EARLY DEVELOPMENT OF BODY-WEIGHT REGULATION SYSTEMS IN THE NONHUMAN PRIMATE: *IN UTERO* EFFECTS OF HIGH-FAT DIET

by

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A DISSERTATION

Presented to the Neuroscience Graduate Program
and the Oregon Health & Science University
School of Medicine
in partial fulfillment of
the requirements of the degree of
Doctor of Philosophy

April 2009

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

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5-HT	5-hydroxytryptamine, serotonin
5-HIAA	5-hydroxyindole acetic acid
5-HTT	5-hydroxytryptamine transporter
αMSH	alpha melanocyte-stimulating hormone
AA	arachadonic acid
ACTH	adrenocorticotropin-releasing hormone
AgRP	agouti-related peptide
ALG9	asparagine-linked glycosylation 9
AP	area postrema
ARH	arcuate nucleus of the hypothalamus
AUC	area under the curve
AVPV	anteroventral periventricular nucleus
bp	base pairs
CART	cocaine and amphetamine related transcript
CHRNA1	cholinergic receptor α1
CHRNA4	cholinergic receptor α4
CHRNB2	cholinergic receptor β2
CHRND	cholinergic receptor δ
CRH	corticotrophin-releasing hormone
CSF	cerebral spinal fluid
CTR	control diet
DBH	dopamine β hydroxylase
DHA	docosahexaenoic acid
DMH	dorsomedial nucleus of the hypothalamus
DMV	dorsal motor nucleus of the vagus
DR	diet-reversal
DR-S	diet-reversal resistant
DR-R	diet-reversal sensitive
DVC	dorsal vagal complex
ELISA	enzyme-linked immuno-sorbent assay
FBP1	fructose-bisphosphatase 1
F-CTR	fetus of mother consuming control diet
F-HFD	fetus of mother consuming high-fat diet
FITC	fluorescein isothiocyanate
G130	gestational day 130
GABA	γ-Aminobutyric acid
GABRA1	γ-Aminobutyric acid, subunit α1
GABRA2	γ-Aminobutyric acid, subunit α2
GABRQ	γ-Aminobutyric acid, subunit Q
GRIA1	glutamate receptor 1, ionotropic
GTT	glucose tolerance test
HAT	histone acetyl transferase
HFD	high-fat diet

HFD-R	high-fat diet resistant
HFD-S	high-fat diet sensitive
HDAC	histone deacetylase
HNE	4-hydroxy-2-nonenal
hPL	human placental lactogen
HSP	heat shock protein
-ir	immunoreactive
ICV	intracerebral ventricular
IGF-1	insulin-like growth factor
IL1β	interleukin 1β
IL1R1	interleukin 1 receptor 1
INSR	insulin receptor
IRS-1	insulin receptor substrate 1
IV	intravenous
J-CTR	juvenile offspring of mother consuming CTR diet
J-HFD	juvenile offspring of mother consuming HFD
KPBS	potassium phosphate buffered saline
LEPR	leptin receptor
LHA	lateral hypothalamic area
MAO	monoamine oxidase A
MCH	melanin-concentrating hormone
MCR	melanocortin receptor
MOR	μ-opioid receptor
MPO	medial preoptic area
NAFLD	non-alcoholic fatty liver disease
NFκB	nuclear factor κB
NHP	nonhuman primate
NO	nitric oxide
NPY	neuropeptide Y
NPY1R	neuropeptide 1 receptor
NTS	nucleus of tractus solitarius
PCR	polymerase chain reaction
PGC-1α	PPARγ coactivator-1α
PEPCK	phosphoenolpyruvate carboxykinase
PFR	perifornical region
POMC	pro-opiomelanocortin
PC1/2	prohormone convertase 1/2
PVH	paraventricular nucleus
PYY	peptide YY
ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction
r.u.	relative units
SCN	superchiasmatic nucleus
SEM	standard error of the mean
SOCS	suppressor of cytokine signaling-3
SON	supraoptic nucleus

SSTR3	somotaostatin 3 receptor
TPH1	tryptophan hydroxylase 1
TPH2	tryptophan hydroxylase 2
VEGF	vascular endothelial growth factor
VLM	ventral lateral medulla
VMH	ventral medial nucleus of the hypothalamus
VTA	ventral tegmental area

ACKNOWLEDGEMENTS

"Nanos gigantum humeris insidentes."

I have truly been blessed by God in being allowed this incredible opportunity to accomplish something like this. I am thankful to all the caring and supporting people in my life.

First, I am grateful to my mentor, Dr. Kevin L. Grove. It is a profound privilege to be his first graduate student and one that I have and will never carry lightly. He has provided for me a rich environment to strive to become an independent thinker as well as a wealth of resources to answer the questions which have become this dissertation. Without his unreserved support and generosity, the work of my Masters of Clinical Research would also not have been possible. By his example, he has expanded my view of an academician to include scientist, parent, teacher, people-manager and businessperson. From observing him in each of these roles, I have learned more about being in academic science then textbooks could ever teach me. His benevolence towards me has been evident during every hurdle that I have faced as a student. I am indebted to him.

I am also greatly appreciative of the mentorship I have received from Dr. M. Susan Smith. Nearly a decade ago, she and Kevin chose to hire me for a job, but what they have provided for me is a career and a passion. Throughout my education, Susan has been the voice of reason amidst confusion, unyielding support and encouragement amidst odds and compassion amidst difficulties. It has been a privilege to work with her and glean from her hard-gained wisdom and experience. She has provided for me the penultimate example of what it is to be a "lady in science." I am sincerely grateful.

I would also like to thank the members of my various graduate committees: Drs. Michael Cowley, Sergio Ojeda, Shaun Morrison, Daniel Marks and Cynthia Bethea. Their input and good judgment has improved the quality of my education as well as the caliber of my studies.

I would sincerely like to thank Dr. Maria Glavas for all the brainstorming about our projects and careers as well as sharing her gift of eloquent writing specifically in editing my manuscripts, grants and dissertation. I am also grateful to Dr. Paul Kievit for assistance in editing my dissertation as well as his encouragement of me during the difficult days of interviewing and writing of this dissertation.

I have been blessed with a very caring and supportive lab. These studies would not be possible without the diligent work of Sarah Williams who has poured herself into the management of the animals described in this dissertation. Along with her, Diana Takahashi, Christopher Osman, Lindsay Pranger, Rebecca Hudson and Anne Evans have provided excellent support in carrying out the protocols for this study. In addition, I am grateful to Melissa Kirigiti for managing the needs of the lab and helping me procure the tools I needed to carry out these studies. I am thankful to Dr. Puspha Sinnayah and Dr. Elinor Sullivan as well as Shin Draper and Cadence True for being caring lab members. To all the past members of the lab, Dr. Rebecca Campbell, Dr. Xiao Qiu Xiao, Summer Allen and Betty Jiang, I am grateful for your support.

I am grateful to my parents, Ryszard and Barbara Kluz, for giving my life and stressing the importance of education. I am also grateful to my siblings Dorothy, Elizabeth and Christopher for our shared love of learning and their support and love throughout my life.

And last and yet foremost, I would not have embarked on the path to attain these degrees without the support of my husband, George Grayson. Without his encouragement, I would surely have lost heart along the way. I am grateful for his steadfast optimism and unconditional faith in me through these years. I am also thankful for my little Isla, whose life began and flourished amidst the difficult months of finishing these degrees.

PREFACE

In accordance with the guidelines set forth by the Graduate Program of the School of Medicine, Oregon Health & Science University, Portland, Oregon, I have prepared my dissertation, consisting of a general introduction, description of the model used, three chapters of original data and a general conclusion. The references sited are listed separately in alphabetical order.

Chapter II contains data, figures and text as they appear in the original paper that has been published previously. Chapters IV and V are being prepared for submission. The Appendix includes a brief description of preliminary data not yet submitted for publication.

ABSTRACT

The prevalence and severity of obesity is on the rise in adults and also devastatingly in children. Despite the similarities to adult obesity, a critical difference with childhood obesity is that the resulting metabolic disturbance may permanently disrupt normal development and increase susceptibility to these diseases independently of genetic factors and nutrition in adulthood.

Critical periods exist in child development during which nutrition and health status may predispose the individual to body-weight regulation difficulties. Among these suggested periods is the *in utero*. Most of what we know about the development of metabolic systems and the impact of early maternal/nutritional manipulations has been obtained in rodent models which have a different ontogeny of these systems. The nonhuman primate, on the other hand, is an ideal model for such studies due to the remarkable similarities in development to the human. Specifically, we do not know the degree of impact of maternal consumption of high fat/calorie diets and maternal obesity on the development of fetal appetitive mechanisms in the brain.

The goal of this dissertation was two-fold. First, I determined the normal ontogeny of the feeding-related neurocircuitry using a nonhuman primate model. Then, using a nonhuman primate model of diet-induced obesity, I determined the impact of maternal obesity on the fetal hypothalamic and midbrain neurocircuitry with a specific focus on body-weight regulation.

Chapter 1 provides a review of the seminal literature that guided the direction of this work. The regulation of body-weight homeostasis is discussed with particular

emphasis on hormonal and neuropeptide controls. The development of the pertinent homeostatic neurocircuitry is also reviewed in rodent models.

Chapter 2 establishes the normal ontogeny of the important neuropeptides that regulate body-weight homeostasis. Since many of these hypothalamic neurons develop and begin to establish projections to other nuclei during the later trimesters (2^{nd} and 3^{rd}).

Chapter 3 introduces the model of maternal diet-induced obesity. Nonhuman primates consumed either a normal fat diet (CTR) or a high fat/high calorie diet (HFD) for four years. In the fifth year, a subset of the HFD cohort was returned to CTR chow for a final pregnancy to determine the impact of "healthful eating" despite overt maternal obesity.

Chapter 4 focuses on the influence of the maternal HFD on hypothalamic expression of feeding-related peptides and how the development of the projections is affected.

Chapter 5 focuses on the midbrain contributions of forward projecting serotonin neurons. In these studies, pathways regulating serotonin metabolism are altered. Since serotonin neurons and the ensuing projections develop very early, serotonergic projections may be far more sensitive to the effects of diet manipulations than thought previously.

Finally, Chapter 6 integrates the findings to one another and the relevance of these findings to human childhood obesity and modern clinical guidelines for pregnant women. In summary, these findings show that maternal high-fat diet consumption during pregnancy has ramifications on the *in utero* growth of the fetus. Even in the absence of

gestational diabetes in these animals, maternal consumption of HFD may be more harmful than previously surmised.

Chapter 1
Introduction

BACKGROUND

In recent decades, obesity during infancy and childhood has become a devastating consequence of the "obesity epidemic" in our country and around the world. According to the recent National Health Examination Survey (NHANES), 33.6% of children within the U.S. are considered overweight, as are 12% of infants (Ogden et al., 2006). Similar to that in adults, the increased prevalence of childhood obesity has led to an increased risk of hypertension, type II diabetes, dyslipidemia, left ventricular hypertrophy, nonalcoholic steatohepatitis, obstructive sleep apnea, orthopedic problems, and psychosocial stress (Taylor et al., 2006) (Sorof and Daniels, 2002).

