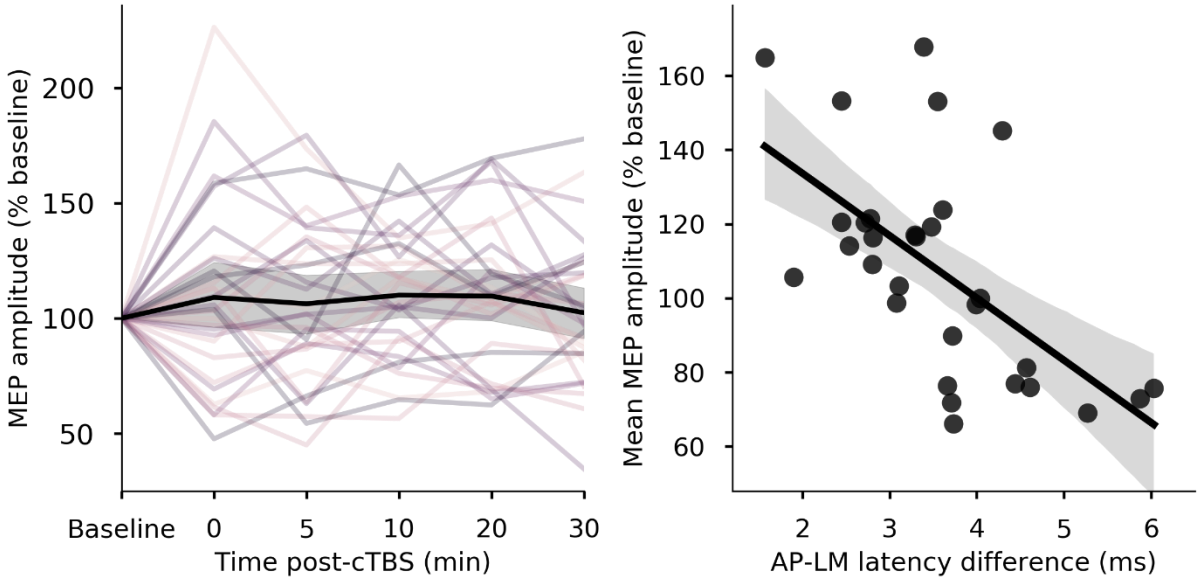


Figure 4.1: cTBS response and I-wave recruitment latencies. *Individual responses to cTBS varied in magnitude and direction, while the group mean (black line) remained near baseline. Shaded area represents SD of group mean (left). Greater late I-wave recruitment, as indicated by longer AP-LM latency difference, was strongly associated with the magnitude and direction of the neuroplastic response to cTBS (*p *< 0.001,* R^2 *= 0.36; right).*

4.5.3 Group comparisons of I-waves and neuroplasticity

Mean post-cTBS MEP amplitudes (% baseline) did not differ between the GDM (104.0 ± 28.0) and control (114.4 ± 31.5) groups ($t_{(28)}$ = -0.922, p = 0.364) (Figure 4.3, right), including when correcting for cortisol concentration (p = 0.424). Similarly, there was no effect of Group (p = 0.667) and no Group*Time interaction (p = 0.945) in a repeated measures ANOVA of MEP amplitudes. Mean AP-LM latency differences did not differ between the GDM (3.53 ± 0.79 ms) and control (3.61 ± 1.48 ms) groups (Welch's $t_{(28)}$ = -0.205, p = 0.869) (Figure 4.4, left). However, there did appear to be a greater spread of AP-LM differences in the control group, with more extreme minimum and maximum values (Figure 4.4), despite the smaller number of participants in this group. Levene's test indicated a significant difference in variances in AP-LM between groups (p = 0.042).

The association between I-wave recruitment and neuroplasticity appeared stronger in the control group, with AP-LM latency difference explaining 86% of the variation in post-cTBS MEP amplitudes (β = -19.7751, p < 0.001, R^2 = 0.858) in this group compared with 13% in the GDM group (β = -12.5061, p = 0.13, R^2 = 0.125).

Despite the non-significant result in the GDM group, the association between AP-LM and plasticity was visually present (Figure 4.3, left), but appeared to be weakened statistically by a greater proportion of AP-LM values clustered between approximately 3.5–4 ms (i.e. on the border of late I-waves), which appears to be a region where the predictive value is low regarding neuroplastic response.

The difference in magnitude of the AP-LM and neuroplasticity association between groups was not statistically significant, as there was not a significant Group*AP-LM interaction when predicting post-cTBS MEP amplitudes (Group, p = 0.20; AP-LM, p < 0.001; Group*AP-LM, p = 0.409). Neither the response to cTBS (p = 0.344) nor mean AP-LM latency (p = 0.162) differed between the maternal GDM treatment groups (metformin or insulin).

Resting motor threshold did not differ between the GDM (45.6 ± 6.0 % MSO) and control (49.1 ± 9.6 % MSO) groups (p = 0.379).

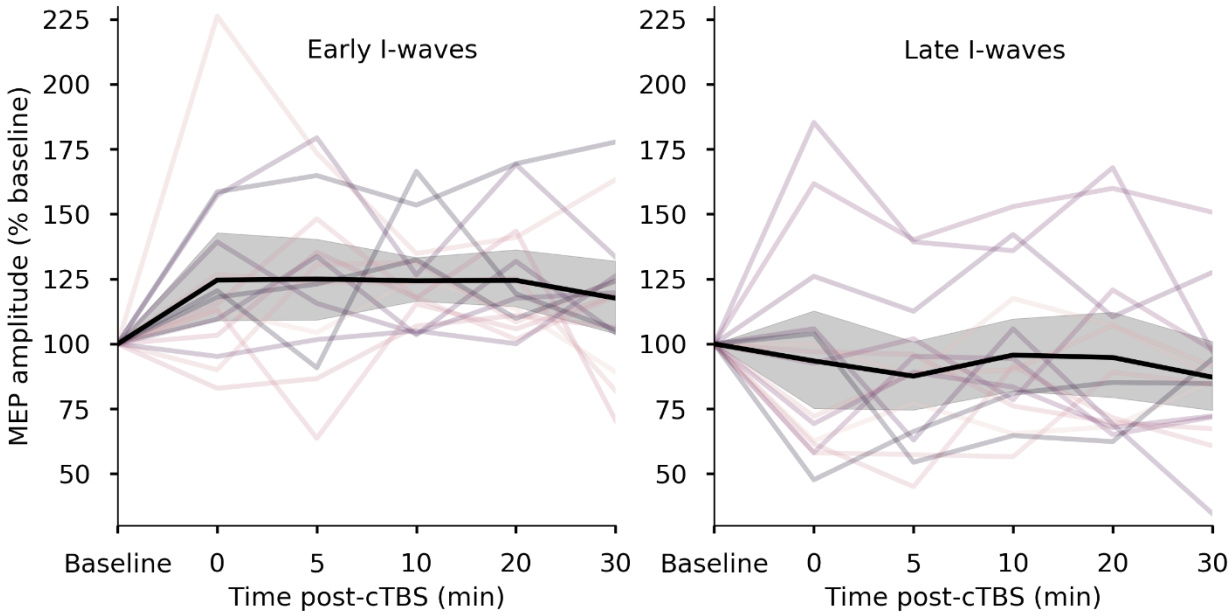


Figure 4.2: cTBS response by I-waves group. *Mean post-cTBS MEP amplitudes were lower in individuals who recruited late I-waves (median split on AP-LM latency difference;* p *= 0.002). In the late I-waves group there was no group-level effect of cTBS, but most individuals showed inhibition-like responses (right), while in the early I-waves group there was a response consistent with facilitation of MEPs (*p *= 0.006; left).*

