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# Specific Aim 1

**Determining the effects of glucocorticoid-induced stress on placental** **transport of nutrients and endocrine function.**

The placenta is the direct and only site of communication between mother and fetus during *in utero* development (Brett *et al.*, 2014). The placenta is the rate-limiting step for fetal nutrient and gas acquisition (Brett *et al.*, 2014). Additionally, the placenta plays an important endocrine role to promote fetal growth and nutrient supply (Malassine *et al.*, 2003). The placenta is highly regulated to ensure adequate growth of the fetus in normal pregnancies (Napso *et al.*, 2018). In cases of maternal glucocorticoid-induced stress, placental nutrient transport and endocrine function are compromised leading to potentially suboptimal fetal growth (Kipmen-Korgun *et al.*, 2012; Waffarn & Davis, 2012). In Denmark, 20% of women reported use of corticosteroids from 4 weeks prior to delivery until delivery between 1996-2008 (Hviid & Mølgaard-Nielsen, 2011). The mechanisms by which maternal corticosteroids influence fetal health and placental function are understudied (Kemp *et al.*, 2015). Some side effects like reduced birthweight, offspring hypertension, mental illness and higher childhood HPA axis activity remain controversial (Alexander *et al.*, 2012; Waffarn & Davis, 2012; Duthie & Reynolds, 2013; Reynolds, 2013; Braun *et al.*, 2013; Moisiadis & Matthews, 2014). My hypothesis is that glucocorticoid treatments prior to conception and/or during conception cause altered placental transport and hormonal function in a time-dependent manner by which an early and prolonged exposure during pregnancy has more prominent side effects on the placenta and fetus. To test this hypothesis, we will examine a) how maternal dexamethasone effects on placental development and function, b) how maternal dexamethasone affects fetal and offspring development and health, and c) the role of placental glucocorticoid receptor (GR) in mitigating the effects of maternal dexamethasone exposure.

# Rationale and Background

## Murine Placental Development and Physiology

The definitive structure of the mouse placenta is (Malassine *et al.*, 2003). The placenta encompasses two sides, an arc-shaped surface facing the maternal side and another flat surface facing the fetal side (Georgiades *et al.*, 2002). The mouse placenta has three distinct compartments, a decidual maternal zone which is the outermost compartment, a fetal-derived junctional zone that mediates placental endocrine function, and a fetal-derived labyrinth zone that comprises the majority of the placenta and is the main site for nutrient and gas exchange (Woods *et al.*, 2018). Three exchange barriers exist moving inwards from the decidua to the fetal compartment including two syncytiotrophoblast layers (in the labyrinth layer) and one fetal endothelial cell layer (Georgiades *et al.*, 2002). The two syncytiotrophoblast barriers comprise the microvillous membrane facing the maternal circulation and the basal membrane facing fetal circulation (Brett *et al.*, 2014). Figure 1 represents the mouse placenta (Bronson & Bale, 2016).

At midgestation, placental invasion of the maternal uterine cavity occurs to allow maternal blood flow into the placental cavity (Malassine *et al.*, 2003; Woods *et al.*, 2018). This invasion permits direct nutrient uptake from the maternal circulation to the fetus through the placenta. Prior to this invasion, the embryo acquires nutrients from the yolk sac, the initial placental structure that absorbs nutrients from maternal circulation (Malassine *et al.*, 2003; Woods *et al.*, 2018).

### Figure 1: Diagram representing the mouse placental cell types and zones



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## Cortisol/Corticosterone Levels in Pregnancy

During human pregnancy, mean cortisol rises gradually as pregnancy progresses (Carr *et al.*, 1981). Mean cortisol levels increase in humans during the first, second and third trimester by 1.6, 2.4 and 2.9 folds, respectively (Jung *et al.*, 2011). The increased cortisol levels may be explained by placental secretions of estrogen stimulating maternal cortisol production and mitigating maternal negative feedback (Lindsay & Nieman, 2005; Duthie & Reynolds, 2013) and/or by placental production of corticotropin-releasing hormone (CRH) into the maternal circulation in mid- and late gestation (Duthie & Reynolds, 2013). Maternal cortisol promotes placental CRH production, which in turn promotes maternal HPA axis activity thus acting as a feed-forward positive mechanism.

However, in mouse pregnancy, corticosterone levels do not increase as much as humans near term although there are still increases. In pregnant control mice, corticosterone levels were not significantly different at E11 and E17 despite slightly higher levels at E17 (Jafari *et al.*, 2017). Other studies showed an increase in corticosterone levels at E19 compared to E16 in control unstressed mice (Vaughan *et al.*, 2012). Unstressed pregnant mice had higher corticosterone levels with peak levels at E16 being 60 times higher than non-pregnant mice (Barlow *et al.*, 1973). The levels then dropped after E16 until delivery at E19 (Barlow *et al.*, 1973).