Genetic shifts are not the at the root of the surge in adult obesity

In the United States, it is estimated that nearly two-thirds of adults are either overweight or obese (Baskin et al., 2005; Flegal, 2005). Among adults aged 20–74 years, the prevalence of obesity increased from 15.0% in the 1980's to 32.9% in the 2000. Worldwide, excess body-weight affects 1.1 billion adults (Haslam and James, 2005). Obesity is generally characterized as the accumulation of excess body fat as a result of overconsumption and underutilization of energy intake. In adults, obesity is diagnosed as a body mass index (BMI) of 30 kg/m^2 or higher (calculated by dividing body-weight by height squared). Obesity is part of a larger disease process termed "metabolic syndrome" which generally includes insulin resistance, impaired glucose tolerance, hypertension, elevated triglycerides and/or cholesterol, and elevated urinary albumin excretion rate, an indicator of renal failure (De Ferranti and Osganian, 2007). Eventually these symptoms lead to diabetes, cardiovascular disease and neuropathies.

Considering the sheer numbers of individuals dealing with issues of body-weight regulation, only about 5% of obesity is linked to known single-locus gene mutations. Taking into account the dramatic rise in incidence of obesity in recent decades, Mendelian genetic shifts must be ruled out as the direct causative factor of this incidence. The growing population of obese individuals indicates that the majority of cases of obesity are likely either polygenic, epigenetic or environmental in origin leading to a susceptibility to weight gain under a calorie-rich diet (Farooqi et al., 2003).

Escalating prevalence of obesity affects child health

In children, metabolic disease has increased concomitant with increases in adult populations. Among U.S. children the overall prevalence of risk for overweight is estimated to be 33.6% for the years 2003-2004 (Ogden et al., 2006). The actual prevalence of overweight was 17.1% in children from ages 2-19 years.

Childhood overweight/obesity is defined using BMI in the same calculation as previously described for adults. However, for children the BMI is then plotted on a growth chart – with greater than the 95^{th} percentile considered overweight or obese. A BMI between the 85^{th} percentile and 95^{th} percentile is considered at risk for becoming overweight. The serious issue in our modern times is that nearly 15% of the US child population is in the 95% percentile. Many risk factors have been identified for childhood obesity. These include parental obesity, higher birth weight, higher weight gain during the first year and short sleep duration at age 3, neonatal adiposity rebound due to low weight or weight gain at birth, more than 8 hours of television per week at age three, and presence of catch-up growth phases (Reilly et al., 2005). Maternal undernutrition during pregnancy, maternal obesity during pregnancy, low birth weight and high birth weight all

appear to predispose individuals to obesity and type 2 diabetes later in life (Wei et al., 2003). In addition, formula feeding as opposed to breastfeeding has a higher association with long-term obesity (Mayer-Davis et al., 2006; Moreno and Rodriguez, 2007). Smoking during pregnancy is also linked with obesity in offspring (Salsberry and Reagan, 2007). Importantly, population-based human studies reveal correlations between fetal high-fat diet exposure and adult weight regulation (Hedley et al., 2004; Ong, 2006; Schaefer-Graf et al., 2005). With as many factors that appear to have a significant impact on long-term body-weight maintenance, it is difficult to estimate the relative contribution of each to the childhood obesity epidemic.

Obesity during childhood negatively impacts many facets of a child's well-being encompassing cardiovascular, musculoskeletal, reproductive and psychological health. Though these problems affect the peripheral organs and functions, these are all controlled functions of the central nervous system. In the Bogulusa Heart Study, a study of the long-term cardiovascular health of over 16,000 children and young adults, overweight children were reported to be 4.5 and 2.4 times more likely to have elevated systolic and diastolic blood pressures, respectively (Freedman et al., 1999). Hypertension was found to be 22% more likely in obese than nonobese children (Sorof et al., 2002). Overweight children report a greater prevalence of fractures and musculoskeletal discomfort as well as greater impairment of mobility (Sorof and Daniels, 2002). Various parameters of childhood metabolic syndrome such as elevated cholesterol, glucose and triglyceride levels are associated with pre-pubertal sleep disordered breathing (Verhulst et al., 2007). Children with high body-weight, either at birth or later in childhood, are at increased risk for future asthma (Flaherman and Rutherford, 2006).

The most immediate yet underemphasized consequences of obesity during childhood are psychosocial, resulting in discrimination and negative self-image during adolescence (Dietz, 1998; Eisenberg et al., 2003). Obese adolescent females are at especially high risk for major depressive disorders and anxiety disorders (Anderson et al., 2007). Together, these findings indicate that childhood obesity has damaging implications in the immediate as well as long-term.

Increased prevalence of metabolic syndrome is supported by the *fetal origins hypothesis*

The *fetal origins of disease hypothesis* or Barker Hypothesis purports that adverse intrauterine conditions can cause permanent changes in the embryo/fetus, predisposing it to chronic disease later in adult life (Barker et al., 2002; Hales and Barker, 2001). Cohort studies have shown that disturbed intrauterine growth has a negative influence on the development of the cardiovascular system and favors the occurrence of hypertension, insulin resistance, hypercholesterolemia, and hyperuricemia in adult life (Barker et al., 1989; Hales et al., 1991). The most well reported studies are the Dutch Famine Studies. Evidence from these cohorts revealed that the early nutritional deprivation in pregnant mothers as a result of famine, produced small-for-gestational-age (SGA) babies. The consequences were irreversible health issues in the offspring. Through longitudinal studies of this population, it came to light that exposure to famine during any period of gestation can lead to glucose intolerance during mid-life (50-60 years) (Ravelli et al., 1998). The worst consequences exist for individuals who experienced famine during early gestation.

These early studies linking low-birth weight and late-onset obesity and diabetes laid the groundwork for the *thrifty phenotype hypothesis,* which proposes that poor nutritional conditions during gestation and early infancy can modify metabolic systems in the offspring to adapt to expectations of chronic undernutrition. With a subsequent affluent environment, these offspring are poorly equipped to cope with energy-dense diets and are possibly wired to store as much energy reserves as possible, leading to late-onset metabolic syndrome (Hales and Barker, 2001). Insults such as poor nutrition and decreased maternal calorie or protein consumption are known to cause intrauterine growth retardation in various mammals (Desai et al., 2007; Louey and Thornburg, 2005; Morrison et al., 2007; Schlabritz-Loutsevitch et al., 2007). However, the effects of insults such as high dietary fat, low polyunsaturated fatty acid (PUFA) intake and excessive overall calorie consumption have been less studied.

Importance of the Prenatal Period

Growth during the prenatal period affects long-term metabolic health (Dietz, 1994; Dietz, 1997). High maternal BMI, even in the absence of maternal gestational diabetes, is a risk factor for large-for-gestational-age (LGA) infants who are highly susceptible to lifelong disease (Boney et al., 2005b; Ehrenberg et al., 2004). Fetal exposure to maternal obesity increases the risk of childhood metabolic syndrome by approximately two-fold (Boney et al., 2005a; Wei et al., 2003) . In addition, obesity during pregnancy has implications for morbidity and mortality for the mother and the baby. Maternal obesity more than doubles the risk of stillbirth and neonatal death (Cnattingius et al., 1998; Kristensen et al., 2005). Overweight mothers also have a greater risk of hypertensive complications and preeclampsia (Sattar et al., 2001). Finally,

women with poor glycemic control and/or gestational diabetes have a high risk of giving birth to a macrosomic baby that is more likely to develop obesity and insulin resistance later in life (Gillman et al., 2003; Lindsay et al., 2000; Plagemann et al., 1997). Though much work has been done to document the trends and associations between maternal nutrition and health during gestation and long-term metabolic health of offspring, it is still not known which mechanisms are responsible for the increased risk for obesity in adulthood.

Critical periods exist in child development during which nutrition and health status may predispose the individual to life-long body-weight regulation difficulties. Among these suggested periods is the *in utero*/early infancy period (Dietz, 1994). Hypothalamic appetite regulatory systems develop and mature during the *in utero*/early infancy period. A primary goal of this introduction is to describe the normal development of hypothalamic appetitive systems as well as their development in the context of genetic, nutritional and environmental manipulations to further understand their impact on body-weight regulation.

OVERVIEW OF NORMAL BODY-WEIGHT REGULATION

Appetite regulation is a complex system with both homeostatic and hedonic aspects as well as central and peripheral components integrated at the level of the hypothalamus (Saper et al., 2002a) *(Figure 1-1)*. At the crossroads of the central nervous system and peripheral components is the arcuate nucleus of the hypothalamus (ARH). A critical aspect of ARH neurocircuitry is the interplay between the anorexigenic peptide-containing neurons such as α melanocyte-stimulating hormone (αMSH) (a cleavage product of the pro-opiomelanocortin, or POMC, gene) and cocaine and amphetamine-

related transcript (CART), and orexigenic peptide-containing neurons namely neuropeptide Y(NPY) and agouti-related peptide (AgRP) *(Figure 1-1, A)*. These neurons are sensitive to blood-borne indicators of energy status (leptin, insulin, glucose, fatty acids, amino acids and gut hormones) and project to other feeding centers in the hypothalamus such as the dorsomedial nucleus of the hypothalamus (DMH) and lateral hypothalamic area (LHA) *(Figure 1-1, B)*. Projections from these nuclei re-converge on the paraventricular nucleus (PVH), which is a site of integration of metabolic signals from many parts of the brain *(Figure 1-1, C)*. The release of the ARH-derived peptides, αMSH, NPY and AGRP, play a key role in the feed-back mechanisms that suppress and enhance feeding according to peripheral substrate availability. Importantly, the ability of the appetite-suppressing peptides to function, particularly αMSH, is often blunted in animals sensitive to weight gain.

In addition to the critical role of the hypothalamus, various nuclei in the hindbrain contribute to the control of food intake particularly through motor and gustatory mechanisms *(Figure 1-1, D)*. Brainstem control of food intake is largely mediated through the dorsal vagal complex (DVC) which includes the dorsal motor nucleus of the vagus (DMV), nucleus of the solitary tract (NTS) and area postrema (AP) (see reviews (Berthoud et al., 2006; Grill, 2006)). For example, mastication, swallowing, and salivation are controlled by the caudal brainstem. The DVC acts as a relay site for short-acting gastrointestinal signals, mediated predominantly through the vagus nerve. In addition, gut peptides communicate with other hindbrain nuclei at the AP, a circumventricular organ with decreased blood brain barrier. These gut-hindbrain

connections are sufficient to produce satiety on their own, but can be overridden by more cognitive inputs of body-weight regulation.

Superimposed on the homeostatic neurocircuitry of body-weight regulation are the hedonic aspects of food intake (*see review* (Saper et al., 2002b)). This behavioral aspect, based on the palatability and reward of food, can override the normal requirements of daily metabolic rate and energy expenditure. These hedonic neurocircuits span brain areas such as the nucleus accumbens (NAc), ventral pallidus (VP) ventral tegmental area (VTA) and higher cortical areas such as the prefrontal cortex, converging on the LHA. These pathways utilize biological substrates such as glutamate, opioids, endocannabinoids and dopamine. The well-known effects of these compounds on mood likely contribute significantly to the powerful and overwhelming impact of the hedonic pathways on food intake.

