Glucocorticoid Regulation of the Placental System A Amino Acid Transporter

by

Melanie Chantal Audette

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy (Ph.D.)

Department of Physiology
University of Toronto

© Copyright by Melanie Chantal Audette (2012)
Glucocorticoid Regulation of the Placental System A Amino Acid Transporter

Melanie Chantal Audette
Doctor of Philosophy, 2012
Department of Physiology, University of Toronto

The system A transporter transfers small neutral amino acids across the placenta and is essential for normal fetal growth. Synthetic glucocorticoids (sGCs) are routinely given to women with threatened preterm labour (PTL) and have been linked to fetal growth restriction. sGCs differentially regulate the system A amino acid transporter depending on organ and cell type, but there are no comparable data in the placenta. Since ~30% of women who receive sGCs carry to term, our objective was to examine the short and longer-term consequences of antenatal sGCs on placental system A transport using human *in vitro* model, mouse *in vivo* and human placenta *ex vivo*. We hypothesized that sGCs would inhibit system A activity, thereby adversely affecting placental function by reducing nutrient transport. Additionally, we hypothesized that the system A transport would be regulated based on timing from sGC exposure. Contrary to our hypothesis, acute sGC treatment (48 h) stimulated, rather than inhibited, system A activity in human placental explants *in vitro*. Increased system A activity was not mediated by changes in mRNA expression; however, it was associated with increased syncytial differentiation and maturation. In the mouse, there were no short-term effects of mid-gestation sGC treatment on murine placental system A transport *in vivo*. However, a substantial reduction in system A mediated transport occurred at term. When examining the effects of sGC treatment given to women at risk of PTL, reductions in system A transport were found in placentae delivered at term following treatment compared to placentae that were delivered less than 14 days and between 14 days and term following treatment and term controls. Taken together, these findings strongly demonstrate that sGC treatment regulates placental system A activity. As such, prenatal use of sGC therapy may lead to a reduction in availability of neutral amino acids if gestation persists until term.
This thesis is dedicated to CP, who has unconditionally supported me from the very beginning.

I would not be where I am today without you.
Acknowledgments

I once heard that great science comes from things you do not expect. I believe this principle also applies to each chapter throughout life. As such, I would like to acknowledge and thank everyone who has shaped my journey throughout graduate school:

First and foremost, I would like to thank my supervisors: Dr. Stephen Matthews and Dr. John Challis both for taking me into their labs. I thank them for their guidance, insight, encouragement and words of wisdom. It is through many discussions that they have challenged me far beyond my comfort levels, which has helped to me to develop both professionally and personally. It is through their examples that I have learned what the standard of excellent science is and the value of a truly great question.

I would like to thank Dr. Rebecca Lee Jones and Dr. Colin Sibley for their invaluable mentorship and technical teachings at the University of Manchester. It was very exciting traveling to England, and the supervision I received truly made me feel part of the lab. Dr. Jones, an intuitive teacher and incredible scientist and has provided continued support, support that was both inspirational and critical to this 5-year long journey.

I would like to thank my committee members, Dr. Isabella Caniggia and Dr. Stephen Lye for their invaluable comments and helpful criticisms, which helped to shape and guide these studies.

I would like to thank all the members of my labs. First to the Challis Lab, members whom I started this journey with, especially Jim Johnstone, Michelle Fantauzzi and Kristin Connor. They have each given me an amazing introduction to the lab and never-ending friendship. Secondly to the past and present members of the Matthews Lab including: the kids; the new guys; Alice, Sophie and Amita – together were a constant source of entertainment and guidance; Elena – for always being helpful and a true pleasure to work with; Jeff – who always listened to me in the BR; Vasilis – for being a great friend who is always a caring and willing to help; and most of all Majid – for providing invaluable guidance throughout all of the lab’s and life’s quarrels. I would have not been able to graduate without our many discussions; you are a wonderful person to work with everyday and a great example of a true friend.

I would also like to thank my friends in the Dept. of Physiology who have made this journey a lot more fun, especially my good friend Nasrin Nejatbakhsh.

I would like to thank my incredible family, especially: CP, my sisters, Mike, Dad and Paquerette and friends, especially my dear friend Sabrina, for the continued encouragement. I would like to thank Matty Timmons, for his support and love throughout this final year of school. Your work ethic, intelligence and ambition are a constant inspiration to me. Thank-you all for listening to me on a daily basis and providing the encouragement I needed to climb that last hill.

Lastly, and most importantly, to my mother. “There never was a woman like her. She was gentle as a dove and brave as a lioness. The memory of my mother and her teachings were, after all, the only capital I had to start life with, and on that capital I have made my way.”

~ Andrew Jackson
Table of Contents

Chapter 1 - Introduction
1.1 Overview.................................................................................................................. 2
1.2 Fetal growth, Perinatal Outcomes and Long-Term Programming of Health and Disease.................................................................................................................. 3
1.3 The Placenta
   1.3.1 The Human Placenta......................................................................................... 5
   1.3.2 The Mouse Placenta......................................................................................... 5
   1.3.3 The Role of the Placenta in Sex-Specific Differences in Fetal Growth......... 7
1.4 The System A Transporter
   1.4.1 Overview.......................................................................................................... 8
   1.4.2 System A Transport Throughout Gestation.................................................... 9
   1.4.3 The Role of System A Transport in Fetal Growth........................................... 10
   1.4.4 Nutrient Availability Regulation of System A Transport............................ 12
   1.4.5 Endocrine Regulation of System A Transport................................................. 15
1.5 Synthetic Glucocorticoids
   1.5.1 Glucocorticoid Signaling................................................................................. 17
   1.5.2 Synthetic Glucocorticoids for the Management of Preterm Labour......... 18
     1.5.2.1 Evidence for Treatment Regime............................................................... 19
   1.5.3 Synthetic Glucocorticoid Effects on Fetal Growth....................................... 22
   1.5.4 Synthetic Glucocorticoid Effects on the Placenta......................................... 24
   1.5.5 Synthetic Glucocorticoid Regulation of System A Transport.................... 30

Figures.................................................................................................................................. 31

Chapter 2 - Objectives and Hypotheses
2.1 Objectives.................................................................................................................. 36
2.2 Hypotheses.................................................................................................................. 37

Chapter 3: In Vitro Regulation of Synthetic Glucocorticoid Treatment on System A Transport in Human Placental Explants in Culture
3.1 Introduction.................................................................................................................. 39
3.2 Hypothesis.................................................................................................................. 41
3.3 Methods
   3.3.1 Placental Collection and Tissue Culture....................................................... 41
   3.3.2 System A Uptake Activity Assay................................................................. 42
   3.3.3 System A mRNA Expression.......................................................................... 43
   3.3.4 Immunohistochemistry of SNAT Proteins.................................................... 44
   3.3.5 Analysis of Placental Cell Turnover and Syncytial Regeneration
     3.3.5.1 hCG, hPL and LDH Quantification.............................................................. 45
     3.3.5.2 Immunohistochemistry............................................................................ 45
Chapter 4 – In Vivo Regulation of Mid-Gestation Synthetic Glucocorticoid Treatment on Murine Placental System A Transport

4.1 Introduction................................................................................................................. 76
4.2 Hypotheses.................................................................................................................... 78
4.3 Methods
4.3.1 Animal Breeding and Treatment.............................................................................. 79
4.3.2 Tissue Collection and System A Mediated Transplacental Transfer.......................... 80
4.3.3 Sex-Determination................................................................................................... 81
4.3.4 qRT-PCR.................................................................................................................... 82
4.3.5 Microvillous Membrane Extraction......................................................................... 83
4.3.6 Alkaline Phosphatase Activity Assay......................................................................... 84
4.3.7 Western Blot............................................................................................................... 84
4.3.8 Morphometric Analyses........................................................................................... 85
4.3.9 Corticosterone Measurements.................................................................................. 86
4.4 Statistical Analyses......................................................................................................... 86
4.5 Results
4.5.1 Ontogeny of System A Activity, Protein and mRNA Expression.............................. 87
4.5.2 The Effects of DEX on System A Activity, Protein and mRNA Expression................ 88
4.5.3 The Effects of DEX on Fetal and Placental Growth.................................................... 89
4.5.4 The Effects of DEX on Maternal and Fetal Plasma Corticosterone............................. 90
4.6 Discussion....................................................................................................................... 90
4.7 Acknowledgements........................................................................................................ 96

Tables.............................................................................................................................. 97
Figures.............................................................................................................................. 100
Chapter 5 - Synthetic Glucocorticoid (sGC) Regulation of Placental System A Transport in Women Treated with Antenatal sGC Therapy

5.1 Introduction.................................................................................................................. 114
5.2 Hypotheses.................................................................................................................... 116
5.3 Methods
   5.3.1 Patient Recruitment............................................................................................... 117
   5.3.2 Placental Tissue Collection.................................................................................. 118
   5.3.3 System A Uptake Activity Assay.......................................................................... 118
5.4 Statistical Analyses....................................................................................................... 118
5.5 Results
   5.5.1 Treatment Subdivisions....................................................................................... 119
   5.5.2 Maternal Parameters ......................................................................................... 119
   5.5.3 Fetal and Placental Parameters ......................................................................... 120
   5.5.4 The Effect of Antenatal sGC on System A Mediated Uptake ......................... 120
5.6 Discussion..................................................................................................................... 121
5.7 Acknowledgements...................................................................................................... 126

Tables................................................................................................................................ 127
Figures............................................................................................................................... 130

Chapter 6 – Discussion

6.1 Summary...................................................................................................................... 134
6.2 Supporting and Conflicting Evidence......................................................................... 137
6.3 Relationship between Gene, Protein and Activity of the System A Transporter...... 139
6.4 Placental Uptake vs. Transplacental System A Activity............................................ 140
6.5 Species Comparisons............................................................................................... 141
6.6 Potential Mechanisms............................................................................................... 143
6.7 Limitations.................................................................................................................. 148
6.8 Significance to Current Knowledge........................................................................... 150
6.9 Conclusions................................................................................................................ 153

Figures............................................................................................................................... 154

References......................................................................................................................... 156
Tables

Chapter 3
Table 3.1 qRT-PCR Primer Sequences and Protocols…………………………………….. 69
Table 3.2 Immunohistochemical Primary and Secondary Antibody Details……………. 70

Chapter 4
Table 4.1 Western Blot Primary and Secondary Antibody Details…………………….. 109
Table 4.2 Placental Zone Fractions on Embryonic Day 18.5……………………………. 110
Table 4.3 Maternal and Fetal Plasma Corticosterone Levels on Embryonic Day 18.5... 111

Chapter 5
Table 5.1 Collection Subdivisions………………………………………………………….. 139
Table 5.2 Maternal Parameters……………………………………………………………. 140
Table 5.3 Fetal and Placental Parameters………………………………………………... 141
Figures

Chapter 1
Figure 1.1 Nutrient Transport Across the Placenta ........................................ 43
Figure 1.2 The Mouse Placenta .................................................................. 44
Figure 1.3 Structure, Function and Location of SNATs ............................. 45
Figure 1.4 Structure and Action of Glucocorticoids .................................. 46

Chapter 3
Figure 3.1 In Vitro Culture Model and Methodology for Term Placental Explants ...... 71
Figure 3.2 Sections of Cultured Explants Stained with Toluidine Blue ............ 72
Figure 3.3 Placental Explant Culture and Uptake Methods .......................... 73
Figure 3.4 The Effects of DEX on Placental System A Transport ................. 74
Figure 3.5 The Effects of DEX on Placental Slc38a Gene Expression ............. 75
Figure 3.6 Reference Gene mRNA Expression Levels in Placental Explants .... 76
Figure 3.7 SNAT1 Localization in Cultured and Uncultured Placenta ........... 77
Figure 3.8 SNAT2 Localization in Cultured and Uncultured Placenta .......... 78
Figure 3.9 SNAT4 Localization in Cultured and Uncultured Placenta .......... 79
Figure 3.10 The Effects of DEX on Placental Explant Cell Turnover: Proliferation ........................................... 80
Figure 3.11 The Effects of DEX on Placental Explant Cell Turnover: Apoptosis and Necrosis ........................................................................ 81
Figure 3.12 The Effects of DEX on Syncytial Regeneration: hCG Secretion ...... 82
Figure 3.13 hCG Localization in Cultured and Uncultured Placenta ............. 83
Figure 3.14 The Effects of DEX on Syncytial Regeneration: hPL Secretion .... 84
Figure 3.15 The Effects of DEX on Syncytial Regeneration: 11β-HSD2 and Syncytin Expression and Localization .............................................. 85
Figure 3.16 The Effects of DEX on Placental Ultrastructure ......................... 86

Chapter 4
Figure 4.1 Experimental Overview ......................................................... 112
Figure 4.2 Measurement of Murine Placental Regions ................................. 113
Figure 4.3 The Ontogenic Profile of Placental System A-Mediated Transfer .... 114
Figure 4.4 The Ontogenic Profile of Placental System A Slc38a mRNA Expression .... 115
Figure 4.5 The Ontogenic Profile of Reference Genes ................................ 116
Figure 4.6 The Ontogenic Profile of Placental SNAT Proteins ..................... 117
Figure 4.7 Positive Control Western Blots for SNAT Antibodies .................. 118
Figure 4.8 The Short- and Longer-Term Effects of Mid-Gestation DEX Treatment on System A-Mediated and Paracellular Transfer .......................... 119
Figure 4.9 The Effects of Mid-Gestation DEX Treatment on System A Gene Expression at E18.5 ................................................................. 120
Figure 4.10 The Effects of Mid-Gestation DEX Treatment on Housekeeping Gene Expression at E18.5 ................................................................. 121
Figure 4.11 The Effects of Mid-Gestation DEX Treatment on Alkaline Phosphatase Activity at E18.5 ................................................................. 122
Figure 4.12 The Effects of Mid-Gestation DEX Treatment on SNATs at E18.5 ... 123
| Figure 4.13 | The Effects of Mid-gestation DEX Treatment on Fetal and Placental Weight | 124 |
| Chapter 5 |
| Figure 5.1 | The Effect of Antenatal sGC Treatment Given to Women at Risk of Preterm Labour on System A-Mediated Transfer | 142 |
| Figure 5.2 | System A Mediated Uptake Linear Regressions | 143 |
| Figure 5.3 | Sex-specific Analyses | 144 |
| Chapter 6 |
| Figure 6.1 | Data Summary | 166 |
| Figure 6.2 | SNAT and Facultative Transporters | 167 |
Abbreviations

11β-HSD2  11beta-hydroxysteroid dehydrogenase type-II
~  approximately
ACTORDS  Australasian Collaborative Trial of Repeat Doses of Steroids
ANOVA  analysis of variance
ATF4  activating transcription factor 4
BCRP  breast cancer resistance protein
hCG  human chorionic gonadotropin
BETA  betamethasone
cDNA  copy deoxyribonucleic acid
CRH  corticotropin releasing hormone
DEX  dexamethasone
DNA  deoxyribonucleic acid
E  embryonic day
GC  glucocorticoid
GCM-1  glial cell missing -1
GDM  gestational diabetes mellitus
GLUT  glucose transporter
GR  glucocorticoid receptor
GRE  glucocorticoid response element
h  hour
HPA  hypothalamic-pituitary-adrenal (axis)
hPL  human placental lactogen
Igf1  insulin-like growth factor-I
Igf2  insulin-like growth factor-II
Igf2P0  placental specific (P0) transcript of insulin-like growth factor-II
IHC  immunohistochemistry
IL-1β  interleukin-1β
IL-6  interleukin-6
i.p.  intraperitoneal
IUGR  intrauterine growth restriction
i.v.  intravenous
IVH  intraventricular hemorrhage
LGA  large for gestational age
MACS  Multiple courses of Antenatal Corticosteroid for preterm birth Study
MeAIB  N-methylated aminoisobutyric acid
min  minute
mRNA  messenger ribonucleic acid
mTOR  mammalian target of rapamycin
mTORC  mammalian target of rapamycin complex
MVM  microvillous membrane
NIH  National Institutes of Health
NEC  necrotizing enterocolitis
PCR  polymerase chain reaction
P-gp  P-glycoprotein
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARγ</td>
<td>peroxisome proliferators-activated receptor gamma</td>
</tr>
<tr>
<td>PTL</td>
<td>preterm labour</td>
</tr>
<tr>
<td>PVL</td>
<td>periventricular leukomalacia</td>
</tr>
<tr>
<td>RDS</td>
<td>respiratory distress syndrome</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interference ribonucleic acid</td>
</tr>
<tr>
<td>SGA</td>
<td>small for gestational age</td>
</tr>
<tr>
<td>sGC</td>
<td>synthetic glucocorticoid</td>
</tr>
<tr>
<td>Slc38a</td>
<td>Solute Carrier family 38, member</td>
</tr>
<tr>
<td>SNAT</td>
<td>sodium couple neutral amino acid transporter</td>
</tr>
<tr>
<td>TBP</td>
<td>Tata-box binding protein</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>vascular endothelial growth factor A</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction
Chapter 1: Introduction

1.1 OVERVIEW

This literature review will focus on the placenta’s role in transferring nutrients to the fetus and how corticosteroid therapy given in pregnancy may alter this ability. Approximately 10% of pregnant women are at risk of preterm labour and are administered synthetic glucocorticoids (sGCs), which act to accelerate fetal lung and organ maturation, thus, dramatically reducing neonatal morbidity and mortality. A number of animal models, as well as human clinical trials using repeated courses of sGC therapy, have demonstrated potential adverse effects on fetal growth. A key function of the placenta is adequate nutrient transport, largely contributing to normal fetal growth, in utero. Specifically, this doctoral thesis will focus on the sodium-dependent system A transporter. Placental system A is located within the syncytiotrophoblast and is responsible for transporting small neutral amino acids. System A activity is comprised of three independent isoforms (SNAT1, SNAT2 and SNAT4). Consistently, studies have demonstrated the critical importance of system A transport for normal fetal growth - as reductions (or increases) contribute to fetal growth restriction (or fetal macrosomia). The effects of sGC treatment on placental structure and function including system A regulation will also be examined. Investigating the effects of sGC administration at the level of the placenta and corresponding fetal phenotypic outcomes is critical as excess exposure to sGCs may contribute to altered health and disease trajectories that persist into adulthood. As such, understanding the mechanisms for altered fetal growth in the context of placental nutrient transfer is critical, not only for decreasing perinatal complications, but also to reduce the potential risk of disease in adulthood.
Healthy fetal growth *in utero* is dependent on a multitude of factors including: maternal nutrient availability, blood flow, metabolism, endocrine signaling and placental transfer. The 2003 Canadian Perinatal Health Report indicated that 8% of Canadian births are small for gestational age (SGA), which is defined as being born <10\textsuperscript{th} percentile of birth weight for a given geographically defined population\textsuperscript{1}. Proper fetal growth is crucial to the health of an individual, as growth restriction is associated with an increased risk of prematurity (defined as birth before 37 weeks of gestation) and is present in almost 80% of neonatal mortalities\textsuperscript{2}. Growth restriction *in utero* is associated with an increased risk of chronic lung disease, impaired postnatal growth and neurodevelopmental problems in early childhood\textsuperscript{3}. Not only do health care threats exist immediately following parturition if born prematurely, but also *in utero* adaptations can alter growth rates, which can result in adverse health outcomes in adult life. Dr. David Barker first developed the concept of early life programming of adult health and disease by initial observations linking low birthweight to increased risk of cardiovascular disease in adulthood\textsuperscript{4}. This observation was a breakthrough study and the term ‘fetal programming’ has come to describe adaptations that occur *in utero* during sensitive developmental windows, which ultimately produce alterations that persist throughout life\textsuperscript{4,5}. The key factors that determine the extent of an adaptive response are the timing, nature and intensity of an environmental adversity experienced *in utero*. It is well characterized that growth restricted fetuses show a predisposition for increased risk of disease, including hypertension, ischemic heart disease and insulin resistance in adulthood\textsuperscript{6}. The programming impact of growth restriction is evident even when infants catch-up in size postnatally, since they still display a predisposition to cardiovascular disease in adulthood\textsuperscript{7}. At the other end of the spectrum, ~10% of all pregnancies give rise to babies that are large for gestational
age (LGA) - defined as birth weight >90\textsuperscript{th} percentile\textsuperscript{8}. Fetal overgrowth or macrosomia poses a threat to immediate neonatal outcomes, as LGA babies are at risk for operative delivery or traumatic birth injury\textsuperscript{9}. Similarly, LGA babies are also at increased risk for disease in adulthood, including metabolic syndrome, obesity and diabetes\textsuperscript{10-12}. Women who are overweight or obese are more likely to give birth to LGA babies\textsuperscript{8, 13}, and as rates of obesity continue to rise, it is evident that inappropriate fetal growth poses a major long-term health care threat. Macrosomia is often reported in diabetic pregnancies, because maternal hyperglycemia contributes to excess glucose delivery to the fetus. Many factors that have been extensively studied and contribute to long-term programming of the neonate include maternal undernutrition, hypoxia, maternal stress and excess exposure to synthetic glucocorticoids (sGCs) \textit{in utero}\textsuperscript{14, 15}. This thesis will focus on the effects of excess sGC exposure, specifically how it affects the placenta’s ability to transport nutrients across gestation and the effects on fetal growth.

Sex-specific neonatal outcomes have been observed in cases of preterm labour (PTL) and in other pathological pregnancy outcomes. Male fetuses have higher rates of mortality and incidence of PTL compared to females\textsuperscript{16}. Moreover, male fetuses experience worse outcomes when faced with the pathological conditions of pre-eclampsia and intrauterine growth restriction (IUGR)\textsuperscript{17-19}, while female fetuses tend to exhibit reduced growth\textsuperscript{16}. The underlying mechanisms of sex-specific outcomes are not fully understood; however, in recent years they have become an increased focus in reproductive and fetal developmental research.
1.3 THE PLACENTA

1.3.1. The Human Placenta

Healthy fetal development in utero requires proper placental attachment and establishment. As such, the placenta represents the dynamic interface between the maternal and fetal environment. Throughout gestation, the placenta transfers nutrients and gases, removes wastes, acts as a protective barrier and produces and secretes hormones, cytokines and signaling molecules into the maternal and fetal circulation. Although each of these functions is critical for a successful pregnancy, this thesis will focus primarily on the function of nutrient transfer.

The human placenta is hemochorial in structure: transfer occurs across the villous trophoblast of the placenta. Within the villi, nutrients must cross the microvillous membrane (MVM; maternal-facing), travel across the trophoblast cytoplasm and cross the basal membrane (BM; fetal-facing) of the multinucleated syncytiotrophoblast layer to enter the fetal circulation \(^{20-22}\) (Fig. 1.1A). Net transfer of nutrients depends on the concentration gradients between the maternal and fetal circulations, blood flow to the placenta, placental metabolism and expression and activity of nutrient and drug transporters.

1.3.2. The Mouse Placenta

The murine placenta is an excellent model to study placental nutrient transport, as both humans and mice have hemochorial placentation\(^ 23\). Murine gestation is approximately 19-20 days depending on strain of mouse (compared to \(~38\) weeks in humans). After conception, implantation of the blastocyst into the uterus occurs at embryonic day (E)\(4.5^{24}\). The definitive murine placenta
is established between E10.5 and E12.5\textsuperscript{23, 25}. Nutrient delivery relies on passive diffusion before this time\textsuperscript{36}.

Two major regions categorize the functional murine placenta (Figure 1.2). Firstly, the junctional zone of the mouse placenta separates the labyrinth from the decidua and maternal uterine tissue. Following E12.5, this region consists mostly of spongiotrophoblast and glycogen cells\textsuperscript{27}. Bordering the junctional zone is a discontinuous layer of trophoblast giant cells\textsuperscript{28}. Second is the nutrient exchange region - the labyrinth zone - that functions in a similar manner to the villi within the human placenta. The murine placenta is trichorial, consisting of two multinucleated syncytial layers (layer II and III)\textsuperscript{29} and an overlying mononuclear layer (layer I)\textsuperscript{27}. During mid-gestation, the trophoblast layer I becomes highly perforated, allowing the maternal blood space to interface directly with the syncytiotrophoblast layer II by E12.5\textsuperscript{25, 27}. Layers II and III are closely apposed with gap junctions and are thought to act in concert to represent the single trophoblast cell layer in the human placenta\textsuperscript{27}; thus, nutrients transfer across layer II and III into the fetal circulation\textsuperscript{30} (Fig. 1.1B). During the second half of gestation from E12.5 to E18.5, the labyrinth zone increases as a proportion of the whole placenta at the expense of the junctional zone and decidua basalis\textsuperscript{25}. This increases the percentage of the placenta that can transport nutrients to the fetus.

In rodents, the amnion and yolk sac are also important in maternal-fetal exchange. In early gestation, solutes are believed to be transported across the amniotic membrane, which makes up a large portion of the amniotic fluid. Later in gestation, amniotic fluid is largely composed of fetal urine and secretion of fluid from the fetal lungs\textsuperscript{31}. The fetus’s first vascular and hematopoietic system is established within the yolk sac\textsuperscript{24}. There are differences between the role of the yolk sac between rodents and humans. In humans, the visceral yolk sac is present only until the first
trimester; in rodents, the yolk sac persists until term\textsuperscript{32}. Furthermore, in the rodent, the yolk sac is involved in embryonic nutrition through macromolecule breakdown and the transfer of ions and $\gamma$ globulin antibodies\textsuperscript{32}.

Overall, the choice of a murine model for placental investigations must weigh the similarities and differences in structure and function compared to humans. The primary benefit of using a mouse model is that studies can be conducted across gestation and significant insight into placental function can be gained using transgenic mice. However, differences do exist between murine and human placentae, therefore extrapolating results from mouse models must be done with caution.

1.3.3 The Role of the Placenta in Sex-Specific Differences in Fetal Growth

When assessing placental function, most studies pool data from placentae of male and female fetuses. Although this provides a representative measure of an entire litter, it potentially masks any differences between sexes. Clifton and colleagues have examined the role of the placenta in sex-specific differences in neonatal outcome in cases of maternal asthma. Their data suggests that male and female fetuses exhibit different coping mechanisms to adverse intrauterine conditions\textsuperscript{33-35}. Female fetuses display reduced growth rates, while males grow normally \textit{in utero} despite adversities. This female response appears to confer protection to subsequent insults, while male offspring display increased susceptibility\textsuperscript{33, 34, 36, 37}. Sex-specific placental cytokine\textsuperscript{38}, protein\textsuperscript{39, 40} and mRNA levels have been identified in cases of maternal asthma\textsuperscript{36}. This suggests that sex-specific regulation of transcription and translation in the placenta may contribute to adaptations responsible for altered fetal outcome.
1.4 THE SYSTEM A TRANSPORTER

1.4.1 Overview

Amino acids are crucial for fetal development, as they are required for protein synthesis, tissue development, energy supply\textsuperscript{41,42} and are essential for metabolic and growth support of the placenta\textsuperscript{20}. There are approximately 15-20 different types of amino acid transporters in the human placenta, which differ in membrane orientation, substrate affinity and kinetic properties. All of these transporters function in concert to coordinate maternal-fetal exchange of nutrients and each has specific patterns of expression and activity across gestation. Amino acid transporters can be categorized into two main classes: accumulative and exchangers. Accumulative transporters mediate amino acid influx, thereby increasing the net concentrations of amino acids within the syncytiotrophoblast. Amino acid exchangers swap one amino acid from outside the cell for another that is inside the cell, without changing the net concentration of amino acids in the placenta\textsuperscript{20,43}.