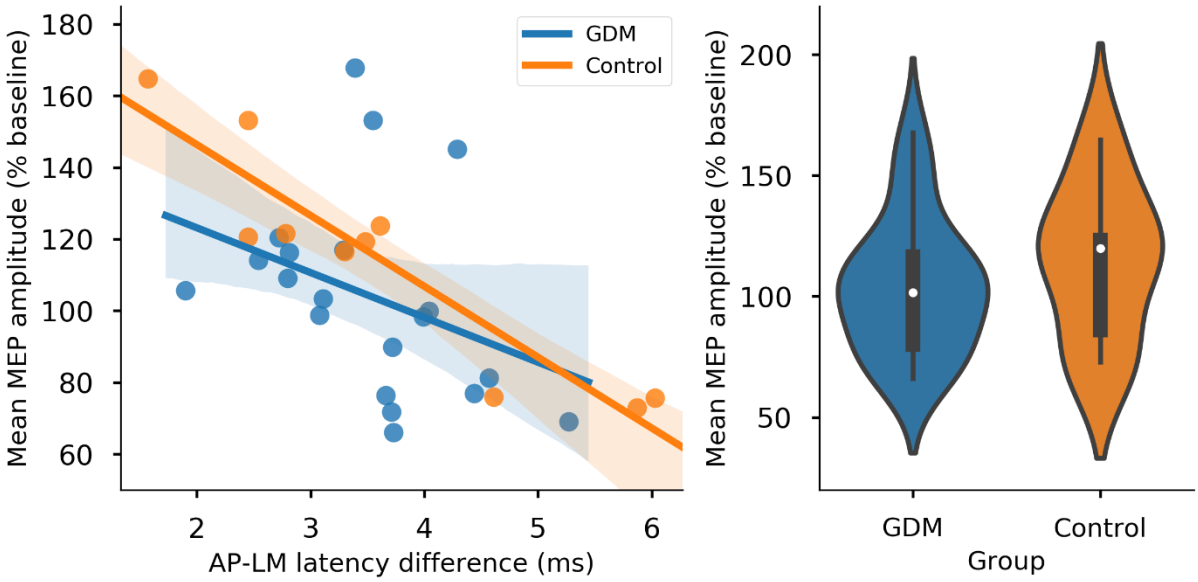


Figure 4.3: Comparison of cTBS response and I-waves-plasticity association by GDM group. *The association between I-wave recruitment and response to cTBS appeared stronger in the control group (*$p < 0.001$*,* $R^2 = 0.858$*) than in the GDM group (*$p = 0.13$*,* $R^2 = 0.125$*), but this difference was not statistically significant (Group*AP-LM interaction,* $p = 0.41$*). Mean post-cTBS MEP amplitudes did not differ between groups (right; inner boxplots). Shaded areas on the scatterplot represent 95% confidence intervals for the regression lines.*

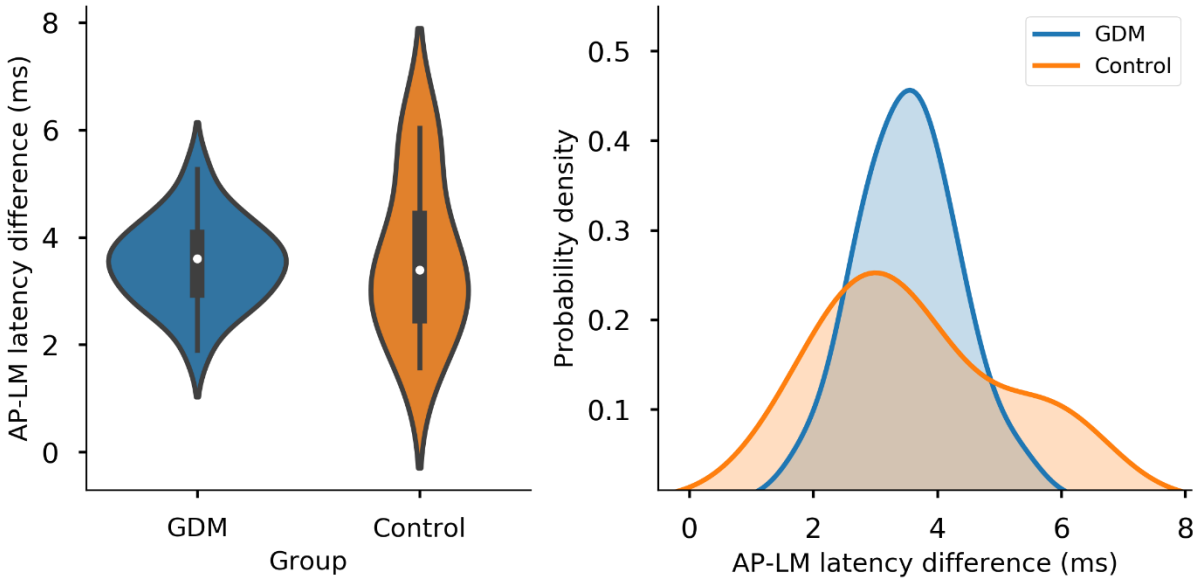


Figure 4.4: I-wave latencies by GDM group. *The central tendency of AP-LM latency differences did not differ between groups (left), but latencies were more variable in the control group (*p *= 0.042; kernel density estimation plot, right).*

4.5.4 Cortisol

The mean (± SD) salivary cortisol concentration at the time of testing was 2.12 ± 1.43 nmol/L. The GDM group (2.46 ± 1.59) had higher but more variable cortisol values compared with the control group (1.39 ± 0.50), but there was no difference in mean cortisol concentration ($t_{(27)}$ = 1.957, p = 0.06, ω^2 = 0.09)

The control group completed the TMS experiment later in the day (IQR: 14:30 – 16:30 hr) compared with the GDM group (IQR: 10:45 – 12:50 hr) ($t_{(26)}$ = -3.987, p < 0.001). However, time of test did not impact cortisol values (β = -0.002, R^2 = 0.09, p = 0.118). Neither time (p = 0.471) nor group (p = 0.687) predicted cortisol concentration in an ANCOVA model ($F_{(2,25)}$ = 1.662, Adj. R^2 = 0.047, p = 0.238). There was no sex difference in cortisol concentrations (Welch's $t_{(23.7)}$ = -1.285, p = 0.211), and maternal treatment (metformin or insulin) did not affect cortisol (p = 0.485). The cortisol values in both the GDM and control groups were consistent with normative data for this age group and for the time of day of assessment (Miller et al., 2016).

Cortisol did not predict mean post-cTBS MEP amplitudes ($F_{(27,1)}$ = 2.254, R^2 = 0.08, p = 0.128) or RMT (β = -1.595, R^2 = 0.095, p = 0.095).

There was a near-significant association between cortisol and AP-LM latency difference, whereby higher cortisol concentrations were associated with greater AP-LM difference (β = 0.243, R^2 = 0.134, p = 0.051), but cortisol as a covariate did not alter any of the associations between group, AP-LM, and response to cTBS described above.

4.6 DISCUSSION

We have provided evidence indicating that the association between interneuron recruitment and the neuroplastic response to cTBS, whereby individuals who exhibit motor responses produced by late I-wave-generating circuitry have stronger and more predictable neuroplastic responses, is present in adolescents to a similar degree to that previously observed in adults (Hamada et al., 2013, Hordacre et al., 2017, Huang and Mouraux, 2015). Although I-wave recruitment was less variable in GDM-exposed participants, mean MEP latencies were similar. The association between I-wave recruitment and the neuroplastic response in this group appeared weaker, but this difference was not statistically significant. Thus, it does not appear that *in utero* GDM exposure fundamentally alters the relationship between I-wave recruitment and the neuroplastic response to cTBS.

In the present study we did not observe a net, group-level neuroplastic response to cTBS. Given the variability often observed in the response to cTBS (Hamada et al., 2013, Vallence et al., 2015, Hordacre et al., 2017, Rocchi et al., 2018) this was not entirely unexpected and largely reflects variability in the direction of responses. That is, whereas some individuals exhibit the expected suppression-like response, often a similar number exhibit responses consistent with facilitation of MEPs, such that the group average post-cTBS MEP amplitude is near baseline. However, in contrast to our previous study (Van Dam et al., 2018), we did not observe any systematic difference in response to cTBS in the GDM group compared with the control group. There are several possible explanations for this result. Firstly, given the responses observed in the current control group, our previous control group may have been particularly responsive to cTBS. That is, by chance we may have recruited subjects in the initial

study who exhibited particularly robust LTD-like responses to cTBS, resulting in inflation of the difference between groups. Nevertheless, we do believe our original conclusion—that there is an effect of GDM on child neurophysiology—to be valid, because there was a range of supporting evidence within the GDM group, including associations between child neurophysiology and maternal insulin resistance during pregnancy (Van Dam et al., 2018). However, the effect size in the group comparison is likely smaller than originally estimated and it may require a larger sample size to make reliable conclusions.

There may also be an effect of age: as our GDM group subjects have aged approximately two years since first assessment, and given the rapid stage of their development, the neurophysiological effects we observed previously may have normalised and may now be relatively subtle, if still present.