NORMAL NEUROPEPTIDE, MONOAMINE AND HORMONAL CONTROL

In the ARH, feeding-related peptidergic neurons respond to both metabolic cues sensed from the periphery and to excitatory and inhibitory inputs from ascending afferents and interneurons, particularly from the brainstem. Within the ARH, the major populations of interest are NPY/AgRP and POMC/CART neurons, as discussed above. The peripheral hormones related to body-weight regulation and the ARH that have received the most attention are leptin and insulin; these hormones can modulate activity of both NPY/AgRP and POMC/CART neurons.

NPY and AGRP provide a powerful orexigenic drive

NPY is one of the most widely distributed and potent orexigenic peptides in the CNS (Allen et al., 1984; Chronwall et al., 1985; Levine and Morley, 1984). Discovered

in 1982 within the brain, (Tatemoto et al., 1982), NPY is expressed in the cortex, hippocampus, hindbrain, thalamus and hypothalamus (Allen et al., 1984; Chronwall et al., 1985; Gray and Morley, 1986) and is associated with functions in the normal adult that include the cardiovascular system (Levine and Morley, 1984), reproduction (Aubert et al., 1998), stress (Heilig and Thorsell, 2002; Thorsell and Heilig, 2002), neuroendocrine regulation (Kalra and Kalra, 2003) and cognition (Kask et al., 2002; Redrobe et al., 1999). Within the hypothalamus, NPY is produced primarily by neurons in the ARH. The ARH-NPY producing neurons also are exclusively co-localized with AgRP (Hahn et al., 1998a).

Injection of NPY peptide into third, fourth, and lateral ventricles, as well as into specific hypothalamic nuclei such as the anterior ventromedial nucleus (VMH), PVH , and the posterior LHA, robustly increases food intake (Morley et al., 1987). Deprivation of food from 24-96 hours results in profound upregulation of NPY mRNA and protein content, particularly within the ARH (Chua et al., 1991; Sahu et al., 1988). Conversely, NPY-specific lesioning of the ARH immunologically (Burlet et al., 1995) or chemically (Stricker-Krongrad et al., 1996) decreases food intake. Infusion of NPY antibodies (Shibasaki et al., 1993) or use of NPY antisense oligonucleotides inhibits food intake (Akabayashi et al., 1994).

Knockout of NPY and receptors have produced controversial results, however. Originally, the NPY-null mouse was produced on a background resistant to obesity and little effect of genetic ablation was reported other than hyperphagia following food deprivation (Erickson et al., 1996). Subsequent studies have shown NPY ablation on a C57BL/6 background leads to increased body-weight and mild obesity, despite

apparently normal food intake, as well as to a diminished refeeding response to starvation (Segal-Lieberman et al., 2003). However, this increase is modest and it appears that developmental adaptations and increased levels of co-expressed AgRP may rescue the expected phenotype (Patel et al., 2006). In diphtheria toxin-targeted ablation of NPY/AgRP neurons in adult mice, starvation ensues (Gropp et al., 2005; Luquet et al., 2005). If the targeted ablation occurs in neonatal mice, early compensation occurs resulting in a nearly normal growth and feeding (Luquet et al., 2005). In light of these experiments, one must consider that NPY/AgRP-expressing ARH neurons express the neurotransmitter GABA. Therefore, KO of one or both peptides does not ablate the inhibitory presence these neurons exhibit within the ARH. However, total ablation of the neuron does remove the endogenous inhibitory tone of this neuropeptide expressing cell. The potential consequences of development and maturation of the ARH in the absence of the neuropeptides (NPY and AgRP) in addition to inhibitory action would be dramatically altered. Overall, these studies exemplify the pervading orexigenic role of central NPY neurons.

AGRP is another powerful orexigenic peptide that also has the ability to block the hypothalamic satiety response. Within the ARH, greater than 95% of the NPY neurons coexpress AgRP in the adult (Chen et al., 1999; Hahn et al., 1998a). Centrally, AgRP is exclusively expressed in the ARH; however it is also expressed and released from the adrenal gland (Hahn et al., 1998a; Shutter et al., 1997). The importance of AgRP in the regulation of energy homeostasis has been demonstrated by numerous groups (Ellacott and Cone, 2004; Small et al., 2003; van den Top et al., 2004). Briefly, AgRP levels are increased by fasting (Hahn et al., 1998a), and intracerebroventricular (ICV) injections of

synthetic analogs of AgRP result in robust long-lasting hyperphagia (Hagan et al., 2000). AgRP was long thought to be solely a melanocortin receptor antagonist, simply involved in the blockade of αMSH effects (Fong et al., 1997; Haskell-Luevano and Monck, 2001); however, AgRP has been more recently characterized as an inverse agonist, specifically for the melanocortin 4 receptor (MC4R) (Nijenhuis et al., 2001). Approximately 50% of the NPY/AgRP neurons within the ARH express leptin receptors (Mercer et al., 1996). Decreased circulating leptin (as accompanied with fasting) leads to an up-regulation of NPY/AgRP mRNA and protein (Rohner-Jeanrenaud et al., 1996; Sainsbury et al., 1996). AgRP also contributes to the suppression of energy expenditure by inhibiting sympathetic outflow to peripheral metabolic tissues (Yasuda et al., 2004). Reduction of AgRP through RNA interference increases energy expenditure and loss of body-weight with no change in food intake (Makimura et al., 2002). AgRP null mice have a normal phenotype (Qian et al., 2002). Additionally AgRP and AgRP/NPY double knock-out mice have both a normal feeding phenotype, and plasticity of the homeostatic feeding circuitry appears to compensate for the loss of these neurons (Qian et al., 2002). However, targeted post-embryonic ablation of the neurons containing NPY/AgRP, results in hypophagia, hypoinsulinemia and increased brown adipose tissue UCP1 expression resulting in a lean phenotype. These data suggest that in additional to the plasticity of the circuits, the other constituents of these neurons such as receptors and peptides are also important.

POMC and CART provide cues for satiety

Pro-opiomelanocortin (POMC) exerts a negative impact on food intake through its various cleavage products such as ACTH, α, β and γMSH and β-endorphin. POMC is produced centrally in the intermediate lobe of the pituitary and in populations of neurons

in the ARH and nucleus of tractus solitarius (NTS) (Joseph et al., 1983; Knigge et al., 1981). Peripherally, POMC products are expressed in the skin, gut, placenta and pancreas (Smith and Funder, 1988). These products work through five melanocortin receptor subtypes. MC1 receptors have the highest affinity for αMSH and are involved in melanogenesis of the skin. MC3 and 4 receptors have the broadest CNS distribution in areas involved in energy balance (Mountjoy et al., 1994; Roselli-Rehfuss et al., 1993). MC2 receptors are involved in adrenal production and release of ACTH whereas MC5 receptors are involved the function of exocrine glands as well as implicated in thermogenesis. Central administration of αMSH peptides or synthetic agonists such as Melanotan II (MTII) results in decreased food intake and body-weight reduction mediated primarily through the MC4 receptor (Fan et al., 1997; Koegler et al., 2001b; Thiele et al., 1998; Trivedi et al., 2003). POMC neurons are known to be stimulated by leptin (Schwartz et al., 1997) and their firing is suppressed by NPY (Cowley et al., 2001).

POMC gene mutations are a source of monogenic obesity and lead to early onset-adiposity, adrenal insufficiency and red hair pigmentation (Farooqi et al., 2006; Krude et al., 1998; Krude et al., 2003). The most common of the monogenic obese mutations is in the MC4R gene (Alharbi et al., 2007; Yeo et al., 1998; Yeo et al., 2003). The prevalence of MC4R mutations has varied from 0.5% of obese adults to 6% (Farooqi et al., 2003). Over 70 different frame-shift mutations in MC4R have been recorded in humans, leading to varying levels of obesity (Hinney et al., 1999; Lubrano-Berthelier et al., 2006; Yeo et al., 2003). This mutation results predominantly in mature-onset obesity concomitant with hyperphagia, hyperinsulinemia, and hyperglycemia. Overall, perturbations of the melanocortin system lead to inevitable weight management difficulties.

CART is another neurotransmitter implicated in diverse physiological functions, which include stress response, feeding, reward and autonomic function. CART is abundantly expressed in the brain, including hypothalamic regions such as the VMH, PVH, ARH, but also the nucleus accumbens, VTA, thalamic nuclei, dorsal raphe, amygdala, olfactory bulbs, spinal cord and NTS (Ersin O. Koylu, 1998; Smith et al., 1997). In the periphery, CART is localized to the adrenal medulla, gut and pancreas (Koylu et al., 1997; Wierup et al., 2004). In the rodent, CART has been found to co-localize with other prominent neuropeptides and neurotransmitters such as POMC, tyrosine hydroxylase and MCH in the hypothalamus, GABA in the nucleus accumbens and acetylcholine in the gut myenteric system (Couceyro et al., 1998; Elias et al., 2001b; F. Brischoux, 2002; Koylu et al., 1999). The function of CART was elucidated when cocaine and/or amphetamine administration resulted in a rise in CART mRNA levels in the rat ventral striatum (Douglass et al., 1995). However it has distinguished itself far more as a feeding-related peptide. CART is an anorexigenic peptide which decreases with fasting and ICV injection inhibits food intake in freely feeding and fasted rodents (Kristensen et al., 1998; Robson et al., 2002). Exogenously administered CART can induce *c-fos* expression in feeding-related hypothalamic nuclei (Vrang et al., 1999c) and CART neurons are known to be sensitive to leptin as well as glucocorticoids (Elias et al., 1998a; Kristensen et al., 1998; Vrang et al., 2003). Unfortunately, the receptor for CART still remains unidentified and elusive and has therefore hampered the study of this neuropeptide. Though clearly involved in food-intake regulation, the physiological role of CART still needs to be elucidated.

MCH and Orexin

In general, the LHA resides at the juncture between the homeostatic pathways that govern body-weight regulation and the hedonic pathways that modulate arousal and reward surrounding food intake. The LHA contains two very separate sub-populations of feeding-related neurons, orexin and melanin-concentrating hormone (MCH). Despite their distinct phenotypes, they share many similar sites of projection (Bittencourt et al., 1992; Elias et al., 1998b). MCH and orexin neurons exhibit complex efferent projections throughout the hypothalamus also with extensive projections throughout the brain to areas such as the hippocampus, thalamus, nucleus accumbens, VTA, locus coeruleus (LC) and raphe. They also provide orexigenic stimuli to sites of feeding modulation such as the PVH, DMH and ARH. Stimulation of the LHA results in hyperphagia and destruction of this nucleus results in decreased food intake and weight loss (Pissios et al., 2006).

Orexin, also called hypocretin, is involved primarily in arousal, the sleep/wake cycle and autonomic control of glucose homeostasis and thermoregulation (Cai et al., 1999; Hagan et al., 1999; Huang et al., 2001). The actions of orexin are mediated through two G-protein coupled receptors (GPCRs) OXR1 and 2. Transgenic knockout of orexin results in narcolepsy as well as hypophagia (Chemelli et al., 1999; Hara et al., 2001). In humans, orexin mutations are uncommon but narcoleptics appear to have suppressed CSF levels of orexin (Dalal et al., 2001) as well as a degeneration of orexin-containing neurons (Peyron et al., 2000). Narcoleptics have also been shown to appear to have altered energy balance and a tendency to late-onset obesity (Schuld et al., 2000). Orexin has also been implicated in the reward-motivated aspects of food-seeking behavior (Harris et al., 2005). This important aspect of orexin physiology which integrates the

addictive and reward-based aspects of food ingestion is very important in the study of obesity.