## Fetal HPA Axis Development

The human fetal hypothalamic-pituitary axis activity is detected as early as 8-12 weeks of gestation (Ng, 2000) and is fully developed in the second trimester of pregnancy (Moisiadis & Matthews, 2014). In early pregnancy, fetal cortisol is thought to primarily be attained from maternal cortisol, as the fetus is believed to sufficiently produce cortisol at 22 weeks of gestation (Buss *et al.*, 2012). Given the critical developmental window by which fetal organs and HPA axis are developing, it is possible that increased maternal cortisol levels in early pregnancy compared to late pregnancy may have more deleterious effects on fetal development (Barker, 2007; Braun *et al.*, 2013). In mice, the offspring HPA develops postnatal in two phases. On postnatal day (PND) 1 through 12, the mouse HPA is considered hypo-responsive, and after PND 12 the HPA system matures (Schmidt *et al.*, 2003).

## Glucocorticoid Treatments in Pregnancy

In addition to the naturally increasing cortisol levels in pregnancy, glucocorticoid (GC) treatments are further prescribed during pregnancy for multiple reasons. A single course of synthetic corticosteroid treatment is prescribed to women who are at risk of delivering premature babies. The treatment is proven to increase offspring chances of survival post-delivery (Doyle *et al.*, 2000; Baisden *et al.*, 2007). Glucocorticoid treatments are prescribed as they enhance fetal growth, specifically fetal lung maturation to prevent respiratory distress syndrome (RDS), and aid in overall embryogenesis to prevent perinatal death due to hemorrhages, heart failure and other underlying causes associated with preterm birth (Lunghi *et al.*, 2010; Singh *et al.*, 2012). Specifically, betamethasone, dexamethasone, prednisolone, corticosteroids, or cortisol are prescribed to women who have acute asthma or asthma, hyperemesis gravidarum, depression, stress, or are at risk of delivering preterm babies (Singh *et al.*, 2012). The use of corticosteroids is widespread. In a Danish cohort study encompassing all births in Denmark from 1996-2008, about 20% of women reported use of corticosteroids from 4 weeks prior to delivery until delivery (Hviid & Mølgaard-Nielsen, 2011). In an American cohort study including 152,531 pregnancies between 1996-2000, 3.5% of pregnant women who had a documented diagnosis associated with preterm birth used corticosteroids, while 1.7% of pregnant women who did not have a documented diagnosis used corticosteroid (Andrade *et al.*, 2004). Despite the placenta’s function to protect the fetus from excess maternal corticosteroid, synthetic corticosteroids used in preterm treatments can readily cross the placenta bypassing inactivation by HSD11B2(Cuffe *et al.*, 2011; Singh *et al.*, 2012).

## Effects of Glucocorticoid Exposure on Placental and Fetal Development

Pregnant rats treated with dexamethasone at E13 until E20 showed reduced placental and fetal weights (Ain *et al.*, 2005). Despite the evident placental and fetal growth restriction, dexamethasone did not affect litter size or fetal viability (Ain *et al.*, 2005). Rats exposed to triamcinolone once at E16 had 53% reduction in placental weight and 73% reduction in fetal weights (Hahn *et al.*, 1999). Mice exposed to a sound stressor on E10.5, E12.5, and E14.5 showed reduced fetal body weight and had growth restriction that was more evident in female fetuses (Wieczorek *et al.*, 2019). Pregnant mice exposed to dexamethasone on E15, E16, and E17 had reduced placental and fetal weights and trophoblast swelling in the junctional and labyrinth zones (Baisden *et al.*, 2007). Furthermore, mice given dexamethasone at E11-E16 had reduced fetal and placental weights at E16, but the volume of the placental junctional and labyrinth zone was unchanged despite less fetal capillaries in the labyrinth zone (Vaughan *et al.*, 2012). On the contrary to the evident reduction in placental and fetal weights seen in some papers, mice treated with dexamethasone on E13.5 and E14.5 showed no effect on fetal weight at E15.5, E18.5, or at birth (Audette *et al.*, 2011). There was also no effect on placental weights at E15.5, E17.5 in male or female placentas, but at E18.5 female placentas had reduced weight but male placentas were not different (Audette *et al.*, 2011). However, placental junctional and labyrinth zone proportions with respect to the total placental area was unchanged (Audette *et al.*, 2011).

## Effect of Glucocorticoid Exposure on Placental Nutrient Transporters

Since the placenta is the only source of fetal nutrient acquisition, transporter expression may reflect the efficiency at which maternal nutrients pass through the placenta to the fetus. To pass to the fetus, the nutrients need to bypass the three placental exchange barriers including two syncytiotrophoblast layers and the fetal endothelial cell layer (Georgiades *et al.*, 2002). The findings below highlight the conflicting evidence within species and between models regarding placental transporter expression, which emphasizes the need for further studies.

### Glucose Transporters

Rats exposed to triamcinolone (TA) at E16 had reduced mRNA and protein expression of GLUT1 and GLUT3 at E21 (Hahn *et al.*, 1999). Inversely, pregnant rats exposed to 100 or 200 ug/kg body weight/ day of dexamethasone starting at E15 showed increased placental GLUT1 protein expression by 1.6 and 1.9 fold, respectively at E21 indicating a dose-dependent effect (Langdown & Sugden, 2001).

Mice exposed to corticosterone in their drinking water at E11-E16 had increased placental GLUT1 and GLUT3 gene expression at E16, while mice exposed at E14-E19 showed unchanged expression (Vaughan *et al.*, 2015). Unlike the transporter expression at both timepoints, transplacental clearance of radiolabeled glucose was unchanged at E16 after the exposure from E11-E16, but clearance was reduced by 33% at E19 after exposure from E14-E19 (Vaughan *et al.*, 2015). This suggests that other glucose transporters may be involved in placental glucose uptake (Vaughan *et al.*, 2015). Opposite results show that mice treated with dexamethasone for 60 hours via a minipump starting at E12.5 showed unaltered gene expression of GLUT1 and GLUT3 at E14.5 and at E17.5 (Cuffe *et al.*, 2011).