Although all amino acid transporters are critical for normal fetal growth and development, this thesis will focus on the system A amino acid transporter. The sodium-dependent system A transporter is pH sensitive and transports small, unbranched, zwitterionic neutral amino acids, such as alanine, glutamine, glycine, threonine, proline, methionine and serine\textsuperscript{44}. It also has the unique ability to transport N-methyl aminoisobutyric acid (MeAIB)\textsuperscript{45}. Amino acids that are substrates of system A are co-transported with Na\textsuperscript{+} (1:1) against their concentration gradient, thus using secondary active transport to accumulate inside the syncytiotrophoblast\textsuperscript{46-48}. System A is comprised of three sodium-coupled neutral amino acid transporter (SNAT) protein isoforms (SNAT1, SNAT2 and SNAT4) that function independently and are each encoded by a distinct gene: Solute carrier family 38, member 1 (Slc38a1), Slc38a2 and Slc38a4, respectively\textsuperscript{49}. All three
isoforms possess homologies in amino acid sequences (SNAT1 and SNAT2 (52%), SNAT1 and SNAT4 (48%) and SNAT2 and SNAT4 (57%))\textsuperscript{46-48, 50}. SNAT4 has a lower substrate affinity for neutral amino acids compared to SNAT1 and SNAT2 and can transport cationic amino acid independent of sodium\textsuperscript{47, 51}. There is limited information regarding the precise molecular structures of the system A transporters. It is postulated that SNAT1 and SNAT2 contain 11 transmembrane domain regions\textsuperscript{49, 52} (Fig. 1.3A), while it has been reported that SNAT4 has 10 transmembrane domain regions\textsuperscript{53}. SNAT1 is expressed predominantly in the brain, retina, placenta and heart. SNAT2 is ubiquitously expressed in most tissues including the brain, spinal cord, placenta, adrenal glands, thymus, testis, intestine, liver, muscle, lung, kidney, spleen, adipose and skin\textsuperscript{49}. Together SNAT1 and SNAT2 mediate glutamine transport in the brain by shuttling glutamine from astrocytes to neurons\textsuperscript{49, 54}. Prior to 2005, SNAT4 was believed to be expressed exclusively in the liver, predominantly in hepatocytes around the central vein\textsuperscript{47, 51}. However, recent work by Desforges \textit{et. al} (2006) demonstrated expression of SNAT4 in the placenta (both in the MVM and BM)\textsuperscript{55}. System A activity is present in both MVM and BM vesicles isolated from human syncytiotrophoblast\textsuperscript{44, 56, 57} (Fig. 1.3B) but activity per milligram of protein is higher in the MVM compared to the BM\textsuperscript{57}. All three isoforms are present in first trimester and term placental MVM\textsuperscript{58}. Each of the three isoforms have also been demonstrated in rodent placentae\textsuperscript{59-61}, especially within the labyrinth zone\textsuperscript{60}.

1.4.2 System A Transport Across Gestation

Nutrient transport systems are specifically coordinated to meet the needs of the growing fetus throughout different developmental stages of gestation. In the second half of gestation, fetal weight increases dramatically compared to placental weight\textsuperscript{62, 63}. Therefore, increased nutrient supply is required to support the growth of the fetus. This is achieved by increasing the transfer of
nutrients from the maternal to fetal circulation. Increased placental transport occurs by increasing blood flow to the placenta, differentiation of the MVM and BM (which increases the transporting surface area and thins the barrier for exchange) and altered nutrient transporter expression and activity. System A activity is higher in MVM vesicles isolated at term compared to first trimester. Examination of gene expression at less than 13 weeks of gestation compared to term demonstrated that Slc38a1 and Slc38a2 gene expression does not change across gestation. Yet, Slc38a4 expression was higher in first trimester tissue compared to term - demonstrating an isoform specific mRNA expression pattern across gestation. However, Slc38a4 gene expression did not correlate to SNAT4 protein expression when measured in total placental homogenates. SNAT4 protein was increased at term compared to preterm in whole placental homogenates. SNAT4 protein measured exclusively from isolated MVM displayed a similar trend to mRNA expression with SNAT4 being higher in first trimester compared to term in the MVM. Since active transporters are located in the membrane of the syncytiotrophoblast, measurements of protein content at the level of the MVM are more likely to reflect protein that is available for transport compared to total cellular protein content. mRNA and activity levels in rat placenta are increased at E20 (term ~ 21 days) compared to E14. Overall, isoforms of system A demonstrate time-specific changes in mRNA and protein, which may contribute to the increased activity levels across pregnancy.

1.4.3 The Role of System A Transport in Fetal Growth

Research groups have reported that placental system A activity is reduced in the MVMs of SGA and pathologically growth restricted fetuses. The extent to which system A in the MVM is reduced correlates with the severity of growth restriction in fetus. Furthermore, amino acid concentrations, including the system A substrate serine, are reduced in cord blood from SGA
fetuses. System A activity and mRNA are unaltered in fetal T-lymphocytes isolated from growth restricted fetuses. Since system A is not constitutively reduced in all cell types in cases of IUGR, this demonstrates the specific reduction in placental system A associated with the reduced fetal growth phenotype. Furthermore, blood flow to the placenta (maternal and fetal surface) is reduced in pregnancies that give rise to IUGR infants. However, no difference in passive permeability or glucose transport occurs in placentae from growth restricted fetuses compared to healthy control infants. Reduced oxygen delivery to the placenta may also contribute to altered system A transport in cases of IUGR. Cytotrophoblast cells cultured with low oxygen have reduced system A activity which is mediated by reduced gene expression of \textit{Slc38a1} and \textit{Slc38a2}. Using a rat model, Cramer \textit{et al.} (2002) inhibited system A activity by infusing the non-metabolizable synthetic amino acid analog 2-MeAIB during gestation and consequently observed reduced fetal weight at term. Further studies examining the effects of nutrient restriction on placental transport demonstrated that rats fed a low-protein diet have reduced placental weight and system A-mediated transfer, and that these effects preceded fetal growth restriction. Collectively, there is evidence to demonstrate that IUGR is not mediated by a decrease in placental transfer capacity, but rather specific decreases in transport systems, including system A. Furthermore, these data suggest that lowered system A activity may be a primary cause of fetal growth restriction rather than a secondary outcome.

In contrast to the situation of IUGR, it has been demonstrated that system A transport is increased in cases of fetal overgrowth. Mice fed a high-fat diet (32% energy from fat compared to 11% in control diet) before and during pregnancy had a 10-fold increased system A activity and 5-fold glucose transport prior to term at E18.5, which was associated with a 43% increased fetal weight. This specific diet increased maternal adiposity but did not alter maternal insulin levels or
glucose tolerance. By using a high-fat diet model, which did not induce diabetes, this allowed for the examination of system A strictly in cases of high maternal body mass index and thus, nutrient over supply, without significantly altering maternal glucose homeostasis. However, studies examining the effects in human placentae that give rise to LGA infants have conflicting results especially in pregnancies complicated with diabetes. During pregnancy, women can have Type-I or Type-II diabetes or develop gestational diabetes mellitus (GDM). Approximately 40% of women who have diabetes during pregnancy give birth to infants who are LGA or macrosomic. Kuruvilla et al. reported that system A activity at the level of the MVM was reduced in women with Type-I diabetes who delivered LGA infants. In 2002, Jansson et al. measured system A activity in placentae from women who had LGA babies as well as from women who had gestational diabetes that gave birth to average or LGA fetuses. System A activity measured in the MVM was unaffected in normal placentae giving rise to LGA fetuses, however, activity was increased in all diabetic pregnancies (gestational diabetes mellitus and Type-I diabetes) regardless of fetal outcome. There was no effect of system A activity in the BM. Amino acid concentrations found in umbilical cord blood were reported to be significantly increased with GDM, even when there was no difference in maternal substrate availability. Investigating excess nutrient delivery is critical as rates of diabetes and obesity continue to rise in the 21st century, which increases the risk of macrosomic babies and long-term disease. Collectively, these studies demonstrate the importance of both increased and decreased system A transport to the development of the fetal phenotype in a variety of pathological pregnancy outcomes.

1.4.4 Nutrient Availability Regulation of System A Transport

Increasing evidence demonstrates that regulation of system A is affected by nutrient availability. Evidence from in vitro studies suggests that system A follows the principle of adaptive regulation,
in which transport is up- or down-regulated when amino acid availability is low or high respectively\textsuperscript{83-86}. To illustrate this property, Jones \textit{et al.} (2006) demonstrated that the depletion of amino acids from culture media stimulates system A activity in BeWo cells by causing an increased relocalization of SNAT2 protein to the cell membrane followed by increased \textit{Slc38a2} mRNA and SNAT2 protein expression\textsuperscript{85}. The mechanism of adaptive regulation is believed to occur in two phases: transporter activity is immediately increased to quickly establish proper amino acid concentrations and then transporter expression is increased over time to maintain longer-term homeostasis. Furthermore, in cultured human fibroblasts, amino acid starvation increased SNAT2 mRNA expression with subsequent reductions in mRNA levels after re-feeding\textsuperscript{83}. Increased expression that occurs with amino acid deprivation is mediated by the binding of activating transcription factor 4 (ATF4) to the first intron in \textit{Slc38a2}\textsuperscript{87, 88}. One proposed mechanism of adaptive regulation is the dual ability of a transporter to act as a transporter and receptor - or ‘transceptor’ - which modulates signal transduction cascades\textsuperscript{89}. The dual transporter/receptor function has been demonstrated in lower organisms such as yeast\textsuperscript{90} and drosophila\textsuperscript{91} and more recently in mammalian systems\textsuperscript{92}. Hyde \textit{et al.} (2007) found that amino acid withdrawal stimulates JNK activation and increases the expression of SNAT2 in L6 myotubes. The increase in SNAT2 expression was abolished when the SNAT2 receptor itself was ablated using RNA interference with short hairpin RNA\textsuperscript{92}.

The placenta itself shows adaptations in nutrient transfer in order to meet fetal demands. Natural intra-litter variation in mice demonstrates that small placentae undergo morphological adaptations by increasing volume of the labyrinth zone and functional adaptations of increased system A activity and \textit{Slc38a2} mRNA expression compared to larger placentae\textsuperscript{93}. This increase in system A transfer capacity allows for smaller placentae to deliver the same amount of nutrients compared to
larger placentae within the litter, which is demonstrated specifically as the rate of diffusion was unaltered with differences in natural litter variation\textsuperscript{94}. Similar adaptations occur in human placentae from infants in the normal birthweight range. System A activity is reported to be increased in placentae that give rise to babies at the lower end of the healthy birthweight range\textsuperscript{95}. Despite being smaller in size, the placenta adapts strategies that help support normal fetal growth.

\textit{In vivo} it has been demonstrated that placental transport may be regulated to mirror maternal nutritional status by either reducing growth in response to decreased substrate availability or increasing growth when substrates are plentiful. Numerous rat models have demonstrated that low-protein diets decreased placental system A uptake and transfer to the fetus\textsuperscript{96-98}. Recently, Jansson \textit{et al.} demonstrated that a 4\% protein diet compared to isocaloric controls of 18\% protein diet reduced system A activity, which preceded reductions in fetal growth. Conversely, mice fed a high-fat diet before and during gestation had increased system A transport and SNAT2 protein expression which was associated with fetal overgrowth\textsuperscript{79}. These studies demonstrate that nutrient availability \textit{in vivo} acts to regulate placental transport to match rates of fetal growth to a sustainable level based on maternal nutritional status.

One molecular target that may be involved in regulating system A in altered nutrient conditions is the mammalian target of rapamycin (mTOR)\textsuperscript{99}. mTOR is a phosphatidylinositol kinase that is associated in two separate complexes. mTOR complex 1 (mTORC1) controls translation, cell proliferation and growth, and is stimulated by insulin-like growth factor-I (Igf\textsuperscript{-1})/insulin activation of the PI3k-Akt pathway, whereas mTOR complex 2 (mTORC2) promotes cell survival\textsuperscript{100}. Inhibition of mTOR using rapamycin decreases system A activity by 17\% in primary trophoblast cells, demonstrating that mTOR positively regulates system A activity\textsuperscript{101}. It has recently been
suggested that mTORC1 and mTORC2 are involved in SNAT protein trafficking to the membrane. siRNA targeted to mTOR adaptor proteins raptor and rictor (proteins critical for normal mTORC function) does not alter total SNAT protein levels but decreases SNAT protein expression at the MVM\(^{102}\). Recent studies have demonstrated that mTORC1 is regulated through Nedd4-2 signaling, a ubiquitin ligase involved in ubiquitination of membrane proteins\(^{103}\). Follow-up studies investigating the underlying mechanisms of reduced system A transport in mice fed a low-protein diet, found that mTOR and insulin (stimulators of system A) were also reduced in this model\(^{102}\). This study highlights the role of mTOR signaling in regulating cellular nutrient uptake by altered level of SNAT recruitment to the MVM.

Overall, studies in rodents have identified key molecular pathways that regulate system A transport in cases of maternal nutrient availability. Whether the same occurs in humans has yet to be investigated. It is important to understand key system A regulatory factors because endocrine, nutrient and maternal circulatory profiles change dramatically in cases of pathological pregnancies.

1.4.5 Endocrine Regulation of System A Transport

Endocrine factors also have potent effects on modulating system A transport\(^{76, 104-109}\). *In vivo*, placental nutrient transport is affected by fetal endocrine demand signals. Using knockout mice for insulin-like growth factor 2 (Igf2) and the placental-specific (P0) transcript of the Igf2 gene (Igf2P0), Constância *et al.* (2002) demonstrated that Igf2P0 is required for normal placental growth. Furthermore, Igf2 from the fetus stimulates system A amino acid transport during mid-late gestation E16, in order to increase nutrient transfer at a time of placental insufficiency\(^{110, 111}\). However, this upregulation of system A during placental insufficiency is not maintained until the
end of term and fetal growth restriction occurs by E18.5. Altered system A transport in this model of growth restriction was associated with changes in the *Slc38a4*. *Slc38a4*, an imprinted gene in the mouse, is critical for proper fetal development as knocking it out results in fetal growth restriction of ~20%\textsuperscript{112}.

Additional studies have implicated placental Igf2P0 in regulating system A activity in cases of maternal nutrient restriction. Deletion of the Igf2P0 gene abolished the initial up-regulation of system A activity in maternally undernourished pregnant mice at E19 and ablated changes to *Slc38a2* gene expression\textsuperscript{113}.

*In vitro* evidence has demonstrated that leptin, insulin and insulin-like growth factor-1 (Igf1) stimulate system A transport in cultured trophoblast or placental villous explants\textsuperscript{104, 107, 108}. Conversely, hypoxia and interleukin-1β inhibit system A transport, *Slc38a1* mRNA and *Slc38a2* mRNA expression in trophoblast cells\textsuperscript{76, 109}. However, transporter function may vary depending on timing and dose of endocrine exposure. For example, insulin and Igf1 stimulate the uptake of the specific system A substrate aminoisobutyric acid (AIB) in cultured placental human trophoblast cells\textsuperscript{104, 108}, while no effect was seen after 1 hour treatment in first trimester villous explants\textsuperscript{114}. Therefore, interpretations and extrapolating results must be done in a time-dependent context. Moreover, differences in regulatory effects may be due to differential regulation of the three SNAT isoforms.
1.5 SYNTHETIC GLUCOCORTICOIDS

1.5.1 Glucocorticoid Signaling

Glucocorticoids (GCs) are lipophilic molecules that cross plasma membranes by cellular diffusion. GC signaling in the body plays a large role in various processes including carbohydrate and protein metabolism, stress response, inflammation and immune suppression. Due to alternative splicing, the human glucocorticoid receptor (GR) has two isoforms, GRα and GRβ\textsuperscript{115}, although GRβ is believed to be transcriptionally inactive\textsuperscript{116}. GC binding to GR can mediate genomic and non-genomic actions. Genomically, GCs bind to cytosolic GRs, which causes associated heat shock proteins to dissociate. This results in a conformational change, which allows the GC-GR complex to translocate to the nucleus and interact with DNA\textsuperscript{117, 118}. Many genes contain glucocorticoid response elements (GREs) – DNA regions possessing the consensus sequence AGAACANNNTGTTCT – to which the GC-GR complex binds. This binding can increase or decrease transcription depending on the target gene\textsuperscript{119}. Evidence of genomic effects (i.e. altered levels of transcription, translation, etc.) can take hours to days to appear at the cellular or tissue level\textsuperscript{120}, whereas non-genomic actions of GC signaling may occur more rapidly (within seconds to minutes). Non-genomic effects involve the alteration of intracellular signaling cascades by affecting ion fluxes, protein kinases and/or cAMP-dependent pathways. Non-genomic actions can be mediated by the binding of GCs to membrane bound or associated receptors in order to alter signal transduction cascades, or by the direct interaction of GCs with the cellular membrane (Fig. 1.4A). Non-genomic effects may indirectly modulate gene expression by the downstream activation/inhibition of other transcription factors that affect gene transcription\textsuperscript{120}.

Several genes, which have known GREs in their promoters regions, have been shown to be regulated by GCs (i.e. Igf2), however there are also genes that are regulated by GCs that do not
have identified GREs (i.e. Igf1)\textsuperscript{121, 122}. In this case, GCs may bind to half-sites of the GRE or modulate the stability of transcripts, translation and post-translational processes\textsuperscript{117, 123}. GR expression is regulated tissue-specifically, thus GCs have widespread effects around the body.

1.5.2 Synthetic Glucocorticoids Treatment for the Management of Preterm Labour

Towards the end of pregnancy, GC concentrations in the fetus increase exponentially. This late-gestation fetal cortisol surge occurs in most species, and acts to mature fetal tissues, especially the fetal lungs, liver, kidney, adrenal and brain\textsuperscript{124-126}. Specifically in the lung, cortisol promotes fetal alveolarization and lung compliance, thinning of the septae, stimulates surfactant production and release from type II pneumocytes into the alveoli and increases the elastin and collagen content of the lungs\textsuperscript{118, 127-130}. However in cases of PTL, fetuses are left susceptible to pulmonary complications, such as respiratory distress syndrome (RDS), due to underdeveloped lung function. PTL is the largest cause of neonatal morbidity and mortality and affects \sim10\% of all pregnancies\textsuperscript{131}. In 1972, Liggins and Howie first established the practice for the use of synthetic glucocorticoid (sGC) treatment to reduce RDS in premature neonates (from 15.6\% to 10\%) in cases of PTL\textsuperscript{132}. Since then, many clinical trials have supported Liggins and Howie’s findings and have demonstrated that this practice also decreases the risk of intraventricular hemorrhage (IVH), periventricular leukomalacia (PVL) and necrotizing enterocolitis (NEC) in the neonate, thus dramatically reducing morbidity and mortality\textsuperscript{132, 133}. In 1994, the National Institutes of Health (NIH) reviewed the evidence for the use of sGCs in cases of PTL. The panel supported the use of sGCs which consists of a single course of either betamethasone (BETA; two doses of 12 mg of given intramuscularly 24 hours apart) or dexamethasone (DEX; four doses of 6 mg of given intramuscularly 12 hours apart) between 24-34 weeks of gestation\textsuperscript{134}. Following this report, the use of sGCs drastically increased worldwide.
1.5.2.1 Evidence for Treatment Regime

Since the initial trials and treatment regime of sGC therapy for the management of threatened PTL established by Liggins\textsuperscript{132}, studies have investigated differences between type of sGC (BETA vs. DEX), doses and gestational time frames for treatment.

\textit{BETA vs. DEX}: Both sGCs are similar in molecular structure (fluorinated GCs) and differ only by a methyl group positioned on ring D (Fig. 1.4B and 1.4C). Both sGCs cross the placenta in their active forms, have similar biological activity and do not activate mineralocorticoid receptors\textsuperscript{135}. The commonly used formula of BETA (50% BETA-phosphate – half-life 4 hours; 50% BETA-acetate – half-life 14 hours) gives it longer overall half-life compared to DEX phosphate, which has a half life of \textasciitilde 3.6 hours\textsuperscript{136}. Conflicting evidence exists regarding the use of one sGC over the other. Three studies have supported the use of BETA over DEX by reporting that BETA exposed infants had lower rates of RDS, PVL, bronchopulmonary dysplasia and neonatal death\textsuperscript{137-139}. However, the Betacode trial (2007) found that neonates exposed to BETA had higher rates of IVH, with no differences in other perinatal outcomes such as RDS, PVL, NEC, sepsis or mortality\textsuperscript{140}. Currently, there is no definitive evidence to support the use of one sGC over the other. The American College of Obstetrics and Gynecologists has supported the use of either agent for the management of PTL\textsuperscript{135}.

\textit{Single vs. Multiple Courses}: Initially, it was proposed that sGC therapy was maximally beneficial between 24 hours and 7 days following treatment\textsuperscript{118}. However, approximately 70\% of women remain at risk of PTL 7 days following their last dose of sGCs\textsuperscript{141}. This led to the administration of repeat courses of sGCs if the risk of prematurity persisted beyond a 7-day period\textsuperscript{142}. In the late 1990s, greater than 85\% of obstetricians from the United States, United Kingdom and Australia
reported that they prescribed more than one course of sGCs, with some administering up to 11 doses. Two major clinical trials have tested the effectiveness of multiple sGC treatments. In 2006, Crowther et al. conducted the Australasian Collaborative Trial of Repeat Doses of Steroids (ACTORDS), which was a randomized trial investigating the efficacy of weekly repeat single doses of BETA on 982 women who were at continued risk of PTL. Their findings supported the use of repeated corticosteroid treatment as exposure to multiple doses significantly reduced neonatal RDS with no differences found in rates of IVH, PVL, NEC or fetal weight. In 2008, the Multiple courses of Antenatal Corticosteroids for preterm birth Study (MACS) led by Murphy et al. also assessed the efficacy of repeat courses of corticosteroids. However, women were given a less frequent intervention of sGC or placebo every 14 days if the fetus remained undelivered until week 33 or birth. The results from this trial identified similar rates of morbidity and mortality, yet discovered adverse effects on fetal growth in those infants exposed to multiple courses. The aforementioned studies have been the largest randomized controlled trials examining the effects of repeat corticosteroid use in pregnancy. Although the acute neonatal benefits may support the use of repeat courses of sGCs, potential adverse effects on fetal growth are a large deterrent for the widespread use of this practice.

**Gestational Windows and Time course:** The use of corticosteroids for the management of PTL is recommended between 24-34 weeks of gestation, although sGCs are also administered to women prior to 24 weeks. The efficacy and safety of this treatment for very preterm infants has recently been called into question. Hayes et al. (2008) conducted a retrospective review to examine the outcomes of infants treated with sGCs at 23 weeks of gestation. Although all neonates suffered from RDS, a full course (2 doses of BETA or 4 doses of DEX) was associated with a decreased odds ratio of death and there was no effect on the risk of RDS. Therefore, the authors postulated
that the beneficial effect of corticosteroids on neonatal survival likely occurred in other organ systems. To determine whether effects of sGC treatment persisted if women remained undelivered for a prolonged period, Vermillion et al. (2001) conducted a retrospective analysis of neonates treated with antenatal corticosteroids and delivered between 28 and 34 weeks’ gestation. They found no difference in perinatal outcome in pregnancies delivered between 8-14 days after treatment compared to those who delivered within 7 days following treatment. Peaceman et al. (2005) conducted a retrospective chart review of corticosteroid exposed infants that delivered before 34 weeks and found no difference in outcomes for those delivered less than 7 days after treatment to those who delivered greater than 7 days after treatment, with the exception of increased need for short term respiratory support in the latter group. Even in very low birth weight infants, longer intervals from exposure to delivery did not appear to affect neonatal outcome. These studies would suggest that the effect of a single course of antenatal corticosteroid persists beyond 7 days. However, gestational age at administration and gestational age at delivery may also determine the length of steroid effect. Ring et al. (2007) examined the outcomes of infants delivered between 26 and 34 weeks after a single course of corticosteroids. Analysis was subdivided between two groups including those infants who were exposed to corticosteroid therapy within 14 days of delivery and those who were exposed for greater than 14 days. The investigators found that those infants who delivered equal to or greater than 28 weeks of gestation and were exposed to corticosteroid for greater than 14 days were associated with increased need for ventilator support and surfactant use.

Although critical for the neonate in cases of PTL, more studies are needed to identify the best dosing regimen to optimally promote fetal maturation and minimize potential growth restriction or developmental adversities.
1.5.3 Synthetic Glucocorticoid Effects on Fetal Growth

Prenatal administration of sGCs has been shown to cause growth restriction in various animals, including mice, rats, sheep and monkeys\textsuperscript{124, 152-158}. In sheep, Pua et al. (2005) demonstrated a decreased fetal weight with both single and repeated doses of DEX\textsuperscript{153}. A single course of BETA reduced birthweight in preterm lambs by 15%, and repeat course (3-4) caused further reductions of up to 27%\textsuperscript{154}. There are conflicting results regarding the adverse effects of sGCs on fetal growth in humans. In 1999, French et al. conducted an observational study following 447 women over the course of three years. They found that increasing courses of antenatal corticosteroids corresponded with decreased birthweight and head circumference\textsuperscript{159}. In 2001, Bloom et al. reported that DEX exposed infants (combined single or multiple course treatment) had significantly reduced birthweight compared to age-matched control infants\textsuperscript{160}. In the ACTORDS trial (2006) previously discussed, no adverse effects on mean weight, length and head circumference at birth or at the time of hospital discharge were reported with multiple dose treatment\textsuperscript{146}. However, the MACS trial (2008) found significant adverse effects on fetal growth with reductions in birthweight, length and head circumference\textsuperscript{141}. Outcomes at 2 years of age have been reported for both the ACTORDS and MACS trials. The ACTORDS trial reported no difference in body size, lung disease or disability with sGC exposure at 2 years of age. However, follow-up reports stated that children who were exposed to multiple courses were more likely to require consideration for attention problems\textsuperscript{161}. At 2 years, follow-up in the MACS cohort demonstrated that there were no differences in neurological function, weight, height or head circumference in children exposed to multiple courses of sGCs\textsuperscript{162}. The Cochrane Review conducted by Roberts and Dalziel (2006) reviewed 21 studies (which included 3885 women and 4269 infants) using antenatal corticosteroid treatment on various maternal and fetal outcomes. Of the studies reviewed, 5 reported a significant effect on birthweight due to sGC treatment.
However, birthweight was significantly reduced at specific times following sGC exposure including: 1-7 days following sGC treatment (1 study - 529 infants), greater than 7 days after GC treatment (1 study - 485 infants), after 24 hours following treatment (2 studies - 242 infants) or after 48 hours following treatment (1 study - 373 infants). In 2009, Davis et al. conducted a study in 105 women and reported that a single course of sGCs was associated with reduced birth size at term compared to control infants\textsuperscript{163}. Although this trial examined a smaller number of women, the findings demonstrated that a single course of sGCs might have adverse effects on infants who are carried to term. Such studies highlight the importance of considering the longitudinal effects of sGC exposure on fetal growth, because \(~30\%\) of women at risk of PTL during gestation are administered sGCs, yet carry to term\textsuperscript{141}.