MCH regulates both food intake as well as stress response, primarily through two GPCR receptors MCH-R1 and R2. ICV administration of MCH leads to increased body-weight gain (Ludwig et al., 1998) and MCH transgenic overexpression results in obesity and insulin resistance (Ludwig et al., 2001). Ablation of the MCH neurons leads to leanness through hypophagia and an inappropriately increased metabolic rate (Shimada et al., 1998). Furthermore, KO of the MCH-R1 leads to hyperphagia, and both increased metabolic rate and locomotor activity (Carol F. Elias, 1998).

Overall, both MCH and orexin involve aspects of body-weight regulation that are involved in the pleasurable aspects of food intake, as well as control of energy metabolism through sleep and metabolic activity.

Ubiquitous monoamine expression exerts influence over body-weight regulation

The role of monoamines such as dopamine, norepinephrine, histamine and serotonin, is critical in a variety of vital physiological processes including food intake and energy balance. The focus in the studies presented in this dissertation is on the role of serotonin; however preliminary evidence suggests possible dysregulation in the other monoamines.

Dopamine

Briefly, dopamine is produced from L-tyrosine through the partial actions of tyrosine hydroxylase. Two major populations of dopamine neurons exist. The first is within the ARH where it functions to inhibit the release of prolactin secretion from the pituitary (Kamberi et al., 1970). These are referred to as tuberoinfundibular dopamine

(TIDA) neurons. The second population spans the substantia nigra and VTA sending projections forward to the forebrain. Dopamine activity is modulated through functions of 5 subtypes of G-coupled receptors, D_{1-5}. Dopamine is clearly involved in reward, motivation and appetite regulation particularly through the mesocortical-mesolimbic pathway (Nelson and Gehlert, 2006). Increased dopamine and dopamine receptor agonists are associated with hypophagia partially through actions to inhibit NPY gene expression (Bina and Cincotta, 2000; Kuo, 2002) and increase POMC expression (Pelletier, 1993; Tong and Pelletier, 1992). Therefore, hypothalamic suppression of dopamine is thought to be a possible mechanism for hyperphagia and weight gain.

Norepinephrine

Norepinephrine (or noradrenaline) is classic biogenic amine neurotransmitter with extensive actions in the sympathetic nervous system in the "fight or flight" response causing increased heart rate, papillary dilation, energy mobilization and shunting of blood from non-essential organs to skeletal muscle. It is also produced by neurons in the caudal ventrolateral medulla (VLM) (predominantly A2 region) and LC in the brainstem and is released from the adrenal medulla into the blood as a hormone (Sawchenko et al., 1985). Norepinephrine is synthesized from dopamine via action of dopamine β hydroxylase (DβH) and acts through two major subtypes of adrenergic receptors, namely α and β. Ascending noradrenergic fibers project to a variety of brain regions which include the limbic system, hippocampus, thalamus hypothalamus and frontal cortex. The VLM-derived projections are notably important in control of cardiovascular function and the baroreceptor reflex as well as modulating the HPA-axis responsiveness to a variety of stressors (Day, 1989; Radley et al., 2008). Descending projections from the VLM to the

spinal cord are involved in glucose sensing (Ritter et al., 2006). The LC-derived projections are largely colocalized with NPY in the rodent and synapse on NPY/AgRP neurons within the ARH as well as other forebrain sites such as the PVH and prefrontal cortex (PFC) (Fuzesi et al., 2007; Sawchenko et al., 1985). Activation of α1-, β2- and β3-adrenoceptors is involved in the suppression of food intake, whereas stimulation of the α2-adrenoceptor increases food intake. Targeting of norepinephrine transporters, together with serotonin transporters through combined SSRI/NRI drugs have provided some success in effecting loss in body adiposity.

Histamine

Histamine is not only produced by mast cells in the immune response but also by neurons in the tuberomammillary nuclei of the posterior hypothalamus (Ishizuka et al., 2006). Histamine plays a role in immune responses peripherally as well as in the gut, sleep regulation, sexual response as well as its role in food intake. These neurons project forward to VMH, PVH, and cortical sites through the medial forebrain bundle where they affect their actions on food intake (Ishizuka et al., 2006; Watanabe et al., 1983). ICV administration of histamine results in a suppression of food intake (Lecklin et al., 1998). These actions are thought to be modulated through the H_1 receptor and are part of the pathway of leptin-induced suppression of food intake (Ishizuka et al., 2006).

Serotonin

The serotonin system has long been associated with psychological well-being, cognition, temperature control, sleep and sexuality as well as appetitive systems. Perturbation of the 5-HT system results in mood, anxiety and depressive, panic, and obsessive-compulsive disorders. Generally, 5-HT modulates food intake through action

as a satiety factor (Heisler et al., 2003; Lam and Heisler, 2007). The 5-HT system in the rodent modulates food intake through widespread reciprocal innervation of many orexigenic and anorexigenic systems including the NPY and melanocortin systems. Chemical depletion of 5-HT in the brain has long been known to increase food intake and adiposity in rodents (Breisch et al., 1976; Samanin et al., 1977). In states of positive energy balance, such as the diet-induced obese rat, a decreased central turnover of 5-HT has been shown (Hassanain and Levin, 2002). In addition, obese humans are reported to have low plasma tryptophan indicating a depressed serotonergic tone (Ashley et al., 1985; Breum et al., 2003).

The principal source of brain 5-HT is the midbrain neurons of the nine raphe nuclei (Lam and Heisler, 2007). 5-HT is synthesized from tryptophan by the rate-limiting enzyme tryptophan hydroxylase (TPH). TPH2 is the predominant isoform in the brain, though TPH1 is expressed developmentally within the rodent hypothalamus as well as peripherally (Nakamura et al., 2006). After its release, 5-HT is shuttled back into neurons via the 5-HT transporter (5-HTT) and is eventually degraded through action of monoamine oxidase (MAO) to 5-HIAA. The 5-HT system is complex, regulated by autoreceptors as well as a host of pre- and post-synaptic receptor subtypes. Within the raphe, 5-$HT1_A$ autoreceptors modulate feedback of 5-HT stimulation from various inputs. Within the hypothalamus of rodents, 5-HT effects on food intake are mediated predominantly by presynaptic stimulation of anorexigenic POMC neurons via 5-$HT2_C$ receptors (Heisler et al., 2002) and a reduced inhibitory action through 5-$HT1_B$ receptors on NPY/AgRP neurons. In addition, postsynaptic 5-$HT1_A$ receptors, present in the VMH

also appear to have a role in the suppression of food intake and increased energy expenditure.

Though each of these monoamines exhibits ubiquitous central and peripheral expression, they have a genuine effect on food intake either directly or through modulation of ingestive-specific pathways. Because they affect so many different systems that have to do with psychological health, cognition and mood, they are particularly challenging to study in animal models because of the difficulty in segregating the various effects. However, this emphasizes even more strongly their overall importance in human health.

Hormonal influences

Blood-borne signals such as leptin, insulin and gut hormones exert influence on body-weight regulation, and much work has been done to elucidate their specific roles. Though experimentally, leptin levels are manipulated acutely as well as large pharmacological doses, leptin more accurately reflects long-term fat reserves whereas insulin is a short-term indicator of energy availability. Gut hormones also are part of the short-term feedback pathway that relays information concerning nutrient status through the GI tract.

Leptin is a 16kDa hormone product of the ob gene produced by adipocytes in proportion to adiposity reserves (Zhang et al., 1994). Deletions in the gene encoding leptin ($LEP^{(ob/ob)}$) that abolish leptin production result in hyperphagia, profound obesity and a variety of endocrine abnormalities both in rodents and humans (Montague et al., 1997; Ozata et al., 1999; Zhang et al., 1994). Leptin replacement in these animals and individuals ameliorates most of the abnormalities (Farooqi et al., 1999; Licinio et al.,

2004; Pelleymounter et al., 1995). Homozygous mutation of the receptor for leptin ($LEPR^{(db/db)}$) also causes early onset-obesity, a lack of pubertal development, reduced secretion of growth hormone and thyrotropin and immune dysfunction (Clement et al., 1998). Central and peripheral injections of leptin decrease food intake and even decrease the re-feed response after 48 hours of fasting. In obesity, leptin levels are high. However, with chronic hyperleptinemia, resistance develops. It is believed that in lean individuals, the transport of leptin across the BBB is already saturated and therefore, changes in peripheral leptin appear to be ineffective at producing hypophagia (Banks et al., 2006; Banks et al., 1996). Though issues of abnormal transport of leptin across the BBB in obesity have been considered (Banks et al., 1996; Kurrimbux et al., 2004), many hypothesize that it is an intracellular defect that sustains the leptin resistance (Enriori et al., 2007).

Insulin levels also directly correlate with levels of fat reserves and together with leptin convey a more complete picture of nutrient storage conditions (Bagdade et al., 1967). Insulin is a 5.7 kDa hormone produced by the β cells of the islets of Langerhans in the pancreas and released in response to rising levels of glucose and amino acids to regulate plasma glucose levels in response to a meal. The major actions of insulin are in the liver, adipose tissue and muscle though nearly all cells possess insulin receptors. Insulin acts to increase glucose uptake and storage at the cellular level. Insulin also functions to increase glycogen and fatty acid synthesis and decrease lipolysis and gluconeogenesis. Insulin effects in the brain are anorexigenic, as determined by administration of the hormone in the CNS and through central disruption of insulin receptors (Air et al., 2002; McGowan et al., 1990; Schwartz et al., 1992). Long-term

obesity is often tantamount to insulin resistance in both animal models and humans. As a result type II diabetes mellitus is typically preceded by marked hyperinsulinemia and increased food intake resulting in resistance to the effects of rising peripheral glucose. Ultimately the pancreas is taxed to the point of β-cell exhaustion.

Gut peptides are part of central short-term as well as paracrine feedback pathways

Though not pursued in the work of this dissertation, the role of gut peptides in the control of food intake cannot be understated. As more attention is given to the actions of the GI tract, more central-acting hormones and peptides are discovered. Among the most elucidated are ghrelin, cholecytokinin (CCK), peptide YY (PYY) and glucagon-like peptide (GLP-1). They have received prominence in recent years for their actions on the brain and periphery, though much is still unknown about their central effects. Furthermore, the role that dysfunction in these systems plays in the development of metabolic disease is yet to be fully elucidated.

DEVELOPMENT OF HOMEOSTATIC CIRUCUITS

Though many of the neuropeptide systems which control food intake are “born” *in utero*, in the rodent the maturation of these neurons and their participation in functional circuits occurs postnatally whereas in the primate, the maturation occurs *in utero*. This shift in timing potentially has many implications for the impact that maternal health and nutritional status versus milk quality and availability has on the developing offspring. Because rodents are used most extensively as the model system for work in energy balance and food intake, the ontogeny of the peptide-producing neurons has been most extensively studied in rodents and is greatly incomplete in NHP or human models.