Human placental extracts from term deliveries further showed reductions in GLUT1 mRNA and protein expression after TA treatment along with reduced protein expression of GLUT3 but unaltered GLUT3 mRNA expression (Hahn *et al.*, 1999). Another study showed that human placental extracts from term deliveries had unaltered GLUT1 mRNA expression but increased GLUT1 protein expression when treated with 1, 2, and 20 mg/ml of hydrocortisone (Mateos *et al.*, 2018). GLUT3 mRNA expression was increased when placental explants were treated with 2mg/ml hydrocortisone only (Mateos *et al.*, 2018). Despite the increased GLUT3 mRNA expression at 2mg/ml and the increased GLUT1 protein expression at all doses of hydrocortisone, placental uptake of radiolabeled glucose was decreased by 30-40% when explants were treated with 2 and 20 mg/ml hydrocortisone (Mateos *et al.*, 2018).

### Amino Acid Transporters

System A is sodium-dependent and allows transport of small non-branched amino acids like alanine and glycine (Jones *et al.*, 2006). Midgestation administration of dexamethasone in mice at E13.5 and E14.5 caused unaltered placental System A transfer of radiolabeled amino acid at E15.5 and E17.5 along with unchanged mRNA expression of SNAT1, SNAT2 and SNAT4 in male and female placentas (Audette *et al.*, 2011). However, at E18.5, system A mediated amino acid transfer of radiolabeled amino acid was reduced in male and female placentas despite no significant changes in SNAT1, SNAT2, and SNAT4 transporter expression at E18.5 (Audette *et al.*, 2011). This indicates a potential long-term effect of midgestational dexamethasone exposure on placental system A amino acid transfer (Audette *et al.*, 2011). Furthermore, at E16, mice treated with dexamethasone in their drinking water at E11-E16 showed unchanged fetal accumulation of radiolabeled amino acid despite a 35% increase in placental radiolabeled amino acid accumulation and increased SNAT1 and SNAT2 gene expression but unaltered SNAT4 expression (Vaughan *et al.*, 2012). At E19, mice given dexamethasone at E14-E19 had reduced fetal and placental accumulation of radiolabeled amino acid by 40-50% despite showing increased SNAT1 expression with unchanged SNAT 2 and SNAT4 expression (Vaughan *et al.*, 2012). Mouse placentas at E19 from those given dexamethasone at E11-E16 had a 38% increase in fetal accumulation of amino acid despite no change in placental accumulation and no change in SNAT1, SNAT2, and SNAT4 expression (Vaughan *et al.*, 2012). This further indicates a time-dependent effect on amino acid transporter expression, placental transport, and fetal accumulation. No change in gene expression of SNAT1, SNAT2, and SNAT4 at E14.5 and at E17.5 was detected in mice given dexamethasone for 60 hours starting at E12.5 (Cuffe *et al.*, 2011).

Human placental explants from term pregnancies were incubated with dexamethasone for 48 hours showed 30% increase in placental uptake of radiolabeled amino acid at 10-6 M but not at 10-8 M despite no changes in mRNA expression of SNAT1, SNAT2 or SNAT4 at both concentrations (Audette *et al.*, 2010). Human term placental extracts from women who were treated with glucocorticoids during gestation showed varied effects depending on the intermittent time between the treatment and delivery (Audette *et al.*, 2014). Uptake of radiolabeled amino acid by placentas of mothers who delivered 14 days after the GC treatment but prior to term was lower than uptake from delivered placentas within less than 14 days of treatment (Audette *et al.*, 2014). Compared to term controls of untreated mothers, term placentas from GC treated mothers had significantly lower system A transport (Audette *et al.*, 2014). Gene expression revealed no effect on SNAT1 and SNAT2 across all treatment groups and the control, but GC-treated term placentas had reduced SNAT4 expression compared to GC-treated placentas delivered after 14 days of treatment but prior to term (Audette *et al.*, 2014). This further suggests that long-term effects of dexamethasone may be more critical given that the reduced transport was amplified in GC-treated placentas delivered after 14 days of treatment but prior to term and in GC-treated term placentas compared to placentas delivered within 14 days of treatment (Audette *et al.*, 2014).

BeWo choriocarcinoma human placental cell lines showed higher radiolabeled sodium-dependent amino acid transfer between membranes when incubated with 1000nM cortisol (Jones *et al.*, 2006). SNAT1 mRNA expression was unchanged when BeWo cells were incubated with cortisol at 20, 50, 1000, and 2500 nM, but SNAT2 mRNA expression increased by 21% and 30% when incubated with cortisol at 1000nM and 2500nM (Jones *et al.*, 2006). SNAT2 protein expression further showed an 11% increase with 1000nM cortisol (Jones *et al.*, 2006).