Given these results, we were unlikely to find support for our original hypothesis that differential I-wave recruitment would explain the difference in neuroplasticity between groups that we previously observed. Indeed, the mean late I-wave recruitment (AP-LM latency) was similar in the GDM group when compared with controls. However, there was an apparent difference in the distribution of I-waves, as the GDM group, despite having a larger sample, had a smaller range of responses, with both smaller group minimum and maximum AP-LM latency differences. The effect of this was a clustering of AP-LM latencies around the 4 ms mark, which represents approximately the border between early and late I-waves (Day et al., 1989, Hamada et al., 2013). Responses in this range appear to have lower predictive value of the neuroplastic response to cTBS, and the association between I-wave recruitment and neuroplastic response therefore appeared weaker (and non-significant) in the GDM group when compared with the robust association seen in the control group. However, this comparison did not prove to be significant and we must therefore conclude that there is no difference in interneuron recruitment by TMS, or in the relationship between late I-wave recruitment and neuroplastic responses to cTBS, in children exposed to GDM, unless such an effect is smaller than we can detect with our sample size. Although the control group was small, the effect size therein was extremely strong. Thus, a larger control group would almost certainly result in a reduction in the magnitude of the difference between groups; therefore, the sample is biased towards a false positive, so we can accept a negative result with relative confidence.

The precise mechanisms underlying the inter-individual differences in late I-wave recruitment are unclear and likely complex. Presumably, individuals differ with respect to the relative excitability of early and late I-wave-generating circuitry, and consequently their responsivity to cTBS. Given that the recruitment of late I-waves is highly consistent within individuals (Hamada et al., 2013), this excitability bias must be relatively fixed. That is, individuals who do not recruit late I-waves should have consistently lower excitability of late I-wave-generating circuitry. Alternatively, these individuals may have structural differences in neural circuitry in the motor cortex, possibly possessing a lower proportion of complex, oligosynaptic inputs to pyramidal cells. In any case, I-wave recruitment, given its reliability, is likely not the primary driver of cTBS response because this would necessitate that similarly consistent neuroplastic responses be observed; test-retest reliability of TBS is relatively modest, especially when not accounting for circadian patterns (Jannati et al., 2019, Schilberg et al., 2017, Vallence et al., 2015, Guerra et al., 2017), and indeed we did not find a correlation between neuroplastic responses measured here and in our previous study. Thus, variation in the association between I-wave recruitment and response to cTBS may explain the apparent (albeit non-significant) difference in this relationship between groups seen in this study, rather than any intrinsic difference in physiology. Nevertheless, future research may extend this analysis with a larger sample to determine whether GDM-exposed individuals exhibit differences in the excitability or structure of I-wave-generating circuitry.

In contrast to our previous study (Van Dam et al., 2018), we did not observe lower cortisol concentrations in the GDM group. Although mean cortisol was higher in the GDM group, median cortisol was similar in each group, and the GDM group had higher variability and several large values, possibly reflecting stress responses not observed in the control group. This variability may also explain the lack of associations between cortisol and both neuroplasticity and cortical excitability that have been observed previously (Van Dam et al., 2018, Pitcher et al., 2012a). In the present study we observed clear trends following the expected patterns, so a lack of statistical power is a likely explanation. Nevertheless, a single afternoon cortisol measurement is not sufficient to reliably characterise hypothalamic-pituitary-adrenal activity. In Chapter 5, we examine the cortisol awakening response and diurnal cortisol profile in children born to women with GDM.

Although our results surprised us in several ways, they may be considered positive: they suggest that GDM, when properly diagnosed and treated, does not result in significant dysfunction in cortical excitability or in the forms of synaptic plasticity measured using cTBS lasting into adolescence. Further, maternal treatment with metformin, a drug that crosses the placenta (Charles et al., 2006), was not associated with adverse outcomes, supporting its safety and efficacy in treating GDM with respect to child neurodevelopment (Wouldes et al., 2016, Rowan et al., 2018, Rowan et al., 2011). However, given our past findings regarding maternal insulin resistance and child neurophysiological outcomes (Van Dam et al., 2018), and the known functional neurological outcomes in individuals exposed to GDM (e.g. Nomura et al., 2012, Xu et al., 2014, Ornoy et al., 1999), improvements to prevention, screening, diagnosis, and treatment practices for GDM remain important areas of investigation. Further, an analysis of forms of facilitatory synaptic plasticity may be warranted. Questions regarding the possible physiological mechanisms underlying the functional neurological consequences of GDM remain open and should be investigated further.

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Chapter 5: Lower morning cortisol secretion in adolescents born to women with gestational diabetes mellitus

5.1 ABSTRACT

Purpose

Gestational diabetes mellitus (GDM) affects approximately 10% of successful pregnancies worldwide. Individuals exposed to GDM *in utero* are at increased risk of developing type II diabetes during childhood and later in life. The hormone cortisol has important roles in metabolism, and cortisol dysregulation is associated with metabolic dysfunction, thus representing a possible mechanistic link between GDM exposure and later diabetes. We aimed to determine whether adolescents exposed to GDM exhibited dysregulation in diurnal cortisol and the cortisol awakening response.

Methods

Salivary cortisol was measured in 22 GDM-exposed adolescents (aged 11–14 years) and 10 controls at 0, 15, 30, and 45 minutes after awakening over 2 consecutive days. Afternoon and night samples were also collected. Data were represented as area under the curve with respect to ground (AUCg) and increase (AUCi) and averaged between days.

Results

The control group had lower AUCg ($p = 0.025$, $\omega^2 = 0.124$), representing lower total morning cortisol secretion, but similar AUCi ($p = 0.662$, $\omega^2 = -0.025$), indicating that the hormonal response to awakening is comparable. These findings may partially reflect altered pre-awakening cortisol secretion.

Conclusions

Given that a dysregulated cortisol profile, including sustained, low cortisol production, is associated with metabolic dysfunction and the development of type II

diabetes, we suggest that this may be one mechanism by which exposure to maternal GDM increases the risk of these problems in offspring.

5.2 INTRODUCTION

Gestational diabetes mellitus (GDM) refers to a state of insulin resistance and hyperglycaemia occurring during pregnancy, affecting approximately 10% of pregnancies, with higher prevalence in overweight and obese women (WHO, 2014). Recently, we reported (Van Dam et al., 2018) that adolescents whose mothers had GDM during pregnancy had lower salivary cortisol in the early afternoon than did a control group. In the present study we aimed to further explore this finding by characterising the cortisol awakening response and daily cortisol secretion in GDM-exposed adolescents.

The hypothalamic-pituitary-adrenal (HPA) axis is a major endocrine system responsible for facilitating adaptation to changing bodily and environmental conditions by regulating behavioural and physiological changes. Cortisol, a glucocorticoid hormone, is the primary adrenal product of the HPA axis and binds to receptors in almost every tissue in the body. In addition to its prominent role in regulating the response to stress, cortisol is involved in many metabolic processes, including gluconeogenesis, insulin regulation, glucose utilization, and cerebral perfusion (Fries et al., 2009).

In addition to stress-related activity, the HPA axis follows a distinct diurnal rhythm, with peak cortisol secretion found in the early morning hours during sleep. Superimposed on this diurnal rhythm is a burst of secretion, known as the cortisol awakening response (CAR), which occurs immediately upon awakening and lasts 30–45 minutes, before declining gradually throughout the day. Although the exact functions of the CAR are unclear, it is believed to be involved prominently in 'priming' an individual's behavioural and metabolic state in line with the expected demands of the day ahead (Fries et al., 2009). For example, individuals anticipating a demanding cognitive test have a more pronounced CAR than those not anticipating a task (Elder et al., 2017), and 'anticipatory stress' appears to be a predominant state variable influencing the CAR.

Dysregulation of the HPA axis, including the diurnal cortisol rhythm and the CAR, is associated with metabolic dysfunction. Specifically, a blunted CAR and flatter cortisol curves have been robustly associated with greater metabolic disturbance, metabolic syndrome, and type 2 diabetes (T2DM) (Lasikiewicz et al., 2008, Bruehl et al., 2009, Lamers et al., 2013, Kuehl et al., 2015, Champaneri et al., 2012). The relationship between the HPA axis and metabolic function may be bidirectional and is certainly complex, involving chronic and acute stress, psychological factors including depression, genetics, and other factors. However, HPA dysfunction can predict the development of T2DM later in life (Hackett et al., 2014, Hackett et al., 2016). Given that children born to mothers with diabetes are at increased risk of developing obesity, insulin resistance, and diabetes both during childhood and later in life (e.g. Silverman et al., 1995, Boney et al., 2005, Lawlor et al., 2011, Nehring et al., 2013, Landon et al., 2018, Kawasaki et al., 2018), we aimed in the present study to determine whether children born to women with GDM have altered CARs or diurnal cortisol secretion, as this may represent one mechanism underlying their increased risk of future metabolic dysfunction.