Hypothalamic neurocircuitry

Considering the nutrient demands to meet the rapid growth of a neonate, the early appearance of NPY within development is not surprising. In the rodent, NPY mRNA is present in the brainstem and forebrain as early as embryonic day 14 (E14) (Allen et al., 1984; Woodhams et al., 1985). ARH NPY mRNA expression rapidly peaks at P15–16, and returns to levels observed in the adult by P30 (Singer et al., 2000a). Additionally within the hypothalamus, NPY mRNA is transiently expressed during development DMH, PVH, anteroventral periventricular (AVPV) and suprachiasmatic (SCN) nuclei as well as the medial preoptic area (MPO) and perifornical region (PFR) (Grove et al., 2001a; Grove et al., 2005a; Grove and Smith, 2003; Singer et al., 2000b). The significance of this largely developmental-specific expression of NPY in multiple hypothalamic nuclei has not been elucidated. Presumably in this context, NPY may act as a growth factor, further promoting neurogenesis and synaptogenesis in the target nuclei. In any event, the NPY projections of the ARH to other hypothalamic areas occur in a time-dependent manner, starting with projections to the DMH complete at P10-11, PVH complete at P15-16 and finally the LHA near weaning (Grove et al., 2001a; Singer et al., 2000a). Using DiI as a anterograde tract tracer, the progression of the outgrowth of these projections from the ARH to various hypothalamic nuclei was followed in a time-dependent manner through development (Bouret et al., 2004a). This illustrated the expansion of projections from the ARH to the DMH around P6, PVH at P8 and LHA thereafter. The DiI labeling of the fibers was indiscriminate of phenotype and therefore characterizes the projections from the nuclei as a whole.

Surprisingly, though AgRP is colocalized with NPY and its only site of localization within the CNS is the ARH, AgRP mRNA levels appear to be first detectible

during the first few postnatal days whereas NPY mRNA levels are detected earlier in gestation (Grove et al., 2003a; Nilsson et al., 2005). This emphasizes a possibly unique role for AgRP apart from providing an orexigenic drive earlier in development. Since these fibers are present as early as P2 , they do follow the projection pattern of innervation of NPY with innervation occurring first in the DMH at P10, then the PVH at P15 and finally the LHA at P21 (Grove et al., 2003a). However, because of their dual role in modulating the actions of satiety-producing peptides such as αMSH, their actions may refine the anorexigenic inputs of melanocortin innervation.

In a like manner to NPY, POMC neurons are present within the hypothalamus in the developing rat at E12-13, several days before their appearance in the pituitary (Bugnon et al., 1982; Khachaturian et al., 1983). Though the projections of POMC neurons become evident towards the end of gestation they do not reach their full maturity until weaning (Khachaturian et al., 1983). This perceived "delay" in maturation in comparison to NPY accentuates the primal orexigenic drive that exists in the developing animal with satiety projections maturing after the orexigenic projections. Early work in developmental biology showed that during gestation, endogenous αMSH functions to stimulate fetal growth (Swaab et al., 1978; Swaab et al., 1976). When antibodies to αMSH are administered in late gestation, fetal growth retardation results (Swaab et al., 1976). These data reveal substantial differences in αMSH actions during early development and adulthood, the former being growth promoting and the latter resulting in satiation. These actions have not been further pursued.

In the rodent MC4R mRNA is also detectible as early as E14 (Mountjoy and Wild, 1998). MC4R is considered the dominant melanocortin receptor in the fetal central

nervous system whereas MC3R becomes detectable postnatally (Kistler-Heer, 1998). Consequently, this developmental difference may reveal why such profound differences in phenotype exist between the MC3R and MC4R null animals. MC4R null animals are hyperphagic, hyperinsulenimic and hyperleptinemic resulting in overt obesity and diabetes. On the other hand, though MC3R animals are hyperleptinemic and hyperinsulinemic, they exhibit a more mild obesity in the presence of hypophagia (Butler and Cone, 2002).The late appearance of MC3R developmentally may underscore the divergence between its roles from that of MC4R. It is unknown however, what role MC4R plays in the sculpting of the appetitive circuits.

Also with anorexigenic functions, CART neurons appear as early as E11 (Brischoux et al., 2001). CART expression appears transiently in other brain areas until approximately E18; they are present solely along the ventricle as well as with a robust presence in the Edinger Westphal nucleus (Brischoux et al., 2001). It is reported that CART neurons may aid in neuronal migration of dopamine neurons during development (Brischoux et al., 2001). CART neurons are present in the LHA and produce peptide during the earliest postnatal days, however the population in the ARH produces very little peptide early-on and appears to mature between P11 and P15 (Grayson, unpublished). In the adult rodent, CART is known to colocalize with αMSH in the ARH and MCH in the LHA (Broberger, 1999).

Within the LHA, orexin and MCH also exert orexigenic influence over the hypothalamus. Orexin neurons appear to develop in the LHA of the rodent as early as E19 (Yamamoto et al., 2000). They have robust synaptic activity around parturition (Anthony N. Van Den Pol, 2001) but the projections do not fully develop until the 3rd

postnatal week (Yamamoto et al., 2000). Within the LHA of the rodent, MCH mRNA expression is detectible as early as E13 (Brischoux et al., 2001) and while fiber expression is detectible as early as E18, fiber projections are not readily detectable throughout the brain until P5 (Presse et al., 1992).

Brainstem Circuitry

In direct contrast to hypothalamic circuit maturation in the rodent, the brainstem neurocircuitry matures prenatally and with further refining and direct input from hypothalamic nuclei postnatally. Therefore, in the rodent, these circuits are conceivably far more sensitive to the maternal environment in comparison to the hypothalamic development which appears to be influenced more by postnatal milk availability. Because of the scope of this dissertation, the ontogeny of serotonin and norepinephine are discussed here.

In the rodent, serotonin-*ir* appears in the raphe at E12 (Lidov and Molliver, 1982) and subsequent expression of the serotonin transporter (5HTT) ensues shortly thereafter (Hansson et al., 1998; Zhou et al., 2000). In general, 5-HT and 5-HTT are richly colocalized throughout the axonal length which emphasizes the volume transmission release (Zhou et al., 2000). By E18-20, 5-HT-*ir* can be visualized throughout the brain (Zhou et al., 2000). Because of the ubiquitous expression of 5-HT in the brain, nearly every neuropeptide and neurotransmitter system is impacted by 5-HT terminal differentiation (Whitaker-Azmitia, 2001).

Similar to serotonin, noradrenergic neurons appear on E12 in the rat (Herlenius and Lagercrantz, 2004). In general noradrenergic-*ir* is far more robust earlier on in development in comparison to 5-HT-*ir* considering both phenotypes of neurons are born

at the same time (Loizou, 1972). In the rat, a wave of proliferation in fiber density and intensity occurs during the first postnatal week, with adult levels being reached by the third postnatal week (Loizou, 1972). In general, noradrenergic innervation is crucial to the proliferation of brain astrocytes and glia and therefore neuronal migration is affected throughout the brain when noradrenergic development is affected (Herlenius and Lagercrantz, 2004).

Leptin and insulin

Though leptin is largely considered an anorectic hormone, ARH neurons do not transmit a response to downstream sites in response to leptin injection during the first postnatal weeks (Ahima and Hileman, 2000). All in all, leptin does not appear to exert its effect on body-weight until after the third postnatal week corresponding with the development of ARH circuits (Proulx et al., 2002). Early studies in the ontogeny of leptin in the developing neonatal rodent showed that leptin levels increase more than 5 fold during the second postnatal week, independent of the amount of fat present (Ahima et al., 1998b). The physiological significance of this leptin surge on the development of hypothalamic circuits was refined through the use of the leptin-deficient mouse (Lep^{ob}/Lep^{ob}) (Bouret et al., 2004b). Overall, projections from the ARH are permanently disrupted in the Lep^{ob}/Lep^{ob} adult. However, in the neonate, treatment with exogenous leptin during development rescues the outgrowth of these projections suggesting the critical role of leptin during a critical window of development (Bouret et al., 2004b). Consequently, the natural leptin surge early in development is now interpreted to have neurotrophic actions on the maturation of hypothalamic circuits rather than the narrow interpretation of leptin as a satiation signal.

Recent work using a recombinant leptin antagonist during the time of the postnatal leptin surge results in adult-onset leptin resistance as well as increased overall adiposity and hyperleptinemia in addition to greater propensity to obesity and diet-induced weight gain (Attig et al., 2008). Though hypothalamic circuitry was not specifically studied, the importance of leptin to the development of these circuits in the rodent is further highlighted.

In addition to its other roles, insulin is also an important neurotrophic factor during development along with insulin-like growth factor (IGF) and brain derived nerve factor (BDNF) (Gerozissis, 2003). The local production of insulin within the hypothalamus and high level of receptor expression confirms the importance of insulin signaling for neuronal maturation in addition to its neuromodulatory functions. Insulin immunoreactivity and insulin receptors are present very early during fetal life (Kozak et al., 2000). However, the function of insulin development within the hypothalamus has not been emphasized in the literature predominately because of the difficulties in separating central actions versus the robust peripheral expression and activity of insulin.

All in all, the birth of the major neurotransmitter- and neuropeptide-producing neurons occurs during early gestation. However, their postnatal proliferation, obviously sensitive to the neurotrophic actions of leptin and potentially other growth factors occurs during the early postnatal period. The ramifications of the nutrition received during this period have direct implications for growth of the feeding neurocircuitry.

NEUROCIRCUITRY PUT TO THE TEST: DIETARY MANIPULATIONS EXACERBATE THE SYSTEM

Animal models

Many animal models have been developed to answer the questions concerning fetal and prenatal development and growth. Models resulting in an adult obese phenotype have relied on maternal overnutrition and undernutrition during gestation. These models change the hormonal and nutrient status of the mother are then reflected in fetal growth and development. Conversely, models of placental insufficiency result in suppressed blood flow and hence nutrients to the growing fetus in the absence of maternal health status being altered. This also leads to maturity-onset obesity due to a series of catch-up growth periods. Alternatively, obesity can also result from neonatal overnutrition and undernutrition. This can be accomplished by creating either very small litters of several pups where an abundance of milk is available, or very large litters where by milk availability is spread thinly among litter mates, thus limiting developmental growth but again spurring catch-up growth phases. Other groups have formula fed pups milk of varying compositions, thus controlling the nutritional quality of the milk. These are less natural models but do mimic the altered nutrition that might be received in formula-fed infants. In the following sections, each of these manipulations will be described in light of their impact on the origin of developmental obesity.