Fetal cortisol levels are also increased in infants that are IUGR\textsuperscript{164}. Exposure to excess GCs during gestation alters fetal growth and has been implicated with long-term programming effects. Prenatal stress and anxiety increases plasma corticosteroids and has been implicated in the development of psychiatric disorders (attention deficit hyperactivity disorder, depression and psychosis)\textsuperscript{165-167} and altered hypothalamic-pituitary-adrenal (HPA) axis function\textsuperscript{168}, birth parameters\textsuperscript{169}, glucose intolerance\textsuperscript{170} and cardiovascular\textsuperscript{171}, liver\textsuperscript{172} and metabolic function\textsuperscript{171}. In animal models, administration of exogenous sGCs, not only reduced birthweight but has also led to hypertension\textsuperscript{173} and altered HPA axis\textsuperscript{174}, altered metabolic function and glucose intolerance\textsuperscript{175}. The molecular mechanisms underlying fetal programming have not been fully established; however, changes to the HPA axis have been implicated in animal models - as this axis is particularly sensitive to the actions of GCs\textsuperscript{176-178}. Further investigations are needed to resolve the conflicting evidence examining the effects of sGC administration on fetal weight, especially in light of the potential sGC-induced long-term changes to growth trajectories.
1.5.4 Synthetic Glucocorticoid Effects on the Placenta

sGCs freely cross the placenta and act directly on fetal tissues expressing GR. However, sGCs may also exert their effects indirectly via the placenta. The placenta is a GC-responsive organ. In the human placenta, GRs are expressed in stromal fibroblasts of the terminal villi, perivascular fibroblasts, vascular smooth muscle cells and cytotrophoblast cells. Syncytiotrophoblast has relatively low expression compared to underlying cytotrophoblasts. In the mouse, GRs are ubiquitously expressed throughout the second half of gestation, in the placenta and uterus, including the labyrinth trophoblast. Regulating the balance of GCs in utero is critical for proper fetal growth as excess exposure of GCs results in fetal growth restriction. Mice that completely lack the GR gene die within a few hours after birth due to respiratory failure.

In addition to being GC-responsive, the placenta also acts as a protective barrier to limit fetal exposure to excess endogenous maternal corticosteroids – through the expression of the enzyme 11beta-hydroxysteroid dehydrogenase type II (11β-HSD2) within the syncytiotrophoblast. 11β-HSD2 rapidly converts cortisol (corticosterone in rodents) to inactive cortisone (11-dehydrocorticosterone in rodents). Thus, 11β-HSD2 regulates the transplacental transfer and local actions of GCs by regulating the level of GCs that activate intracellular placental receptors. The actions of placental 11β-HSD2 result in maternal levels of active GC that are much higher than in the fetus. The human placenta is exposed to increased levels of maternal cortisol as term approaches and corresponds with increased expression of 11β-HSD2 mRNA. In humans and rats, birthweight is correlated with the level of placental 11β-HSD2 expression. Reduced expression occurs in cases of IUGR. Furthermore, babies who are either homozygous or heterozygous for deleterious mutations of 11β-HSD2 also display very low birthweight. In the murine placenta, 11β-HSD2 mRNA is expressed exclusively in the labyrinth zone, which is the
major site of nutrient exchange\textsuperscript{190}, and is highly prominent in trophoblast cells\textsuperscript{180}. mRNA expression is tightly regulated across gestation in the mouse; it is highly expressed at E12.5, decreases across the second half of gestation until mRNA is barely detectable at E16.5 and prior to term (E18.5) expression is undetectable\textsuperscript{180}. Thus, the placental 11β-HSD2 mRNA expression profile in mice is the inverse to that of corticosteroid: 11β-HSD2 decreases while corticosteroids increase with advancing gestation. Furthermore, 11β-HSD2\textsuperscript{-/-} null mice, in which the fetus and placenta are exposed to increased levels of maternal GCs, have significantly reduced birthweight compared to wildtype controls\textsuperscript{191}. sGCs are highly effective in the treatment of PTL because they are not substrates for 11β-HSD2, therefore escape inactivation by this enzyme. However, both endogenous and synthetic GCs, can alter the expression of 11β-HSD2, thereby having an indirect effect on endogenous GC concentrations at the level of the placenta. Investigations in the sheep by Clarke et al. (2002) revealed that 11βHSD-2 activity decreases after exogenous administration of maternal cortisol in late gestation\textsuperscript{192}. Prenatal administration of sGCs reduces fetal weight, 11β-HSD2 expression and function\textsuperscript{193}. However, regulation of 11β-HSD2 by GCs is species-specific and timing of exposure-dependent. Kerzner et al. (2002) found decreased mRNA with maternal DEX administration in sheep\textsuperscript{193}. However, in the rat DEX and BETA did not affect 11β-HSD2 expression or protein yet reduced the functional activity of 11β-HSD2\textsuperscript{194}. DEX treatment of cultured human term placental trophoblast stimulated the expression and activity of 11β-HSD2\textsuperscript{195}. and also stimulated the expression of 11β-HSD2 in primary cytotrophoblasts cultured from preterm placenta\textsuperscript{197}. Both endogenous GCs and exogenous sGCs regulate 11β-HSD2 expression and activity. Therefore, the capacity of sGCs administered for PTL to alter barrier function and GC content of the placenta dictates fetal and placental exposure to GCs.
Another mechanism by which the placenta limits the passage of sGCs is by the expression of drug transporters, particularly, the multidrug resistance transporter P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP). P-gp pumps endogenous and foreign substances (including BETA and DEX) out of the syncytiotrophoblast back into the maternal circulation\textsuperscript{198}. In humans, \textit{ABCB1} mRNA and the encoded P-gp protein are highly expressed in the first and second trimesters, but levels dramatically decline near term\textsuperscript{199, 200}. Findings regarding the pattern of \textit{ABCG2} (encoding BCRP) expression over gestation are varied\textsuperscript{201–203}. Some studies report an increase\textsuperscript{201} while others report a decrease\textsuperscript{202} in expression throughout gestation. In the mouse, P-gp expression also decreases during the second half of gestation\textsuperscript{204} and this is coincident with increased fetal accumulation of P-gp substrates\textsuperscript{205}. In the mouse, mRNA levels decrease as gestation progresses, while protein levels do not change\textsuperscript{206}. In addition to being substrates for P-gp and BCRP, DEX regulates P-gp and BCRP expression and activity in a dose- and sex-dependent manner\textsuperscript{158, 207}. Thus, sGCs given in pregnancy may play a role in altered fetal susceptibility to other therapies, teratogens and endogenous and exogenous substrates transported by these efflux transporters.

In addition to the direct effects of GCs on the fetus and/or maternal metabolism, there is evidence that the placenta mediates the GC-induced reduction in birthweight, as maternal but not fetal infusion of BETA resulted in ovine fetal growth restriction\textsuperscript{155, 156}. No major clinical studies have investigated whether transplacental (given to the maternal circulation, thus must cross the placenta to reach the fetus) or direct fetal corticosteroid injection is the best route for administration in humans\textsuperscript{208}, thus maternal intramuscular injection and transplacental transfer remains the mode of corticosteroid delivery to date. The major action of sGC treatment is to mature fetal organs by shifting tissues from a proliferative to a differentiated state. However, the placenta is also greatly
impacted by this treatment. In the mouse placenta, DEX treatment (0.5 mg/kg i.p.) given on E14.5-E16.5 (mid-gestation) causes the down-regulation of 1212 genes and the up-regulation of 1382 genes – genes involved in cell division, growth promotion and many other signalling cascades required for normal placental development and function\textsuperscript{209}. Therefore, GR actions within the placenta have widespread transcriptional effects, which can alter placental stereology and functional capabilities and thus indirectly affect fetal outcome.

Elevated levels of maternal GCs have detrimental effects on placental development. Maternal infusion of cortisol in sheep in late gestation decreased placental weight by 25\%\textsuperscript{210}. sGC treatment given to pregnant sheep also reduced placental weight with reductions ranging up to \approx 35\% with single course treatment and \approx 43\% with multiple courses\textsuperscript{156, 211, 212}. 11\beta-HSD2\textsuperscript{−/−} mice, which have elevated levels of maternal corticosterone, had reduced placental weight (up to 11\%) at E15 and this reduction was sustained until prior to term at E18\textsuperscript{59}.

sGC treatment alters placental structure including decreasing binucleate cell number\textsuperscript{211} in sheep and increased placental apoptosis\textsuperscript{213, 214} in the rat. Studies investigating changes in cell signaling linked to survival in the rat found that DEX treatment significantly reduced cellular Akt and ERK1/2 activation\textsuperscript{215}. This may account for inhibited cell survival or stimulated apoptosis observed in growth-restricted placentae. In human trophoblast cell lines, the sGC triamcinolone acetonide altered functions related to proliferation and invasion\textsuperscript{216}. sGC treatment may also alter vascular development, thus affecting the ability of the maternal circulation to deliver nutrients to the placenta. DEX treatment in rats restricts placental vascularity, by reducing vascular endothelial growth factor-A (VEGF-A)\textsuperscript{217}, possibly by reducing peroxisome proliferators-activated receptor gamma (PPAR\textsubscript{γ}) – a key regulator of VEGF-A\textsuperscript{218}. Similarly, 11\beta-HSD-2\textsuperscript{−/−} mice had reduced
vascularization and mRNA expression of VEGF-A and PPARγ due to levels of elevated maternal corticosterone. In cases of PTL, female fetuses born within 72 hours of sGC exposure had higher levels of neonate urinary normetanephrine (metabolite of norepinephrine - a vasoconstrictor). Normetanephrine was inversely correlated to peripheral microvascular blood flow measured by laser Doppler flowmetry. Altering vascularity in the placenta or vascular tone of the fetus is likely to profoundly impact blood flow and the availability of substrates to the fetus. Thus, there is a clear potential for sGC treatment to negatively impact fetal nutrient delivery and fetal circulation, which may contribute to differential sex-specific outcomes in pathological pregnancies.

Excess GC exposure has also been shown to alter aspects of placental function, especially transport systems. Along with the previously described effects of sGCs on placental drug transporters, sGCs also alter nutrient transport in the placenta. The synthetic GC triamcinolone acetonide downregulated expression of the glucose transporters (GLUT)1 and GLUT3 in isolated human trophoblast cells and in the labyrinth of rat placenta. This suggests that GCs reduce placental nutrient transfer, which could have significant implications for subsequent fetal growth and development. However, DEX has shown the opposite effect when given daily from E15-E21 by causing a dose-dependent increase in placental GLUT1 and GLUT3 protein expression. This increase in GLUT expression may reflect an attempt to compensate for dose-dependent reductions in placental and fetal weight. In 11β-HSD2−/− mice, which have elevated levels of circulating maternal corticosterone, placental glucose transport is unaffected at mid-gestation, yet is markedly reduced in late gestation (during the time which fetal growth restriction occurs). Therefore, the potent effects of GCs on glucose transport may contribute to the phenotypic changes observed in the fetus after sGC treatment. The effects of antenatal GC
treatment on human placental function, in particular the transplacental transfer of amino acids from maternal to fetal circulation, are currently unknown. They will be further investigated in this doctoral thesis.

Not only is the transport function of the placenta affected, hormone production and secretion is also affected by sGC treatment. Reduced levels of placental lactogen (measure of endocrine function) occurs when pregnant sheep are treated with BETA$^{211}$. In rats, DEX-induced growth restriction is also associated with altered circulating maternal and fetal leptin concentrations and reduced placental leptin protein expression$^{222}$. In the rat, DEX given between E13 and E20 of gestation, not only induced fetal growth restriction and reduced placental weight by ~50%, it was also was associated with dysregulated placental prolactin family and Igf2 gene expression$^{214}$. In the human placenta, DEX also modifies endocrine function by altering levels of corticotrophin releasing hormone (CRH) secretion$^{223}$ as well as human chorionic gonadotropin (hCG) in cytotrophoblast cells$^{224}$. In summary, GCs play a major role in altering weight, structure and function of the placenta. Although the effects of sGCs depend on dose and timing of use throughout gestation, changes to the placenta due to treatment may directly or indirectly contribute to changes in fetal phenotype,
1.5.4 Synthetic Glucocorticoid Regulation of System A

System A can be stimulated or inhibited by GCs depending on cell type. *In vitro* studies have shown that cortisol (1 µM) stimulates system A amino acid transport in BeWo cells with increases in activity mediated by upregulated *Slc38a2* mRNA expression\(^\text{106}\). DEX (5 x 10\(^{-6}\) M) also stimulated AIB uptake in cultured human trophoblast cells\(^\text{108}\). However, DEX inhibits the initial rate of uptake of neutral amino acids transported by system A including AIB in rat hepatoma cells and hepatocytes\(^\text{225, 226}\). Cortisol does not affect system A activity after acute treatment in first trimester villous explants\(^\text{114}\). Whether sGCs used for the management of PTL play a regulatory role on system A transport in gestation remains to be investigated and is the focus of this doctoral thesis.
Figure 1.1: Nutrient transport route across the placenta in A) human (term) and B) mouse (E18.5). A) In humans, nutrients pass from maternal circulation in the intervillous space (IVS) where they are actively transported across the microvillous membrane (MV) into the syncytiotrophoblast (ST). Nutrients then cross the basal membrane (BM), where they can diffuse through the fetal connective tissue and the fetal capillary endothelium (FCE) into the fetal capillary (FCL), which contains fetal red blood cells (FRBC). In some placentae, a BM is present on the FCE which remains separated by villous core material (VC), while in others these two merge to give the appearance of one BM. B) In the mouse, nutrients pass from the maternal blood space (MBS) across the trophoblastic compartment that consists of three layers. Trophoblast layer I (tI) is a mononuclear cell layer and does not have microvilli. Layer II (tII) is loosely attached to tI and together with layer III (tIII) functions similar to the human ST. (*) indicate spaces between tI and tII, where MBS is found between the two layers. [Scale bars = 2 µm. Adapted from 30]
Figure 1.2: Diagrammatic Representation of Regions of the Mouse Placenta.
Figure 1.3: SNAT Transporter proteins structure, location and function. (A) Proposed Amino Acid Structure of SNAT1. The SNAT1 transporter is composed of 11 transmembrane domains (I-XI). The N-terminus is present in the cytoplasm where the C-terminus extends into the extracellular compartment. * indicates potential sites for N-linked glycosylation. [Adapted from49]. (B) Diagrammatic representation of SNAT1, SNAT2 and SNAT4 proteins transporting amino acids (A.A.) into the syncytiotrophoblast using Na⁺ co-transport on both the microvillous (apical) and basal membranes.
Figure 1.4: Signaling and structure of glucocorticoids (GCs). (A) Genomic and non-genomic mechanisms of GC action. GCs may act genomically by passing through the plasma membrane and bind to cytosolic glucocorticoid receptors (GRs) and mediate actions within the nucleus (genomic) or through other signal transduction cascades (non-genomically). GCs may also bind to membrane bound receptors or associate with the plasma membrane to mediate non-genomic actions. Structure of synthetic glucocorticoids B) betamethasone and C) dexamethasone.
Chapter 2: OBJECTIVES & HYPOTHESES
Chapter 2: OBJECTIVES AND HYPOTHESES

2.1 OBJECTIVES

sGCs are administered to women with threatened PTL to enhance fetal lung maturation. Repeat courses of sGCs have been shown to reduce infant birthweight, although this practice is no longer recommended. A possible mechanism for birthweight reduction is through alteration of placental nutrient transport, a critical determinant of fetal growth. Activity of the system A amino acid transporter has been largely implicated in altered fetal growth. Since 70% of women treated with sGCs do not deliver within 7 days and in fact ~30% of pregnant women who receive sGCs carry to term, the objective of the outlined studies is to investigate alterations in placental function, specifically system A transport, that occur with antenatal sGC treatment across gestation. These studies explore the potential role of sGC treatment on altered placental system A transporter function using a human *in vitro* model with DEX treatment (Chapter 3), a mouse *in vivo* model with DEX treatment (Chapter 4) and human *ex vivo* measurements following antenatal therapy in women at risk of PTL (Chapter 5). This doctoral thesis, also examines the association of system A transport with fetal and placental growth outcomes in both the mouse and human with antenatal sGC treatment. Furthermore, the underlying mechanisms of altered transport and potential sex-specific differences in system A transport due to sGC therapy are examined.
2.2 HYPOTHESES

It is hypothesized that sGCs inhibit system A activity, thereby adversely affecting placental function by reducing nutrient transport.

Our specific hypotheses include:

1. System A activity will be inhibited *in vitro* in human term placental explants by sGC treatment (Chapter 3);

2. System A transcripts, protein and activity will increase from mid-late gestation in the murine placenta (Chapter 4);

3. System A transport and expression will be affected *in vivo* with mid-gestation DEX treatment in a sex-dependent manner (Chapter 4).

4. System A activity will be regulated by antenatal sGC therapy given to women at risk of PTL. We hypothesize that initially system A activity will be stimulated by antenatal sGC treatment; however, the activity will be reduced in term placenta of women exposed to maternal sGCs compared to term placenta from women who did not receive antenatal sGC therapy (Chapter 5).
Chapter 3: *In Vitro* Regulation of Synthetic Glucocorticoid Treatment on System A Transport in Human Placental Explants in Culture
Chapter 3: *In Vitro* Regulation of Synthetic Glucocorticoid Treatment on System A Transport in Human Placental Explants in Culture

**Audette MC**, Greenwood SL, Sibley CP, Jones CJ, Challis JR, Matthews SG, and Jones RL. 2010


### 3.1 INTRODUCTION

Approximately 10% of all pregnancies are at risk of PTL, hence, a substantial number of pregnant women receive synthetic glucocorticoid (GC) treatment\(^{142}\). This practice accelerates fetal organ maturation and decreases the risk of RDS and IVH, thus, dramatically reducing neonatal morbidity and mortality\(^{132,133}\). GC effects are maximally beneficial within 7 days of treatment\(^{142}\). However, past obstetrical practice consisted of administering multiple GC courses if the risk of PTL persisted beyond the 7-day period. The NIH consensus no longer recommends repeat administration as trials have demonstrated that increasing the number of GC courses leads to reductions in birthweight with no additional benefit to the fetus\(^{141, 227}\). Low birth weight is a serious concern as it is correlated with an increased risk of disease in adulthood, such as hypertension, ischemic heart disease, and insulin resistance\(^6\). Thus, alterations in fetal development due to GC treatment may be a contributor to long-term fetal programming.

The adverse effects of GCs on fetal growth have been substantiated by animal studies\(^{211, 228}\). Although GCs may have direct effects on the fetus and/or maternal metabolism, there is evidence that the placenta mediates the GC-induced reduction in birthweight, as infusion of BETA directly
into the ovine fetus does not result in growth restriction\textsuperscript{156}. Studies in sheep and rats have demonstrated that administration of synthetic GCs reduces not only placental growth and development, with decreased placental weight\textsuperscript{156} and binucleate cell number\textsuperscript{211}, and increased placental apoptosis\textsuperscript{213}, but also shows impaired endocrine function, with reduced circulating levels of placental lactogen\textsuperscript{211}.

The effects of antenatal GC treatment on human placental function, in particular the transplacental transfer of nutrients from maternal to fetal circulation, are currently unknown. The synthetic GC triamcinolone downregulates expression of glucose transporters GLUT1 and GLUT3 in isolated human trophoblast cells and in the labyrinth of rat placenta\textsuperscript{220}. This suggests that GCs may act to reduce placental nutrient transfer, which could have significant implications for subsequent fetal growth and development.

The sodium-dependent system A transporter, located on both MVM and BM within syncytiotrophoblast transports small neutral amino acids and uniquely, the non-metabolized synthetic analogue N-methylated aminoisobutyric acid (MeAIB), which is used as a specific substrate to assess system A activity\textsuperscript{45}. System A comprises three independent isoforms: SNAT1, SNAT2 and SNAT4, encoded by genes \textit{Slc38a1}, \textit{Slc38a2} and \textit{Slc38a4}, respectively\textsuperscript{49}. Studies have consistently demonstrated the critical importance of system A for normal fetal growth, as its activity is reduced in pregnancies where the infant is small for gestational age or growth restricted\textsuperscript{66, 69}. Using a rat model, Cramer et al. (2002) demonstrated that inhibition of system A directly leads to decreased fetal weight, suggesting that reduced activity may be a primary cause of fetal growth restriction rather than a secondary outcome\textsuperscript{77}. 
Evidence from animal models has demonstrated that sGC treatment can affect placental structure and function. GCs can modulate amino acid transport, although the effects on system A transport differ according to cell type. DEX treatment inhibits AIB uptake in freshly isolated rat hepatocytes, but has opposing effects in rat hepatoma cell lines\textsuperscript{225, 229} and human fibroblasts\textsuperscript{230}. The aim of the current study was to elucidate the regulatory actions of GCs on placental system A activity in human pregnancy, with decreased amino acid transport proposed as a potential mechanism for GC-induced growth restriction. Previous studies investigating GC effects on placental system A have utilized either isolated term cytotrophoblasts or the BeWo cell line, and have demonstrated a stimulatory effect of acute DEX (5 µM) or longer term cortisol (1 µM) exposure\textsuperscript{106, 108}. The placental explant model provides a more physiological method, retaining complex cellular relationships, to assess the effects of chronic (48h) GC exposure on placental system A in intact placental villi\textsuperscript{231}.

3.2 HYPOTHESIS

We hypothesized that the sGC dexamethasone would inhibit system A activity \textit{in vitro}, thereby adversely affecting placental function by reducing nutrient transport.

3.3 METHODS

3.3.1 Placental Collection and Tissue Culture

Experimental design is illustrated in Figure 3.1. General laboratory reagents were purchased from Sigma (Poole, Dorset, UK) unless otherwise stated. Term placentae (n = 7; vaginal delivery) were
collected within 30 min of delivery from women with healthy pregnancies at St. Mary’s Hospital (Manchester, UK). Written informed consent was obtained from all subjects and ethical approval was obtained from the Central Manchester Research Ethics Committee. Biopsies of villous tissue 1 cm³ in size were randomly taken and washed in culture medium [CMRL-1066 (100 ml/L), NaHCO₃ (2.2 mg/ml), streptomycin sulphate (100 mg/ml), penicillin G (100 IU/ml), insulin (1 mg/ml), retinol acetate (1 mg/ml), L-glutamine (100 mg/ml) and 5% fetal bovine serum (pH 7.2, Invitrogen Corporation, Paisley, UK)]. Explants (~ 5 mg) were dissected and cultured in individual Costar Netwells (Corning, Corning, NY) at 37°C (21% O₂, 5% CO₂) as previously described²³¹ (Fig. 3.2A). Culture medium was collected and replaced every 24 h across a 6-day period. Cultured term placental explants undergo a unique process by which the outer syncytial layer is shed and the underlying cytotrophoblasts differentiate and regenerate the syncytial layer²³¹ (Fig. 3.3). This process occurs over 3-4 days in culture. Explants were treated on days 4 and 5 of the culture period with vehicle (ethanol 0.01%), dexamethasone (DEX) 10⁻⁸ M or DEX 10⁻⁶ M. DEX treatments were designed to span the range of concentrations the placenta is exposed to following antenatal administration in vivo. The maximal theoretical concentration of sGC exposure in maternal plasma is 1.5–3 mM, with betamethasone concentrations falling to 250 nM 1 h after treatment¹¹⁸. These concentrations are also consistent with GC concentrations commonly used for in vitro studies²³².

3.3.2 System A Uptake Activity Assay

On day 6, placental explants were incubated for 10, 20 or 30 min in either Na⁺- containing or Na⁺-free Tyrode’s buffer (NaCl replaced by an equimolar concentration of choline chloride) containing 0.5 mCi/ml ¹⁴C-MeAIB (~ 8.5 mM) at 37°C²³³ (Fig. 3.2B). ¹⁴C-MeAIB has a specific affinity for the system A transporter and is not metabolized⁴⁵. It has been used extensively to study system A
transport in the placenta. The fragments were then immersed in dH$_2$O for 16–18 h at room temperature (RT). Liquid scintillation fluid (16 ml) was added to the water lysate and mixed thoroughly. The radioactive content (DPM) of the water lysate was measured using a β-counter. Efficiencies of radioactive $^{14}$C measurement were $\geq 93\%$. Na$^+$-dependent system A activity was calculated by subtracting uptake in Na$^+$-free Tyrode’s from that in Na$^+$-containing Tyrode’s, with correction for fragment protein content, measured using Bio-Rad protein assay according to manufacturers instructions (Bio-Rad Laboratories Ltd, Hertfordshire, UK).

### 3.3.3 System A mRNA Expression

On day 6 of the culture period, RNA was extracted from a further group of cultured explants using the Absolutely RNA Miniprep Kit (Stratagene, La Jolla, USA) according to the manufacturer’s instructions. RNA integrity was verified with gel electrophoresis.

cDNA was prepared from 1 µg of RNA in reaction mix containing Multiscribe Reverse Transcriptase (50 U/mL), deoxynucleotide triphosphate mix, and random (hexameric) primers (Applied Biosystems, Foster City, USA). Samples were incubated at 25°C for 10 min, 37°C for 120 min and 85°C for 5 min (DNA Engine DYAD; MJ Research, Waltham, MA).

Quantitative reverse transcriptase PCR (qRT-PCR) reaction mix contained Platinum Quantitative PCR SuperMix (Invitrogen, Burlington, ON, Canada), SYBR Green I (0.0032% v/v, Molecular Probes, Invitrogen), 10 ml of cDNA (diluted 1:10) and sense and antisense primers (300 pmol/ml) (Table 3.1).
PCR protocol included 40 cycles of denaturation for 30 s at 95°C, 30 s at a specific annealing temperature for each primer set (Table 3.1), and extension for 30 s at 72°C. For each run a non-template-control (containing H2O in place of template RNA) and a non-amplification control (containing H2O in place of template cDNA) was included as a negative controls. qRT-PCR was performed using Rotor Gene SG-3000 (Corbett Research, Montreal, Canada). Samples were measured in duplicate and normalized to the housekeeping gene TATA box binding protein (TBP)234. All PCRs were repeated in triplicate. A single qRT-PCR amplification product of the correct size was obtained by melting curve analysis and agarose gel electrophoresis for each gene product (Table 3.1).

3.3.4 Immunohistochemistry of SNAT Proteins

IHC was performed to localize SNAT isoforms as previously described58. On day 6 of the culture period, a further group of placental explants, as well as, uncultured first trimester and term explants were fixed overnight in 10% formalin overnight (4°C), washed in TBS, and paraffin embedded. 6 µm sections of the explants were used for all IHC staining. Sections were de-waxed and rehydrated using a series of graded ethanol submersions. Antigen retrieval was conducted by heating sections in a microwave (2 x 5 min) in citrate buffer (0.01 M). Sections were incubated with H2O2 (3%) to eliminate any endogenous peroxidase activity before blocking with non-immune block (30 min in 0.1% Tween-20 in TBS containing 10% normal swine serum and 2% normal human serum). Primary antibodies were incubated overnight (4°C). Primary and secondary antibody details are shown in Table 3.2. The sections were washed in TBS Tween-20 (0.6%) before applying secondary antibody. Chromogen diaminobenzidine was used to develop colour staining on the sections before counter staining with Harris’ haematoxylin. Lastly, sections were dehydrated and mounted. Negative controls were performed by substitution of primary antibody
with non-immune rabbit IgG at a matching concentration or by preabsorption of primary antibody with a 10-fold excess of antigenic peptide. Sections were observed using a Leica DMRB microscope and images were captured using a HyperHAD Sony camera at 40x original magnification.

3.3.5 Analysis of Placental Cell Turnover and Syncytial Regeneration

3.3.5.1 Human Chorionic Gonadotropin (hCG), Human Placental Lactogen (hPL) and Lactate Dehydrogenase (LDH) Quantification

hCG and hPL secretion into culture medium was assayed by ELISA (DRG Diagnostics, Germany) according to the manufactures’ instructions. hCG ELISA had a lower detection limit of 5 mIU. The intraassay variability was 6.82%. LDH release into culture medium was quantified using the Cytotoxicity Detection kit (Roche Applied Science, Indianapolis, USA) according to manufacturer’s instructions. A standard curve of L-lactic dehydrogenase from rabbit muscle verified a linear relationship ($R^2=0.9638$).