5.3 METHODS

5.3.1 Ethical Approval

All procedures were approved by the Women's and Children's Health Network and University of Adelaide human research ethics committees and conducted in accordance with the Declaration of Helsinki (2008 revision). Parents and subjects provided written, informed consent.

5.3.2 Subjects

22 GDM-exposed subjects (10 female) aged 11–14 years were recruited from the Adelaide arm of the Metformin in Gestational diabetes (MiG) trial (Rowan et al., 2008). Their mothers had been treated for GDM not responding to lifestyle intervention, with a 1:1 random allocation at study entry to receive either insulin (n = 14) or metformin (n = 8) treatment (at 30 ± 2.6 weeks gestation). 10 control subjects (3 female) matched as closely as possible for age, gestational age at birth, parity, and

birthweight were also recruited. Mothers of control participants did not have GDM or any other major complication during pregnancy.

5.3.3 Salivary cortisol and the CAR

Saliva was collected by participants at home at 0, 15, 30, and 45 minutes after awakening in order to reliably capture the features of the CAR (Stalder et al., 2016). Samples were collected over 2 consecutive days. On day 1, an additional 2 samples were collected at approximately 12pm and 8pm (or before bedtime) to examine the wider diurnal slope. Samples were collected using cortisol-specific Salivettes (Sarstedt) and standardized collection procedures. Participants were instructed to collect the first sample immediately after awakening in the morning, to record the time of each sample on a log-sheet provided to them, and to store samples in a freezer until collected by the researcher. Wake times were also recorded using digital actigraphy (Actiwatch 2; Philips Respironics). Due to device errors valid actigraphy was only recorded for 19 participants (8 controls). Salivettes were subsequently stored at -20°C until assayed. Twenty-five microliter aliquots of saliva were assayed in duplicate for cortisol by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (HS-Cortisol; Salimetrics).

5.3.4 Statistical analysis

To assess cortisol dynamics, the areas under the curve with respect to both ground (AUCg) and increase (AUCi) were calculated as described by Pruessner et al. (2003). AUCg and AUCi were calculated using the morning cortisol samples on each day, and mean measures were also calculated (AUCgmean and AUCimean) across both days. Additionally, on day 1 AUCg was calculated with all samples (including afternoon and night) to estimate total daily cortisol secretion (AUCtotal). AUCtotal was calculated using individual self-reported sample times to account for variability in inter-sample duration. The sampling duration (hours between first and last sample) was also calculated to account for individual differences in the duration of the sampled cortisol slope and thus total cortisol secretion. The diurnal cortisol slope was calculated by fitting a regression line through cortisol data at awakening, afternoon, and night (Hackett et al., 2016) for each participant (day 1). Diurnal slope was very strongly

correlated with S1 (R = 0.978) but is reported here in order to aid comparison with other literature. Sampling latency was calculated as the difference (in minutes) between objective wake time (determined by actigraphy) and self-reported time of first sample. A repeated-measures ANOVA was performed on raw morning cortisol data with the within-subjects factors Day (2 levels) and Sample (4 levels; 0, 15, 30, 45 minutes). As there was no effect of Day (p = 0.947), and no Day*Sample interaction (p = 0.440), subsequent analyses were performed using AUCgmean and AUCimean for the purpose of simplicity. In separate repeated-measures ANOVAs for each day there were no effects of sampling latency or wake time, and no interaction effects (all $p > 0.455$). Thus, sampling latency and wake time were not included in subsequent analyses of AUCi or AUCg.

Data were analysed using Python 3.7. Independent samples *t*-tests were used to compare means between groups. Linear regressions were used to examine the relationships between continuous variables. Potential covariates, including age, gestational age at birth, and sex, were added to ANCOVA models to examine their effects on the CAR and potential interactions with GDM group. All data and residuals were normally distributed as determined by the Shapiro-Wilk test (all $p > 0.247$). All *t*-tests and ANOVA models satisfied the assumption of homogeneity of variances (Levene's test; all $p > 0.112$).

5.4 RESULTS

5.4.1 Baseline characteristics

The mean (± SD) age of participants was 13.1 ± 1.0 years. Although control participants were matched for date of birth, age at assessment was higher in the control group (13.6 ± 1.2 years) than in the GDM group (12.8 ± 0.8 years) (p = 0.036). The mean gestational age at birth was 273 ± 9 days (39 weeks), and mean birth weight was 3487 ± 411 grams. Maternal age at birth was slightly higher in the GDM group (34.3 ± 4.4 years) than the control group (30.9 ± 5.2 years), but this was not statistically significant (p = 0.084; grand mean 33.4 ± 4.8 years). None of the above variables differed between groups ($p > 0.331$), and none were associated with the cortisol awakening response independently or when treated as covariates (all $p > 0.124$). Mean

sampling latency (n = 19) was 4 ± 3.51 minutes (median = 3.5 minutes), and median wake time was 07:20 hrs. These did not differ between groups ($p > 0.429$).

***Table 5.1: Descriptive statistics for each cortisol metric**. AUCi and AUCg measures are calculated from morning cortisol samples, and AUCimean and AUCgmean represent 2-day means. AUC values represent nmol/L. AUCtotal is calculated on day 1 using all 6 samples (including afternoon and night), representing total daily cortisol secretion. Diurnal slope units are regression slopes. SD = standard deviation. 25%, 50%, and 75% represent percentiles.*

	AUCimean	AUCgmean	AUCtotal	Diurnal slope
N	32	32	31	30
Mean	8.87	416.70	4667.15	-0.07
SD	159.66	158.25	1866.87	0.05
Min	-455.65	122.91	957.69	-0.20
25%	-74.86	307.57	2968.29	-0.10
50%	-2.28	447.48	4721.62	-0.07
75%	101.37	518.50	6114.94	-0.04
Max	318.46	763.45	8072.60	0.01

5.4.2 Maternal GDM is associated with lower total morning cortisol secretion

Summary statistics for all cortisol measures are presented in Table 5.1. In a repeated-measures ANOVA there was a strong effect of Sample ($p < 0.001$), indicating the presence of the CAR (Figure 5.1). There were significant increases in cortisol concentrations relative to S1 at 15 ($p < 0.001$), 30 ($p < 0.0001$), and 45 ($p < 0.00001$) minutes post-awakening. Cortisol increased by 130 ± 128 % (median = 74 %). The

group means of AUCg (p = 0.791), AUCi (p = 0.819), and percentage increase (p = 0.880) did not differ between days. The GDM group had lower cortisol concentrations at waking (S1, p = 0.046, ω^2 = 0.163, corrected for sampling latency), 30 minutes (p = 0.047, ω^2 = 0.094), and 45 minutes (p = 0.0008, ω^2 = 0.285) post-awakening (Figure 5.1).

Participants in the GDM group (375.3) had lower AUCgmean than control participants (507.9) (p = 0.025, ω^2 = 0.124), indicating lower total morning cortisol secretion. Similarly, in a repeated measures ANOVA on raw cortisol values there was a significant main effect of GDM Group (p = 0.012, η_p^2 = 0.192). AUCimean was similar in the GDM and control groups (p = 0.662, ω^2 = -0.025).

There was a strong negative association between S1 concentration and AUCi magnitude (p = 0.00004, ω^2 = 0.420). This association was not affected by sampling latency (p = 0.884) and did not differ between groups (Group*S1 interaction, p = 0.540).

5.4.3 Maternal GDM is associated with lower daily cortisol secretion and flatter diurnal slope

When controlling for time of sample, the GDM and control groups did not differ in afternoon (p = 0.832) or evening cortisol concentrations (p = 0.296). The strongest predictor of AUCtotal was the time interval between the samples collected at 45 minutes post-awakening and in the afternoon; afternoon sample times were variable and thus altered the shape and area under the curve. When correcting for morning-to-afternoon sample interval, the GDM group had lower AUCtotal (4258.9) than the control group (5665.1) (Group, p = 0.031, ω^2 = 0.081; Interval, p = 0.0003, ω^2 = 0.310), indicating lower total daily cortisol secretion. Sampling duration (time between first and last samples) did not add significantly to this model (p = 0.874, ω^2 = -0.024), but duration and interval were strongly correlated (R = 0.702, p = 0.0001).