Maternal overnutrition

Models in which overall content and quality of the maternal diet is altered prior to and during gestation, result in long-lasting effects on energy balance in the offspring. Very specific and reproducible effects on brain neurochemistry and hormone expression occur with maternal high-fat diet (HFD) consumption in the rodent. Even in the rat fetuses exposed to maternal HFD, serum leptin and insulin levels are increased in addition to increased levels of hypothalamic NPY, AgRP, POMC and MC4R (Gupta et

al., 2008). Offspring in this type of model have higher insulin/glucose ratios, higher body fat percentage, and greater triglyceride levels at birth (Guo and Jen, 1995b). Early postnatal effects include elevated leptin and corticosterone and increased body-weight and adiposity (Walker et al., 2008). Another group using this model shows brain neuropeptide alterations including galanin, dynorphin and enkephalin level changes in the PVH and MCH and orexin in LHA (Chang et al., 2008). In addition, HFD consumption stimulated neuroepithelium to proliferate and preferentially become orexigenic peptide-producing neurons of the LHA of the offspring (Chang et al., 2008). Some groups have used a "junk food diet" which is high not only in fat but preferentially higher in sugars and salt. This diet results in enhanced adiposity in female offspring as well as a host of changes in metabolic parameters (Bayol et al., 2008). The use of sheep allows for the impact of maternal diet to be investigated during the time of hypothalamic development since the sheep is another model where hypothalamic development occurs *in utero*. In a study of 10 day infusion of glucose, circulating glucose and insulin levels were increased, in addition to POMC mRNA within the ARH (Muhlhausler et al., 2005). This indicates that the ability to respond to nutrient cues as well as eliciting early measures of a satiety response is intact. Following a diet of 40% increased nutrition only during the third trimester in sheep, adiposity increased as well as POMC levels (Muhlhausler et al., 2006). Though many groups have done studies similar to this investigating the critical developmental time points: fetal, neonatal, juvenile and adult life, what is certain from these studies is that a host of central as well as peripheral metabolic pathways are permanently dysregulated with maternal HFD consumption. Though the changes

observed in these models are described here, we do not know the mechanism by which these changes are triggered.

Maternal undernutrition

In studies in which maternal nutrient restriction is imposed during early and mid-gestation, obesity and energy balance dysregulation also results. This in many ways is paradoxical considering that overnutrition also results in obesity. However, though the result is similar, the mechanism may indeed be different. Offspring of dams placed on a 50% food restriction during gestation have a dampened leptin surge during the first two postnatal weeks in comparison to CTR animals and have suppressed POMC as well as reduction in endorphin-*ir* at weaning (Delahaye et al., 2008). These animals show decreased body-weight during early postnatal life. Since leptin has been shown to be crucial to the maturation of these circuits, dampened leptin appears to have disturbed wiring of the hypothalamus. Pregnant dams exposed to low protein diets during pregnancy give rise to offspring who have a preference for fatty foods as opposed to high protein and carbohydrate (Bellinger et al., 2004). By reducing caloric intake by the pregnant dam, the sparing that occurs to support fetal growth appears to result in wiring that favors fuel storage, perhaps for a future time of decreased energy availability.

Placental Insufficiency

Intrauterine growth retardation (IUGR) is a common gestational condition that results in increased infant morbidity and mortality. In animal models IUGR is accomplished through bilateral uterine artery ligation resulting in reduced blood flow to the placenta and placental insufficiency. This model has been characterized in the rodent as well as the sheep. IUGR rodents become glucose intolerant and insulin resistant at an

early age which eventually results in diabetes (Simmons et al., 2001). Another group has shown up to a 40% reduction in pancreatic β cell mass corresponding to the reduction in insulin content (Styrud et al., 2005). In the sheep, IUGR results in variety of metabolic alterations in the offspring which include accelerated "catch-up" growth after birth, reduction in lean body mass and adult obesity (De Blasio et al., 2007a; De Blasio et al., 2006; De Blasio et al., 2007b).

Neonatal overnutrition

By modifying litter sizes and thus making smaller litters, pups can overconsume maternal milk and thus gain weight rapidly during the early postnatal period (Duff and Snell, 1982; Faust et al., 1980). Maintenance of the dams on a normal diet allows for targeting of early neonatal overconsumption independent of maternal diet (Plagemann, 1999). Pups postnatally overfed in this manner have been characterized extensively; however the functional reason for their resulting tendency towards obesity has not been elucidated fully. These animals are hyperphagic and hyperleptinemic and hyperinsulinemic as early as P10 (Plagemann et al., 1992). They continued to have significantly increased body-weight in adulthood as well as increased blood pressure (Zippel et al., 2001). Young adult offspring from small litters appear to have a reduction in sensitivity to leptin (Davidowa and Plagemann, 2000a). We have also been able to demonstrate this as early as P16 (Glavas et al., 2008). Using p-STAT as an indicator of neuronal activation, we reported diminished neuronal activation in animals reared in a small litter (Glavas et al., 2008). Offspring reared in small litters also have a dampened suppression of firing in ARH neurons when exposed to insulin versus a more complete suppression in ARH neurons from control litters (Davidowa and Plagemann, 2007). This

indicates that early life overnutrition has functionally altered the tone of the neurons within the ARH by overall sensitivity to leptin and insulin and perhaps other nutrients such as fatty acids and glucose. Their altered ability to respond to incoming signals is further reflected in diminished fiber projections to sites of integration such as the PVH (Bouret, unpublished data). Other groups have entirely altered the content of milk by removing the pups from their dams and feeding them artificially. In this model pups are switched to a high carbohydrate milk during the postnatal period, they demonstrate hyperphagia and increased body-weight on a chow diet (Srinivasan et al., 2008). These effects are present in the next generation which requires a greater study of the possibility of genetic imprinting and epigenetic alteration (Srinivasan et al., 2008)

Neonatal undernutrition

One of the most widely used models of neonatal undernutrition is to alter pup milk availability by placing an extraordinary demand on maternal milk production through maintenance of very large litter (14-24 pups)(Crnic and Chase, 1978; Crnic and Chase, 1980). This model describes early stunted growth and overall lower body-weight early in life with catch-up growth and late-onset insulin resistance and glucose intolerance (Codo and Carlini, 1979; Nagy et al., 1977). These animals are thus exposed to inadequate nutrition during the time of hypothalamic development and have dampened levels of leptin during this critical time period.

Hypothalamic-specific changes as a result of diet-induced obesity during adulthood

In an attempt to understand the mechanisms of diet-induced obesity, research has focused on characterizing changes in the hypothalamus as a result of fat and calorie overconsumption. Differential expression of neuropeptides in the course of high-fat diet

consumption appears to occur in two phases: namely an early phase where feedback responses are still intact and a latter phase where counter-regulatory responses appear ineffective. In the short term, NPY/AgRP mRNA is decreased and POMC mRNA is greatly elevated (Torri et al., 2002; Ziotopoulou et al., 2000) However with long-term overconsumption, differences in feeding-related neuropeptides often diminish or are of a small magnitude (Elliott et al., 2004; Gao et al., 2002), which would be expected to promote or exacerbate body-weight gain .

Moreover, in rats selectively bred for polygenic traits predisposing them to obesity, a decreased projection pattern of ARH projections is seen in comparison to controls. In explants from these DIO animals, leptin fails to cause neurite outgrowth to the same extent as control animals. Thus, further evidence points to the anatomic disruption of ARH projections into adulthood due to polygenic cause of obesity on a moderate fat diet (Bouret et al., 2008).

Because the hypothalamus and specifically the ARH is at the crossroad between central and peripheral homeostatic mechanisms for body-weight regulation, the ARH responds to and conveys signals concerning nutrient availability and fat stores to downstream regions. Therefore monitoring of ARH-responsivity to these metabolic and hormonal cues is important for discerning the metabolic status of the animal.

CONCLUSION

Although numerous rodent studies have examined the effects of maternal nutrition on offspring, limitations exist in the usage of rodent models to study human forms of obesity. The critical period for feeding-related neurocircuitry in the hypothalamus occurs during the third trimester of fetal life in the human. At this juncture, the mother provides

a flux of nutrients as the fetus is unable to independently ingest. In contrast in rodents, this critical hypothalamic period occurs postnatally in the first three weeks of life and is therefore more susceptible to postnatal compared to prenatal maternal diet. This temporal difference supports the necessity of studies in a model more similar to the human. In the case of this dissertation, the nonhuman primate model was chosen for its similarity to the developmental patterns in the human.

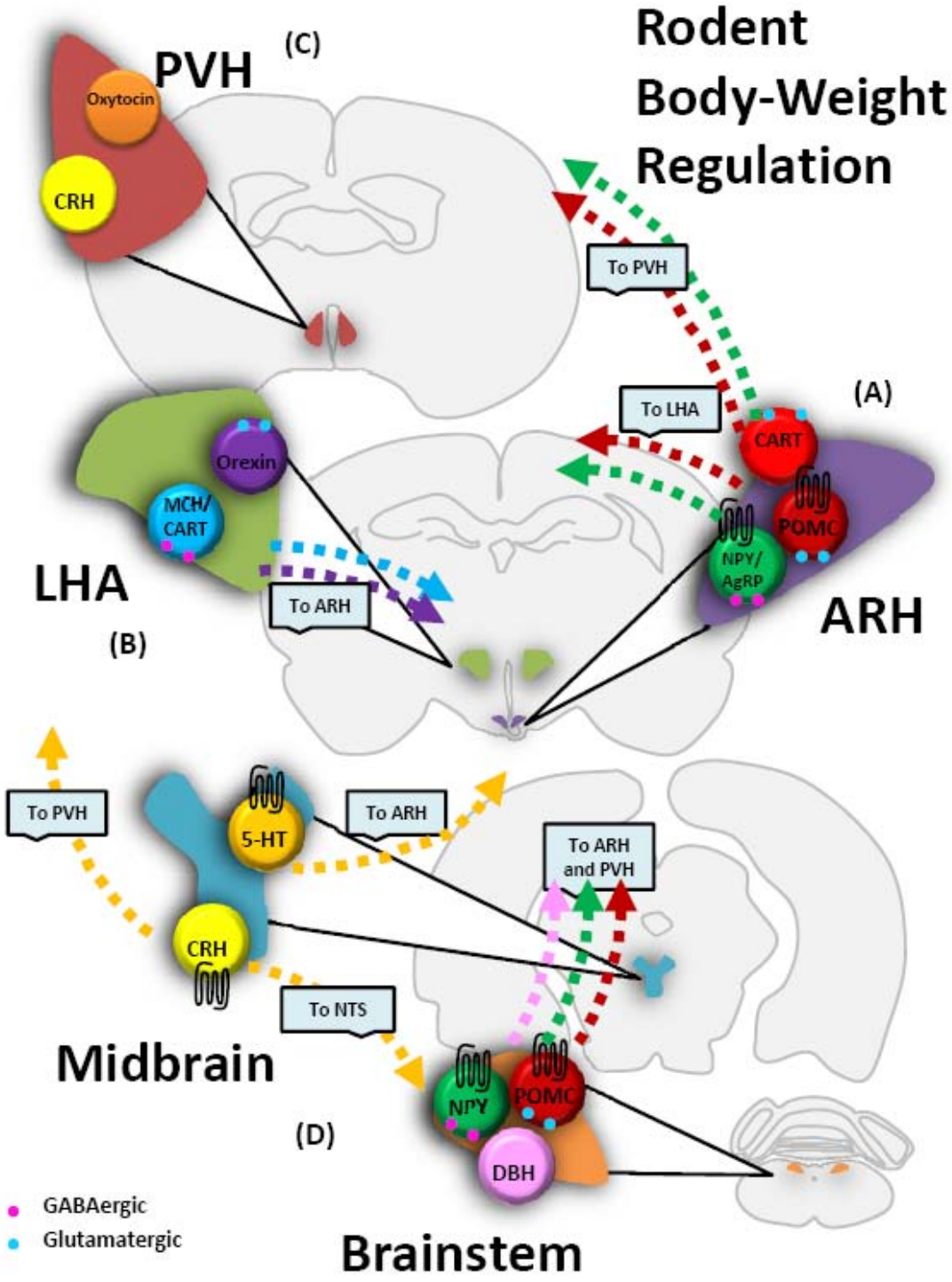


Figure 1-1: Body-weight regulation neurocircuitry in the rodent. Neuronal populations and projections in the ARH (A), LHA (B) PVH (C) midbrain and brainstem (D)

AIMS OF DISSERTATION AND APPROACH

The increase in obesity in prevalence and severity among children necessitates a need to better understand the development of the early origins of metabolic disease and the effects of the *in utero* environment, maternal diet and maternal health status on the growing fetus. Data from the rodent as well as growing evidence from epidemiological data suggests that the *in utero* and early postnatal environment have a significant sphere of influence on the maturation and function of the neurocircuitry governing body-weight regulation. Though a growing body of literature exists concerning the ontogeny of the neuropeptides and hormones which impact body-weight regulation in the rodent, critical differences in the timing of their maturation suggest the need for a model that mimics the human more closely.