3.3.5.2 Immunohistochemistry

A similar protocol as described above was used to assess markers of placental cell turnover and differentiation with antibodies specific for Ki67, M30, β-hCG, 11β- HSD2 and syncytin (Table 2). Negative controls were obtained by incubation in non-immunized mouse, rabbit or goat IgG at matching concentrations to the primary antibody.
For quantitative immunohistochemical Ki67 analysis, 10 images were taken randomly of slides from each placental sample (n = 7). The proportion of Ki67\(^+\) stained cytotrophoblast nuclei out of the total nuclei present per image was calculated and averaged per placenta per treatment.

3.3.5.3 Quantitative Real-time PCR

An identical protocol to that described for system A gene expression studies was used to quantify mRNA expression of syncytial markers (β-hCG, hPL, 11β-HSD2 and syncytin). Primer details are shown in Table 3.1.

3.3.5.4 Electron Microscopy

On day 6 of the culture period, a further group of explants were fixed and embedded according to the following protocol\(^{235}\). A Reichert Ultracut microtome was used to cut semi-thin sections (0.5 \(\mu\)m) and photographed using light microscopy. Blocks were sectioned at 70 nm with a diamond knife, mounted on copper grids and stained with uranyl acetate and lead citrate. A Philips CM10 electron microscope was used to observe the explants at an accelerating voltage of 80 kV. High-resolution digital images were captured using a Deben camera.

3.4 STATISTICAL ANALYSES

Statistical analyses were performed using GraphPad software (San Diego, CA). System A activity was analyzed using linear regression to assess differences in the rate of MeAIB uptake between treatments. hCG, hPL, and LDH concentrations were log transformed and analyzed using two-way repeated measure ANOVA with Tukey’s post-test. Day 1 values were excluded for hCG secretion.
due to maternal blood contamination. To account for inter-placental variability, fold-change comparisons were made from day 6 (day of activity measurement) compared to day 4 (initial day of treatment) and analyzed using Friedman test with Dunn’s post-test. qRT-PCR mRNA results were analyzed using Kruskal–Wallis with Dunn’s post-test. Ki67 staining differences in response to DEX treatments were analyzed as fold-change from vehicle controls due to inter-placental variability using Wilcoxon Signed Rank test.

3.5 RESULTS

3.5.1 The Effects of DEX on System A Mediated Uptake

$Na^+$-dependent and $Na^+$-independent uptake of $^{14}$C-MeAIB by placental explants was linear over 30 min and extrapolation of the linear regression intercepted the x-,y-axis close to the origin, indicating measurement of the initial rate of activity of the system A transporter. DEX $10^{-6}$ M treatment significantly increased $Na^+$-dependent $^{14}$C-MeAIB placental uptake compared to vehicle ($p < 0.05$) and DEX $10^{-8}$ M ($p < 0.01$) treatment. No significant difference was detected between vehicle and the lower DEX treatment ($10^{-8}$ M) (Fig. 3.4A). $Na^+$-independent uptake was not significantly different between vehicle and DEX treatments (Fig. 3.4B).

3.5.2 The Effects of DEX on System A Isoform mRNA and Protein Expression

No significant differences in $Slc38a1$, $Slc38a2$ and $Slc38a4$ mRNA expression were detected with DEX treatment when compared to vehicle (Fig. 3.5A–C). System A gene expression was measured relative to the reference gene $TBP$. $TBP$ was not affected by DEX treatment (Fig. 3.6A). A similar gene expression pattern was found with reference gene β-actin (Fig. 3.6B).
demonstrating the validity for use of relative expression. IHC demonstrated that all three SNAT isoforms were specifically localized to the newly regenerating syncytiotrophoblast layer, with no apparent differences in staining intensity or distribution with DEX treatment (Fig. 3.7-3.9; A-C). Negative controls performed by peptide preabsorption or isotype antibody exhibited no staining (Fig. 3.7-3.9; D and F). Uncultured placental tissue was included as a positive control for each isoform and demonstrated staining in syncytiotrophoblast and underlying cytotrophoblast cells (Fig. 3.7-3.9; E) as previously reported. Staining was also present in stromal Hofbauer cells in cultured and uncultured placenta as previously reported.

3.5.3 The Effects of DEX on Placental Cell Turnover and Viability

To determine whether the effect of DEX on system A was mediated through alterations in placental cell turnover or viability, analyses of proliferation, apoptosis and necrosis were performed.

3.5.3.1 Proliferation

Intense nuclear immunostaining for Ki67 identified proliferating cytotrophoblast cells. Representative images are shown for vehicle (Fig. 3.10A), DEX 10\(^{-8}\) M (Fig. 3.10B) and DEX 10\(^{-6}\) M (Fig. 3.10C) treated explants. Negative controls exhibited no staining (Fig. 3.10D). DEX 10\(^{-6}\) M significantly decreased cytotrophoblast proliferation compared to vehicle control (p<0.05), however, no effect was detected with DEX 10\(^{-8}\) M treatment (Fig. 3.10E).
3.5.3.2 Apoptosis

M30 immunostaining detected cleaved cytokeratin-18, a marker of apoptosis. Cytoplasmic immunostaining detected apoptotic cytotrophoblasts and regions of apoptotic syncytiotrophoblast. Representative images are shown for vehicle (Fig. 3.11A) and DEX $10^{-6}$ M (Fig. 3.11B) treated explants. No differences were apparent between treatments. Negative controls exhibited no staining (Fig. 3.11B - inset).

3.5.3.3 Necrosis

LDH release from placental explants, a marker of necrosis, decreased across the 6-day culture period in all treatment groups (Fig. 3.11C). Compared to vehicle levels, DEX $10^{-6}$ M treatment significantly reduced LDH secretion on day 6 (day of activity measurement) as a fold change from day 4 (initial day of treatment) ($p < 0.05$, Fig. 3.11D).

3.5.4 The Effects of DEX on Cytotrophoblast Differentiation and Syncytial Regeneration

To monitor syncytial shedding and regeneration, medium was collected every 24 h across the 6 day culture period and assayed for hCG secretion, a marker of syncytiotrophoblast endocrine function. From day 1 to day 2, hCG secretion decreased due to shedding of syncytiotrophoblast and loss of contaminating maternal blood. The syncytiotrophoblast of both vehicle and DEX treated explants regenerated, with significantly increased hCG levels by day 5 and 6 compared to day 2 ($p < 0.05$, Fig. 3.12A). Analysis of hCG secretion on day 6 (day of activity measurement) as a fold change from day 4 (initial day of treatment) demonstrated a stimulatory effect of both DEX treatments compared to vehicle ($p < 0.05$, Fig. 3.12B). This was unrelated to changes in $\beta$-hCG
mRNA expression (Fig. 3.12C). β-hCG was localized primarily to the old shedding syncytial layer, with staining also present in the newly regenerating layer in both vehicle (Fig. 3.13B) and DEX (Fig. 3.13C and D) treated explants. Negative controls for cultured explants exhibited no staining (Fig. 3.13E). Uncultured term placental explants used as a positive control, exhibited β-hCG staining in the syncytiotrophoblast (Fig. 3.13A). No significant differences in hPL secretion or mRNA expression occurred due to DEX treatment (Fig. 3.14).

To further investigate the effect of DEX on syncytial differentiation, analysis of mRNA expression and protein localization of syncytiotrophoblast-specific factors, 11β-HSD2 and syncytin were measured by qRT-PCR and IHC. DEX treatment (10⁻⁶ M) elevated 11β-HSD2 mRNA expression significantly compared to vehicle (p < 0.05, Fig. 3.15A). In explants 11β-HSD2 protein was primarily localized to the newly regenerating layer, with no apparent differences in staining intensity or localization with DEX treatment (Fig. 3.15C and D). No staining was present in negative controls (Fig. 3.15D). In uncultured term placenta, 11β-HSD2 was exclusively localized to the syncytiotrophoblast layer (Fig. 3.15E). There was a non-significant trend towards increased syncytin mRNA expression with DEX (p = 0.14, Fig. 3.15B). In uncultured placental explants, syncytin protein was localized to the microvillous membrane in first trimester (Fig. 3.15F) and term (Fig. 3.15G) placenta. However, very little immunoreactive staining was present in term placental explants or after DEX (10⁻⁶ M) treatment (Fig. 3.15H). Negative controls exhibited no staining (Fig. 3.15H).

Electron microscopy was used to investigate differences in placental explant morphology due to DEX treatment. In vehicle treated explants, a new layer of mononuclear cytotrophoblast cells was detected underlying the shedding syncytiotrophoblast. These cells were in the early stages of
syncytial regeneration, as illustrated by organelle abundance and initial microvilli formation (Fig. 3.16A). Placental explants treated with DEX $10^6$ M possessed densely distributed organelles, such as endoplasmic reticulum and secretory droplets, and extensive microvilli formation (Fig. 3.16C). Despite areas of fused syncytiotrophoblast present in only a minority of samples on day 6, differences in nuclear maturation were evident. Vehicle treated explants contained light and evenly coloured circular nuclei (Fig. 3.16A), whereas DEX $10^6$ M treatment exhibited features of heightened maturation with highly irregularly shaped and basophilic nuclei with increased heterochromatin formation (Fig. 3.16C). DEX $10^8$ M treated explants exhibited a degree of syncytial differentiation and microvilli formation that was intermediate compared to vehicle and DEX $10^6$ M treated explants (Fig. 3.16B).

3.6 DISCUSSION

Contrary to our hypothesis, the present study demonstrates that DEX ($10^6$ M) alters placental nutrient transfer capacity by stimulating system A activity. This increase in activity is specific to system A Na$^+$-dependent activity, as there was no effect on Na$^+$-independent transport. This increase in activity was independent of changes in mRNA expression and protein localization. GC administration did not adversely affect explant viability as DEX $10^6$ M treatment lowered LDH release and did not affect apoptosis rates. Moreover, DEX $10^6$ M administration enhanced placental explant differentiation, as demonstrated by morphological and ultrastructural evidence, heightened syncytiotrophoblast endocrine function and elevated $11\beta$-HSD2 mRNA expression. This increased differentiation occurred at the expense of cytotrophoblast proliferation.
The stimulatory effect of DEX $10^{-6}$ M on system A activity was in the region of $\sim 30\%$. This increase in MeAIB uptake is consistent with reported differences in system A activity between placentae from normal pregnancies and those complicated by fetal growth restriction (which range from 16 to 45 $\%$)$^{66, 236}$. Lowered system A activity in growth-restricted pregnancies is thought to be a significant contributor to reduced fetal growth; hence an upregulation of the same magnitude by DEX treatment may be of relevance. DEX $10^{-6}$ M treatment caused an average increase of $^{14}$C-MeAIB uptake of 20 pmol/mg/30 min into placental explants. If these data are extrapolated from placental fragments to the intact placenta, this could represent an increase in amino acid transport of up to 576 µmol for a 600 g placenta per day. Increased system A transport with GC administration is consistent with *in vitro* studies using BeWo choriocarcinoma cells where 24 h treatment with 1 µM cortisol stimulated system A activity$^{85}$. Cortisol would be unlikely to induce the same effect in placental explants, due to the effects of the metabolizing enzyme 11$\beta$-HSD2 in syncytiotrophoblast$^{237}$. Synthetic GCs are poorly metabolized by 11$\beta$-HSD2 and thus evade the normal protective mechanisms that reduce GC effects on the placenta and fetus. Studies using 11$\beta$-HSD2$^{-/-}$ mice, where the placenta is exposed to excess endogenous maternal cortisol, demonstrate increased system A activity at E15 of gestation$^{59}$. In human pregnancy, Verhaeghe *et al.* (2007) reported elevated concentrations of amino acids (including system A substrates glycine and alanine) in fetal blood within 48h of maternal GC administration, which may reflect elevated placental system A activity$^{238}$. Overall, both *in vitro* and *in vivo* findings suggest that GCs have a stimulatory effect on placental system A and promote uptake of amino acids from maternal to fetal circulation.

Although DEX treatment stimulated system A activity after 48 h, this increase may be due to an acute upregulation rather than permanent alterations in the rate of placental transport. Increased
system A activity with GC exposure occurred in 11β-HSD2<sup>−/−</sup> mice at E15, however, there were no differences at E18. Therefore, despite the reduced placental weight across gestation, the upregulation in system A activity is only transient and may reflect a placental attempt to increase nutrient transport<sup>59</sup>. A transient effect of antenatal GCs on placental function has also been detected in sheep treated with DEX on day 40 of gestation, with elevated <i>11β-HSD2</i> mRNA expression on day 50, which is not sustained to term (140 days)<sup>239</sup>.

The Cochrane systematic review by Roberts and Dalziel (2006) examined 21 trials of antenatal GC treatment for threatened PTL and reported no difference between mean birthweight within 48 h of treatment<sup>240</sup>. However, when delivery occurred &gt;7 days following treatment, reductions in birthweight occurred. Approximately 70% of women who receive GCs do not deliver within 7 days, and approximately 30% of those women deliver at term<sup>141</sup>. Therefore, fully understanding the physiological role of the system A transporter, in relation to fetal growth, for women treated with synthetic GCs must include investigation of both immediate and longer term effects of GCs on the placenta.

The localization of SNAT isoforms to the newly regenerating syncytiotrophoblast layer is a novel finding and identifies the cellular layer responsible for system A transport. This suggests that, despite the lack of complete syncytial fusion, the newly regenerating layer is functionally active, further validating the placental explant model for the study of nutrient transfer in response to chronic <i>in vitro</i> treatments. Although we have focused on SNATs in the syncytiotrophoblast, future studies are also needed to determine the role of SNAT proteins across pregnancy in other cell types in the placenta.
Although changes to amino acid transporter expression was not reported, maternal antenatal DEX treatment (0.5 mg/kg) given to mice on E15–E17 caused a downregulation of 1212 genes and an upregulation of 1382 genes in the murine placenta at E20\textsuperscript{209}. Although DEX has been shown to be a potent regulator of gene expression in the mouse, increased system A transport by high dose DEX treatment in the current study was independent of effects on mRNA expression of system A transcripts. The lack of correlation between gene expression and activity levels was not unexpected as disparity between protein and gene expression of placental transporters has been previously reported\textsuperscript{58}. There was however, wide intra-placental variability in system A isoform mRNA expression, thus we cannot exclude an effect on mRNA expression with larger group sizes. The SNAT antibodies are not suitable for western blotting techniques, thus we were unable to assess GC effects on translation quantitatively. Moreover, DEX may affect post-translational modifications that regulate translocation of the SNAT proteins to the microvillous membrane. The heightened differentiation in response to DEX is also likely to contribute to the enhanced system A activity, potentially through increasing microvillous membrane maturation and surface area for transport.

The effect of DEX treatment on placental cell turnover and morphology was investigated due to the potent actions of GCs promoting cell differentiation\textsuperscript{118}. In vitro GC treatment decreases proliferation of the choriocarcinoma JEG-3 cells, whilst decreased proliferation in vivo has also demonstrated in various animal models, coincident with reduced placental weight following GC administration\textsuperscript{59, 211, 216}. The increased hCG secretion, an established syncytiotrophoblast marker, by DEX treatment is consistent with previous studies\textsuperscript{241, 242} and suggests that DEX enhances cytotrophoblast differentiation, thus promoting regeneration of the syncytiotrophoblast. The lack of effect of DEX treatment on hPL secretion, a hormone suppressed by GC treatment in pregnant
sheep, is consistent with other human placental functional endocrine studies\textsuperscript{211,243} and may reflect the lower degree of differentiation of the regenerating syncytial layer at the time point studied. Moreover, ultrastructural studies reveal concrete morphological evidence to support our endocrine data and demonstrate accelerated phenotypic differentiation with DEX treatment, with some evidence of dose dependency. This gain in functional maturity with DEX treatment is also supported by increased expression of the protective barrier enzyme 11β-HSD2, which is exclusively expressed by the syncytiotrophoblast in the uncultured placenta. Its presence in the regenerating syncytiotrophoblast layer suggests the newly formed layer is capable of maintaining syncytial barrier functions, despite being predominantly mononucleated. The lack of increase in syncytin expression, a syncytial protein that promotes fusion, is consistent with the differentiation status of the explants on day 6 of culture. We therefore provide endocrine, enzymatic and morphological evidence demonstrating GC effects on promoting placental differentiation.

Nutrient transport to the fetus is not only dependent on uptake into the placenta but also on the delivery of nutrients to the fetus via the feto-placental circulation. Although we did not examine vasculature in our explant model, studies in 11β-HSD2\textsuperscript{-/-} mice demonstrated reduced placental vascularity and expression of angiogenic growth factors\textsuperscript{59}. In vitro, DEX treatment demonstrated an acute vasodilatory effect with intact lobule placental perfusion\textsuperscript{244}, whilst recent findings from our group suggest longer-term exposure results in enhanced constriction of chorionic plate arteries\textsuperscript{245}. Whether DEX treatment affects maternal blood delivery to the placenta, another important determinant of nutrient transport capacity, has not been examined.

Regulated cytотrophoblast proliferation and syncytial fusion is essential for villous expansion and for maintaining syncytial integrity throughout gestation. Interference with these processes by
preterm GC administration may have the potential to disrupt placental growth and/or syncytial function. Although placental explants *in vitro* provide an exaggerated model of syncytial turnover, these effects may be extrapolated to normal cell turnover processes occurring *in vivo*. Most of the repair/regeneration in reported explant models takes place over days 2–4\textsuperscript{231}, while we observe a delay to this process during our treatment period (days 4 and 5). Therefore, it is likely that the cellular kinetics during the experimental period are slower, more closely resembling the syncytiotrophoblast renewal of normal pregnancy. Moreover, DEX effects on cell turnover are particularly relevant in pathological pregnancies characterized by abnormal cell turnover, such as pre-eclampsia or intrauterine growth restriction\textsuperscript{246}, in which GCs are often administered due to iatrogenic preterm delivery.

This study is the first to examine the effects of the synthetic GC DEX on system A uptake in human term placental explants. Our findings suggest a stimulatory effect of DEX on placental system A transporter activity. Moreover, DEX promotes syncytiotrophoblast differentiation and maturation, which may contribute to the enhanced nutrient transfer capacity.

### 3.7 ACKNOWLEDGMENTS

I would like to thank Dr. Carolyn Jones for assistance in conducting EM experimentation and analyses. I would also like to thank Dr. Susan Greenwood for technical training and guidance. We thank the midwives and clinical research fellows at St. Mary’s hospital for their participation in placental collection. We also thank Professors M. Kilby and P. Stewart (University of Birmingham) for the kind gift of the 11\(\beta\)-HSD2 antibody. Infrastructure support in Manchester was provided by Tommy’s Charity and the NIHR Biomedical Research Centre. This work was supported by the Canadian Institutes of Health Research.
<table>
<thead>
<tr>
<th>Target</th>
<th>Primer Sequence (5'-3')</th>
<th>Annealing Temp (°C)</th>
<th>Size (bp)</th>
<th>Published Reference</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Slc38a1</strong></td>
<td>F:GTGTATGCTTTACCACCATTGC R:GCACGTTGTCATAGAATGTCAAGT</td>
<td>64</td>
<td>187</td>
<td>Desforges et al. 2006&lt;sup&gt;55&lt;/sup&gt;</td>
<td>NM_030674</td>
</tr>
<tr>
<td><strong>Slc38a2</strong></td>
<td>F:ACGAAACAATAAACACCACCTTAA R:AGATCAGAATGGGCACAGCATA</td>
<td>58</td>
<td>141</td>
<td>Desforges et al. 2006&lt;sup&gt;55&lt;/sup&gt;</td>
<td>NM_018976</td>
</tr>
<tr>
<td><strong>Slc38a4</strong></td>
<td>F:TTGCCGCCCTTTTGTTAC R:GAGGACAATGGGCACAGTTAGT</td>
<td>58</td>
<td>152</td>
<td>Desforges et al. 2006&lt;sup&gt;55&lt;/sup&gt;</td>
<td>NM_018018</td>
</tr>
<tr>
<td><strong>TBP</strong></td>
<td>F:CAAGAACCACGCACTGATT R:TTTTCTTGCTGCCCAGTCTGGAC</td>
<td>60</td>
<td>89</td>
<td>Meller et al. 2005&lt;sup&gt;247&lt;/sup&gt;</td>
<td>NM_003194</td>
</tr>
<tr>
<td><strong>β-hCG</strong></td>
<td>F:CTACTGCCCCACCATGACCC R:TGGACTCGAAGCGCAGCTGCAC</td>
<td>58</td>
<td>92</td>
<td>Ng et al. 2003&lt;sup&gt;248&lt;/sup&gt;</td>
<td>NM_000737</td>
</tr>
<tr>
<td><strong>hPL</strong></td>
<td>F:CATGACTCCAGACCTCCTTC R:TGCAGAGCATCAGCTTAGTTGG</td>
<td>56</td>
<td>97</td>
<td>Ng et al. 2003&lt;sup&gt;248&lt;/sup&gt;</td>
<td>NM_001317</td>
</tr>
<tr>
<td><strong>11β-HSD2</strong></td>
<td>F:AGTAGTTGCTGATCGCGGA R:CATGCAAGTGCTCGATGT</td>
<td>56</td>
<td>398</td>
<td>Hardy et al. 1999&lt;sup&gt;249&lt;/sup&gt;</td>
<td>NM_000196</td>
</tr>
<tr>
<td><strong>syncytin</strong></td>
<td>F:GAAGGCCCTTCTAAAACATGA R:GATATTGGCTAAAGGAGTGTGTC</td>
<td>60</td>
<td>83</td>
<td>Chen et al. 2006&lt;sup&gt;234&lt;/sup&gt;</td>
<td>NM_014590</td>
</tr>
<tr>
<td><strong>β-actin</strong></td>
<td>F:TCACCCACACTTGCCCCATCTACGA R:CAGCGGAACCCGCTCATTGCCAATGG</td>
<td>64</td>
<td>295</td>
<td>Casciani et al. 2008&lt;sup&gt;250&lt;/sup&gt;</td>
<td>NM_001101</td>
</tr>
<tr>
<td>Primary Antibody</td>
<td>Source: Concentration</td>
<td>Secondary Antibody- Dilution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>------------------------</td>
<td>------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-SNAT1</td>
<td>Rabbit polyclonal: 2 µg/ml <em>(Eurogentec, Southampton, UK)</em></td>
<td>Goat anti-rabbit IgG- 1:200 <em>(Vector, Burlington, ON)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-SNAT2</td>
<td>Rabbit polyclonal: 2.5 µg/ml <em>(Eurogentec)</em></td>
<td>Goat anti-rabbit IgG- 1:200 <em>(Vector)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-SNAT4</td>
<td>Rabbit polyclonal: 2 µg/ml <em>(Eurogentec)</em></td>
<td>Goat anti-rabbit IgG- 1:200 <em>(Vector)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki67</td>
<td>Mouse monoclonal: 0.16 µg/ml <em>(Dako, Ely, UK)</em></td>
<td>Horse anti-mouse IgG- 1:200 <em>(Dako)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M30</td>
<td>Mouse monoclonal: 0.17 µg/ml <em>(Roche, Indianapolis, USA)</em></td>
<td>Goat anti-mouse IgG- 1:200 <em>(Dako)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-hCG</td>
<td>Rabbit polyclonal: 4 µg/ml <em>(Dako)</em></td>
<td>Goat anti-rabbit IgG- 1:200 <em>(Dako)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11β-HSD2</td>
<td>Sheep polyclonal: 9.6 µg/ml <em>(gift M. Kilby/P.Stewart, Birmingham)</em></td>
<td>Goat anti-rabbit IgG- 1:200 <em>(Dako)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>syncytin</td>
<td>Goat polyclonal: 2 µg/ml <em>(Santa Cruz, Heidelberg, Germany)</em></td>
<td>Rabbit anti-goat IgG- 1:200 <em>(Dako)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Treatment:
- Vehicle
- Dexamethasone (DEX)
- $10^{-8}$ M
- $10^{-6}$ M

System A Activity Measured
- Villous fragment uptake method

RNA Extracted:
- qRT-PCR

Explants Fixed:
- IHC
- Electron & light microscopy

Figure 3.1: *In Vitro* Culture Model and Methodology for Cultured Term Placental Explants.
**Figure 3.2: Placental Explant Culture and Uptake Methods.** Placental fragments were (A) cultured for a 6-day period in individual Costar Netwells and on day 6 (B) system A activity was assessed by measuring uptake of radiolabeled tracer $^{14}$C-Methylamino isobutyric acid (meAIB) into placental fragments.
Figure 3.3: Semi-thin Sections of Glutaraldehyde Fixed Cultured Explants Stained with Toludine Blue. Toludine blue staining illustrates successful shedding of the syncytiotrophoblast (SH ST) layer and underlying regeneration (RG) in two representative images [Scale bar = 50 µm].
Figure 3.4: The Effects of DEX on Placental System A Transport. (A) Na\(^+\)-dependent and (B) Na\(^-\)independent \(^{14}\)C-MeAIB uptake in term explants over 30 min. [Mean ± SEM, n = 7; p < 0.05, **p < 0.01].
Figure 3.5: The Effects of DEX on Placental Slc38a Gene Expression. mRNA expression of system A genes (A) Slc38a1, (B) Slc38a2 and (C) Slc38a4 relative to TATA box binding protein (TBP) expression. [Box and whisker plots show the sample minimum, lower quartile (25%), median, upper quartile (75%) and sample maximum; vehicle (n = 6), DEX $10^{-8}$ M (n = 5) and DEX $10^{-6}$ M (n = 7)].
Figure 3.6: Reference Genes (A) TBP and (B) β-actin mRNA Expression in Placental Explants. No significant differences exist for TBP or β-actin mRNA expression (p>0.05) between vehicle (n=6), DEX 10-8 M (n=5), and DEX 10-6 M (n=7). [Box and whisker plots show the sample minimum, lower quartile (25%), median, upper quartile (75%) and sample maximum; vehicle (n = 6), DEX $10^{-8}$ M (n = 5) and DEX $10^{-6}$ M (n = 7)].
Figure 3.7: SNAT1 Localization in Cultured and Uncultured Placenta. SNAT1 is localized to the regenerating syncytial layer for each treatment. Representative images are demonstrated for A) vehicle, B) DEX 10^{-8} M, C) DEX 10^{-6} M, D) negative control, E) uncultured term placenta and F) preabsorption control [SH = Shedding syncytiotrophoblast, RG = Regenerating syncytial layer; Scale bar = 5 µm].
Figure 3.8: SNAT2 Localization in Cultured and Uncultured Placenta. SNAT2 is localized to the regenerating syncytial layer for each treatment. Representative images are demonstrated for A) vehicle, B) DEX $10^{-8}$ M, C) DEX $10^{-6}$ M, D) negative control, E) uncultured term placenta and F) preabsorption control [SH = Shedding syncytiotrophoblast, RG = Regenerating syncytial layer; Scale bar = 5 µm].
Figure 3.9: SNAT4 Localization in Cultured and Uncultured Placenta. SNAT4 is localized to the regenerating syncytial layer for each treatment. Representative images are demonstrated for A) vehicle, B) DEX $10^{-8}$ M, C) DEX $10^{-6}$ M, D) negative control, E) uncultured term placenta and F) preabsorption control [SH = Shedding syncytiotrophoblast, RG = Regenerating syncytial layer; Scale bar = 5 µm].
Figure 3.10: The Effects of DEX on Placental Explant Cell Turnover: Proliferation. Nuclear Ki67 immunostaining for: (A) vehicle, (B) DEX $10^{-8}$ M, (C) DEX $10^{-6}$ M treated explants and (D) representative negative control [scale bar = 50 µm]. (E) Proliferation index (number of Ki67$^+$ cytotrophoblasts/total nuclear count) in DEX treated explants, presented as fold change from vehicle controls (dashed line at 1.0 represents proliferative index in vehicle controls) [Median + IQR; *p < 0.05].
Figure 3.11: The Effects of DEX on Placental Explant Cell Turnover: Apoptosis and Necrosis. Immunohistochemical staining for M30 in (A) vehicle and (B) DEX $10^{-6}$ M treated explants (negative control - inset). (C) Explant LDH release over the 6-day culture period. [Log transformed data as mean ± SEM (Arrows indicate days of treatment)]. (D) LDH secretion measured as fold change on day 6 (day of activity measurement) from day 4 (initial day of treatment). [Box and whisker plots show the sample minimum, lower quartile (25%), median, upper quartile (75%) and sample maximum, n = 7, *p < 0.05, scale bar = 50 µm.]
Figure 3.12: The Effects of DEX on Syncytial Regeneration: hCG Secretion. (A) hCG secretion profile over the 6-day culture period. [Log transformed data as mean ± SEM, *p < 0.05 compared to day 2 (arrows indicate days of treatment)]. (B) hCG secretion measured as fold change on day 6 (day of activity measurement) from day 4 (initial day of treatment). (C) hCG mRNA expression relative to TBP. DEX did not significantly affect hCG mRNA expression. [Box and whisker plots show the sample minimum, lower quartile (25%), median, upper quartile (75%) and sample maximum, *p < 0.05, **p < 0.01; n = 7].
Figure 3.13: hCG Localization in Cultured and Uncultured Placenta. hCG is predominantly in the shedding ST and less abundant in the regenerating syncytial layer. Representative images are demonstrated for A) control uncultured term placenta, cultured term explants treated with (B) vehicle, (C) DEX 10⁻⁸ M, and (D) DEX 10⁻⁶ M and (E) negative control [SH = Shedding syncytiotrophoblast, RG = Regenerating syncytial layer; Scale bar = 50 µm].
Figure 3.14: The Effects of DEX on Syncytial Regeneration: hPL Secretion. (A) hPL secretion profile over the 6-day culture period. [Log transformed data as mean ± SEM, (arrows indicate days of treatment)]. (B) hPL secretion measured as fold change on day 6 (day of activity measurement) from day 4 (initial day of treatment). (C) hPL mRNA expression relative to TBP. DEX did not significantly affect hPL secretion, fold change or mRNA expression. [Box and whisker plots show the sample minimum, lower quartile (25%), median, upper quartile (75%) and sample maximum, n = 7].
Figure 3.15: The Effects of DEX on Syncytial Regeneration: 11β-HSD2 and Syncytin Expression and Localization. (A) 11β-HSD2 and (B) syncytin relative to TATA box binding protein (TBP) expression. [Box and whisker plots; vehicle (n = 6), DEX $10^{-8}$ M (n = 5), and DEX $10^{-6}$ M (n = 7); *p < 0.05]. Immunohistochemical localization of 11β-HSD2 in (C) vehicle and (D) DEX $10^{-6}$ M treated explants (negative control in inset) and (E) uncultured term placenta. Syncytin localization in (F) uncultured first trimester placenta, (G) uncultured term placenta and (H) DEX $10^{-6}$ M treated explants (negative control in inset). [Scale bar = 50 µm].
Figure 3.16: The Effects of DEX on Placental Ultrastructure. Electron microscopy of (A) vehicle, (B) DEX $10^{-8}$ M and (C) DEX $10^{-6}$ M treated cultured placental explants. Right panels show enhanced magnification for each treatment. [MV, microvilli; SH, shedding syncytiotrophoblast; scale bar as indicated].
Chapter 4: *In Vivo* Regulation of Mid-Gestation Synthetic Glucocorticoid Treatment on Murine Placental System A Transport
4.1 INTRODUCTION