When correcting for sampling duration, the GDM group had flatter diurnal cortisol slopes than the control group (GDM, β = -0.064; Control, β = -0.100; Group, p = 0.031, ω^2 = 0.120).

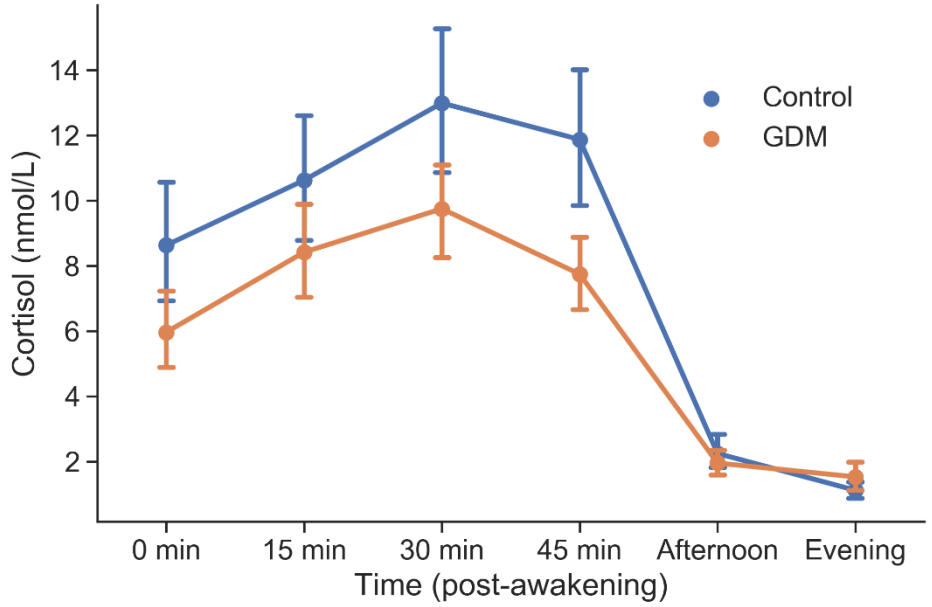


Figure 5.1: Comparison of the cortisol profile between GDM groups. *The shape of the cortisol awakening response (averaged across 2 days) was similar in each group (AUCi, p = 0.662), but the GDM group had lower total morning cortisol secretion (AUCg, p = 0.025). Error bars represent 95% confidence intervals for the means.*

5.4.4 Maternal metformin versus insulin treatment

There was no difference in AUCgmean (p = 0.809), AUCimean (p = 0.992), or diurnal slope (p = 0.609) between subjects born to women treated for GDM with metformin or insulin. However, when correcting for sampling duration, subjects in the metformin group (5459.1) had higher AUCtotal than did those in the insulin group (3573.1) (Treatment, p = 0.041, ω^2 = 0.140; Duration, p = 0.130, ω^2 = 0.055). This was due primarily to greater cortisol under the curve in the afternoon and night, although these samples individually were not different (afternoon, p = 0.157; night, p = 0.116).

5.4.5 Sex did not alter cortisol profile

There were no significant sex differences in S1 (p = 0.099, ω^2 = 0.056), AUCgmean (p = 0.127, ω^2 = 0.044), or AUCimean (p = 0.535, ω^2 = -0.019), and no sex*GDM interactions when predicting AUCg (p = 0.791) or AUCi (p = 0.907).

5.5 DISCUSSION

We have provided evidence indicating that adolescents exposed *in utero* to GDM have significantly lower cortisol secretion during the morning, but no difference in the cortisol response to awakening (CAR), representing a possible mechanism underlying the increased risk for metabolic dysfunction observed in this population.

When interpreting the CAR, it is important to distinguish between the different summary indices commonly used and the components of HPA axis activity that they represent. AUCi is sensitive to the pattern or rate of change of hormonal output (Fekedulegn et al., 2007), and thus reflects the CAR as it is commonly understood: the pattern of change in cortisol output in response to awakening. In contrast, the AUCg largely reflects total hormonal output, and is less sensitive to temporal patterns. Thus, it has been suggested that AUCg does not truly measure the CAR and should be considered a separate but related phenomenon (Clow et al., 2010, Stalder et al., 2016). Another important component of the circadian cortisol system is pre-awakening cortisol (PAC). Cortisol rises gradually throughout the night during sleep, reaching a circadian peak in the early hours pre-awakening. This circadian system is distinct from the CAR, but measurement of the CAR nevertheless relies on this process; the absolute cortisol concentrations are set relative to the starting point (S1) established during the pre-awakening period. Furthermore, in the pre-awakening period, there appears to be a de-coupling of cortisol from its secratagogue, ACTH, as cortisol sensitivity to ACTH decreases, which has been speculated to provide steroidogenic capacity with which to initiate a marked increase in cortisol secretion in the immediate post-awakening period (Born et al., 1999, Clow et al., 2010).

The awakening cortisol sample (S1) provides information about PAC, and a negative association between S1 and AUCi is often observed (Adam et al., 2006, Dahlgren et al., 2009, Stalder et al., 2009, Vreeburg et al., 2009, Wilhelm et al., 2007). This can be amplified by long S1 sampling latency but may also reflect variation in the magnitude of SCN mediation of ACTH sensitivity. Unfortunately, in many studies a distinction between these measures is not made, contributing to the heterogeneity of CAR research. In the present study the CAR is represented by AUCi, AUCg is taken to represent total hormone secretion, and S1 can be used as an index of PAC.

Our findings suggest that individuals exposed *in utero* to GDM do not have an impaired CAR, but do exhibit lower total cortisol output during the morning, which subsequently normalises during the afternoon. Given that awakening cortisol is lower in this group, this effect may represent a difference in early morning, pre-awakening cortisol secretion reflecting altered circadian and sleep-regulated cortisol production.

Dysregulation of the HPA axis, including the diurnal cortisol curve and the CAR, is associated with metabolic dysfunction. For example, Kuehl et al. (2015) found that lower total morning cortisol (AUCg) was associated with higher risk of metabolic syndrome, especially higher blood pressure. Bruehl et al. (2009) found a blunted CAR (AUCi) in type 2 diabetes mellitus (T2DM) patients, and Lasikiewicz et al. (2008) also found that 'blunted' CARs were associated with metabolic disturbance, but did not specify which AUC measure was used. Hackett et al. (2014) reported that flatter diurnal cortisol slopes were associated with T2DM and, in a related study, found flatter diurnal slopes to predict the development of T2DM around 10 years later Hackett et al. (2016).

Importantly, individuals born to mothers with diabetes are at increased risk of developing obesity, insulin resistance, and T2DM both during childhood and later in life (e.g. Silverman et al., 1995, Boney et al., 2005, Lawlor et al., 2011, Nehring et al., 2013, Landon et al., 2018, Kawasaki et al., 2018). Thus, our findings suggest that altered function of the HPA axis—specifically, lower total morning cortisol secretion and possibly PAC—may represent one mechanism underlying the increased risk of future metabolic dysfunction in this group.

The mechanisms linking GDM and offspring HPA axis function are unclear. However, there is evidence from both animals and humans indicating that an adverse intrauterine metabolic environment programs the fetal HPA axis (Sullivan et al., 2015). For example, Sasaki et al. (2013) reported that rats exposed to maternal high-fat diet had increased expression of corticosteroid receptors in the amygdala, decreased basal corticosterone production, and slower responsivity to stress. In humans, higher maternal obesity, triglycerides, and cholesterol are associated with greater cortisol reactivity in children at 3-5 years (Mina et al., 2017).

Similarly, the mechanisms underlying the link between HPA axis function and current or future metabolic dysfunction are not entirely understood. However, cortisol plays an important role in many metabolic processes. Cortisol functions to increase

glucose through gluconeogenesis and directly regulates insulin sensitivity through activation of receptors on pancreatic β cells. Further, cortisol can induce lipolysis and fatty acid release into the blood, and promote triglyceride build-up in adipose tissue—both processes associated with increased diabetes risk (Di Dalmazi et al., 2012, Pankow et al., 2004). Abnormal diurnal cortisol patterns may also disrupt immune and inflammatory processes and thereby alter metabolic function (Wang et al., 2013), and altered stress regulation is associated with greater risk of T2DM (Pouwer et al., 2010).

Besides slightly higher total cortisol secretion in the metformin group, there were no significant differences in cortisol characteristics between participants whose mothers were treated with metformin versus those treated with insulin, supporting the safety of metformin for treating GDM (Rowan et al., 2008, Rowan et al., 2011, Wouldes et al., 2016, Rowan et al., 2018).