The goal of this dissertation is to provide a characterization of the ontogeny of the major neuropeptide systems in a nonhuman primate model and then determine the impact of maternal HFD during gestation day 130, a critical time point for hypothalamic development (Figure 1-2).

Specific Aim 1: Characterize the ontogeny of feeding circuitry during normal NHP development with specific focus on NPY/AgRP and POMC development. (Chapter 2 and Appendix A and D)

A. When do the ARH projections develop in the NHP normally?

B. What species differences exist?

Hypothesis: The maturation of the feeding-related circuits would occur largely *in utero*.

***Approach*:** Using *in situ* hybridization and fluorescent immunohistochemistry, three developmental time points: G100, G130 and G170 were investigated.

Specific Aim 2: Examine feeding circuitry in the G130 fetus exposed to maternal high-fat diet (HFD) in comparison to control (CTR).

A. What hypothalamic neuropeptide and receptor systems are affected by HFD *in utero*? (Figure 1-2, Chapter 4, Appendix B and C)
B. Determine if hypothalamic changes can be prevented with maternal consumption of a low-fat diet during pregnancy? (Figure 1-2, Chapter 4)
C. How is the serotonin system affected in the midbrain and hypothalamus in fetal offspring of obese mothers? (Figure 1-2, Chapter 5, Appendix E)

Hypothesis: Major feeding neuropeptides will be affected by maternal overconsumption of an energy dense diet during gestation day 130. Reversal to a CTR chow diet during pregnancy will only partially ameliorate the neuropeptide expression in the hypothalamus due to continued maternal obesity.

Approach: Quantitative real-time PCR, *in situ* hybridization and fluorescent immunohistochemistry will be used to determine changes in expression on the mRNA and protein level between CTR and HFD.

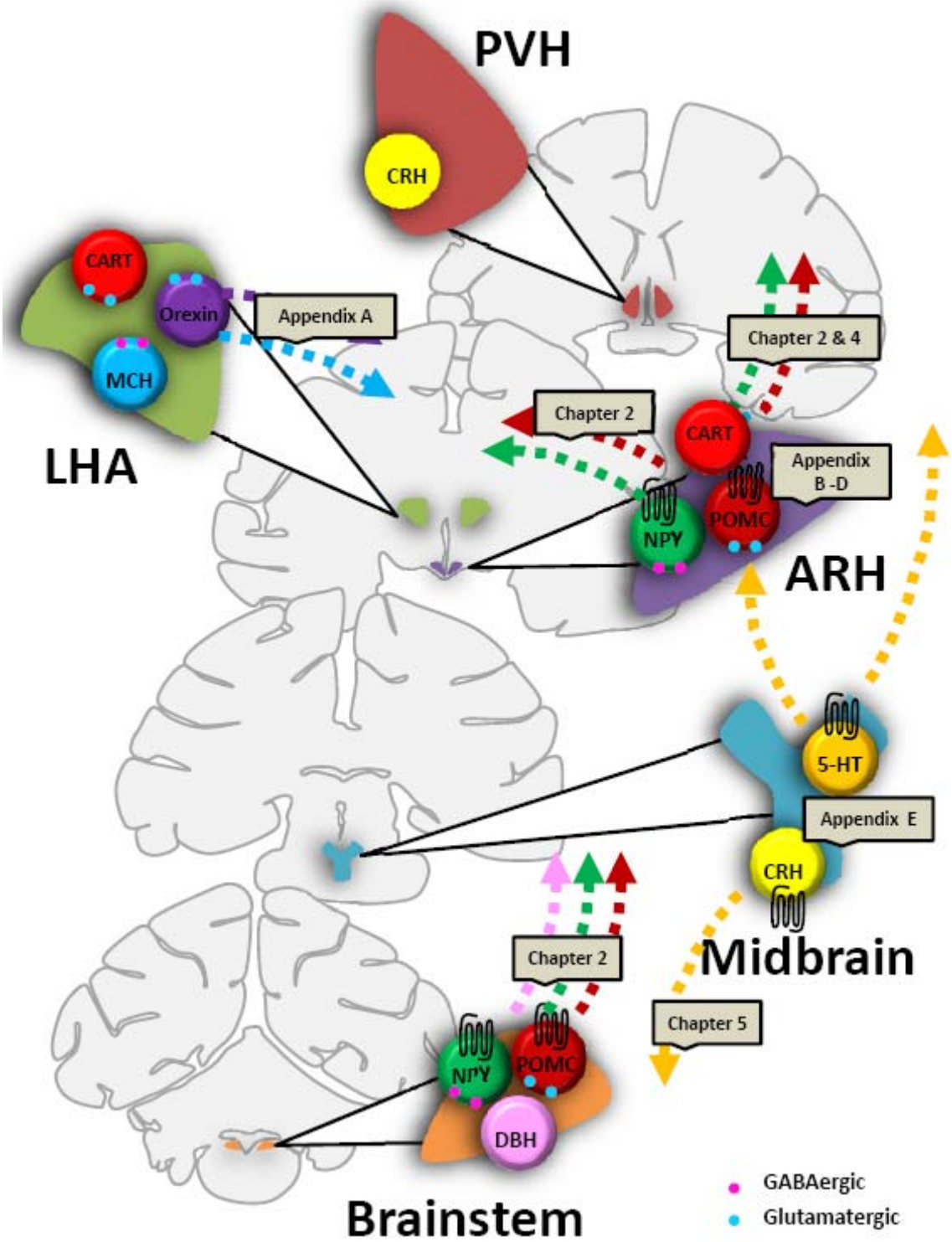


Figure 1-2. Summary of overall work encompassing the development of Body-Weight Regulation Systems in the Fetal NHP

Chapter 2

Prenatal Development of Hypothalamic Neuropeptide Systems in the Nonhuman Primate

Grayson, B. E., Allen, S. E., Billes, S. K., Williams, S. M., Smith, M. S., and Grove, K.L.(2006). Prenatal development of hypothalamic neuropeptide systems in the nonhuman primate. *Neuroscience* **143,** 975-86.

ABSTRACT

In the rodent, ARH-derived NPY and POMC neurons have efferent projections throughout the hypothalamus that do not fully mature until the second and third postnatal weeks. Since this process is likely completed by birth in primates we characterized the ontogeny of NPY and melanocortin systems in the fetal Japanese Macaque during the late second (G100), early third (G130) and late third trimesters (G170). NPY mRNA was expressed in the ARH, PVH, and DMH as early as G100. ARH-derived NPY projections to the PVH were initiated at G100 but were limited and variable; however, there was a modest increase in density and number by G130. ARH-NPY/AgRP fiber projections to efferent target sites were completely developed by G170, but the density continued to increase in the postnatal period. In contrast to NPY/AgRP projections, αMSH fibers were minimal at G100 and G130 but were moderate at G170. This study also revealed several significant species differences between rodent and the NHP. There were few NPY/catecholamine projections to the PVH and ARH prior to birth, while projections were increased in the adult. A substantial proportion of the catecholamine fibers did not coexpress NPY. In addition, CART and αMSH were not colocalized in fibers or cell bodies. As a consequence of the prenatal development of these neuropeptide systems in the NHP, the maternal environment may critically influence these circuits. Additionally, because differences exist in the neuroanatomy of NPY and melanocortin circuitry the regulation of these systems may be different in primates than in rodents.

INTRODUCTION

Neuropeptide Y/agouti-related peptide (NPY/AgRP) and pro-opiomelanocortin (POMC) neurons in the arcuate nucleus of the hypothalamus (ARH) function as key central regulators of food intake and homeostatic feedback control of energy balance in both rodents and nonhuman primates (NHP) (Grove et al., 2003b; Kagotani et al., 1989; Kalra et al., 1999; Koegler et al., 2001a; Porte et al., 1998; Schwartz et al., 2000), in addition to playing a role in the reproductive, neuroendocrine and stress axes (Bell et al., 2000; Campbell et al., 2001; Conrad and McEwen, 2000; Hokfelt et al., 1998; Kalra, 1986; Li et al., 1999; Li et al., 2000). Although NPY neurons are present throughout the brain in both the normal adult rodent and NHP, the major origin of hypothalamic NPY production is the ARH. The unique feature of ARH-NPY neurons is that they are the sole source of AgRP, which has become a useful marker of ARH-NPY neurons (Broberger et al., 1998b; Chen et al., 1999; Grove et al., 2003a; Hahn et al., 1998b). ARH-NPY/AgRP neurons have a broad range of projections throughout the hypothalamus and forebrain; however, it is the projections to the paraventricular nucleus (PVH), dorsomedial hypothalamic nucleus (DMH), perfornical region (PFR) and lateral hypothalamic area (LHA) that have received the most study in regard to their role in the regulation of energy balance. In the rat, a low level of NPY peptide is discernible as early as embryonic day 14, and immunoreactive (-*ir*) fibers in the ARH are present two days after birth. However, the projections of ARH neurons, including NPY/AgRP and POMC neurons, to downstream target sites do not start developing in the rodent until the end of the first postnatal week and do not fully develop until the third postnatal week (Bouret et al., 2004a; Grove et al., 2003a; Grove and Smith, 2003). Recently, it was also

demonstrated that ARH projections to the PVH do not fully develop in the leptin deficient *ob/ob* or the leptin receptor mutant *db/db* mice, suggesting that leptin is a critical factor for the development of ARH circuits (Bouret et al., 2004b). There is a natural leptin surge during the second postnatal week (Ahima et al., 1998a) of rodents, supporting its physiological role, and leptin replacement to *ob/ob* mice specifically during the second to third postnatal week restores the development of ARH projections (Bouret et al., 2004b).

While these circuits have been extensively studied in adult rodents, and more recently in the developing rodent, little has been done to elucidate development of these important energy homeostatic circuits in primates. In the present study, we report the presence of NPY mRNA in the ARH during late second and third trimesters of pregnancy. We characterized the development of ARH and brainstem NPY efferent projections during the late second and third trimester in the NHP, with a focus on the PVH as an important "feeding center". We also characterized the development of α melanocyte-stimulating hormone (αMSH) and cocaine and amphetamine related transcript (CART) projections from the ARH. Finally, we measured fetal and maternal leptin during late pregnancy to determine if there is a surge of leptin that correlates with the development of ARH circuits.