Administration of sGC for threatened PTL has become standard obstetrical practice\textsuperscript{132, 134, 251}. Treatment with sGC promotes fetal lung maturation and decreases the incidence of RDS in the neonate\textsuperscript{132}. Initially, it was proposed that sGC therapy was maximally beneficial between 24 hours and 7 days after treatment\textsuperscript{118}. This observation led to the administration of repeat courses of sGC if the risk of preterm delivery persisted after initial treatment. Although sGC treatment has clear neonatal benefit, exogenous GC exposure may also adversely affect the growth of the fetus. Controlled clinical trials have shown that multiple courses of sGC treatment are associated with decreased fetal weight, height, and head circumference\textsuperscript{141}. Birth weight within a healthy range is critical, because deviations from the normal growth trajectory, such as those that occur in fetal growth restriction and/or macrosomia, predict a predisposition to hypertension, glucose intolerance, and the metabolic syndrome in later life\textsuperscript{6}. Furthermore, evidence from animal models indicate that exposure to excess GC in utero can contribute to the programming of disease in adulthood\textsuperscript{168, 251-253}. 

Although the mechanisms of fetal growth restriction remains to be elucidated, current evidence suggests that changes in fetal development are mediated by alterations in placental structure and/or function. In pregnant sheep, maternal, but not fetal, administration of sGC resulted in fetal growth restriction. In sheep and mice, elevations in maternal GC during pregnancy reduced fetal and placental growth, which was associated with altered placental nutrient transfer, gene expression, and endocrine signaling.

One critical determinant of fetal growth is placental amino acid transfer. The placental system A amino acid transporter is composed of three functionally independent protein/gene isoforms sodium-coupled neutral amino acid transporter (SNAT)1/Slc38a1, SNAT2/Slc38a2, and SNAT4/Slc38a4, that transport small zwitterionic neutral unbranched amino acids. System A activity is reduced in conditions of fetal growth restriction and in pregnancies of SGA infants. In vitro experiments have shown that human placental system A activity is increased by Igfs, leptin and GC treatment (endogenous and synthetic) and down-regulated by hypoxemia and nutrient deficiency. In vitro, sGC administration (48-h treatment) resulted in a stimulatory effect on system A activity in term placental villous explants in culture. This increase in system A transfer was also associated with a promotion of syncytial regeneration and differentiation. This may represent an artifact of the culture model used and thus may not reflect the effects of sGC on placental function in vivo. There are no data concerning the acute effects of sGC in vivo or the potential longer-term effects of treatment. However, in 11β-HSD2 knockout mice, placental system A activity is differentially regulated at E15 compared with E18, suggesting a gestational-specific response. In these mice, placental metabolism of maternal corticosterone is substantially reduced, which elevates the level of endogenous GC across gestation. Because approximately 70% of pregnant women who receive sGC for threatened PTL do not deliver within...
7 days and greater than 30% carry to term\textsuperscript{141}, it is important to investigate the longitudinal effects of sGC treatment on placental system A transfer \textit{in vivo}.

Male fetuses grow faster from the first trimester, tend to weigh more, are longer, and have larger placentae compared with female fetuses. Male fetuses also have increased mortality rates and worse outcomes reported in PTL\textsuperscript{37, 255}. Whether a sexual dimorphic function of placental nutrient transporters contributes to the differences in male and female growth across gestation and/or in cases of maternal sGC treatment has yet to be determined. Hence, the objectives of the present study were to 1) delineate changes in system A activity and isoform gene expression during the second half of murine gestation, 2) determine the acute and longer-term effects of exogenous sGC on system A and 3) examine whether these effects depend upon the sex of the fetus, because sex differences are clearly emerging as being of importance in the expression of key placental regulatory activities\textsuperscript{36, 256, 257}.

4.2 HYPOTHESES

\textit{We hypothesize that:}

1. System A expression, protein and activity will increase across mid-late gestation;

2. System A transport and expression will be altered \textit{in vivo} with mid-gestation DEX treatment and that the nature of this outcome will be sex-dependent.
4.3 METHODS

Diagrammatic representation of experimental aims is illustrated in Figure 4.1.

Aim 1: To characterize the ontogenic profile of system A in the murine placenta, pregnant dams were examined at E12.5, E15.5 and E18.5 (term ~E19-E20). E12.5 was examined as the first timepoint because prior to E12.5 the placenta does not rely on active transport of nutrients\(^{25}\). System A transport was also measured at E15.5 to determine differences across mid gestation (E12.5-E15.5) and prior to term at E18.5 to determine differences across late gestation (E15.5-E18.5).

Aim 2: To examine the effects of sGC treatment on system A transport, pregnant dams were injected subcutaneously with either vehicle control (saline) or dexamethasone (DEX; 0.1 mg/kg) at mid-gestation on E13.5 and E14.5. Treatment during mid-gestation allowed for the acute and longer-term measurements of system A transport. Following DEX treatment pregnant dams were examined on E15.5 (acute; 24 h following DEX), E17.5 (longer-term; 72 h following DEX) and E18.5 (prior to term; 96 h following DEX).

4.3.1 Animal Breeding and Treatment

C57BL/6 mice (6–8 weeks; Charles River, Germantown, NY) were bred overnight and the morning in which a vaginal plug was found was designated embryonic day (E)0.5. Litters with fewer than five were excluded to avoid small litter effects. As such, no significant differences in litter size were present in any experiment. All protocols were approved by the Animal Care Committee at the University of Toronto and in accordance with the Canadian Council for Animal Care. Animals were fed \textit{ad libitum} with standard rodent chow and the lights cycled on a 0700 h lights on and 1900 h lights off cycle. A subset of mice was used to examine system A transport
across gestation and received no handling before tissue collection and transplacental transfer assay. To determine any longitudinal effects of exogenous sGC, pregnant dams were injected subcutaneously with either saline (control) or 0.1 mg/kg of DEX (Vétoquinol N.-A., Inc., Lavaltrie, Quebec, Canada) at mid-gestation on E13.5 and E14.5. This dose has been commonly used in rodent models of antenatal sGC therapy. Administration at mid-gestation allows for the examination of acute and longer-term effects of sGC treatment on placental system A.

4.3.2 Tissue Collection and System A-Mediated Transplacental Transfer Assay

To characterize the developmental profile of system A-mediated transplacental transfer in the murine placenta during the second half of gestation, pregnant dams were examined at E12.5, E15.5 and E18.5 (term ~E19–E20; n = 6–8 dams per gestational age). To examine any potential timing effects of sGC treatment on system A-mediated transfer, pregnant dams (n = 6 –11 dams per treatment and gestational age) were examined 24 h (E15.5; acute), 72 h (E17.5; longer term) and 96 h (E18.5; longer term) after treatment.

System A-mediated transfer was determined using established protocols. At each gestational time point, pregnant dams were administered (i.v.) the specific system A substrate $^{14}$C-N-methylated aminoisobutyric acid (MeAIB) (3.5 µCi, NEC671; PerkinElmer, Inc., Boston, MA) in saline (100 µl). A subset of mice (n = 4 – 6 dams per treatment and gestational age) was used to examine placental permeability using the paracellular diffusion marker $^{14}$C-mannitol [1.75 µCi, NEC377050UC; PerkinElmer, Inc.] in saline (100 µl). Animals were anesthetized with isoflurane and euthanized by cardiac puncture, 4 min after injection. Maternal blood was collected, and plasma was separated by centrifugation at 10,000 rpm for 2 min at 4°C. Whole concepti were dissected out from the maternal uterus. Fetal units (consisting of fetus, yolk sac,
amniotic sac, and amniotic fluid) were collected by removal of the placenta from the whole conceptus. Fetal units for system A-mediated activity measurements were arbitrarily removed from various positions throughout the uterine horn to avoid any positional effects on growth\textsuperscript{259}. In any experiment, half the litter (three to five fetal units) was weighed, and tissues were collected for transplacental transfer assay. The remaining fetuses (approximately three to five per litter) were removed from the amniotic sac and collected to measure fetal weight. Placental tissue was collected, weighed, frozen, and stored at -80°C. Fetal tails were collected for DNA extraction needed for sex determination. A subset of dams (n = 4 – 5) was allowed to carry to term (~E19.5). Mice were monitored from E19 to E20. Immediately after delivery, neonates were removed and weighed.

Fetal units were homogenized in PBS. Homogenate (200 µl) was added to SOLVABLE (1 ml; PerkinElmer, Inc.) along with H\textsubscript{2}O\textsubscript{2} (30%; 100 µl) and scintillation fluid (10 ml, Ultima-Gold; PerkinElmer, Inc.). Radioactivity was measured using a Tri-Carb \(\beta\)-Counter (PerkinElmer, Inc.) in both homogenates of fetal units and maternal plasma, with a counting efficiency of more than or equal to 93%. System A-mediated transplacental transfer was calculated as a ratio of radioactivity present in the fetal unit (disintegrations per minute) relative to maternal plasma (disintegrations per minute) standardized per gram of placental protein (as determined by Bradford Assay (Sigma))\textsuperscript{205}.

4.3.3 Sex-Determination

DNA was extracted and amplified using Sigma REDExtract- N-AMP Tissue PCR kit (XNAT; Sigma Chemical Co., St. Louis, MO) according to manufacturers’ instructions. PCR primer sequences for the male sex-determining region Y were forward, TCA TGA GAC TGC CAA CCA
CAG and reverse, CAT GAC CAC CAC CAC CAC CAA. Sex determining region Y gene amplicon was determined by visualization of a 441-bp band on a 1% agarose gel.260

4.3.4 Quantitative Real-Time PCR

Placentae from male and female fetuses (one to two arbitrarily chosen per litter) were homogenized in TRIzol reagent (1 ml; Invitrogen, Burlington, Ontario, Canada) and total RNA extracted as described in the manufacturer’s protocol. Contaminating genomic DNA was removed by treating RNA samples with DNA-free deoxyribonuclease treatment (Ambion, Austin, TX). RNA purity and concentration were assessed using spectrophotometric analysis, and RNA integrity was verified using gel electrophoresis. RNA samples were combined to give a mean male and female sample per litter. RNA was stored at -80°C until further use.

RNA was converted to cDNA using Multiscribe Reverse Transcriptase (50 U/µl), deoxynucleotide triphosphate mix, and random (hexameric) primers (Applied Biosystems, Foster City, CA). Samples were incubated at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min using the C1000 Thermal Cycler (Bio-Rad, Hercules, CA).

Real-time PCR was performed using the C1000 Thermal Cycler and quantified using the CFX96 Real-Time System (Bio-Rad). Samples were prepared using TaqMan Universal PCR Master Mix (Applied Biosystems, Hammonton, NJ), primer-probes sets for the following genes: GAPDH (lot no. 4352932E), Slc38a1 (Mm00506391_m1), Slc38a2 (Mm00628416_m1), Slc38a4 (Mm00459056_m1) and TBP (Mm00446971_m1) (Applied Biosystems) and cDNA template (50 ng) using ratios according to manufactures instructions. Data analysis was undertaken using CFX Manager Software (Bio-Rad). For each primer probe set, a standard curve was generated by serial
dilution of a pooled reference sample with a minimum efficiency more than or equal to 90%.

Samples were run in triplicate. Relative mRNA expression was calculated as gene of interest expression normalized [ΔΔc(t)] to reference gene expression (GAPDH and TBP)\(^{158,261}\). For each plate, a non-template control (containing H\(_2\)O in place of template cDNA) and non-amplification control (containing H\(_2\)O in place of template RNA) was run to verify amplification, RT specificity by lack of genomic contamination.

4.3.5 Microvillous Membrane Extraction

A subset of mice was used to examine SNAT protein expression. Pregnant dams were treated with DEX (0.1mg/kg, n = 6) or saline (n = 6) on E13.5 and E14.5. Dams were euthanized at E18.5 and placental tissue was collected. Protein expression was measured in total placental lysate and extracted MVM (as described\(^{79}\)). All placental tissue was pooled per litter. Pooled placental tissue was washed (3x in Hepes-Tris buffered saline). Tissue was homogenized in Buffer D (250 mM sucrose in 10 mM Hepes-Tris, phosphatase inhibitor cocktail 2 & 3 (Sigma-Aldrich) and protease inhibitor cocktail (Sigma-Aldrich)). Aliquots of placental homogenate were removed for protein and alkaline phosphatase activity analyses to verify MVM enrichment\(^{79}\). The remaining lysate was centrifuged (10,000 g, 15 min, 4ºC). The supernatant was filtered and centrifuged (108,500 g, 30 min, 4ºC). The pellet was retained and resuspended using Buffer D. MgCl\(_2\) (12 mM) was added to the suspension and stirred over ice (20 min). The solution was centrifuged (2,500 g, 10 min, 4ºC) and following the supernatant was retained and subsequently centrifuged (108,500 g, 30 min, 4ºC). This final step produced the MVM extracted as the pellet, which was resuspended in a total volume of 100 µl in Buffer D and was stored at -80ºC until further use. MVM enrichment was assessed by examining alkaline phosphatase activity as a ratio in MVM/homogenate\(^{79}\).
4.3.6 Alkaline Phosphatase Activity Assay

Alkaline phosphatase activity assay was used to verify enrichment of placental MVM compared to total placental homogenates. This assay was done according to manufacturers instructions (Sigma Diagnostics; St. Louis, USA). Samples were incubated with alkaline buffer solution and stock substrate solution (p-nitrophenol phosphate) for 15 min at 37ºC. NaOH (0.05N) was added to each sample to stop the reaction. The phosphatase activity of alkaline phosphatase can be measured in the production of p-nitrophenol from the p-nitrophenol phosphate substrate solution. P-nitrophenol is a bright yellow colour and therefore can be measured using a colourmetric assay. The amount of alkaline phosphatase activity was determined by measuring the absorbance of each sample at 410 nm relative to a standard curve. The activity values obtained were standardized per µg of protein. Assay limits were defined between 1-10 sigma units/ml. The intraassay variability was 1.07%.

4.3.7 Western Blot

To examine protein expression across gestation, total placentas (~1 female and ~1 male, were pooled per litter from collected placental tissue of pregnant dams outlined in sections 2.2.1 and 2.2.2. Pooled litters were used to measure protein levels of SNAT1, SNAT2 and SNAT4 in total placental homogenates (across gestation and with DEX treatment) and in isolated MVM (with DEX treatment) using western blot technique (described in reference 262). Protein was homogenized in 3x (weight/volume) radioimmunoprecipitation assay lysis buffer. Following, homogenate was centrifuged twice (10000 g, 10 min, 4ºC), each time the supernatant was retained. Extracted protein was stored at -80ºC until use. For DEX-treated placentae, placental homogenates and extracted MVM were isolated in Buffer D as described above. Protein was loaded (50 µg: Ontogeny and 10 µg: DEX treatment) and separated using 8% poly acrylamide gels and transferred to nitrocellulose membranes (iBlot transfer apparatus, Invitrogen). Membranes
were incubated overnight at 4°C) in blocking buffer, 5% skim milk powder in 0.01 M PBS with Tween [PBS-Tween; 0.1% Tween 20 (pH 7.5)] (Sigma). Membranes were washed and incubated with primary antibodies (SNAT1, SNAT2, SNAT4, alkaline phosphatase and actin) for 1 h. Primary antibody details are listed in Table 4.1. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody against the corresponding primary antibody (Table 4.1). Membranes were washed and incubated (1 min) in enhanced chemiluminescence substrate (PerkinElmer) and exposed to chemiluminescence film (General Electric Healthcare, Baie d’Urfe, Quebec, Canada). Glycine stripping buffer (0.01 M glycine (pH 2.8)) (Wisent, Inc., Saint-Jean-Baptiste de Rouville, Quebec, Canada) was used to strip membranes of antibodies prior to re-blotting. Densitometric analysis of protein expression was undertaken using MCID Core imaging software (InterFocus Imaging Ltd., Linton, England). Expression of SNAT1, SNAT2 and SNAT4 in placental homogenates was measured as a ratio over actin. Expression of SNAT1, SNAT2 and SNAT4 in extracted MVMs was measured as a ratio over alkaline phosphatase. To examine recruitment to the MVM from the total placental pool ratios of SNAT proteins in MVM over the total placental homogenate were calculated. Antibodies at the correct molecular weight were verified using positive control tissues (placenta, liver and brain) for each of the three isoforms.

4.3.8 Morphometric Analyses

An additional group of pregnant dams (n = 7–9) was treated on E13.5 and E14.5 with either vehicle or DEX as described above. On E18.5, placentae were collected for morphometric analyses. One male and one female placenta closest to the mean litter placental weight were used as a representative sample. Placentae were hemisected along the midline using the umbilical cord insertion as the central reference. Tissue was fixed in paraformaldehyde (4%, 24 h), then
dehydration and embedded with paraffin. Sections were prepared from the full sagittal central plane of the placenta and stained with hematoxylin and eosin. Analysis was undertaken using NDP.view NanoZoomer (Hamamatsu Photonics K.K., Bridgewater Township, NJ), by an operator blinded to treatment. The total area of both the labyrinth and junctional zone was measured. Final comparisons were made as a regional percentage of the total placental area per section (Fig. 4.2).

4.3.9 Corticosterone Measurements

Maternal plasma was collected from pregnant dams treated with either saline or DEX (E13.5 and E14.5) as stated above at E18.5 via cardiac puncture (n = 10 vehicle and n = 8 DEX). A small sample (~15 µl) of fetal blood was collected from each fetus. Once removed from the uterus, the jugular veins and arteries from each fetus were cut and blood was pooled per litter. Corticosterone was measured using a RIA (MP Biomedicals, Solon, OH) according to the manufacturers instructions. The samples were analyzed in a single assay (intraassay variability ~8%), where the lower limit of detection was 25 ng/ml.

4.4 STATISTICAL ANALYSES

Statistical analysis was conducted using SPSS/WIN version 18.0 (SPSS, Inc., Chicago, IL) and GraphPad software. No significant intralitter variation occurred; therefore, sex-matched siblings were averaged per litter.

*Aim 1: Characterizing the ontogenic profile of system A* - Two-way ANOVA was used to examine differences in transport and mRNA due to sex across gestation with Bonferroni post-hoc analyses.
ANOVA was used to examine differences in protein levels across gestation with Tukey’s Multiple Comparison post-hoc.

Aim 2: Examining the effects of sGC treatment on system A transport - Two-way ANOVA was used to examine differences in transport and mRNA due to treatment across gestation with Bonferroni post-hoc analyses. Unpaired t-test was used to compare alkaline phosphatase fold enrichment, SNAT protein levels, corticosterone concentrations and placental zone fraction regional percentages.

Statistical outliers greater than two standard deviations from the mean were excluded from the analyses. Datasets that failed to meet normality or with unequal variances were log transformed before analyses. Significance was determined at P<0.05, and values are presented as mean ± SEM.

4.5 RESULTS

4.5.1 Ontogeny of System A Activity, Protein and mRNA Expression

System A activity increased from E12.5 to E18.5 (P<0.01), with no difference between placentae from male and female fetuses (Fig. 4.3A). Fetal weight increased significantly from E12.5 to E18.5 (P<0.05), with no significant differences between male and female fetuses at any gestational age (Fig. 4.3B). Placental weight increased significantly from E12.5 to E15.5 (P<0.01), with no further significant difference between E15.5 and E18.5. There was a strong trend toward an effect of sex on placental weight irrespective of gestational age, with female fetuses having reduced placental weight compared to males (P=0.0532) (Fig. 4.3C). Maternal weight increased
significantly across gestation (P<0.05) from E12.5 (26.9 ± 0.4 g) to E15.5 (32.4 ± 0.7 g) and E18.5 (37.2 ± 0.9 g).

Consistent with increased activity across mid to late gestation, mRNA expression of system A genes Slc38a1, Slc38a2 and Slc38a4 increased from E12.5 to E18.5 (P<0.05) relative to GAPDH and TBP reference gene expression (Fig. 4.4A–C). No sex-specific difference in mRNA expression occurred for any one of the system A isoforms. No sex-specific or gestational differences in TBP or GAPDH gene expression (Fig. 4.5) were found, thus validating these genes for use as reference genes.

SNAT1 protein levels increased significantly from E12.5 compared to E18.5 (P<0.05; Fig. 4.6A) in the whole placenta. No difference occurred in SNAT2 (Fig. 4.6B) or SNAT4 (Fig. 4.6C) expression across gestation at the level of the whole placenta. Actin expression was unaffected by gestational age (Fig. 4.6). Positive control expression was verified for SNAT antibodies with SNAT1 expressed in the placenta and brain, SNAT2 expressed in the placenta, liver and brain and SNAT4 expressed in the placenta and liver (Fig. 4.7).

4.5.2 The Effects of DEX on System A Activity, Protein and mRNA Expression
DEX treatment did not significantly affect system A-mediated transfer 24 or 72 h after treatment at E15.5 and E17.5 in placentae from either male or female fetuses. However, DEX significantly reduced system A-mediated placental transfer transport just prior to term on E18.5 (Fig. 4.8A and B). To determine the specificity of sGC treatment on system A transfer, we examined the paracellular transfer of $^{14}$C-mannitol per gram of placenta. $^{14}$C-mannitol transfer increased from
E15.5 to E18.5 in placentae from male and female fetuses (P<0.05). However, DEX did not affect passive transfer in placentae from either sex at E15.5 or E18.5 (Fig. 4.8C and D).

Antenatal DEX treatment had no effect on the levels of mRNA for Slc38a1, Slc38a2 or Slc38a4 at E15.5, E17.5 (data not shown) or E18.5 (Fig. 4.9) in placentae of male or female fetuses despite the significant decrease in system A-mediated transfer at E18.5. DEX did not affect reference gene expression of TBP or GAPDH in male or female placentae (Fig. 4.10).

Alkaline phosphatase protein enrichment between placental homogenates and extracted MVM was verified using western blot. Alkaline phosphatase activity demonstrated similar fold enrichment between vehicle and DEX treated placentae (Fig. 4.11A). A representative image is shown in Figure 4.11B. Protein levels of SNAT1, SNAT2 and SNAT4 were demonstrated in total placental homogenates but, were not affected by DEX treatment (Fig. 4.12A, D and G). Similarly, SNAT1, SNAT2 and SNAT4 demonstrated in MVM vesicles were also unaffected by DEX treatment (Fig. 4.12B, E and H). When examining the SNAT protein ratio in MVM/Total placenta as a measure of recruitment to the membrane, we found that no SNAT ratios were significantly different due to DEX treatment (Fig. 4.12C, F and I).

4.5.3 The Effects of DEX on Fetal and Placental Growth

DEX treatment did not affect fetal weight in utero for either male or female fetuses between E15.5 and E18.5, nor did it affect fetal birth weight at term (Fig. 4.13A and B). Antenatal DEX treatment did not significantly affect placental weight at E15.5 or E17.5. On E18.5, DEX did not significantly affect placental weight with a male fetus (Fig. 4.13C), but it reduced placental weight with a female fetus at E18.5 (P<0.05) (Fig. 4.13D). Placental efficiency, measured as the ratio of
fetal weight to placental weight, was not significantly affected by DEX treatment in males (Fig. 4.13E). Female placental efficiency was not affected by DEX at E15.5 or E17.5; however, the ratio of fetal to placental weight was significantly increased before term at E18.5 after DEX treatment (Fig. 4.13F). DEX did not alter the relative proportion of either the labyrinth or junctional zone in placentae giving rise to male or female fetuses on E18.5 (Table 4.2).

4.5.4 The Effects of DEX on Maternal and Fetal Plasma Corticosterone

There was no difference in maternal or fetal plasma corticosterone concentrations between the vehicle and sGC-treated animals by E18.5 (Table 4.3).