To conclude, there are well-established links between maternal metabolic dysfunction and offspring HPA axis activity, and between altered HPA axis activity and later metabolic dysfunction. We have provided evidence that maternal GDM is associated with lower morning cortisol secretion, and possibly pre-awakening cortisol, and suggest that this may be a mechanism underlying the increased risk of metabolic disturbance and T2DM in this population. Although our study is small and relied on subjective sample times, it involved multiple days of sampling, high protocol adherence, and comprehensive analyses, including a variety of potential confounders and control measures. However, we cannot determine which specific features of GDM affect the developing HPA axis. GDM is a complex disorder involving changes in endocrine and metabolic function both superimposed on and modulated by pregnancy-related processes. We would speculate that insulin resistance, hyperglycaemia, and resulting inflammation are important mechanisms, but future research should investigate specific markers in both GDM and control mothers. Further, it will be important to examine overnight cortisol secretion, the neuroendocrine response to stress, and potential correlates of altered HPA function, including stress regulation and sleep, in individuals exposed *in utero* to GDM.

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Chapter 6: General discussion

GDM affects more than one in ten live births globally, and incidence rates are increasing alongside the obesity epidemic. There is a substantial body of evidence indicating that children born to women with GDM often experience altered neurodevelopmental trajectories. Neurodevelopmental disorders such as autism and attention-deficit hyperactivity disorder, impaired intelligence and motor function, and language disturbances are all more common following *in utero* exposure to GDM. Further, these individuals are at much higher risk of developing T2DM during childhood or later in life.

Despite the knowledge about functional outcomes of GDM exposure, very little is known about the underlying physiology. Indeed, I am not aware of any previous research directly examining neurophysiology in relation to GDM in humans. Similarly, there is a lack of research into the physiological, non-genetic mechanisms underlying increased diabetes risk following exposure to GDM. Thus, the studies described in this thesis reflect the first efforts towards characterising neurophysiological and neuroendocrine development in humans born to women with GDM.

6.1 GDM MAY HAVE SUBTLE EFFECTS ON CHILD NEUROPLASTICITY

In Chapter 2, I presented evidence suggesting that children born to women with GDM have a more variable and, on average, smaller LTD-like response to cTBS. However, in Chapter 4 these findings were not replicated. That is, neuroplasticity did not differ between the GDM group and the control group. The strength of the group comparison in Chapter 2 may be partially explained by an unrepresentative control sample; the responses in this group were particularly strong and consistent. Nevertheless, there is reason to believe that the effect observed in Chapter 2 does exist. These findings did not consist solely of a between-group comparison, but were supported by a range of physiological markers, most prominently the association between plasticity and maternal insulin resistance. That is, within the GDM group,

those individuals whose mothers had more severe GDM were more likely to have abnormal neuroplastic responses to cTBS, an effect that is consistent with animal research (Jing et al., 2014, Janthakhin et al., 2017, Bae-Gartz et al., 2019) and research in individuals born preterm (Pitcher et al., 2012a). Further, the GDM group had lower cortical excitability, further indicating a detrimental influence of GDM on neurodevelopment. Therefore, I would suggest that the most likely explanation is that GDM does indeed affect a child's cortical physiology, including mechanisms underlying synaptic plasticity and excitability, but that these effects are generally subtle and would require larger samples to detect reliably with current technology. Indeed, it is important to remember that the sample under investigation here, which is representative of most the relevant first-world population, experienced by definition relatively mild complications of GDM; their medical care was of the highest quality and their GDM was diagnosed as early as contemporary guidelines allowed. Thus, the consequences of GDM to the children in this sample are necessarily correspondingly mild. As I mentioned in Chapter 1, however, the majority of GDM cases worldwide occur in developing nations and are not so well managed. Thus, the mild and inconsistent effects observed here may belie more serious neurological and neurophysiological consequences that may affect many thousands of children globally each year. GDM is an inherently complex disorder, and cases differentially involve various internal and external factors, including diet, lifestyle, obesity, genetics, and epigenetics. Thus, elucidating clear mechanistic links between pathophysiological aspects of GDM and specific neurodevelopmental factors is a challenging task. Indeed, inter-individual variation in the time-course of the development of GDM is likely integral to subsequent neurodevelopmental consequences, as even milder cases may lead to more serious harm if they occur earlier, during more sensitive periods of fetal development.

Given the available evidence, we cannot conclude that GDM exposure does or does not influence the child's capacity for synaptic plasticity. However, assuming such an effect exists, animal research provides some potential mechanisms. For example, as discussed in Chapter 1, rodents born to dams with adverse metabolic phenotypes exhibit atrophy of pyramidal neuron dendrites in both the hippocampus and amygdala and have significantly different global gene expression profiles—including in a number of key pathways involved in neurogenesis, axogenesis, and regulation of

synaptic plasticity (Bae-Gartz et al., 2019)—which may partially explain the alterations in synaptic plasticity and learning in these animals (Janthakhin et al., 2017).

Throughout the course of the research presented in this thesis, it became increasingly clear that cTBS suffers from significant variability. Indeed, several recent studies have reported considerable intra- and inter-subject variability in responses (Vallence et al., 2015, Jannati et al., 2017, Pellegrini et al., 2018, Jannati et al., 2019). Importantly, responses to TBS vary not only in magnitude but also in the direction of excitability change. That is, cTBS, which is designed to induce inhibition-like changes in excitability, has been observed to induce changes consistent with facilitation of excitability, as was found in Chapters 2 and 4. Therefore, to detect differences in the response to cTBS between groups reliably would likely require sample sizes much larger, ideally in the hundreds, that are far beyond what was possible during the research presented here. This research, therefore, may be considered a preliminary investigation into the possible physiological mechanisms underlying altered neurodevelopment following GDM, and should be extended both with larger samples and using additional technologies. TMS and TBS have recently become common supplements to neuroimaging techniques, and combinations of such tools, for example concurrent TMS-EEG (Rogasch and Fitzgerald, 2013, Chung et al., 2015b), offer promising pathways for future research into the neurophysiological consequences of GDM.

6.2 ROLE OF INTERNEURON RECRUITMENT

Several studies have attempted to identify and quantify factors contributing to variability in the response to cTBS. Of these factors, the most important are believed to include age, gender, cortisol, attention, genetics, and muscle activity (when assessing the motor cortex) (Ridding and Ziemann, 2010). Following the completion of the research presented in Chapter 2, I learned that the measurement of I-wave recruitment had recently been identified as a novel and potentially powerful predictive marker of the response to cTBS and that it may be useful in explaining high variability of cTBS responses (Hamada et al., 2013). Given the variability observed in the GDM group in Chapter 2, I formulated the hypothesis that interneuron recruitment may be able to explain the results observed in this study. Indeed, the results presented by

Hamada et al. (2013) and Hordacre et al. (2017) raised the possibility that the difference in responses to cTBS reflected, at least in part, differences in cortical interneuron recruitment rather than in the mechanisms underlying synaptic plasticity. Although the relationship between I-wave latencies and the neuroplastic response to cTBS (and iTBS) was compelling, evidence was limited, and the effect had not been examined in children or adolescents. Further, there had not yet been an investigation into a potential interaction with either age or sex. Thus, in Chapter 3 I examined these factors and presented some surprising results.

Contrary to previous studies (Hamada et al., 2013, Huang and Mouraux, 2015, Hordacre et al., 2017), we did not find a significant association between I-wave recruitment and the response to cTBS when assessing the full sample. However, we did observe significant interactions between I-wave recruitment and both age and sex. Closer inspection revealed that preferential late I-wave recruitment only predicted cTBS response in females, especially young females. The association was not detectable to any degree in males, regardless of age. While the association was not significant in adult females, a trend was observed in the expected direction, and in females of all ages the effect remained, so it may be premature to conclude that the association is not present in adult females. In any case, these results were surprising, and we are not able to explain them fully. Although the presence of a relationship between interneuron recruitment and the response to TBS is highly probable, our findings suggest that the specificity of this effect, namely to females, may limit the utility and generalisability of this factor as a predictive marker or control variable.