### Fatty Acid Metabolism

To my knowledge, lipid transporter expression and transport has not been assessed after antenatal GC exposure. One study did assess lipoprotein lipase activity along with fatty acid esterification and oxidation (Mateos *et al.*, 2018). Using placental explants from term deliveries, fatty acid utilization and storage was altered when cells were treated with hydrocortisone. Fatty acid oxidation was reduced by 25, 50 and 75% in explants treated with 1, 2 and 20 mg/ml hydrocortisone (Mateos *et al.*, 2018). Fatty acid esterification was also reduced at all doses used. Lipoprotein lipase (LPL) activity showed reductions by 40 and 80% when cells were incubated with 2 and 20 mg/ml hydrocortisone (Mateos *et al.*, 2018). Hence, fatty acid uptake, storage and oxidation were all impaired. This same study showed reductions in radiolabeled glucose uptake at 2 and 20 mg/ml doses, and the reduced fatty acid metabolism capacity further suggests failed placental compensatory mechanism to utilize fatty acids when glucose uptake is compromised, despite the availability of nutrients (Mateos *et al.*, 2018). In humans, LDL receptor protein levels were reduced in placentas from pregnancies with IUGR (Wadsack *et al.*, 2007).

Finally, ultrasound images of human growth-restricted fetuses showed reduced fat and lean mass, suggesting fetal nutrient deprivation (Padoan *et al.*, 2004).

## Effect of Glucocorticoid Exposure on Placental mTORC1 Function

mTORC1 is a crucial nutrient sensor that plays a role in integrating maternal and fetal signals to ensure adequate nutrient transport to the fetus through the placenta (Wen *et al.*, 2005; Roos *et al.*, 2007; Mparmpakas *et al.*, 2012; Jansson & Powell, 2013). Fewer studies have assessed the relationship between maternal GC exposure and placental mTORC1 activity in rodents or humans, but its activity is reduced in intrauterine growth restriction (Roos *et al.*, 2007). Mice exposed to corticosterone at E14-E19 had reduced mTORC1 activity at E19 evident by the reduced p4E-BP1 and pS6K expression, downstream targets of mTORC1, and increased REDD1 expression which is an inhibitor of mTORC1 signaling (Vaughan *et al.*, 2015). Mice exposed to corticosterone at E11-E16 had reduced pAKT levels but unchanged total AKT levels and unchanged REDD1 expression, suggesting a minimal effect on mTORC1 function at E16 (Vaughan *et al.*, 2015).

## Effect of Glucocorticoid Exposure on Placental Endocrine Function

Pregnant rats treated with dexamethasone at E13 had reduced *Igf2* mRNA expression in the junctional zone but unaltered expression in the labyrinth zone (Ain *et al.*, 2005). Conversely, pregnant mice exposed to glucocorticoids at midgestation showed no change in placental *Igf2* gene expression (Baisden *et al.*, 2007; Cuffe *et al.*, 2011; Vaughan *et al.*, 2015).

Growth differentiation factor 15 is produced in the placenta, and changes are associated with a variety of complications including miscarriage, nausea and hypertension (Tong *et al.*, 2004; Chen *et al.*, 2016; Petry *et al.*, 2018). There are no studies assessing placental GDF15 activity in response to GC or psychological stress exposures. Placental GDF15 levels are positively correlated with maternal and fetal GDF15 levels, suggesting that the placenta is the primary source of this hormone during pregnancy (Sugulle *et al.*, 2009). Based on our results, other placental hormones may be assessed in the future.

## Effect of In Utero Glucocorticoid Exposure on Offspring

Women with higher corticotropin-releasing hormone at midgestation, were 7.5 fold more likely to deliver preterm (Inder *et al.*, 2001). However, offspring outcome remains conflicting. In humans, antenatal corticosteroid exposure caused higher systolic and diastolic blood pressure in children ages 14 years (Doyle *et al.*, 2000). At 30 years of age, offspring of mothers who received antenatal betamethasone had higher insulin levels 30 minutes after a glucose tolerance test with lower glucose concentrations at 120 minutes, but offspring did not have altered cortisol levels, lipid profile or blood pressure (Dalziel *et al.*, 2005). This suggests an impaired insulin sensitivity (Dalziel *et al.*, 2005). Additionally, body composition of children of mothers who had higher cortisol levels during pregnancy showed increased fat mass index in girls but a lower fat mass index in boys indicating a sex-difference (Van Dijk *et al.*, 2012). Maternal third trimester cortisol levels were positively associated with infant body fat percent increase from age 1-6 month suggesting programmed adiposity that can contribute to childhood obesity (Entringer *et al.*, 2016). Antenatal GC treatment showed a blunted HPA axis activity in infants ages 3-6 days after a stressful exposure (Davis *et al.*, 2004). Despite showing reduced cortisol levels in newborns exposed to antenatal glucocorticoids, long-term effects vary. Children ages 6-11 years who were exposed to antenatal glucocorticoids had higher cortisol levels in response after a standardized stressful test, and this difference was mainly influenced by higher salivary cortisol in females, indicating a potential sex-dependent elevation in HPA axis activity effect (Alexander *et al.*, 2012). Studies have shown multiple offspring outcomes including increased blood pressure in children, increased risk of preeclampsia, impaired mental development in infants, increased infant cortisol, reduced fetal weight, and other symptoms associated with timing, dosage and type of corticosteroid treatment during pregnancy (Singh *et al.*, 2012).