4.6 DISCUSSION

System A-mediated transfer and mRNA expression increased between mid- and late-gestation in placentae from murine pregnancies with either male or female fetuses, consistent with increased fetal growth during this period. Placental system A-mediated transfer was differentially affected by the timing of exposure from maternal sGC treatment. Although DEX given at mid-gestation did not have acute effects on system A-mediated transfer, system A mRNA expression, fetal weight, or placental weight, it significantly reduced system A transport before term (E18.5) in placentae giving rise to both male and female fetuses. DEX did not affect passive permeability or regional fractions of the placenta, thus demonstrating the specificity of effect on system A. Changes in system A-mediated transfer due to DEX treatment were not mediated via alterations in system A Slc38a1, Slc38a2 or Slc38a4 gene expression or SNAT1, SNAT2 or SNAT4 protein expression in the total placenta or at the level of the MVM. Despite the effect of DEX on system A, there was no effect on male or female fetal weight across gestation or weight at term. DEX
treatment significantly reduced placental weight in females before term (E18.5) but did not significantly affect placental weight in males. Alterations in system A-mediated transfer cannot be attributed to chronic alterations in endogenous GC levels, because DEX treatment (E13.5 and E14.5) did not result in altered circulating levels of fetal or maternal corticosterone at E18.5.

This is the first study to investigate the effects of antenatal sGC treatment on the placental system A transporter, *in vivo*. Recently, it has been demonstrated that initial rate system A activity is comparable in microvillous membrane vesicles isolated from both human and mouse placenta. We have demonstrated that system A-mediated transfer increases across gestation in the murine placenta and follows a similar trend to the change in system A activity in the human placenta across gestation: system A activity measured in microvillous membrane at term is significantly increased compared with first trimester. Therefore, both murine and human placental system A activities increase across gestation, most likely to meet the growing demands of the fetus.

In the present study, we have used a murine model to examine the regulatory effects of sGC *in vivo*, without any confounding effects of PTL or other pregnancy complications on placental nutrient transport as would occur in human pregnancies. In contrast to previous studies in human term placental explants, in which we have shown that sGC stimulate the system A transporter after 48 h treatment *in vitro*, the present data demonstrate a longer-term inhibition on system A activity levels in the mouse. A possible reason for the contrast may reflect the difference between model systems used. Investigation of human placental explants *in vitro* requires a 7-day culture period, during which the outer syncytiotrophoblast layer is shed and regenerated before exogenous sGC can be administered. Treatment with the sGC DEX not only stimulated system A activity but also heightened syncytialization of explants in a dose-dependent manner during the culture
period\textsuperscript{105}. Thus, the potent differentiating actions of DEX also increased microvillous membrane formation of the newly generated syncytial layer, which may contribute to increased system A activity and transport capacity within explants.

By examining sGC treatment \textit{in vivo} using a murine model, Baisden \textit{et al.} (2007) demonstrated that antenatal DEX treatment (0.5 mg/kg) on E14.5–E16.5 down-regulates 1212 genes and up-regulates 1382 genes in the murine placenta at term\textsuperscript{209}. Therefore, DEX treatment given in an \textit{in vivo} animal model may alter placental gene expression and indirectly contribute to longer-term changes in nutrient transport. However, we were unable to detect direct changes in any of the system A transporter isoforms at the level of gene expression in either the \textit{in vitro} studies (human placental explants)\textsuperscript{105} or the current \textit{in vivo} studies (mouse). Recent studies conducted in the mouse support our findings, which administered 60 h of DEX treatment starting on E12.5 and found no effect of treatment on \textit{Slc38a1}, \textit{Slc38a2} or \textit{Slc38a4} gene expression\textsuperscript{264}. It is not uncommon to report disconnect between placental transporter activity, protein, and mRNA expression for system A and other placental transport systems\textsuperscript{58, 158}. Also, mRNA and protein levels measured at the level of whole placenta may not reflect activity levels found in the membrane as SNAT proteins have also been localized outside of the labyrinth with SNAT1 in endothelial cells of the junctional zone and SNAT2 found in marginal giant cells\textsuperscript{61}. The authors did not comment on SNAT4 expression due to no suitable antibody for use. Although system A is found largely in the transporting region of the labyrinth, measurements at the level of the whole placenta takes into account message and protein in other cell types that are not responsible for transport. Protein measurements made in isolated microvillous membrane best reflect protein levels available for transport. However, since we did not see any changes in SNATs at the level of the MVM, it is possible that reductions in activity may be due to post-translational regulation.
Since sGC treatment did not chronically alter maternal hypothalamic-pituitary-adrenal function, the mechanism of decreased system A activity 96 h after exposure may be mediated by long-term alterations to SNAT proteins at the level of the MVM, thus rendering the transporters functionally inactive. This aspect underlying sGC-induced changes in placental transport warrants further investigation.

We have demonstrated that approximately 40 – 50 % reduction of system A transport did not affect fetal weight outcome. Transport activity rates are reduced by 15 – 40 % in human microvillous membrane vesicles isolated from both small for gestational aged infants as well as infants that are pathologically growth restricted. However, studies in rodent models demonstrate that reductions in system A-mediated transfer \textit{in vivo} precede fetal growth restriction. Thus, it is perhaps not surprising that in our model, where system A-mediated transfer is only decreased just before term, the fetus escapes an effect on growth. Across gestation, system A activity follows a similar trend to fetal weight, because both parameters significantly increase across the second half of gestation. The largest increase in fetal growth occurs from E15.5 to E18.5 (267 % increase in fetal weight) compared with E18.5 to term (18 % increase in fetal weight). Therefore, if reductions in system A-mediated transfer had occurred earlier in gestation, it is quite possible that this would translate to reductions in fetal growth.

Currently, the National Institutes of Health recommends a single course of antenatal corticosteroid treatment as the standard of care for the management of the patient presenting with apparent PTL. This practice has been shown to decrease the rate of RDS immediately after treatment, and clinical trials have demonstrated no effect of a single course of sGC administration on fetal growth. However, a recent smaller study by Davis et al. (2009) was the first to examine
longer-term exposure of sGC. In this study, a single course of sGC treatment caused a reduction in fetal length, weight and head circumference in sGC-exposed infants who carried to term. These reductions were not related to fetal size before antenatal mid-gestation treatment. A mouse model has allowed for the investigation of differences in placental system A nutrient transport across gestation based on duration since sGC exposure. Clearly, additional studies are required to further map the effects of sGC exposure over time after treatment in human pregnancies treated with antenatal sGC therapy.

In the present study, it is possible that a single course sGC treatment may cause a compensatory up-regulation of other nutrient transport systems, which could maintain fetal weight. Dose-dependent increases in glucose transporter (GLUT)1 and GLUT3 protein expression occur before term in rats given DEX (0.1 – 0.2 mg/kg) starting at day 15 of gestation. In 11β-HSD2/−/− knockout mice, system A activity increased at E15 and fetal weight was protected despite reduced placental weight. It was only later in gestation on E18 that a fall in fetal weight occurred coincident with a decrease in placental glucose transport and GLUT3 mRNA expression and a return of system A activity to control levels. Therefore, it is possible that the placenta up-regulates glucose transport in an attempt to maintain fetal weight after exposure to sGC.

When assessing placental function, most studies pool data from placentae of both male and female fetuses. Although this provides a representative measure, analyzing data by this method may mask any opposing sex-dependent effects that may be present. The present study is novel in that it is one of the first to examine sex-specific differences in murine placental system A transport. Placentae from both male and female fetuses exhibited similar rates of transport during normal gestation and after antenatal sGC treatment. Clifton and Murphy suggest that male and female fetuses exhibit
differential mechanisms to cope with adverse intrauterine environments\textsuperscript{257}. In adverse uterine conditions, female fetuses reduce growth rate, which confers a protective mechanism for any secondary adversities\textsuperscript{36, 257}. In contrast, male fetuses grow normally \textit{in utero} despite adversities, and the lack of compensation in growth rates increases their susceptibility to altered environmental factors\textsuperscript{36, 37}. Our data suggest that female placentae may be more susceptible to reduced growth when treated with a single course of sGC. Similar findings have demonstrated female placentae show sex-specific reductions in weight when exposed to DEX treatment\textsuperscript{264}. As such, female placentae exposed to mid-gestation sGC treatment have significantly increased fetal to placental weight ratio, which may predict an increased placental efficiency in the female when exposed to sGC therapy.

Repeat courses of sGC reduce human and rodent placental growth in a dose-dependent manner\textsuperscript{266, 267}. In rodent models, various dosing regimes have targeted placental growth with specific reductions in both the labyrinth and junctional zone. In rats, chronic DEX treatment on d 13–22 (1 µg/ml) administered in drinking water caused reductions in both the labyrinth and junctional zones\textsuperscript{268}. We have demonstrated that although DEX (0.1 mg/kg on E13.5 and E14.5) reduced female placental weight at E18.5, these reductions were proportionally distributed to both the labyrinth (transport region) and junctional zone (region of hormonal secretion). Because DEX did not affect mannitol transfer (marker of flow-limited diffusion)\textsuperscript{254, 258}, we can eliminate any profound alteration on vascularity contributing to reduced $^{14}$C-MeAIB acid transport. Overall, this demonstrates specificity for the DEX-induced reduction of system A-mediated transfer per gram of placental tissue before term at E18.5.
Currently, there is a significant increase in the number of women who are exposed to sGC therapy for risk of preterm labour who carry to term. In 2004, Polyakov et al. (2007) report approximately 11.2 % of all pregnant women received sGC, which was increased from 8.4 % in 1998. This increase in sGC exposure also includes a larger proportion of women who received sGC yet delivered after 34 weeks of gestation. If our data can be extrapolated to the clinical situation, they would suggest that the system A transporter is differentially regulated based on interval of time after sGC exposure. There are no short-term regulatory effects of mid-gestation sGC treatment. However, a substantial reduction in system A-mediated transport may occur if pregnancy continues to term. Whether this reduction in placental system A transport occurs in women who are treated with antenatal therapy requires further investigation.

4.7 ACKNOWLEDGEMENTS

I would like to thank Dr. Sophie Petropoulos for her assistance with the transplacental transfer experiments, Dr. Susan L. Greenwood for her expert advice regarding experimental design, Abhijeet Minhas for his assistance in murine placental morphometry analyses, Dr. Theresa Powell and Dr. Thomas Jansson for providing a protocol for the microvillous membrane extraction and Majid Iqbal and Elena Burdett for their assistance in optimizing and performing western blot experiments.
<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Source: Dilution</th>
<th>Secondary Antibody- Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SNAT1 (H-60): sc-67080</strong></td>
<td>Rabbit Polyclonal: 1:1000 ((Santa Cruz Biotechnology, Inc., Santa Cruz, CA))</td>
<td>Goat anti-Rabbit – 1:1000 ((PerkinElmer, Waltham, MA))</td>
</tr>
<tr>
<td><strong>SNAT2 (G-8): sc-166366</strong></td>
<td>Mouse monoclonal: 1:500 ((Santa Cruz Biotechnology, Inc.)</td>
<td>Goat anti-mouse – 1:2000 ((PerkinElmer))</td>
</tr>
<tr>
<td><strong>SNAT4 (M-60): sc-67085</strong></td>
<td>Rabbit polyclonal: 1:500 ((Santa Cruz Biotechnology, Inc.)</td>
<td>Goat anti-rabbit – 1:1000 ((PerkinElmer))</td>
</tr>
<tr>
<td><strong>Anti-mouse Alkaline Phosphatase/ALPL</strong></td>
<td>Goat polyclonal: 1:500 ((R&amp;D Systems, MN; USA))</td>
<td>Donkey anti-goat – 1:5000 ((PerkinElmer))</td>
</tr>
<tr>
<td><strong>Rabbit Anti-Actin</strong></td>
<td>Rabbit Polyclonal 1:1000 (DEX study), 1:5000 (Ontogeny) ((Sigma, St. Louis, MO))</td>
<td>Goat anti-rabbit- 1:1000 ((PerkinElmer))</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>---------------------</td>
<td>--------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td></td>
<td>Vehicle (n=9)</td>
<td>DEX (n=8)</td>
</tr>
<tr>
<td>Labyrinth zone %</td>
<td>74.61 ± 0.8</td>
<td>74.75 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>76.75 ± 1.0</td>
<td>76.83 ± 1.6</td>
</tr>
<tr>
<td>Junction zone %</td>
<td>25.39 ± 0.8</td>
<td>25.25 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>23.25 ± 1.0</td>
<td>23.17 ± 1.6</td>
</tr>
</tbody>
</table>

Data presented as mean±SEM. n-values denoted per group.
Table 4.3: Maternal and Fetal Plasma Corticosterone Levels on Embryonic Day 18.5

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>DEX</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Plasma</td>
<td>823.5 ± 69.24</td>
<td>736.8 ± 153.7</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td>(n=8)</td>
<td>(n=8)</td>
<td></td>
</tr>
<tr>
<td>Fetal Plasma</td>
<td>121.9 ± 6.46</td>
<td>102.8 ± 7.94</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td>(n=10)</td>
<td>(n=6)</td>
<td></td>
</tr>
</tbody>
</table>

Data presented as mean±SEM. n-values denoted per group.
Figure 4.1: Experimental Overview for Investigations of Synthetic Glucocorticoid Treatment on System A Transport in the Mouse Placenta. **Aim 1:** System A transport was characterized across gestation with no treatment from mid-late gestation on embryonic day (E) 12.5, 15.5 and 18.5. **Aim 2:** investigated the effects of mid-gestation dexamethasone (DEX) treatment on acute (E15.5; 24 h following treatment) and longer-term effects (E17.5 and E18.5; 72 and 96 h following treatment) placental system A. The day of vaginal plug detection was designated as E0.5.
Figure 4.2: Measurement of Murine Placental Regions. Mid-sagittal section of mouse placenta stained with hemotoxylin and eosin (H & E). The labyrinth and junctional zone were measured using NDP.view NanoZoomer.
Figure 4.3: The Ontogenic Profile of Placental System A-Mediated Transfer at E12.5, E15.5 and E18.5 for placentae from male (black) and female (gray) fetuses. (A) Placental system A-mediated transfer. (B) Fetal weight. (C) Placental weight. No significant sex-specific differences occurred. Data presented as mean ± SEM; n values are denoted in bars. Letters that are different represent significant differences between embryonic days (E) (P < 0.05).
Figure 4.4: The Ontogenic Profile of System A mRNA Expression at E12.5, E15.5 and E18.5 for placentae from male (black) and female (gray) fetuses. mRNA expression of (A) Slc38a1, (B) Slc38a2 and (C) Slc38a4 relative to TBP and GAPDH reference genes. No significant sex-specific differences occurred. Data presented as mean ± SEM; n values are denoted in bars. Letters that are different represent significant differences between embryonic days (E) (P < 0.05).
Figure 4.5: The Ontogenic Profile of Reference Gene Expression. mRNA expression for reference genes at E12.5, E15.5 and E18.5 for placentae from male (black) and female (gray) fetuses. mRNA expression of (A) Tata-box binding protein (TBP) and (B) GAPDH. No sex-specific or gestational effects on reference gene expression occurred. Data presented as mean ± SEM; n values are denoted in bars.
Figure 4.6: The Ontogenic Profile of Placental SNAT Protein Expression. SNAT proteins measured at E12.5, E15.5 and E18.5 for male and female placentae combined. Protein levels were determined as (A) SNAT1, (B) SNAT2 and (C) SNAT4 relative to actin. Data presented as mean ± SEM; n values are denoted in bars. Letters that are different represent significant differences between embryonic days (P < 0.05).
Figure 4.7: Positive Control Western Blots for SNAT Antibodies. Representative images are shown for SNAT1, SNAT2 and SNAT4. Staining for SNAT1 is found in the placenta and brain. Staining for SNAT2 is found in the placenta, liver and brain. SNAT 4 is predominately found in the placenta and liver.
Figure 4.8: The Short- and Longer-term Effects of Mid-Gestation DEX (0.1 mg/kg administered on E13.5 and E14.5) Treatment on System A-Mediated and Paracellular Transfer. System A-mediated transfer from placentae of (A) male and (B) female fetuses. Placental paracellular diffusional transfer from placentae of (C) male and (D) female fetuses. Vehicle treatment for both sexes is designated in white, and DEX treatment is indicated in black (males) and gray (females). Data presented as mean ± SEM; n values are denoted in bars. An asterisk denotes significant difference between DEX and vehicle treatment (P < 0.05).
Figure 4.9: The Effects of Mid-Gestation DEX (0.1 mg/kg administered on E13.5 and E14.5) Treatment on System A Gene Expression at E18.5. mRNA expression of (A) Slc38a1, (B) Slc38a2 and (C) Slc38a4 relative to TBP and GAPDH reference genes. Vehicle treatment for both sexes is designated in white, and DEX treatment is indicated in black (males) and gray (females). Data presented as mean ± SEM; n values are denoted in bars.
Figure 4.10: The Effects of Mid-Gestation DEX (0.1 mg/kg administered on E13.5 and E14.5) Treatment on Reference Gene Expression at E18.5. mRNA expression of (A) Tata-box binding protein (TBP) and (B) GAPDH. Vehicle treatment for both sexes is designated in white, and DEX treatment is indicated in black (males) and gray (females). Data presented as mean ± SEM; n values are denoted in bars.
Figure 4.11: The Effects of Mid-Gestation DEX (0.1 mg/kg administered on E13.5 and E14.5) Treatment on Placental Alkaline phosphatase Activity and Protein levels at E18.5. A) Alkaline phosphatase activity fold change (MVM/total placental homogenate) in vehicle and DEX treated placentae. B) Alkaline phosphatase protein level demonstrated in representative sample of placental homogenate and isolated MVM. Data presented as mean ± SEM; n values are denoted in bars.
Figure 4.12: The Effect of Mid-Gestation DEX (0.1 mg/kg administered on E13.5 and E14.5) Treatment on (A-C) SNAT1, (D-F) SNAT2 and (G-I) SNAT4 Protein Levels at E18.5. Expression was determined at the ratio of (A, D, G) SNAT/actin (total homogenate) or (B, E, H) SNAT/alkaline phosphatase (AP) (MVM). Representative images are shown for each of the SNATs in the vehicle and DEX treated placenta. Ratio of SNAT protein expression in (C, F, I) MVM/total placental homogenates. Actin and AP expression did not vary with treatment. Data presented as mean ± SEM; n values are denoted in bars.
Figure 4.13: The Effects of Mid-Gestation DEX (0.1 mg/kg administered on E13.5 and E14.5) Treatment on (A and B) Fetal Weight and (C and D) Placental Weight. (E and F) Placental efficiency is determined as the fetal to placental weight ratio (F:P). Vehicle treatment is designated in white, and DEX treatment is designated in black (males: A, C, and E) and gray (females: B, D, and F). Data presented as mean ± SEM; n values are denoted in bars. An asterisk denotes significant difference between vehicle and DEX treatment (P < 0.05). Letters that are different represent significant differences between embryonic days (P < 0.05).
Chapter 5: Synthetic Glucocorticoid (sGC) Regulation of Human Placental System A Transport in Women Treated with Antenatal sGC Therapy
Chapter 5: Synthetic Glucocorticoid (sGC) Regulation of Placental System A Transport in Women Treated with Antenatal sGC Therapy

Audette MC, Challis JRG, Jones RL, Sibley CP and Matthews SG. Antenatal synthetic glucocorticoid treatment in preterm labour reduces system A mediated transport in term placentae. 2012 In preparation

5.1 INTRODUCTION

sGCs are administered to women who are threatened with PTL. This treatment acts to enhance fetal lung and organ maturation, thus significantly reducing neonatal morbidity and mortality\textsuperscript{132,133}. Two major randomized controlled trials have reported conflicting results on the adverse effects of repeated administration of sGCs, for women who remained at risk of PTL if undelivered, on fetal birthweight. The ACTORDS trial, which enrolled 982 women, found no effect of repeated weekly single doses of sGC treatment on fetal birthweight\textsuperscript{146}. However, the MACs trial, which enrolled 1858 women, found significant reductions in birthweight, length and head circumference in infants whose mothers were administered repeated sGCs courses (2 doses per course) once every 14 days\textsuperscript{141}. Differences between the two trials may be due to the amount and frequency of sGC received. Smaller studies have also noted adverse affects on fetal growth specifically when the timing of delivery following sGC treatment exceeded 14 days. French et al. (1999) recruited 477 women into an observational study and reported an association between repeated courses of sGCs and an increased percentage of babies born in the <10\textsuperscript{th} percentile weight range\textsuperscript{159}. Furthermore, subdividing this cohort based on the timing of exposure to sGCs, irrespective of the
number of sGC treatments received, found that reductions in birthweight (~150 g) did not occur until >14 days following exposure\textsuperscript{159}. The administration of repeat courses of sGCs is no longer recommended, while a single course (consisting of 2 doses of BETA (12 mg each) or 4 doses of DEX (6 mg each) given intramuscularly) is currently standard practice of care\textsuperscript{134}. A systematic review of the Cochrane database examined 21 studies, which used both single and multiple course sGC treatments at various gestational ages to determine the effect on maternal and fetal outcomes. This meta-analysis did not find an overall effect on fetal growth due to a single course of sGC treatment. However, 5 studies reported effects on birthweight that vary depending on the number of days between sGC treatment and delivery\textsuperscript{240}. In a small study of 105 women, Davis \textit{et al.} (2009) reported that body length, birthweight and head circumference were reduced in infants exposed to sGC in mid-gestation for threatened PTL but delivered at term, compared to term controls\textsuperscript{163}. Exposure of the fetus to excess sGCs has been shown to contribute to fetal programming of long-term health and disease\textsuperscript{270}. The neonatal benefits regarding the use of a single course of sGCs strongly outweigh the potential adverse effects on fetal growth. However, these studies do emphasize the importance of examining potential adversities on fetal outcome based on gestational length from exposure to sGCs.

The system A transporter is composed of three independent gene/protein isoforms: \textit{Slc38a1}/SNAT, \textit{Slc38a2}/SNAT2 and \textit{Slc38a4}/SNAT4, which are located on the syncytiotrophoblast (human) and labyrinth (rodents) layers of the placenta\textsuperscript{49, 58}. System A regulation has generated an increase focus in recent years, as various nutrient and endocrine factors have been shown to alter transport, both \textit{in vitro} and \textit{in vivo}\textsuperscript{22}. System A is differentially regulated by GC treatment depending on the organ and cell-type studied\textsuperscript{106, 108, 229}. We have previously provided evidence that sGCs regulate placental system A-mediated transfer. \textit{In vitro},
DEX administration ($10^{-6}$ M; 48h exposure) stimulated system A activity in cultured term placental villous explants (Chapter 3). This increase in system A transfer was associated with a promotion of syncytial regeneration and differentiation$^{105}$. The opposite effect of sGC treatment on system A transport was observed in vivo using a mouse model. DEX treatment (0.1 mg/kg) given at mid-gestation (E13.5 and E14.5) did not affect system A transporter expression or function after 24 hours (E15.5) or 72 hours (E17.5) following treatment. However, system A activity was down-regulated prior to term (E18.5) in placentae from both male and female fetuses after maternal DEX treatment in mid-gestation (Chapter 4)$^{271}$. Whether the differences in these effects are due to differences in time measured following exposure (acute vs. longer-term) or differences between species (mouse and human) remains unknown. Therefore, we have collected placentae from women treated with sGCs during pregnancy for risk of PTL to determine the effect of antenatal sGC therapy on system A transport. Since ~70% of pregnant women who receive sGC for threatened PTL do not deliver within 7 days and in fact greater than 30% carry to term$^{141}$, understanding the longitudinal effects of timing from sGC exposure may be critical for women who are potentially misdiagnosed with PTL or who remain undelivered following sGC treatment.

### 5.2 HYPOTHESES

Based on results from the in vitro explant study (Chapter 3) and the in vivo mouse model (Chapter 4), we hypothesized that system A activity would be initially stimulated by sGC treatment. However, we also hypothesized that system A activity would be reduced in term placenta of women exposed to maternal antenatal sGC therapy compared to term placenta from women who did not receive antenatal sGC therapy.
5.3 METHODS

5.3.1 Patient Recruitment

Women were recruited to the study through the Research Centre for Women’s and Infants’ Health (RCWIH) BioBank (Samuel Lunenfeld Research Institute, Mount Sinai Hospital; Toronto, ON) with informed consent in accordance with the policies of the Mount Sinai Hospital Research Ethics Board and in accordance with the University of Toronto Research Ethic Board. Maternal age, ethnicity, gravida, parity and body mass index (BMI; calculated by weight and height measurements) were collected at the time of obstetrical appointments. The inclusion criteria for the treatment groups included women with a singleton pregnancy, at risk of PTL (as diagnosed by the attending obstetrician) and who had received sGC treatment. We also recruited women who delivered healthy infants at term for our control group. All women in the treatment groups received two doses of celestone (BETA - (12 mg) intramuscularly ~12 hours apart), except for one patient who received one dose of BETA and 12 hours following received 1 dose of DEX. The exclusion criteria for all groups included smoking, alcohol use, recreational/illicit drug use and pathological conditions such as gestational diabetes, diabetes (type-I or type-II), preeclampsia or major fetal anomalies. After treatment, women were followed until delivery when placental tissue was collected. Placental collections were subdivided based on elapsed time from sGC treatment to examine various time intervals of antenatal therapy on placental system A-mediated transport. Human studies have shown that sGCs significantly reduce fetal weight after various times from treatment. Time intervals range from >24 h to >14 d post treatment\textsuperscript{159,240}. In our study, there was no significant difference between system A activity in placentae of women who delivered <24 h (n=4) and those who delivered between 24 h and 14 d (n=5); p=0.41). Therefore we combined all deliveries <14 d. Our subdivisions included: women who delivered less than 14 d following treatment, after 14 d but before term following treatment, at term following treatment and at term
without treatment (control group) (Table 5.1). Preterm labour was defined as delivery between 24 and 36 weeks + 6 days of gestation. Term labour was defined as delivery 37 to 42 weeks of gestation. Modes of delivery included both vaginal and cesarean section deliveries. Fetal birthweight was measured at the time of delivery.

5.3.2 Placental Tissue Collection

Placentae were collected less than 30 min following delivery. Placental weight was measured. Sections from each of the 4 quadrants were sampled and pooled. Tissue samples were washed and stored (1x PBS). Immediately following tissue was used to measure system A-mediated uptake.

5.3.3 System A Uptake Activity Assay

The system A uptake activity assay measured over a 30 min time course is described in section 3.3.2. System A-mediated transport measured over 30 min was standardized per gram of protein.

5.4 STATISTICAL ANALYSES

Statistical analyses were performed using GraphPad software (San Diego, CA). System A activity was analyzed using linear regression to assess differences in the rate of MeAIB uptake between treatment exposure length subgroups and based on fetal sex. Activity measurements are presented as mean ± SEM. Differences in gestational age at treatment, maternal age, maternal BMI, gravida and parity were analyzed using ANOVA - Kruskal-Wallis with Dunn's Multiple Comparison post-hoc test, α= p<0.05. Birthweight, placental weight, gestational age of delivery were analyzed between preterm and term grouping using the Mann-Whitney t-test. Comparisons between male
and female placental weight and birthweight were also made using the Mann-Whitney t-test. Maternal, fetal and placental parameters are presented as median (range). Maternal, fetal and placental parameters were analyzed using linear regressions against system A mediated uptake calculated as area under the curve (AUC). Differences in birthweight and placental weight based on sex are presented as Box and whisker plots [data display the sample minimum, lower quartile (25%), median, upper quartile (75%) and sample maximum]. Significance was set at P<0.05.

5.5 RESULTS

5.5.1 Treatment Subdivisions: Gestational length from sGC exposure to delivery

No significant differences in the gestational ages at which sGC treatments were given were found between treatment subgroups. All women were treated with the sGC celestone between 23 - 34 weeks of gestation (Table 5.1). No significant differences were found between gestational ages at delivery either preterm or at term. Preterm deliveries occurred between 25.1 and 36.9 weeks of gestation, while term deliveries occurred between 37.3 and 40.6 weeks of gestation (Table 5.1).