Nevertheless, the strong association in young females supported the idea that interneuron recruitment may drive at least some of the difference in cTBS response between the GDM and control groups presented in Chapter 2, at least in females. Thus, in addition to an attempt at replication of the findings in Chapter 2, Chapter 4 acts as an investigation into a potential role of differential interneuron recruitment in explaining those findings. As discussed above, the difference in neuroplastic responses observed between the GDM and control groups was not replicated. As has now been observed in several studies, cTBS did not produce a net, group-level suppression of MEP amplitudes. Thus, given that plasticity did not differ significantly between groups, it was unlikely that we would find a difference in the association between plasticity and interneuron recruitment. Indeed, such an effect was not observed, and

the central tendency of I-wave latencies was similar between groups. However, the GDM group were observed to have a greater spread of latencies, including both more extreme minima and maxima. Thus, there is some evidence for a difference in the recruitment of interneuron networks by TMS in adolescents exposed to GDM, but this difference is unlikely to be related to a difference in synaptic plasticity or to systematically alter the response to cTBS.

6.3 ALTERED CORTISOL PRODUCTION

In Chapter 5, I presented evidence suggesting that adolescents born to women with GDM have lower cortisol secretion during the morning and likely in the period before awakening. Conversely, their CAR was of a similar magnitude and shape to the control group. Dysregulation of the HPA axis, including the diurnal cortisol rhythm and the CAR, is associated with metabolic dysfunction, including T2DM (Lasikiewicz et al., 2008, Bruehl et al., 2009, Lamers et al., 2013, Kuehl et al., 2015, Champaneri et al., 2012, Hackett et al., 2014, Hackett et al., 2016). HPA dysregulation has also been associated with developmental insults; for example, Kuras et al. (2017) found that mild–moderate early-life adversity was strongly associated with a blunted diurnal cortisol profile and low awakening cortisol, but not an altered CAR. Thus, other forms of adverse development are known to result in very similar HPA profiles to those observed in the GDM group in Chapter 5. Indeed, as discussed in Chapter 1, environmental programming during early development is crucially important for later health, and the HPA axis represents a prominent mechanistic pathway between early life exposures and development. Prenatal exposure to smoking, adverse environmental conditions, maternal stress, and non-optimal nutrition all have been found to alter the function of the HPA axis in offspring.

Given that children born to mothers with diabetes are both exposed to adverse prenatal environments and are at increased risk of developing obesity, insulin resistance, and diabetes, both during childhood and later in life (e.g. Silverman et al., 1995, Boney et al., 2005, Lawlor et al., 2011, Nehring et al., 2013, Landon et al., 2018, Kawasaki et al., 2018), an abnormality in the function of the HPA axis may represent a mechanism underlying the increased risk of future metabolic dysfunction in this group. The hippocampus may play an important role in this relationship; adverse

conditions during development change the size and functioning of the hippocampus, thereby altering HPA axis activity. Childhood adversity, for example, is associated with reduced hippocampal volume (Andersen and Teicher, 2009) and GR methylation and transcription (Oberlander et al., 2008, Romens et al., 2015), possibly explaining the subsequent alteration in basal HPA activity (Kuras et al., 2017). The pathway from adverse developmental conditions to altered hippocampal development was recently extended to GDM via the study of rats. Glombik et al. (2019) reported that offspring of rats with induced GDM experienced increased hippocampal inflammation, an effect that was attenuated when mothers were treated with metformin of glyburide. Similarly, offspring of diabetic rats have been shown to exhibit chronic hippocampal inflammation and decreased synaptic integrity which were associated with impaired memory into young adulthood (Vuong et al., 2017).

Cortisol rises gradually throughout the night during sleep, reaching a circadian peak in the early hours pre-awakening. This phenomenon, a part of the circadian cortisol system, is distinct from the CAR. Although not fully understood, pre-awakening cortisol is likely influenced by a range of complex factors. For example, in the pre-awakening period cortisol production appears to be largely de-coupled from its secratagogue, ACTH. That is, cortisol sensitivity to ACTH decreases, which has been speculated to provide steroidogenic capacity with which to initiate a marked increase in cortisol secretion in the immediate post-awakening period. This dissociation probably results from extra-pituitary SCN-mediated mechanisms (Born et al., 1999, Clow et al., 2010). Pre-awakening cortisol concentrations are likely subject to a range of other inputs, including negative feedback inhibition of the HPA axis via glucocorticoid receptors which operate at the level of the hypothalamus, pituitary, and hippocampus (De Kloet, 2004). The findings in Chapter 5 suggest that individuals exposed *in utero* to GDM exhibit a difference in the processes that regulate pre-awakening cortisol, resulting in lower output in this period. For example, it may be speculated that the dissociation of cortisol from ACTH is of a greater magnitude and that greater cortisol desensitivity to ACTH results in lower early morning cortisol. Such a process itself may result from any of a range of factors that contribute to cortisol-ACTH dissociation, including neuropeptides, neurotransmitters, cytokines, and adipokines, which all are known to modulate adrenal glucocorticoid release independently of pituitary ACTH (Bornstein et al., 2008).

Whatever mechanisms are affected, our results suggest that they must spare the function of the CAR. This is entirely plausible given the overlapping yet distinct regulatory pathways controlling the CAR. Indeed, integral to the expression of the CAR are SCN-related mechanisms which are opposite in nature to the regulation of pre-awakening cortisol; PAC relies on reduced adrenal sensitivity to ACTH, while the CAR is related to increased adrenal sensitivity to ACTH post-awakening (Clow et al., 2010). This switch in ACTH sensitivity post-awakening is likely mediated by light-sensitive extra-pituitary neural pathways: in mammals, light activates retinal ganglion cells which communicate with the SCN. Indeed, morning awakening in the absence of light decreases the dynamic of the CAR (Scheer and Buijs, 1999), while waking following the simulation of dawn (by gradually increasing light) increases the CAR (Thorn et al., 2004). In rodents, these effects are absent following lesion of the SCN (Buijs et al., 2003). Thus, GDM may alter the mechanisms mediating pre-awakening ACTH sensitivity while sparing those that regulate post-awakening light-sensitivity.

6.4 METFORMIN

Although not a major focus of this thesis, the MiG study (Rowan et al., 2008) was concerned with the safety and efficacy of metformin treatment during pregnancy. Given that the research presented herein comprises follow-up studies of the MiG study, we were able to examine potential differences in outcomes between children whose mothers were treated with metformin compared with those treated with insulin. Although metformin, an oral antihyperglycaemic agent, is a routine treatment for T2DM and has been shown to be safe and effective for use during pregnancy in the short term, concerns remain around its long-term safety due to its ability to cross the placenta (Hague et al., 2003). Although limited, our results do not suggest that metformin is associated with significant differences in neurophysiology or cortisol secretion in offspring when compared with maternal insulin treatment. Thus, these data represent a promising addition to a growing body of evidence regarding metformin's long-term safety (Rowan et al., 2008, Rowan et al., 2011, Barrett et al., 2013, Battin et al., 2015, Wouldes et al., 2016, Coat et al., Unpublished observations, Rowan et al., 2018).

6.5 CONCLUDING REMARKS

Individuals born to women with GDM are at increased risk of a host of neurodevelopmental difficulties. Although there is a large body of evidence describing these functional outcomes, very little is known about the underlying neurophysiology. In this thesis I presented limited evidence that GDM exposure is associated with altered synaptic plasticity and cortical excitability. However, these findings were inconsistent and likely represent very subtle effects that may be difficult to replicate without much larger samples and more sensitive techniques. Nevertheless, they may represent important avenues of research, especially when considering that globally most cases of GDM and GDM-related developmental pathology occur in the developing world and are significantly more severe. Future studies may employ additional techniques, including EEG, MRI, and other neuroimaging modalities, to further explore the neurophysiology of GDM. The research presented here only examined one form of neuroplasticity—LTD—so a natural extension of this work would involve the study of different plasticity mechanisms.

In addition to neurodevelopmental consequences, individuals born to women with GDM are at much higher risk of developing diabetes and other metabolic pathologies during their lives, and the mechanisms underlying these risks are similarly under-studied. In Chapter 5 I presented evidence suggesting that GDM exposure is associated with decreased pre-awakening and morning cortisol secretion but a similar response to awakening (CAR). Given that altered cortisol profiles are associated with metabolic pathology, this may represent an important pathway between GDM exposure and later metabolic dysfunction. Further, cortisol has important roles in the brain, including in synaptic plasticity, so altered cortisol secretion may prove important to the neurodevelopmental outcomes experienced by this group. More detailed examinations of cortisol secretion in this group are warranted and should include measurements during the night and in response to stress and other secretory challenges.

Millions of children are born each year having experienced GDM during gestation, and these numbers are climbing. The research in this thesis contributes to the important and growing body of research describing the pathophysiological features

of GDM exposure with the goal of reducing neurodevelopmental morbidity and the substantial health and economic impacts of GDM.