In mice, male offspring exposed to dexamethasone for 60 hours starting at E12.5 had lower fetal weights at E14.5 but not at E17.5 (O’Sullivan *et al.*, 2013). The male offspring from similarly treated dams had similar weights at 2 weeks, 4 weeks, and 3 and 6 months of age (O’Sullivan *et al.*, 2013). Twenty-one-day-old rats exposed to antenatal dexamethasone at E15 till delivery had 66% lower body weights than controls, lower corticotropin releasing hormone content and concentrations, lower adrenal and plasma corticosterone levels and severe adrenal atrophy (Dupouy *et al.*, 1987). Conversely, rats exposed to antenatal glucocorticoids showed unaltered ACTH and corticosterone plasma levels at PND1,7,9, and 20 but had a suggested increased HPA axis activity when stress was induced during adulthood (Bakker *et al.*, 1995). Offspring of physically stressed rats during gestation showed higher plasma glucose levels at 24 months of age despite similar insulin secretion when challenged with oral glucose tolerance test (Lesage *et al.*, 2004).

# Experimental Design

To determine how glucocorticoid exposure affects placental function, we will obtain n=X females and males /per group 8 week-old C57BL/6 virgin mice from Jackson laboratory. Mice will be given two weeks to acclimatize with *ad libitum* access to normal chow diet and water. After acclimatization, mice will then be single-housed and randomized into one of the following groups, to assess placental morphology (at E14.5) and effects on offspring (at delivery). The experimental design is represented in Figure 2. Pending these results other groups may be evaluated at different gestation timepoints.

Cohort A of groups treated one week prior to gestation:

1. *Water Pre-gestation till E14.5:* control group on water one week prior to conception and until midgestation at embryonic day 14.5
2. *Dexamethasone Pre-gestation till E14.5*: experimental group exposed to dexamethasone in drinking water a week prior to conception and until midgestation at embryonic day 14.5
3. *Water Pre-gestation till Delivery*: control group on water one week prior to conception and until delivery
4. *Dexamethasone Pre-gestation till Delivery*: experimental group exposed to dexamethasone in drinking water a week prior to conception and until delivery

Cohort B of groups treated at conception:

1. *Water Conception till E14.5*: control group on water starting at conception and until midgestation at embryonic day 14.5
2. *Dexamethasone Conception till E14.5*: experimental group exposed to dexamethasone in drinking water starting at conception and until midgestation at embryonic day 14.5
3. *Water Conception till Delivery*: control group on water starting at conception and until delivery
4. *Dexamethasone Conception till Delivery*: experimental group exposed to dexamethasone in drinking water starting at conception and until delivery

All groups will have *ad libitum* access to normal chow diet and water or dexamethasone depending on treatment arm. Experimental groups will receive 1mg/kg/day dexamethasone in their drinking water with *ad libitum* access. For groups of Cohort A (receiving dex or water a week prior to conception), female mice will be mated with age-matched male mice after one week of treatment. A copulatory plug will be checked daily to identify E0.5 day. For groups of Cohort B (receiving dex or Water at conception), mice will be mated with age-matched males immediately after acclimatization while having *ad libitum* access to water. We will check for the presence of copulatory plugs daily to determine treatment initiation. Once a copulatory plug is identified, mice will be placed on dexamethasone or water based on their assigned group.

Males will be removed from the cage after a copulatory plug is detected to minimize male exposure to treatment and to better detect potential miscarriages. Dams from all groups will undergo body mass assessment three times weekly using magnetic resonance to assess body composition. Water and food intake will be recorded weekly. For groups that will be sacrificed prior to delivery (E14.5), placental and fetal extractions will occur midgestation at E14.5, since by midgestation, the placenta is fully developed and mature. Briefly, the dams will be anesthetized using a vaporizer during the placental and fetal extraction. Litter size will be determined per dam and will account for potential resorbed placentas. Placental and fetal weights will be collected. Placentas will be snap frozen in liquid nitrogen while some will be embedded in paraffin for histology. Molecular studies on placental samples will be conducted to determine protein expression.

For the groups that will deliver their pups at E21.5, survival and birth rates will be noted. Water and dex groups that will complete their pregnancy and deliver their pups will have *ad libitum* access to normal chow diet and will be placed on regular water immediately after parturition and during lactation (no dex exposure during lactation). Pups will be sexed and culled to 2 at PND2.5. The offspring will be weighed at PND0.5, PND7.5, 14.5, 16.5, and at 21.5. Pups will be weaned based on sex and treatment group. The weaned pups will have *ad libitum* access to normal chow diet and water. Their water and food intake will be assessed weekly. They will further undergo body composition analysis by echoMRI at weaning and weekly thereafter till 6 weeks of age. At the age of 6 weeks, assess offspring insulin sensitivity will be tested by an insulin tolerance test (ITT) followed by sacrifice and tissue collection of fat pads 3 days after the ITT. Offspring fat pads (gWAT and iWAT) will be collected and weighed to determine adiposity.