5.5.2 Maternal Parameters

Women from various ethnicities including Caucasian, Black, Latin/Hispanic, Asian and East Indian were recruited to participate in this study. Maternal age was not significantly different between sGC treatment groups and ranged from 20-39 years of age (Table 5.2). Maternal BMI was not significantly different between sGC treatment subdivisions (Table 5.2). Gravida and Parita status of each woman involved in the study was not significantly different between all subgroups (Table 5.2).
5.5.3 Fetal and Placental Parameters

Overall 12/16 preterm births were male and 4/16 preterm births were females. No significant differences in birthweight were found between infants born preterm either delivering <14 d or >14 d. There was also no difference in birthweight between infants born at term who were exposed to sGC compared to those who were not (Table 5.3). Placental weight was not significantly different between infants born prematurely or between those at term (Table 5.3). Thus, no significant differences were found in fetal to placental weight ratios between preterm and term infants (Table 5.3).

5.5.4 The Effect of antenatal sGC administration on System A Mediated Uptake

Na$^+$-dependent uptake of $^{14}$C-MeAIB by placental explants was linear over 30 min and extrapolation of the linear regression displayed that the curve intercepted the x- and y-axes close to the origin, indicating measurement of the initial rate of activity of the system A transporter. System A-mediated uptake measured in placenta from women who delivered less than 14 d following sGC administration was significantly elevated compared to women who were treated with sGCs and carried to term (Fig. 5.1A; p<0.05). System A activity was also elevated in women who delivered between 14 d and term following treatment compared to placentae that were exposed to sGCs and delivered at term (Fig. 5.1A; p<0.05). No differences in system A activity were found between those who delivered prematurely and control term placentae. However, sGC exposed placentae that were delivered at term had significantly reduced transfer compared to term control deliveries (Fig. 5.1B; p<0.05). Na$^+$-independent uptake was not significantly different between sGC-exposed placentae or between term controls and sGC-exposed placentae that were delivered at term (Fig. 5.1C and D).
System A mediated uptake measured as area under the curve was not significantly correlated with birthweight, placental weight, maternal age, maternal BMI or gestational age at delivery (Fig. 5.2A-E). No significant correlations for each of the aforementioned parameters were found based on sex, thus, analyses are shown for both males and females combined.

Analyses of system A activity, birthweight and placental weight were conducted irrespective of sGC treatment. No sex-specific differences were found between system A mediated activity (Fig. 5.3A), birthweight (Fig. 5.3B) and placental weight (Fig. 5.3C).

5.6 DISCUSSION

Placental measurements of system A-mediated uptake were made in placentae from women treated with antenatal sGCs for risk of PTL. Contrary to our hypothesis that system A activity would be acutely stimulated after exposure to sGCs, no immediate effect was found in comparison to control term deliveries. The results suggest that there were longer-term reductions in system A-mediated uptake; term deliveries that were exposed to sGC treatment had reduced activity compared to placentae from women who delivered <14 d following treatment, between 14 d and term following treatment and term controls. No significant sex-specific effects were found on system A mediated uptake, birthweight or placental weight. System A area under the curve was not significantly correlated to maternal, fetal or placental parameters. Maternal parameters did not differ between subgroupings. Fetal weight and placental weights were not significantly different between preterm and term groups.
These current data more closely agree with our mouse in vivo findings regarding the effects of sGC treatment on system A-mediated transfer than with the results found in vitro using a placental explant model with human term tissue. Mid-gestation treatment of mice (DEX; E14.5 and E15.5) with sGCs did not elicit any effects at 24 h or 72 h following treatment; however, reduced system A mediated transplacental transfer longer-term at E18.5 (term ~E19.5) in both male and female placentae. No specific differences in transport were found in either murine (Chapter 4) or human studies (Chapter 5) due to sex. Studies using human placental explants in culture may not show the same effect of sGC treatment due to the nature of the model system used. Explants in culture undergo a process by which the outer syncytial layer sheds after culture days 1-2 and regenerates by days 3-4 (Fig. 3.3). DEX treatment of these explants increased system A transport. However treatment also significantly promoted syncytialization and differentiation of the underlying trophoblast. Immunohistochemical analyses in these explants demonstrated each of the three SNAT proteins in the newly regenerated syncytial layer. Thus, it is difficult to determine to what degree the sGC-stimulated syncytialization contributed to increased transport in the explants. It is possible that system A activity is stimulated acutely after sGC treatment in vivo preterm (Chapter 5), although we have no preterm controls to test this directly. From second trimester to term system A activity rates would be expected to be increased in normal human placental tissue. Since there are no significant differences between control term deliveries and preterm following sGC treatment, it is possible that preterm levels following treatment have been stimulated compared to untreated preterm levels. Following treatment, activity levels fall and are significantly reduced in sGC treated term placentae. As such, the increase in preterm activity may mirror what is seen in the human placental explant measures following sGC treatment in culture. However, the current results and the outcome of studies in DEX treated mice suggest that long-term reductions in system A-mediated nutrient transfer occur with exposure to sGCs.
Although the systematic review of the Cochrane database does not report an overall significant effect of sGC therapy on birthweight, there are smaller investigations that report contrary results\textsuperscript{240}. Within the review, 5 studies reported effects of sGCs on birthweight that depended on the amount of time between sGC treatment and delivery. Birthweight was reduced at specific times following sGC exposure including: after 24 hours following treatment (2 studies - 242 infants), after 48 hours following treatment (1 study - 373 infants), 1-7 days following sGC treatment (1 study - 529 infants) and greater than 7 days after GC treatment (1 study - 485 infants)\textsuperscript{240}. There were no significant differences found in birthweight in the cohort of infants studied. However, we do not have a preterm control group that is not sGC treated, and thus can only make comparisons between the sGC treated groups (preterm and term) compared to control term deliveries.

Reduced system A-mediated transfer has been demonstrated in growth restricted pregnancies\textsuperscript{66}. As well, late-term reductions in transfer after sGC administration may have longer-term implications on fetal growth despite the absence of gross changes in birthweight. The system A transporter is an accumulative transporter which brings small unbranched zwitterionic neutral amino acids into the cell\textsuperscript{49}. In the placenta, system A mediates a net influx into the syncytiotrophoblast where substrates require other transporter systems to cross the basal membrane before passing into the fetal circulation. These basolateral transporter systems may include amino acid exchangers (which swap one amino acid for another with no net change in amino acid concentration) or facilitative transporters (which mediate the efflux of amino acids from the cell thereby reducing amino acid concentrations)\textsuperscript{20, 43}. Thus altering the amount of system A substrates in the cell may have downstream effects on exchange or facilitative systems. Further research is needed to elucidate the
effects of altered amino acid balance within the placenta and the fetus, and to identify longer-term programming of the affected offspring.

Maternal risk factors have been shown to contribute to the risk of PTL. Therefore, we have reduced the impact of maternal lifestyle on our study measures by excluding women who partake in smoking, recreational/illicit drug use and alcohol consumption. One benefit of collecting placental tissue from Mount Sinai hospital located in Toronto, Canada, is that Toronto is an extremely multicultural city. This study recruited women from a variety of nationalities such as Black, Caucasian, Latin/Hispanic or Asian. Rates of PTL vary by ethnicity\textsuperscript{272}, and the present study sampled from various cultural populations. No evidence is currently available concerning variation of placental system A transport between women from different cultural and ethnic backgrounds. No differences in median maternal age have been found from the cohort of women recruited, which is important as advanced maternal age has been shown to contribute to PTL\textsuperscript{273}. However, the size of the present cohort is small, and the study would need extending to a larger group of subjects. The placentae of pregnant teenagers have reduced system A activity and \textit{Slc38a1} and \textit{Slc38a2} mRNA expression compared to that of adults\textsuperscript{274}. No evidence exists regarding the effects of adult maternal age on placental system A activity. Maternal body composition has also been shown to affect system A-mediated transfer in placentae of term deliveries. In the teenage cohort, system A activity was higher in placentae of teenagers who were growing compared to non-growing teenagers\textsuperscript{274}. As well, teenagers who had a low BMI had reduced placental system A activity compared to teenagers with a BMI in the normal range\textsuperscript{274}. Studies conducted within the Southampton Women’s Survey found that in women with smaller pre-pregnancy arm muscle area or who reported to exercise heavily compared to those who were more sedentary, had lower placental system A activity\textsuperscript{275}. Although we do not have specific
anthropometric measurements from our cohort to assess arm mass or specific maternal phenotypic attributes, BMI was not significantly different between subgroups. Placental system A activity is also reduced in adult parous pregnancies compared to primiparous pregnancies \(^{274}\), however, no significant effect of parity was found within the cohort of women studied.

Cytokines are expressed in the placenta and play a critical role in mediating responses at the maternal and fetal interface throughout normal gestation \(^{276}\). Infection and inflammation during pregnancy causes the release of proinflammatory cytokines. In fact, acute and chronic infection is present in 25 - 40% of cases underlying PTL \(^{277}\). Due to GC-induced immunosuppression, sGC treatment may decrease maternal circulating hormonal or cytokine concentrations that may indirectly affect system A transport. Cytokines have been shown to regulate system A activity \(\text{in vitro}\) and in an \(\text{in vivo}\) rat model. The proinflammatory cytokines interleukin-6 (IL-6) and tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) stimulated system A activity after 24 h treatment in primary cultured trophoblast cells by increasing SNAT1 and SNAT2 protein levels and \(\text{Slc38a2}\) mRNA expression \(^{278}\). Increased transport of system A by IL-6 treatment was mediated through STAT3 signaling to induce the expression of SNAT2 \(^{278}\). Contrary to this finding, the proinflammatory cytokine interleukin-1\(\beta\) (IL-1\(\beta\)), which is increased in systemic and intrauterine infections \(^{279}\), inhibits system A transport in a time- (after 8 - 24 hours) and dose-dependent (0.01 ng/ml - 10 ng/ml) manner in BeWo cells. Reductions in system A transport were associated with reduced \(\text{Slc38a1}\) and \(\text{Slc38a2}\) mRNA expression \(^{109}\). IL-1\(\beta\) (2 mg/kg; \(n=3\)) given on day 19 of gestation in the rat, decreased system A activity (~40%) in placental MVM vesicles obtained 24 h following treatment on day 20 of gestation \(^{109}\). Some of the women recruited in our study presented with various grades of chorioamnionitis as well as elevated white blood cell count at various points in gestation. Inflammation present in the fetal membranes would alter the level of cytokines to which
the placenta is exposed, potentially affecting the system A transporter. The relative contribution of each cytokine is unknown. Since cytokines mediate changes in system A gene expression, future studies are needed to examine changes in system A gene expression underlying altered activity levels. Moreover, since sGCs are administered in cases of PTL, it will be important to establish how GCs interact with cytokines to alter system A and future studies could be undertaken to address this both with *in vitro* systems (primary trophoblast cells, BeWo cell and/or explant models) and *in vivo* (using a murine model or measured in women treated with antenatal sGCs). It remains unknown whether cytokine levels plays a role in the reduced transporter function in placentae at term following treatment (Chapter 5).

The acknowledged limitation of this study is the low number of women recruited, especially the number of women who received sGCs, yet delivered at term. Women were recruited and consented into this study at Mount Sinai Hospital (Toronto, Canada) at a time of higher risk, thus they received sGC administration for threatened PTL. However, if no other complications arose, the patients returned home and often delivered at neighbouring hospitals. Therefore, they are lost to the study and do not return to Mount Sinai Hospital. More women would need to be recruited in order to determine whether the trend found for reduced system A activity seen in term deliveries exposed to sGCs compared to control term deliveries is significant. This study will be ongoing in order to understand longitudinal effects of sGC administration on system A expression and activity in women who are treated antenatally with sGCs.

5.7 ACKNOWLEDGEMENTS

I would like to thank the nurses and staff at Mount Sinai Hospital and the RCWIH Biobank for collection of the placental tissues and the women who donated their placentae to this study.
<table>
<thead>
<tr>
<th>Group</th>
<th>Delivery (mode: C-section/total)</th>
<th>Treatment</th>
<th>Gestational length following treatment before delivery</th>
<th>Gestational Age of Last Treatment [Median (range)] Weeks</th>
<th>Gestational Age at Delivery [Median (range)] Weeks</th>
<th>N-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Preterm (0/9)</td>
<td>sGC</td>
<td>&lt;14d</td>
<td>27.6 (24.1-32.6)</td>
<td>27.7 (25.1-35.9)</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>Preterm (3/7)</td>
<td>sGC</td>
<td>&gt;14d and &lt; Term</td>
<td>32.0 (23.0-33.3)</td>
<td>35.0 (26.7-36.9)</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>Term (0/4)</td>
<td>sGC</td>
<td>Term</td>
<td>28.3 (26.6 – 33.9)</td>
<td>39.2 (37.3-39.9)</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>Term (0/5)</td>
<td>Control No Treatment</td>
<td></td>
<td></td>
<td>40.1 (39.7-40.6)</td>
<td>5</td>
</tr>
</tbody>
</table>
Table 5.2. Maternal Parameters

<table>
<thead>
<tr>
<th></th>
<th>1 sGCs: &lt;14d Preterm (n=9)</th>
<th>2 sGCs: &gt;14d &amp; &lt;Term Preterm (n=7)</th>
<th>3 sGCs: Term (n=4)</th>
<th>4 Control Term (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age</td>
<td>26 (20-35)</td>
<td>32 (22-39)</td>
<td>31.5 (29-39)</td>
<td>33.0 (31-37)</td>
</tr>
<tr>
<td>Maternal BMI</td>
<td>20.3 (17.0-34.2)</td>
<td>23.9 (21-26.4)</td>
<td>22.1 (16.1-23.9)</td>
<td>24.3 (19.8-26.8)</td>
</tr>
<tr>
<td>Gravida</td>
<td>1 (1-4)</td>
<td>4 (1-7)</td>
<td>3 (1-5)</td>
<td>2 (1-5)</td>
</tr>
<tr>
<td>Parity</td>
<td>0 (0-3)</td>
<td>0 (0-2)</td>
<td>1 (0-2)</td>
<td>0 (0-1)</td>
</tr>
</tbody>
</table>

Data is presented as Median (range). sGCs: synthetic glucocorticoids; BMI: Body Mass Index; d: days.
### Table 5.3. Fetal and Placental Parameters

<table>
<thead>
<tr>
<th></th>
<th>1 sGCs: &lt;14d (n=9)</th>
<th>2 sGCs: &gt;14d &amp; &lt;Term (n=7)</th>
<th>3 sGCs: Term (n=4)</th>
<th>4 Control Term (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fetal Sex</strong></td>
<td>6:3</td>
<td>6:1</td>
<td>3:1</td>
<td>2:3</td>
</tr>
<tr>
<td><strong>Birthweight</strong></td>
<td>970 (710-3010)</td>
<td>2400 (930-2890)</td>
<td>3105 (2780-3660)</td>
<td>3630 (2945-3770)</td>
</tr>
<tr>
<td>(g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Placental</strong></td>
<td>225.0 (191-529)</td>
<td>354.0 (219.0-505.0)</td>
<td>431.4 (355-507.8)</td>
<td>415.5 (315.3-491.0)</td>
</tr>
<tr>
<td>Weight (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fetal/Placental Ratio</strong></td>
<td>4.2 (3.4-6.6)</td>
<td>4.9 (4.2-8.2)</td>
<td>8.2 (7.2-9.2)</td>
<td>8.7 (7.7-9.3)</td>
</tr>
</tbody>
</table>

Data is presented as Median (range). sGCs: synthetic glucocorticoids; d: days; g: grams.
Figure 5.1: The effect of antenatal sGC treatment given to women at risk of preterm labour on system A-mediated $^{14}$C-MeAIB transfer over 30 min. Na$^+$-dependent uptake of (A) sGC exposed placentae and (B) sGC exposed placentae that delivered at term compared to control (vaginal term delivery). (C) Na$^+$-independent $^{14}$C-MeAIB uptake of sGC placentae and (D) sGC exposed that delivered at term placenta compared to control (vaginal term delivery). [Data presented as Mean ± SEM; *p<0.05; n=9 sGCs: delivered <14d, n=7 sGCs: delivered >14d and <term, n=4 sGCs: delivered term and n=5 control term].
Figure 5.2: System A Mediated Uptake Linear Regressions. System A Mediated uptake measured as area under the curve (AUC) against A) birthweight, B) placental weight, C) maternal age, D) maternal body mass index and E) gestational age at delivery.
Figure 5.3: Sex-specific comparisons. A) System A Na\textsuperscript{+}-dependent \textsuperscript{14}C-MeAIB uptake over 30 min. [Data presented as Mean ± SEM] B) Birthweight and C) Placental weight. [Data presented as Box and whisker plots show the sample minimum, lower quartile (25%), median, upper quartile (75%) and sample maximum; n=17 males, n=8 females].
Chapter 6: Discussion
Chapter 6: Discussion

6.1 SUMMARY

This doctoral thesis focused on investigating the role of sGC treatment on placental system A transport, specifically with regards to antenatal therapy given to women at risk of PTL. These studies have examined the effects of sGC treatment on altered functional transport, as well as, the potential mechanisms underlying these effects. Prior to these described studies, only limited information existed regarding the effects of sGCs on the system A transporter in the placenta. We hypothesized that reduced system A activity would contribute to GC-induced reductions in birthweight. These studies were designed to not only investigate the immediate effects on placental system A, but also examined the longitudinal effects with various lengths of time from exposure. Three model systems were used to for these investigations. Results are summarized in Figure 6.1.

In Chapter 3, an in vitro model was used to examine the acute effects of sGCs on system A transport in human placental explants. Term explants were cultured over a 6-day period and treated with DEX (48h treatment; 10^{-8} M and 10^{-6} M doses) given on day 4 and 5. System A-mediated uptake was measured 24 h following treatment on day 6. DEX 10^{-6} M stimulated system A-mediated transporter activity compared to control and the lower dose DEX 10^{-8} M treatment. Changes in activity were not mediated by alterations in \textit{Slc38a1}, \textit{Slc38a2} or \textit{Slc38a4} isoform gene expression. Explants in culture undergo a process in which the syncytial layer is shed around day 2 and the underlying trophoblast layer regenerates to form a new syncytium on days 3-4. DEX treatment was also associated with a dose-dependent increase in morphological markers of syncytialization including accelerated apical microvilli formation, nuclear maturation, and
increased cell organelle number. DEX increased functional markers of syncytiotrophoblast differentiation including elevated hCG secretion, increased 11β-HSD2 mRNA expression and reduced cytotrophoblast proliferation. Taken together, DEX treatment administered in vitro to term placental explants in culture both stimulated system A activity and promoted syncytiotrophoblast differentiation and maturation. Two questions, which arose from this study, were 1) whether these stimulatory changes were an acute effect of treatment and 2) whether system A transporter activity was regulated similarly in vivo.

In chapter 4, we used an in vivo mouse model with DEX treatment, which was designed to address these remaining questions. In order to examine the effects of sGC treatment, we described the ontogenic profiles of the system A transporter (mRNA, protein and activity levels) in the murine placenta from mid-gestation to term (E12.5, E15.5 and E18.5 [term ~E19.5]). Once this profile was characterized in the murine placenta, we administered DEX treatment in mid-gestation (E13.5 and E14.5) and examined the acute (24 h following treatment on E15.5) and longer-term effects (72 hours following treatment on E17.5 and 96 hours following treatment on E18.5). We hypothesized that system A activity would be altered based on timing from sGC exposure in vivo, and that effects of sGC treatment would be sex-dependent. We determined that DEX treatment had no acute effect at E15.5 or longer-term effect at E17.5 but significantly decreased system A-mediated transfer before term (E18.5) in placentae of male and female fetuses. No sex-specific effects of treatment were found on system A activity. There was no effect of DEX on Slc38a gene expression, total SNAT protein levels or SNAT protein levels in the MVM at E18.5. Administration of DEX in this regime also had no effect on fetal or birth weight; however, it significantly reduced placental weight in female fetuses but only at E18.5. By E18.5 no difference in maternal or fetal corticosterone was found, thus reductions in activity were not due to prolonged
changes in the HPA axis due to DEX treatment. Overall, DEX treatment given in vivo during mid-gestation led to a substantial decrease in placental system A-mediated transport in late gestation in the mouse. Given the critical role of reduced system A activity in fetal growth, and the regulatory effects of sGCs in vitro and in our animal model, I questioned whether these findings would translate to women who receive sGCs for risk of PTL and especially in those who do not deliver immediately following treatment.

In the study outlined in chapter 5, placentae were collected from women treated with antenatal sGCs during pregnancy for risk of PTL to determine whether the differences in the effects of sGCs on placental system A activity were due to differences in the time measured following exposure (immediate vs. longer-term interval) and/or the differences between species (mouse and human). It was hypothesized that system A activity would be initially stimulated by antenatal sGC treatment in human pregnancies and reduced in term placenta of women exposed to sGC therapy compared to term placenta from women who did not receive antenatal sGCs. Three different ‘treatment to delivery’ intervals were examined, which included placentae from women: 1) who delivered less than 14 days following treatment, 3) between 14 days and term following treatment and 4) term deliveries that received sGC treatment. These subgroupings were compared to term deliveries that were unexposed to antenatal therapy. When examining the days of antenatal sGC treatments and gestational age at deliveries, no significant differences occurred within preterm and term subgroups. The major results from this study are that sGC treated women who are exposed to antenatal sGCs and deliver at term have reduced activity compared to those who deliver less than 14 days, between 14 days and term and control deliveries. System A was not significantly correlated with birthweight, placental weight, maternal BMI or gestational age at delivery. Maternal age, parity or BMI were not significantly different between subgroups, nor were fetal
birth or placental weights. Taken together, these data strongly suggest that system A mediated transfer is altered in vivo by sGC treatment and the nature of this effect is based on timing from sGC exposure. Evidence from both murine in vivo studies and from women treated with antenatal sGC therapy suggests that prenatal sGC treatment may lead to a reduction in availability of neutral amino acids to the fetus if gestation persists longer-term or is carried to term.

6.2 SUPPORTING AND CONFLICTING EVIDENCE

The studies contained in this thesis are the first to examine the effects of sGC treatment on placental system A transport in human cultured term explants, in the mouse placenta and in women treated for threatened PTL. Similar to results from the current in vitro study, Jones et al. (2006) found that 24-hour cortisol treatment of BeWo cells significantly increased system A mediated transfer. This increase in activity was associated with increased Slc38a2 mRNA expression at cortisol concentration of 1 µM and higher\textsuperscript{106}. Although the BeWo cell line is commonly used to investigate placental functions, this model also has limitations including the lack of SNAT4 expression\textsuperscript{58}. Placental explants were chosen in my study as this model retains more physiological cell interactions and all three isoforms comprising system A activity are expressed in these explants. Nonetheless, both models demonstrate a stimulatory effect on system A mediated transport in vitro. Although, the low dose DEX treatment (10\textsuperscript{-8} M) did not significantly affect transport, it led to a trend to reduced activity compared to vehicle treatment (Fig. 3.4). It is possible this could become significant by increasing the number of experiments. As such, a reduction in activity would be consistent with both murine and ex vivo measurements from women who are treated antenatally. The effect of sGCs on placental system A activity may change as a function of dose, as well as, time from treatment.
A recent study conducted by Cuffe et al. (2011) found that 40 or 60 hour DEX treatment (1 µg/kg/h starting on E12.5) had no effect on Slc38a1, Slc38a2 or Slc38a4 mRNA expression in male or female mouse placentae. Similarly, DEX treatment did not affect murine or human placental Slc38a isoform transcript levels in the outlined studies. Cuffe et al. (2011) only examined gene expression of the system A isoforms after DEX treatment, and it was not known whether these treatments would have an effect on activity. Similar to our results, DEX treatment reduced placental weight from female fetuses only. Reductions in female placental weight were also associated with decreased MAPK1 protein expression, thus suggested altered MAPK pathway signaling. Corticosterone administered in mid-late gestation in mice, demonstrated an effect of treatment on system A mediated transfer based on timing from exposure. Slc38a1 and Slc38a2 gene expression were immediately increased following treatment (corticosterone given E11-E15) on E16. However, increased system A mediated transfer was not apparent until prior to term on E19. Differences in regulation of system A transfer between corticosterone and DEX may be due to their different receptor affinities, as DEX binds predominately to GR whereas corticosterone binds GR and the mineralocorticoid receptor. Differences may also be due to the ability to access and affect the placenta, as corticosterone is rapidly metabolized by 11β-HSD2.
6.3 RELATIONSHIP BETWEEN GENE, PROTEIN AND ACTIVITY OF THE SYSTEM A TRANSPORTER

Transporters can be regulated at the level of transcription, mRNA stability, translation, translocation of membrane bound vesicles into the membrane and/or post-translation modifications affecting transporter activity or turnover. Thus, alterations in transporter function cannot always be attributed to direct changes in mRNA or protein expression. A disconnect between global protein and mRNA is commonly reported with the system A transporter. Furthermore, the contribution of each isoform to total system A activity is currently not known. Functional analyses elucidated that SNAT4 may play a larger role in early gestation whereas, SNAT1 and SNAT2 may be more important in the latter half of gestation. siRNA knockdown of SNAT1 conducted in vitro using term cytotrophoblast cells demonstrated a median 75% knockdown in system A mediated transfer. The study by Desforges et al. (2010) was the first to demonstrate the relative contribution of SNAT1 to overall system A activity. The contribution of SNAT2 and SNAT4 to overall system A activity, and whether this contribution is consistent throughout pregnancy requires further investigation. Out of the three isoforms, SNAT2 has the highest affinity for MeAIB. Since this is the marker used to measure system A-mediated transport, it is likely that SNAT2 activity is the predominant protein being functionally assessed by this assay.

In the murine placenta, measuring system A mRNA levels and SNAT proteins from total placental homogenates, may not correlate to activity measured in the labyrinth, as other cell types within the rodent placenta express system A isoforms. Immunohistochemical staining of the rat placenta revealed that SNAT1 and SNAT2 proteins were localized not only in the labyrinth, but SNAT1 was also found in endothelial cells of the junctional zone and SNAT2 was found in marginal giant
cells. Low levels of SNAT2 staining were also found in endothelial cells. Thus, mRNA or protein measured in total placental homogenates may not reflect activity at the level of the trophoblast membrane. Protein measured from isolated MVM vesicles is more indicative of protein that is available for functional activity. Many studies examining protein levels in the placenta do not measure protein at the level of the MVM, which may be a reason they report no correlation between mRNA, protein and activity. In our murine study, we did not find an effect of DEX treatment at the level of the whole placenta or at the level of the MVM. Thus, we can conclude effects are not mediated at a translational level in the whole placenta or by SNAT protein found in the membrane.