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Appendices

6.7 APPENDIX I: CHAPTER 2 STATEMENT OF AUTHORSHIP

Statement of Authorship

Title of Paper	Reduced cortical excitability, neuroplasticity, and salivary cortisol in 11–13-year-old children born to women with gestational diabetes mellitus
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	VAN DAM, J. M., GARRETT, A. J., SCHNEIDER, L. A., HODYL, N. A., GOLDSWORTHY, M. R., COAT, S., ROWAN, J. A., HAGUE, W. M. & PITCHER, J. B. 2018. Reduced Cortical Excitability, Neuroplasticity, and Salivary Cortisol in 11-13-Year-Old Children Born to Women with Gestational Diabetes Mellitus. EBioMedicine, 31, 143-149.

Principal Author

Name of Principal Author (Candidate)	Jago Van Dam		
Contribution to the Paper	Designed study, recruited participants, collected data, processed and analysed data, prepared figures and wrote the manuscript		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	15/05/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Amy J. Garrett		
Contribution to the Paper	Collected data, performed immunohistochemical analysis		
Signature		Date	15/5/2020

Name of Co-Author	Luke A. Schneider		
Contribution to the Paper	Recruited participants, collected and processed data		
Signature		Date	26/5/2020

Name of Co-Author	Nicolette A. Hodyl		
Contribution to the Paper	Contributed to study design, performed immunohistochemical analysis		
Signature		Date	15/5/2020

Name of Co-Author	Mitchell R. Goldsworthy		
Contribution to the Paper	Assisted data collection and analysis		
Signature		Date	15/5/2020

Name of Co-Author	Suzette Coat		
Contribution to the Paper	Managed parent study and participant registry, assisted recruitment		
Signature		Date	15/5/2020

Name of Co-Author	Janet A. Rowan		
Contribution to the Paper	Conducted parent clinical trial, assisted interpretation of data		
Signature		Date	18/5/2020

Name of Co-Author	Bill M. Hague		
Contribution to the Paper	Conducted parent clinical trial, assisted recruitment and interpretation of data and preparation of manuscript		
Signature		Date	18/5/2020

Name of Co-Author	Julia B. Pitcher		
Contribution to the Paper	Designed study, assisted analysis and interpretation of data, assisted preparation of manuscript, supervised project		
Signature		Date	29/06/2020

6.8 APPENDIX II: CHAPTER 3 STATEMENT OF AUTHORSHIP

Statement of Authorship

Title of Paper	The association between I-wave recruitment and the response to cTBS is age- and sex-dependent
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	VAN DAM, J.M., GOLDSWORTHY, M.R. 2020. The association between I-wave recruitment and the response to cTBS is age- and sex-dependent.

Principal Author

Name of Principal Author (Candidate)	Jago Van Dam		
Contribution to the Paper	Conceived of and designed study, recruited participants, collected data, processed and analysed data, prepared figures and wrote manuscript		
Overall percentage (%)	95		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	20/07/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Mitchell R. Goldsworthy		
Contribution to the Paper	Assisted with interpretation of data and editing of manuscript		
Signature		Date	20/07/2020

6.9 APPENDIX III: CHAPTER 4 STATEMENT OF AUTHORSHIP

Statement of Authorship

Title of Paper	Cortical plasticity and interneuron recruitment in adolescents born to women with gestational diabetes mellitus
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	VAN DAM, J. M., GOLDSWORTHY, M. R., HAGUE, W. M., COAT, S., & PITCHER, J. B. 2020. Cortical plasticity and interneuron recruitment in adolescents born to women with gestational diabetes mellitus.

Principal Author

Name of Principal Author (Candidate)	Jago Van Dam		
Contribution to the Paper	Designed study, recruited participants, collected data, processed and analysed data, prepared figures and wrote the manuscript		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	20/07/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Mitchell R. Goldsworthy		
Contribution to the Paper	Assisted with study design, interpretation of data, and editing of manuscript		
Signature		Date	20/07/2020

Name of Co-Author	Bill M. Hague		
Contribution to the Paper	Identified potential control participants, assisted with editing manuscript		
Signature		Date	17/07/2020

Name of Co-Author	Suzette Coat		
Contribution to the Paper	Assisted with recruitment and editing of manuscript		
Signature		Date	13/07/2020

Name of Co-Author	Julia B. Pitcher		
Contribution to the Paper	Assisted with study design, interpretation of data and editing of manuscript		
Signature		Date	17/07/2020

6.10 APPENDIX IV: CHAPTER 5 STATEMENT OF AUTHORSHIP

Statement of Authorship

Title of Paper	Lower morning cortisol secretion in adolescents born to women with gestational diabetes mellitus
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	VAN DAM, J. M., GOLDSWORTHY, M. R., HAGUE, W. M., COAT, S., & PITCHER, J. B. 2020. Lower morning cortisol secretion in adolescents born to women with gestational diabetes mellitus.

Principal Author

Name of Principal Author (Candidate)			
Contribution to the Paper	Designed study, recruited participants, collected data, processed and analysed data, prepared figures and wrote the manuscript		
Overall percentage (%)	90		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	20/07/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Mitchell R. Goldworthy		
Contribution to the Paper	Assisted with study design, interpretation of data, and editing of manuscript		
Signature		Date	17/07/2020

Name of Co-Author	Bill M. Hague		
Contribution to the Paper	Identified potential control participants, assisted with editing manuscript		
Signature		Date	17/07/2020

Name of Co-Author	Suzette Coat		
Contribution to the Paper	Assisted with recruitment and editing of manuscript		
Signature		Date	13/07/2020

Name of Co-Author	Julia B. Pitcher		
Contribution to the Paper	Assisted with study design, interpretation of data and editing of manuscript		
Signature		Date	17/07/2020

6.11 APPENDIX V: PUBLICATIONS ARISING FROM THIS THESIS

VAN DAM, J. M., GARRETT, A. J., SCHNEIDER, L. A., HODYL, N. A., GOLDSWORTHY, M. R., COAT, S., ROWAN, J. A., HAGUE, W. M. & PITCHER, J. B. 2018. Reduced Cortical Excitability, Neuroplasticity, and Salivary Cortisol in 11-13-Year-Old Children Born to Women with Gestational Diabetes Mellitus. *EBioMedicine,* 31**,** 143-149.

VAN DAM, J. M. & GOLDSWORTHY, M. R. 2020. Case report of a vasovagal pre-syncope event during single-pulse transcranial magnetic stimulation in a healthy adult participant. *Clin Neurophysiol,* 131**,** 981-982.

VAN DAM, J. M., GOLDSWORTHY, M. R. The association between I-wave recruitment and the response to cTBS is age- and sex-dependent. *Submitted for publication.*

VAN DAM, J. M., GOLDSWORTHY, M. R., HAGUE, W. M., COAT, S., & PITCHER, J. B. 2020. Cortical plasticity and interneuron recruitment in adolescents born to women with gestational diabetes mellitus. *Under review.*

VAN DAM, J. M., GOLDSWORTHY, M. R., HAGUE, W. M., COAT, S., & PITCHER, J. B. 2020. Lower morning cortisol secretion in adolescents born to women with gestational diabetes mellitus. *Under review.*

6.12 APPENDIX VI: OTHER WORK PERFORMED DURING THIS PHD

VAN DAM, J. M., GARRETT, A. J., SCHNEIDER, L. A., BUISMAN-PIJLMAN, F. T. A., SHORT, M. A., HODYL, N. A., EDWARDS, H. K., GOLDSWORTHY, M. R. & PITCHER, J. B. 2018. Variability of the cortisol awakening response and morning salivary oxytocin in late adolescence. *J Neuroendocrinol***,** e12645.

GOLDSWORTHY, M. R., ROGASCH, N. C., BALLINGER, S., GRAETZ, L., VAN DAM, J. M., HARRIS, R., YU, S., PITCHER, J. B., BAUNE, B. T. & RIDDING, M. C. 2020. Age-related decline of neuroplasticity to intermittent theta burst stimulation of the lateral prefrontal cortex and its relationship with late-life memory performance. *Clinical Neurophysiology*, 131**,** 2181-2191.

VAN DAM, J. M., GOLDSWORTHY, M. R., SHORT, M. A., GARRETT, A. J., SCHNEIDER, L. A., BUISMAN-PIJLMAN, F. T. A., & PITCHER, J. B. Larger average cortisol awakening response is associated with poorer executive function and processing speed in healthy adolescents. *Submitted for publication.*

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