To determine if the effects of dexamethasone exposure on the placenta and the fetus can be rescued, we will develop a placenta-specific glucocorticoid receptor knockout (KO) model. To isolate placental from fetal and maternal glucocorticoid signaling, our knockout model will ablate GR conditionally in the placenta. To my knowledge, this is the first time such a model has been generated. To generate the GR-KO, we will use the Cre-loxP recombination technology. We will leverage the fact that placental tissue is primarily fetal derived, so the genotype of the offspring will dictate the genotype of most of the placenta. The breeding scheme is represented in Figure 3. First, female mice with homozygously flanked exon 2 of *Nr3c1* will be crossed with a male having placental driver *Cyp19a1-CreTg/+* (Wenzel & Leone, 2007).This *Cyp19a1-Cre* has been also used elsewhere to generate a placental knockout model (Wieczorek *et al.*, 2019). This cross will generate wild-type (WT) and heterozygous (Het) offspring at a 1:1 ratio. The expected timeline between this first breed and the second one is 9-12 weeks. The offspring of this first cross will be bred (WT x Het) to generate the parental strains for this experiment. Briefly, this cross will yield a combination of knockout *Nr3c1* fl/fl;*Cyp19a1-CreTg/ +*, conditionally heterozygous *Nr3c1* fl/+;*Cyp19a1-CreTg/+* , and wild-type *Nr3c1* fl/fl ; *Cyp19a1-Cre* +/+ , *Nr3c1* fl/+ ; *Cyp19a1-Cre* +/+ , *Nr3c1* +/+ ; *Cyp19a1-Cre* +/+ , or *Nr3c1* fl/fl ; *Cyp19a1-Cre* Tg/+ (no Cre transgene) at an expected ratio of 1:2:5 with the knockout and wild-type (*Nr3c1* fl/fl ; *Cyp19a1-Cre* +/+ only) animals only being used for further breeding. The expected timeline for this second cross to generate mature offspring capable of breeding is also 9-12 months. The final parental breed of WT x KO will generate our placental KO model. The final offspring generated from the next generation will all have the floxed allele with the Cre (KO) or without (WT). The offspring generated from the last main parental breed will either be WT with intact placentas or knockout with placental KO and a phenotypically WT embryo.

The dams with GR-KO will be treated with dexamethasone similar to the previous groups in cohorts A and B to determine placental, embryonic and offspring function and growth.

### Figure 2: Diagram representing the experimental design and respective timeline



### Figure 3: Diagram representing the breeding method to generate the knockout placenta



# Methods

## Dexamethasone Exposure

Water-soluble dexamethasone (Sigma) will be prepared at a concentration of 53 mg/L, which our previous work shown results in a dose of approximately 1 mg/kg/day in non-pregnant mice.

If the dam is single housed or nursing pups:

(the new added total water/dexamethasone- the last measurement’s water/dexamethasone) / # of days between measurements

If more than one adult mouse is in the cage (when the male is breeding in the same cage), food intake will be calculated as follows:

(the new added total water/dexamethasone - the last measurement’s water/dexamethasone) \* #of days between measurements / sum of days spent by each mouse in that cage between measurements

## Food Intake

Food will be weighed when the treatment starts and throughout the experiment. The weight of the dam’s food will be recorded three times weekly every Monday, Wednesday, and Friday. Food will also be weighed at delivery for the dam. Food will be topped off to ~400g weekly every Friday. Food intake will be calculated as:

If the dam is single housed or with nursing pups:

(the new added total food weight - the last measurement’s food weight) / # of days between measurements

If more than one adult mouse is in the cage (when the male is breeding in the same cage, or when weaned offspring are housed together), food intake will be calculated as follows:

(the new added total food weight - the last measurement’s food weight) \* #of days between measurements / sum of days spent by each mouse in that cage between measurements

## Body Composition

Mice will be weighed by using dynamic weighing to capture accurate weight using a digital scale. The weight will be recorded along with the mouse ear tag number. The mouse will be gently placed in the MRI tube with the plunger slightly compressing along the mouse body to ensure it cannot move during the measurement. Fat, lean, free water and total water mass (g) will be recorded for each animal.

## Sacrifice and Tissue Collection

Dams of groups E14.5 will be sacrificed on the respective dates based on their treatment group. Dams will be anesthetized using an isoflurane vaporizer. Toe punches will be performed to ensure that the mouse is under anesthesia. A midline incision of the skin from the rectum to the diaphragm will be made while the mouse is still alive and anesthetized using the vaporizer. The uterine horn will be exposed and placental and fetal excision will begin in order along the uterine horn starting from the side (closer to the ovaries). The amniotic sac for each pup will be ruptured using fine scissors. The placenta will be detached from the maternal tissue and the umbilical cord then weighed and immediately snap frozen or cryopreserved and in paraffin for future molecular and histological studies. Fetuses will be weighed after removal from the amniotic sac then they will be immediately sacrificed by decapitation using surgical scissors. After the complete extraction of tissue, dams will be euthanized while under anesthesia by cardiac exsanguination.

Offspring of dams that will be allowed to deliver and nurse (groups of E21.5) will be dissected at 6 weeks of age. Offspring will be first anesthetized using isoflurane drop jar. Offspring will be sacrificed using isoflurane drop jar. Cervical dislocation will be performed as a secondary measure to confirm euthanasia. We will dissect the offspring fat pads by a midline incision of the skin from the rectum to the diaphragm, extract inguinal and gonadal white adipose tissue. Inguinal white adipose tissue (iWAT) will be collected from the mouse right side first by pulling the peritoneum away from the skin. Inguinal fat will be carefully extracted, weighed then snap frozen in liquid nitrogen for further molecular studies. Right gonadal white fat tissue (gWAT) will be collected next by scraping the fat along the gonads (ovaries or testis), weighed, and then snap frozen in liquid nitrogen in 2ml tubes. The fat pads will be stored at a temperature of -80C.