6.4 PLACENTAL UPTAKE VS. TRANSPLACENTAL SYSTEM A ACTIVITY

Differences in regulatory effects identified between the human (Chapter 3 and Chapter 5) and murine studies (Chapter 4) may be attributed in part to the methods used to measure system A-mediated transfer. Although both studies utilized $^{14}$C-MeAIB as the non-metabolizable specific system A substrate, the placental uptake activity assay was used to measure human placental system A-mediated transfer, whereas the in vivo murine study measured system A-mediated transplacental transfer. System A uses the Na$^+$ concentration gradient to drive the uptake of neutral amino acids into the cell. System A is present on the MVM and BM thus, directing substrates intracellularly. Placental uptake studies therefore measure how much substrate accumulates inside the cell. However, examining transplacental transfer is less well understood. It is known that $^{14}$C-MeAIB is detectable in the fetus after maternal administration; however, the mechanism of transfer across the BM needs to be further established. It was first postulated that transfer of system A substrates cross the BM could occur by system L. System L transports a wide range of
substrates in a Na\textsuperscript{+}-independent manner\textsuperscript{44, 284}. Non-transporter mediated transport may also account for system A substrate transfer across the BM\textsuperscript{57, 285, 286}. Three facilitated transporters TAT1, LAT3 and LAT4 have been recently identified on the BM in human placental tissue, which mediate the efflux of substrates from the placenta into the fetal circulation\textsuperscript{43} (Fig. 6.2). Substrates transported by facilitated transporters into the fetal circulation could also be exchanged by placental exchangers on the BM to mediate transport of a range of amino acids. Amino acid exchangers LAT1, LAT2, y+LAT1, y+LAT2 and ASC mRNA and BM activity of LAT, y+LAT and ASC have been identified in human placental tissue. Exchanger ASC1 and ASC2 mRNA have been identified in placental cell lines\textsuperscript{287, 288}. However, the precise distribution of each isoform has not been identified. Further, it is not known how MeAIB is specifically transferred from the placenta to the fetal circulation. Nonetheless, measuring system A-mediated transplacental transfer reveals relevant information regarding total fetal exposure of system A substrates, not just transfer into placental tissue. Direct comparisons cannot be made between system A-mediated placental uptake and transplacental transfer. As an alternative, future studies may include examining system A activity from isolated mouse MVM vesicles to measure exclusively placental uptake rather than total transfer to the fetus.

6.5 SPECIES COMPARISONS

The mouse model used in chapter 4 offers an advantage of examining effects of sGCs across gestation at controlled time points. Mice administered sGC at mid-gestation have no pre-existing conditions that may confound the effects on system A transport; thus, we can examine the effects of sGCs alone. Measurement of system A transplacental transfer using \textsuperscript{14}C-MeAIB over a 4 min time period is a well established methodology\textsuperscript{59, 110, 111, 113, 261} and thus, allows us to measure the
initial rate of activity that is transferred across the placenta and into the fetus. Kusinski et al. (2010) have also demonstrated that initial rate activities of system A in MVM vesicles isolated from human and mouse placentae were similar. The similarity was specific for system A as system beta was significantly lower in the murine placenta compared to the human\textsuperscript{263}. Thus, using a murine model for system A investigations may be a helpful tool to help elucidate underlying changes in human placental function.

Differences are likely to exist in the local GC concentrations at the level of the placenta between humans and mice. Maternal cortisol levels increase as pregnant women near the end of gestation\textsuperscript{124}. This is accompanied by increases in 11\(\beta\)-HSD2 expression\textsuperscript{186} and activity\textsuperscript{188} in the placenta at term compared to first or second trimester levels. However, mice express a different pattern of 11\(\beta\)-HSD2 mRNA in the trophoblast cells of the labyrinth. Expression is high at E12.5, decreases until E16.5 and is undetectable prior to term at E18.5\textsuperscript{180}. Therefore, during late gestation, the local placental GC inactivation is much lower in the mouse compared to the human. However, maternal corticosterone levels in the mouse do not increase as does maternal cortisol in late human gestation. Maternal corticosterone levels increase dramatically from E10.5 and peak at E16.5. Corticosterone levels decrease from E16.5 towards term\textsuperscript{289}. 11\(\beta\)-HSD2 expression and maternal corticosterone are inversely related in the mouse throughout mid-late gestation from E12.5 and E16.5. In the rat, DEX and BETA treatment given from day 16 - 20 reduced 11\(\beta\)-HSD2 activity on day 21\textsuperscript{194}. However, DEX treatment stimulated 11\(\beta\)-HSD2 expression and activity in human term placental trophoblast cells\textsuperscript{196}. Differences in profile expression and sGC regulation of 11\(\beta\)-HSD2 may contribute to the different placental concentrations of GCs between the mouse and human. As a result, these differences may alter system A in a species-specific manner.
6.6 POTENTIAL MECHANISMS

Each model system used throughout these studies has its own underlying mechanism of GC-induced alterations on system A activity. Our in vitro study demonstrated that DEX stimulated system A mediated uptake after a 48-hour treatment. Increased system A activity with cortisol treatment in BeWo cells was mediated by increased transcription of the Slc38a2 gene and translation of the SNAT2 protein. Increased system A activity in our in vitro model was demonstrated without underlying changes to mRNA expression. We could not quantify protein levels in our explants due to the lack of suitable SNAT antibodies available at the time of the conducted studies. Antibodies used in the murine studies from Santa Cruz have only recently become available. Therefore, sGCs may alter activity levels by increasing SNAT protein isoform translation, stability or potentially recruitment of proteins stored intracellularly to the MVM.

When examining the morphology of our cultured explants, DEX increased syncytialization in a dose-dependent manner (Fig. 3.16). GR is expressed in cytotrophoblast cells at a higher level compared to syncytiotrophoblast, thus DEX activation of GR is able to signal the maturation of underlying cells to syncytialize. GCs also cause maturational shifts in other tissues, including the fetal lung, liver, muscle, kidney and gut. In the placenta, cytotrophoblast differentiation occurs under the command of many signals. First, the underlying cytotrophoblasts, which are proliferative, must undergo an arrest of mitosis before they fuse into the overlying syncytium. In order for the cell cycle to switch from a proliferative state to a differentiated state, many biochemical changes must take place, including changes in growth factors, enzymes, ion channels, hormone receptors, structural proteins, binding proteins and transporters. For example, caspase activation, syncytin1 (fusion protein), transcription factor glial cell missing-1 (GCM-1; an upstream regulator of syncytin1), connexin43 (gap junction protein) are a few regulators that have
been identified as critical components required for trophoblast fusion and syncytial differentiation\textsuperscript{294-296}. Another example includes hCG, a hormone secreted by the syncytiotrophoblast, which has also shown to be involved in autoregulation of differentiation\textsuperscript{297,298}. GCs have been shown to alter several intracellular signaling cascades involved in differentiation, although the widespread effects are not fully known. For example, cortisol has been shown to decrease liver Igf2 transcription in fetal sheep\textsuperscript{299}. DEX given from day 13 - 20 in pregnant rats also decreased placental Igf2 and components of the downstream Akt pathway\textsuperscript{214}. DEX given to pregnant mice (0.5 mg/kg; day 15 - 17) also decreased placental expression of genes involved in cell division (cyclin genes A2, B1, D2, cdk 2 and M-phase protein kinase) and genes involved in growth (EGF-R, BMP4 and IGFBP3)\textsuperscript{209}. In human placenta, \textit{in vitro} studies have demonstrated that DEX increased syncytial formation and increased gap junction coupling of the trophoblast\textsuperscript{300}. In our study, we have not identified the molecular pathway involved in DEX promotion of syncytialization. However, we measured several molecular, endocrine and morphological markers that were altered with DEX treatment as a functional readout of syncytialization. Further studies are needed to elucidate the signaling mechanisms involved in this maturational shift. Overall, the differentiation identified with increasing doses of DEX \textit{in vitro} may contribute to the enhanced nutrient transfer. As such, we cannot isolate whether the increase in activity is mediated by increased transporter function alone or depends in part on increased microvillous formation.

GR is ubiquitously expressed in the mouse trophoblast in the labyrinth throughout the second half of gestation\textsuperscript{180}. The mouse is not as susceptible to the effects of differentiation and maturation as the human placenta, which may explain differences seen in the effect on system A transfer after DEX treatment. In the human placenta, the underlying cytotrophoblast stem cell layer proliferates
continuously. The newly generated cells undergo differentiation and fuse with the overlying syncytiotrophoblast multinucleated cell layer. Within the syncytiotrophoblast differentiation continues. Progressively, older nuclei become apoptotic and arrange together in a syncytial knot, which is shed into the maternal circulation. This process is tightly regulated as not all villous cytotrophoblasts fuse, rather they may remain as an underlying stem cell reserve of cytotrophoblasts on the basal lamina in an undifferentiated state until term. As such, there is continual regeneration of the syncytiotrophoblast layer by underlying cytotrophoblasts and continual release of apoptotic material into the maternal circulation. The rate of syncytial turnover takes approximately 3-4 weeks with almost 3 grams of syncytial knots being shed per day. In the explant model we have used, the potent actions of GCs enhance syncytial formation and maturation with extensive microvilli formation, organelle maturation and nuclear condensation in a dose-dependent manner. However, the mouse placenta, although susceptible to the effects of sGCs, may not undergo the same differentiation in response to sGCs as the human placenta. Murine gestation is significantly shorter than that of humans and does not have similar syncytial turnover to that seen in the human placenta. The mouse labyrinth initiates development around E8.5. It is at this time point that the fetal vasculature grows from the allantois, which attaches to the chorion (chorioallantoic fusion). Chorionic trophoblast cells differentiate in the labyrinth trophoblast layers. Layers II and III are multinuclear and act together, directly opposite the fetal endothelium. Layer I is mononuclear and remains outside the syncytiotrophoblast layer. Although layer I is most closely analogous to mononuclear cytotrophoblast it does not give rise to a proliferative pool that generates layers II and III. The outer cytotrophoblast layer thins as gestation progresses and becomes highly perforated by E12.5. Thus, maternal blood travels through the labyrinth-like sinuses of layer I to reach layer II. Once layer II and III are functionally established, the outer layer II becomes increasingly microvillous which increases the amount of
surface area for transport\textsuperscript{27}. However, the same cell populations remain relatively intact from placental establishment (E12.5) until term (~E19.5).

In chapter 4, we examined three potential sites of sGC modulation in the mouse placenta: transcription, translation and recruitment of the transporter to the MVM. Surprisingly, the results indicate that reductions in system A were not mediated by alterations at any of these levels. The recent study by Cuffe \textit{et al.} (2011) also found no effect of short term DEX treatment on system A transporter \textit{Slc38a} genes\textsuperscript{264}. This is the only other known study to examine the effects of GCs on SNAT mRNA levels in the mouse placenta. In our study, regulation has not occurred at the level of transcription, translation or at protein levels at the MVM, and it is possible that post-translational modifications may reduce transporter function. Although we have not examined the glycosylation state of the membrane transporters, it is possible that glycosylation of SNAT proteins could contribute to longer-term reductions seen in both male and female murine placentae. Glycosylation sites are predicted in extracellular hydrophilic loops, especially between SNAT1 transmembrane domain V and VI\textsuperscript{49} (Fig. 1.3) and SNAT4 which also contains a 6 potential N-linked glycosylation sites\textsuperscript{53}. Less is known about the structure of SNAT2. System A activity in rat hepatoma cells is also associated with a glycoprotein\textsuperscript{307}. Inhibition with tunicamycin has shown that the glycoprotein is exposed on the extracellular surface\textsuperscript{307}. Therefore, it is possible that sGC treatment could affect glycosylation or the associated extracellular glycoprotein, causing reductions in functional protein activity.

Although, we have not measured additional potential indirect effects on the HPA axis, it is unlikely that changes to this axis underlie reductions in system A activity in the mouse since maternal and fetal corticosterone levels are not significantly different due to DEX treatment at
E18.5. Among women treated with antenatal sGCs, reductions in system A uptake were found in term placentae compared to control term deliveries. Reductions in activity in these placentae may be mediated by long-term inhibition of Slc38a mRNA expression, mRNA stability, SNAT protein translation, protein stability, recruitment to the MVM or post-translational modifications. It is possible that DEX treatment in mice and BETA treatment in human placenta has down-regulated GR expression and that this loss of signaling is involved in longer-term alterations in transporter function. DEX treatment (100 nM for 24 h) in human term placental tissue demonstrated a decrease in GRα and GRδ mRNA expression. It is possible that GCs stimulate system A; however, a reduction in GR expression due to sGC treatment could account for a loss of stimulation that occurs over time. In both the mouse and in women treated with antenatal sGCs, it is possible that stable reductions in GR expression are mediated by epigenetic signaling. Epigenetic regulation of gene expression is described as mitotically or meiotically derived alterations in gene expression that are not due to changes in DNA sequence. Altered mechanisms of gene expression can be acquired in utero, postnatally and throughout the course of life. These alterations in gene expression may be mediated by DNA methylation, histone modification, chromatin remodeling and/or micro RNAs, all of which act to promote or inhibit transcription or translation. GR has been shown to be epigenetically regulated in the hypothalamus by maternal care in rats. The level of pup licking and grooming altered levels of DNA methylation in the hippocampal GR promoter and this methylation pattern remained stable into adulthood. In the placenta, differential methylation of the GR gene exon 1F was shown to be associated with differences in fetal growth; therefore, evidence exists linking changes in GR expression to a potential determinant of placental function. Epigenetic mechanisms have not been investigated in the outlined studies. However, stable epigenetic marks to cytotrophoblast GR expression or to other factors (such as enzymes involved in glycosylation, phosphorylation) that indirectly
modulate system A may account for reduced activity that manifests days (in the mouse) or weeks (in human) after treatment. Further studies are required to determine the underlying mechanism(s) of reduced transport in term placentae from women who are exposed to antenatal therapy.

6.7 LIMITATIONS

As with many model systems, there are limitations to methodologies and this is especially true with studies using placental tissue for developmental investigations. Chapter 3 employs an in vitro model in which placental tissue was cultured over 6 days. This model using placental explants retains better physiological context with multiple cell types compared to isolated trophoblast cells or the BeWo cell line alone. Thus, we were able to test in vitro treatments over several days. However, to test placental function, explants must be cultured for a minimum of 4 days before treatment. During this time the outer syncytiotrophoblast layer is shed and the underlying cytotrophoblast layer differentiates into a ‘syncytial-like’ layer. Although the underlying layer is not a true syncytiotrophoblast layer, it functions in a manner comparable to that of true syncytiotrophoblast as demonstrated with similar levels of hCG secretion (Fig. 3.12A). In addition, SNAT proteins are localized to the underlying regenerative layer and functional activity can be measured (Fig. 3.7 - 3.9).

Chapter 4 examined changes in placental function longitudinally in an animal model, after sGC treatment, due to the importance of antenatal sGC therapy given to women at risk of PTL. The benefits of using an animal model, such as the C57BL/6 mouse, are that external factors can be controlled more effectively than in human populations. External factors that can be matched in mouse models include maternal age, parity and environmental housing. The effects on system A
can be investigated without any confounding factors of PTL. However, there are limitations of using a mouse model. Gestation in a mouse is relatively short (~19.5 days), and the definitive placenta is only established at E12.5 (~64% of total gestational length)\textsuperscript{25}. We cannot examine sGC treatment at an earlier time point to determine whether changes in transport precede reductions in fetal weight, because the administration of sGC earlier than E13.5 would target early placental development rather than placental function. As such, the developmental profile of murine placental and fetal development cannot be mapped directly to that of humans though it can be extrapolated with caution.

Placental tissue is often difficult to obtain due to availability and/or ethical constraints. It can only be collected post-labour or caesarian section and obtaining second trimester control tissue for comparison is not possible. Thus in Chapter 5, comparisons were not made against age-matched controls because there was no time to consent women who came in and delivered immediately without sGC treatment. Treatment groups were compared based on different delivery intervals as well as to term deliveries unexposed to sGCs. Women who deliver prematurely often have pregnancy pathologies that are present below a detectable level and/or the patients are exposed to substances that are not reported. Thus, the placental tissue we have tested may be confounded by exposure to other variables or pathologies. Lastly, more women will need to be recruited into this study to further investigate the possible trend of reduced activity in women exposed to sGCs yet carry to term compared to unexposed term deliveries. Currently, there is a low sample number for subgroupings, especially in the group of women who were exposed to sGC treatment yet delivered at term, where only 4 placental samples were collected. In order to obtain a difference in system A mediated transport of a range between 20-50% with a power of 80% (\(\beta=0.2\)), we would have to enroll between 49-309 women. It is often difficult to collect these placental samples as women
consent into the study at the time of obstetrical appointment when they receive sGCs. However, in cases where pregnancy ensues and is carried to term, often women do not return to Mount Sinai hospital and deliver at hospitals closer to their place of residence. Therefore, interpretations with negative data must be made with caution, considering the reduced power obtained with sample n values between 4-9.

6.8 SIGNIFICANCE TO CURRENT KNOWLEDGE

Currently, 7-10% of all pregnancies are at risk of PTL, and thus a large proportion of women are exposed to antenatal sGC therapy\textsuperscript{313}. Further almost 30% of these pregnancies carry to term\textsuperscript{141, 142}. It is critical to examine the regulatory effects of sGCs at the level of the placenta as women are exposed in cases of PTL but are also administered GCs throughout pregnancy in cases of maternal asthma or congenital adrenal hyperplasia\textsuperscript{314, 315}. We have undertaken the first studies to examine the acute and longitudinal effects of sGCs on the placental system A transporter both in vitro and in vivo. Although these studies cannot be compared directly to one another, as various factors of dose, timing, species and mode of transport were not consistent throughout each, they do provide critical information as to how nutrient availability may change over time with maternal administration of sGC therapy. Women treated with sGCs before 28 weeks of gestation are more likely to deliver after 7 days following treatment compared to women who were at risk of PTL after 28 weeks of gestation. Furthermore, the underlying cause of PTL may affect the duration between sGC treatment and delivery. Women with idiopathic PTL, hypertension or preeclampsia are more likely to deliver within a 7 day period compared to women with placenta praevia or cervical incompetence, who are more likely to deliver greater than 7 days following sGC
These findings taken together demonstrate the importance for correct diagnoses of PTL. The underlying cause and thus likelihood of delivery following sGC exposure may have implications on placental function, especially nutrient transport.

Evidence from human populations, as well as various animal models, have consistently demonstrated that in utero adaptations associated with altered growth rates can result in adverse health outcomes that persist into adult life. Epidemiological studies have demonstrated that smaller neonates are at greater risk of adult hypertension, heart disease, impaired glucose tolerance and insulin resistance, whereas being born large for gestational age increases the incidence of obesity and metabolic syndrome in adulthood. However, birth weight is a crude marker for in utero adaptations and potentially many adversities may affect the developmental trajectory and not grossly alter birth weight\textsuperscript{317}. Birth weight is commonly used as a proxy of the uterine environment, since adversities experienced in utero contributes to growth restriction or fetal overgrowth. Adversities to both placental structure and function that impair the ability of the placenta to transfer nutrients have a profound impact on the intrauterine environment experienced by the fetus. Although fetal weight can be used as one measure that indicates increased risk of disease in adulthood, programming effects can occur without any change in fetal weight after sGC treatment\textsuperscript{318, 319}. Differential adaptations in placental transfer mechanisms may alter fetal body composition, without changes to newborn weight and these responses may persist in long-term and alter adult phenotype\textsuperscript{261, 320}. Whether the mechanism of sGC-induced long-term programming, with/or without changes to total fetal body weight, is mediated by in utero adaptations caused by altered placental system A nutrient delivery warrants further investigation.
This thesis compared the effects of sGC on placental system A-mediated transport and characterized this in two models (placental explants and murine) compared to women exposed in gestation with PTL. Thus, comparisons between model systems have been outlined and identified. This study also characterized murine placental system A across gestation as a baseline in normal pregnancy, which may be further used when studying other gestational pathologies using a mouse model.

Overall, understanding the underlying changes in placental function with sGC treatment across gestation may allow for the translation of molecular physiology to a potential therapeutic treatment. If reduced nutrient transport leads to growth restriction, then increasing placental function of the system A transporter may help to alleviate changes in fetal phenotype. Stimulation of the system A transporter as demonstrated with various endocrine, nutrient or synthetically synthesized regulators may be a possible therapeutic intervention to resolve changes to transporter function. In order to determine specific therapeutics more research is required to characterize the molecular structure of each of the three SNAT proteins and potential therapeutic agents which would then have to be tested in sufficient pathological models.

Investigating the role of system A transport across gestation is critical due to the increasing amount of evidence in its contribution to fetal growth. However, the system A transporter is amongst a myriad of transporters that function in coordination and overall contribute to fetal phenotype. This thesis does not address the importance of other nutrient systems. Nonetheless, the findings reported here contribute importantly to our understanding of placental system A transport.
6.9 CONCLUSIONS

Despite extensive research, rates of PTL have changed little over the past 50 years\textsuperscript{321}. With the widespread use of sGC treatment for the management of PTL, a substantial number of women and fetuses are exposed to sGCs worldwide. The benefits of reduced neonatal morbidity and mortality due to sGC therapy clearly outweigh the risks of use. Our study does not suggest that the use of antenatal sGC treatment be discontinued but reinforces the need for accurate diagnosis of PTL to avoid excess exposure to women who do not require treatment. Evidence from both murine and human placentae demonstrates that system A transport is regulated \textit{in vivo} based on timing from sGC exposure. Therefore, women who are treated with sGCs, yet deliver at term have reduced placental system A mediated transfer and thus, likely reduced availability of neutral amino acids in late gestation to the fetus. By understanding the functional effects on placental system A due to antenatal GC therapy, a potential therapeutic window may be identified. Interventions during this window can be designed to maintain proper nutrient supply in cases of PTL. Ultimately, these studies will help to promote the health of future generations, not only by immediate benefits of the neonate, but also potentially for the prevention of long-term disease through fetal programming.
### Human in vitro model – 48 h DEX (10^{-8} M and 10^{-6} M) treated term explants

<table>
<thead>
<tr>
<th>Effect on System A</th>
<th>Effect Cell Turnover</th>
<th>Effect on Syncytial Regeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity</td>
<td>↑ DEX 10^{-6} M</td>
<td>Proliferation</td>
</tr>
<tr>
<td>SNAT1, SNAT2, SNAT4</td>
<td>localized to</td>
<td>↓ DEX 10^{-6} M</td>
</tr>
<tr>
<td>Slc38a1, Slc38a2, Slc38a4</td>
<td>No effect</td>
<td>Apoptosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Necrosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓ DEX 10^{-6} M</td>
</tr>
</tbody>
</table>

**Mouse in vivo model – Mid-gestation DEX (E13.5 and E14.5) treatment and effects measured acutely (E15.5) and longer-term (E17.5 and E18.5)**

<table>
<thead>
<tr>
<th>System A Ontogeny (No treatment)</th>
<th>Effect of DEX on system A</th>
<th>Effect on Fetal and Placental Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity</td>
<td>↑ E12.5 to E18.5</td>
<td>Fetal Weight</td>
</tr>
<tr>
<td>SNAT1, SNAT2, SNAT4</td>
<td>↑ E12.5 to E18.5</td>
<td>No effect</td>
</tr>
<tr>
<td>No change</td>
<td>↑ E12.5 to E18.5</td>
<td>Placental Weight</td>
</tr>
<tr>
<td>No change</td>
<td>↑ E12.5 to E18.5</td>
<td>↓ Females only</td>
</tr>
<tr>
<td>Slc38a1, Slc38a2, Slc38a4</td>
<td>↑ E12.5 to E18.5 in all</td>
<td>• Equally affected</td>
</tr>
<tr>
<td></td>
<td>↑ E12.5 to E18.5 in all</td>
<td>labyrinth and junctional zones in</td>
</tr>
<tr>
<td></td>
<td>↑ E12.5 to E18.5 in all</td>
<td>males and females</td>
</tr>
<tr>
<td>mRNA</td>
<td>No effect in whole placenta or at MVM</td>
<td>Cortico-sterolone</td>
</tr>
<tr>
<td>mRNA</td>
<td>No effect in whole placenta or at MVM</td>
<td>No change in maternal or fetal levels</td>
</tr>
</tbody>
</table>

**Ex vivo measurements made from placentae of women treated with sGCs for risk of PTL**

<table>
<thead>
<tr>
<th>Effect on System A</th>
<th>Treatment and Delivery</th>
<th>Effect on Fetal and Placenta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity</td>
<td>↓ in sGC term deliveries</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• No sex-specific differences</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>Not measured</td>
<td></td>
</tr>
<tr>
<td>mRNA</td>
<td>Not measured</td>
<td></td>
</tr>
<tr>
<td>Gestational age at treatment</td>
<td>No difference</td>
<td></td>
</tr>
<tr>
<td>Gestational age at delivery</td>
<td>No difference</td>
<td></td>
</tr>
<tr>
<td>Fetal Weight</td>
<td>• No difference due to treatment</td>
<td></td>
</tr>
<tr>
<td>Placental Weight</td>
<td>• No sex-specific differences</td>
<td></td>
</tr>
</tbody>
</table>

Figure 6.1: Data Summary.
Figure 6.2: SNATs and Facilitative Transporters. Diagrammatic representation of SNAT1, SNAT2 and SNAT4 proteins transporting amino acids (A.A.) into the syncytiotrophoblast using Na\(^+\) co-transport on both the microvillous (apical) and basal membranes. Facilitative transporters TAT1, LAT3 and LAT4 are believed to efflux A.A. substrates across the basal membrane into the fetal circulation.
References


34. Murphy VE GP, Talbot PI, Clifton VL. Severe asthma exacerbations during pregnancy. *Obstet Gynecol.* 2005;106:1046-1054


acids to the fetus across the basal membrane of the placental syncytiotrophoblast. *J Physiol*. 2011


47. Sugawara M, Nakanishi T, Fei YJ, Martindale RG, Ganapathy ME, Leibach FH, Ganapathy V. Structure and function of ata3, a new subtype of amino acid transport system a, primarily expressed in the liver and skeletal muscle. *Biochim Biophys Acta*. 2000;1509:7-13


49. Mackenzie B, Erickson JD. Sodium-coupled neutral amino acid (system n/a) transporters of the slc38 gene family. *Pflugers Arch*. 2004;447:784-795


86. Roos S PTaJT. Leucine deprivation in cultured trophoblast cells up-regulates system a activity. *Placenta*. 2006;27:A36


89. Hundal HS, Taylor PM. Amino acid transceptors: Gate keepers of nutrient exchange and regulators of nutrient signaling. *AJP: Endocrinol Metab*. 2008;296:E603-E613


100. Shaw RJ, Cantley LC. Ras, pi(3)k and mtor signalling controls tumour cell growth. *Nature.* 2006;441:424-430


103. Rosario FJ PT, Jansson T. Regulation of trophoblast amino acid transporter trafficking by mtor complex 1 is mediated by the ubiquitin ligase ned4-2. *Reprod Sci.* 201;3:O-172


164


152. Seckl J. Glucocorticoids and small babies. *Q J Med*. 1994;87:259-262


166. van Os J, Selten JP. Prenatal exposure to maternal stress and subsequent schizophrenia. The may 1940 invasion of the netherlands. *Br J Psychiatry*. 1998;172:324-326


177. Emack J, Matthews SG. Effects of chronic maternal stress on hypothalamo-pituitary-adrenal (hpa) function and behavior: No reversal by environmental enrichment. Horm Behav. 2011;60:589-598


191. Holmes MC, Abrahamsen CT, French KL, Paterson JM, Mullins JJ, Seckl JR. The mother or the fetus? 11beta-hydroxysteroid dehydrogenase type 2 null mice provide evidence for direct fetal programming of behavior by endogenous glucocorticoids. *J Neurosci.* 2006;26:3840-3844


217. Hewitt DP, Mark PJ, Waddell BJ. Glucocorticoids prevent the normal increase in placental vascular endothelial growth factor expression and placental vascularity during late pregnancy in the rat. *Endocrinol.* 2006;147:5568-5574


263. Kusinski LC, Jones CJP, Baker PN, Sibley CP, Glazier JD. Isolation of plasma membrane vesicles from mouse placenta at term and measurement of system a and system beta amino acid transporter activity. *Placenta.* 2010;31:53-59


293. Antonow-Schlorke I, Schwab M, Li C, Nathanielsz PW. Glucocorticoid exposure at the dose used clinically alters cytoskeletal proteins and presynaptic terminals in the fetal baboon brain. *J Physiol (Lond)*. 2003;547:117-123