## Insulin Tolerance Test

Weaned offspring in groups water or dexamethasone till delivery from cohort A (pre-gestation) and cohort B (at conception) will undergo an insulin tolerance test (ITT) being challenged with 1 U/kg body weight after a 6-hour fast with *ad libitum* access to water. The effects of antenatal glucocorticoid exposure on offspring adolescent insulin sensitivity will be determined. Briefly, after the fast, the tail will be cut to allow for blood sampling via AccuCheck Advantage Glucometer. Tail vein blood will be immediately measured at 0minutes after the 6-hour fast to denote fasting blood glucose. Mice will be injected by a syringe into the interperitoneal cavity with the appropriate insulin dosage. The timer will be set as to allow for blood collection every 15 minutes. Blood will be collected at 5, 30, 45, 60, 75, 90 and 120 minutes after injection. After the ITT is done, mice will have *ad libitum* access to normal chow diet and water again. These data will be analyzed by mixed linear models of glucose at each time point.

## Real time qPCR

Using the placental tissues collected from the dams, we will assess RNA expression of macronutrient transporters and endocrine hormones. RNA samples will be prepared from the mouse tissues using the PureLink RNA Mini Kit. Briefly, tissues will be cut to ~50mg samples that will be homogenized and treated to collect the RNA. The RNA will be quantified using a nanodrop. Later, first strand cDNA will be synthesized from the purified RNA samples using High Capacity cDNA Reverse Transcription Kit. The cDNA samples will be diluted and added to the clear 384 well plate in triplicates. A Primer/SYBR Green mix will be prepared for each primer. Briefly, we will use sequence-specific primers to amplify GLUT1, GLUT3, GLUT4, SNAT1, SNAT2, SNAT4, LPL, GDF15 and IGF-II using primer pairs (forward and reverse). This will allow us to assess the overall endocrine and transport function of the placentas of Dex- and Water-treated dams. PCR will be performed for *Sry* to determine the sex of the placentas/fetuses using a piece of the placenta or fetal tails, respectively.

## Genotyping

Maternal and fetal genotyping will be conducted to confirm the GR KO or WT genotype of the dams and fetuses/placentas. To genotype the dams, DNA extraction from tail clips will be done. qPCR analysis of the *Nr3c1* gene will be conducted to determine gene expression. For fetal/placental genotyping, fetal tail will be entirely clipped for DNA analysis along with a section of the placenta to confirm expression of *Nr3c1.*

## Western Blotting

Using the placentas collected at E14.5, mTORC1 activity will be assessed. Validation of glucocorticoid receptor ablation will be validated from collected placentas. Briefly, a portion of the sample will be boiled and loaded into different wells with a ladder control. Proteins will transfer to nitrocellulose overnight. The matrix will be stained for total protein using Revert total protein and scanned by LiCOR to normalize against total protein. Samples will be incubated with the primary then the secondary antibodies. Briefly, antibodies against total and phosphorylated mTORC1 targets (S6K, 4EBP1, S6) and regulators (Akt, IRS and TSC2) and antibodies against GR will be used.

## Histology

Placentas collected from control and experimental at E14.5 will be embedded in paraffin and stained at the Rogel Cancer Center’s Tissue and Molecular Pathology. Slides will be blindly assessed for labyrinth thickness and area.

# Expected Results

## **Aim 1.1:**How does maternal GC exposure affect placental development, fetal growth, and fetal survival?

I hypothesize that our prolonged dexamethasone exposure will reduce placental and fetal weights along with reduced placental labyrinth zone area causing intrauterine growth restriction (IUGR) in group of dexamethasone pre-gestation till E14.5 and in the group of dexamethasone conception till E14.5 with more pronounced effect in the groups treated with dexamethasone pre-gestation over groups treated at conception. Pregnant mice exposed to dexamethasone on E15, E16, and E17 had reduced placental and fetal weights and trophoblast swelling in the junctional and labyrinth zones (Baisden *et al.*, 2007). Furthermore, mice given dexamethasone at E11-E16 had reduced fetal and placental weights at E16, but the volume of the placental junctional and labyrinth zone was unchanged despite less fetal capillaries in the labyrinth zone (Vaughan *et al.*, 2012), which suggests that our prolonged exposure will have more drastic effects on placental labyrinth area. To my knowledge, studies assessing effects of glucocorticoid treatment pre-gestation or very early in gestation are lacking. My preliminary data shows that mice treated with 1 mg/kg of the synthetic glucocorticoid dexamethasone in the drinking water from 1 week prior to mating through pregnancy had 1) dramatically reduced fertility whereby litter size from treated dams was 34% lower and had 2) 37% reduction in offspring birth weight. I thus expect fetal survival to be reduced as evident by resorption more so in the dexamethasone pre-gestation group than in the conception group.

## **Aim 1.2:** How does maternal GC exposure affect the expression of placental nutrient transporters?

The experiments conducted in this aim will examine the effect of dexamethasone treatment on placental transport and transporters. I predict that placental glucose transporters, GLUT1 and GLUT3, will have increased gene expression. This is supported by increased placental GLUT1 protein expression at E21 in rats exposed to 100 and 200 ug/kg body weight/ day of dexamethasone starting at E15 (Langdown & Sugden, 2001). Furthermore, mice exposed to corticosterone in their drinking water at E11-E16 had increased placental GLUT1 and GLUT3 gene expression at E16 (Vaughan *et al.*, 2015).

I also predict increased placental system A amino acid transporter expression of SNAT1, SNAT2 but unchanged SNAT4 with our GC exposures. This hypothesis is supported by increased SNAT1 and SNAT2 gene expression but unaltered SNAT4 expression at E16 in mice treated with dexamethasone in their drinking water at E11-E16 (Vaughan *et al.*, 2012). At E19, mice given dexamethasone at E14-E19 also showed increased SNAT1 expression but unchanged SNAT2 and SNAT4 expression (Vaughan *et al.*, 2012). This further indicates a time-dependent effect on amino acid transporter expression, by which our longer exposure starting at pre-gestation or at conception will yield more similar results to the exposure given at E11-E16.

I hypothesize that LPL will be downregulated in mouse placentas. This hypothesis is based on findings of reduced placental LPL activity in hydrocortisone treated term placental explants (Mateos *et al.*, 2018).

## **Aim 1.3:** Is placental mTORC1 signaling altered after maternal GC exposure?

I hypothesize that placental mTORC1 activity will be reduced in placentas exposed to dexamethasone. This is because mice exposed to corticosterone at E14-E19 had reduced expression of downstream targets of mTORC1 and increased expression of REDD1, an inhibitor of mTORC1 signaling (Vaughan *et al.*, 2015). Furthermore, the expected IUGR (from *Aim 1*) may be supportive of reduced mTORC1 signaling.

## **Aim 1.4:** How does maternal GC exposure affect placental endocrine function?

I hypothesize GDF15, an anorexic hormone, to be downregulated in the placentas of dexamethasone-treated animals. Given that GDF15 levels are increased in muscle with activated mTORC1 activity (Tang *et al.*, 2019) and reduced in plasma after rapamycin treatment , and since placental mTORC1 activity is predicted to be reduced (from *Aim 3*), then GDF15 levels should decrease accordingly.

I expect IGF2 expression to be reduced since pregnant rats treated with dexamethasone at E13 had reduced *Igf2* mRNA expression in the junctional zone despite unaltered expression in the labyrinth zone (Ain *et al.*, 2005). Since our exposure is more chronic and placental weight and development is expected to be reduced (from *Aim 1*), then placental *Igf2* should be reduced.

## **Aim 1.5:** Is offspring survival, weight, body composition and insulin sensitivity altered with maternal GC exposure?

Given the severe expected IUGR, reduced fetal weights, and increased resorption (from *Aim 1*), I predict that offspring survival will be reduced. Our preliminary data shows that pre-gestational exposure will dramatically reduce offspring survival at PND0.5-1 with 100% lethality of all pups from dexamethasone-treated dams. Dexamethasone exposure at conception is similarly predicted to reduce offspring survival within PND0.5-1 but to a lesser degree than the pre-gestational exposure. I predict offspring body weight to be relatively similar at 6 weeks as seen in adult rats (Lesage *et al.*, 2004; O’Sullivan *et al.*, 2013), but the offspring will have higher fat mass as seen in human studies (Van Dijk *et al.*, 2012). Furthermore, since our exposure is longer than that of most studies, offspring may show increased insulin levels during the ITT at 6 weeks of age. In support of this hypothesis, 30-year-old human offspring of mothers who had received antenatal betamethasone had higher insulin levels 30 minutes after a glucose tolerance test (Dalziel *et al.*, 2005). On the other hand, adult rat offspring of antenatally stressed dams had unaffected insulin secretion in response to an OGTT (Lesage *et al.*, 2004), but since our exposure is more chronic prior to or at gestation, then I expect insulin levels to be higher indicating less insulin sensitivity.

## **Aim 1.6:**Does a placental GR-KO model rescue the placental and fetal effects of GC exposure?

Based on the results of Aims 1.1-1.5 we will have identified critical glucocorticoid-induced changes in placental gene expression, signaling, placental and fetal size and offspring health. Those models however do not separate effects of dexamethasone on the mother from those on the placenta. To separate these we will use placental GR knockouts and repeat these studies. We expect that placental-derived glucocorticoid actions will be blocked by GR knockout in the placenta, but maternal glucocorticoid actions will be retained.

# Potential Pitfalls and alternate Approaches (Aims 1.1-1.6)

It is possible that mice in the pre-gestation dexamethasone will not conceive immediately upon mating thus causing their 1 week dexamethasone exposure prior to mating to be prolonged. We will have to eliminate all dams that will be exposed to pre-gestational dexamethasone for more than a week and the half prior to conception, thus we may need more mice. It is also possible for both groups that the mice may have spontaneous abortions and resorptions due to induced dexamethasone-induced stress even prior to the E14.5 timepoint. We may thus need to alter our exposure time and try different exposure windows for shorter periods of time during gestation. It is also possible that our placental GR KO model may prove lethal. In that case, we will use a different parental strain of Hets (Het x Het) to generate a partial knockout that may prove viable.

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