MATERIALS AND METHODS

Animals

All animals were maintained in outdoor/indoor group housing in a natural light schedule. Animals were given water and food *ad libitum* consisting of high protein monkey chow biscuits (Ralston Purina Co., St Louis, MO). All animal procedures were

approved by the Oregon National Primate Research Center (ONPRC) Institutional Animal Care and Use Committee.

Pregnant *Macaca fuscata*, Japanese macaque monkeys were identified by the Department of Animal Resources during routine observations of the animal colony. Initial pregnancy was determined by palpation and then confirmed by ultrasound examination 30 and 60 days following initial knowledge of pregnancy. Animals in the study were all multiparous and no twins were included in the study. Caesarean sections were performed at day 100, 130 or 170 fetal gestational age (full term is 175 days) by the trained surgical staff at ONPRC. The adult animals were allowed to recover under veterinary supervision before they were returned to the outdoor group housing. The fetuses were taken directly to necropsy and were deeply anesthetized with sodium pentobarbital (>30 mg/kg IV). The chest cavity was opened and the ascending aorta was bisected and the cardiovascular system was flushed with 0.9% saline containing 5,000 units of heparin/L to inhibit clotting. Trans-carotid perfusion of the head with 4% paraformaldehyde in buffered sodium phosphate (pH 7.4) was performed. The brain was removed, blocked and post-fixed overnight at 4°C in the same fixative and subsequently transferred for 24 hours to 10% glycerol buffered with NaPO4 and for 48 hours into 20% glycerol buffered with NaPO4. Tissue was frozen by submersion in < -50°C methylbutane and then stored in -80°C until sectioning.

Maternal and fetal leptin was determined by RIA (Linco, St. Charles, MO) as directed by assay protocol. Insulin levels were measured by an electrochemiluminescence immunoassay using an automated instrument (Elecsys 2010 immunoassay analyzer, Roche Diagnostics). The interassay CV was less than 5%.

Experiment 1: *In situ* Hybridization for NPY mRNA

Coronal hypothalamic sections (25 μm) were collected in 1:12 series using a freezing microtome. Sections were stored in ethylene glycol cryoprotectant at –20°C until time of use. One series of sections were wet mounted in RNAse free KPBS on Fisherbrand *Superfrost* slides. Animals were studied at approximate gestational days 100, 130 and 170 and as adults (5-7 years of age) (n = 3/group).

In situ hybridization for NPY mRNA was performed as previously described (Chen et al., 1999; Grove et al., 2001a). Briefly, a human preproNPY cRNA probe was transcribed from a 500bp cDNA in which 50% of the UTP was ^{35}S labeled. The saturating concentration of the probe used in the assay was 0.3 μg/ml/kb. Brain sections were fixed in 4% paraformaldehyde (pH 7.4), treated with protein kinase at 37°C to increase penetration, and then with 0.25% acetic anhydride in 0.1M triethanolamine (pH 8.0). Sections were then rinsed in 2X SSC, dehydrated through graded series of alcohols, delipidated in chloroform, rehydrated through a second series of alcohols and air-dried. The sections were exposed to the labeled probes overnight in a moist chamber at 55°C. After incubation the slides were washed in 4X SSC, in RNase A at 37°C, and in 0.1X SSC at 60°C. Slides were then dehydrated through a graded series of alcohols and dried. For visualization of the probe, labeled sections were exposed to sheet film (Biomax MR, Kodak) overnight. For histological analysis of the distribution of NPY mRNA, slides were dipped in Kodak NBT2 emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 in 600 mM ammonium acetate and then placed in light-tight boxes containing desiccant and stored at 4°C for 7 days. The slides were then developed and counterstained with cresyl violet and the distribution of silver grains analyzed using darkfield microscopy.

Darkfield Microscopy

Darkfield images of silver grains were captured using a Nikon Eclipse E800 Microscope coupled with a CoolSnap HQ camera (Photometrics, Westchester, PA) along with Metamorph Software (Universal Imaging Corp). Objectives used were Nikon Plan Fluor 4X (NA 0.13). The images were further processed using Adobe Photoshop.

Immunofluorescence

Double-label immunofluorescence

Coronal hypothalamic sections (25 μm) from the same animals as above were used for immunofluorescence studies. Animals were studied at approximately gestational days 100, 130 and 170 and as adults (5-7 years of age) (n = 3/group). Double-label immunofluorescence coupled with confocal microscopic analysis was performed as previously described (Campbell et al., 2001; Grove et al., 2000).

Experiment 2: ARH-NPY fiber projections

NPY fibers from the ARH were identified as those colocalized with AgRP. To characterize the development of ARH-NPY/AgRP efferent projections, a cocktail of goat anti-NPY (a kind gift of P. Larsen; 1:5,000), and a guinea pig anti-AgRP (produced by Antibodies Australia, Melbourne, Australia; 1:5000) was used. Briefly, tissue sections were removed from cryoprotectant and washed in 0.05 M potassium phosphate-buffered saline (KPBS), preincubated in blocking buffer (KPBS + 0.4 % triton X-100 + 2% normal donkey serum) for 30 min at room temperature, followed by incubation with AgRP and NPY-specific antibodies in blocking buffer for 48 h at 4°C. Following washes in KPBS, tissue was incubated for 1 h in biotinylated donkey anti-guinea pig antibody (1:600, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and subsequently

washed and incubated in avidin-biotin solution (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA) for 30 min. The AgRP-*ir* signal was further amplified using a commercial kit (tyramide signal amplification-indirect kit; NEN Life Sciences Products, Boston, MA). AgRP-enhanced-*ir* was visualized using Alexa 568 (red) conjugated to streptavidin (1:1,000, Molecular Probes, Eugene, OR). For NPY colocalization, NPY-*ir* was visualized with Alexa 488 (green) conjugated to a donkey anti-goat antibody. (1:200, JacksonImmuno Lab, West Grove, PA)

Experiment 3: Brainstem NPY fiber projections

NPY fiber projections from the brainstem were identified as those colocalized with dopamine β hydroxylase—a marker for catecholamine neurons (DBH). To characterize the development of brainstem derived NPY, a cocktail of mouse DBH (Chemicon; 1:5,000) and rabbit anti-NPY (a kind gift of S. Brown, 1:75,000) was used. DBH-*ir* and NPY-*ir* were visualized by direct labeling with TRITC donkey anti-mouse and FITC donkey anti-rabbit, respectively (1:200, JacksonImmuno Lab, West Grove, PA).

Experiment 4: αMSH and CART fiber projections. To characterize the development of POMC and CART efferent projections, a cocktail of sheep αMSH (Chemicon; 1:5,000) and a rabbit anti-CART (a kind gift of N. Vrang, 1:80,000) was used. The protocol for signal enhancement for CART was similar to that described above for AgRP. CART-*ir* was visualized with streptavidin Alexa 488 (1:1,000, Molecular Probes, Eugene, OR). For αMSH-*ir*, TRITC 568 (red) was used conjugated to a donkey anti-sheep antibody (1:200, JacksonImmuno Lab, West Grove, PA).

Computer Assisted Line Drawings

Single-label immunohistochemistry for AgRP was done using DAB chromagen enhancement. *The Rhesus Macaque Brain Atlas* by Paxinos was used to match hypothalamic levels in the adult and G130 animal at levels -5.0, -5.5, -6.0 and -6.75 caudal to bregma. Using a Nikon Eclipse E800 Microscope coupled with a CoolSnap HQ camera (Photometrics, Westchester, PA) adjacent images of single-labeled AgRP-*ir* were snapped at 20X using Metamorph Software. Montages were constructed using Adobe Illustrator. Lines were drawn over contiguous fiber projections and terminal axonal fields using the pencil tool. AgRP-*ir* was used as a marker of ARH-NPY.

Confocal Laser Microscopy

Confocal laser microscopy, as previously described (Campbell et al., 2001; Grove et al., 2000; Li et al., 1999; Li et al., 2000), was used to analyze the double-label immunofluorescent images. The Leica Corp. (Germany) TSC SP confocal system consisted of a RBE inverted microscope equipped with an Ar laser producing light at 488 nm (for visualization of FITC and Alexa 488) and a Kr laser producing light at 568 nm (for visualization of Alexa 568). Various objectives (25X/NA 0.75 and 40X/NA 1.25) were used to scan the images. For each experiment, fluorophore signals were checked individually for bleed through to the apposing detector. All bleed through was eliminated by adjusting the laser intensity and detector window width. A series of continuous optical sections, 0.5 μm intervals along the z-axis of the tissue section, were scanned for each fluorescent signal. The signals were obtained for each fluorophore on one series of optical sections and stored separately as a series of 512 x 512 or 1024 X 1024 pixel images. The images were then processed with the MetaMorph Imaging System (Universal Imaging Corp., West Chester, PA). The confocal images are presented as

projections of a stack of sequential optical slices totaling 10 μm of the section. The brightness and contrast of the images were adjusted in Photoshop (Adobe Systems Inc., San Jose, CA).

RESULTS

Hormonal environment:

Fetal leptin levels were low at G100 and G130 compared to G170 *(Figure 2-1)*. However, there were dramatic differences in fetal body-weights between the gestational ages (G100, 172g ± 12; G130, 414g ± 37; G170, 636g ± 39). Since body fat levels were not measured, it is difficult to determine if leptin levels correlate with body fat as they do in adult animals. Furthermore, it is unknown how much of the leptin is actually coming from the fetus or from the placenta. Fetal insulin levels did not significantly change at the three gestational ages (G100 – 6.6 μU/ml ± 2.6; G130 – 6.4 μU/ml ± 1.1; G170 – 4.7 μU/ml ± 2.5).

Maternal leptin was elevated throughout the third trimester (G130 and G170) compared to the second trimester (G100), but was highest during the late third trimester (G170) *(Figure 2-1)*. However, like humans, most of this increased leptin secretion during the third trimester is likely due to placental leptin secretion (Sagawa et al., 2002; Schubring et al., 1997).

NPY mRNA expression in the hypothalamus

As previously shown (Grove et al., 2003b), adult animals displayed intense NPY expression in the ARH and low levels in the PVH and scattered cells in the area of the DMH. Similar to the adult, NPY mRNA was expressed within the ARH at all fetal ages investigated, and exhibited intense expression in both G100 and G130 fetuses.

Furthermore, scattered NPY mRNA expressing cells were present in the PVH at G100; however, these neurons were not evident in the older gestational ages. The reason for this is unknown, but even in adult animals the levels are low in the PVH in unfasted animals and, therefore, may be simply due to detection sensitivity.

ARH-NPY/AgRP Cell Bodies

Previously, our group, and others, have shown that AgRP is expressed in the vast majority of ARH-NPY neurons (Chen et al., 1999; Hahn et al., 1998b; Haskell-Luevano et al., 1999). This has been shown for both rodents and NHPs. In the present study, we used AgRP-*ir* as a marker of ARH-NPY projections (Broberger et al., 1998b; Grove et al., 2003a). Similar to previous data (Haskell-Luevano et al., 1999), there was an abundance of NPY/AgRP fibers in the ARH of the adult monkey; however, NPY-*ir* cell bodies were not evident, while many AgRP-*ir* cell bodies could be detected with this antibody *(Figures 2-3 and 2-4)*. This is a common limitation of this technique in which the peptide is rapidly transported from the cells bodies and not accumulated. It is difficult in the normal adult animal to detect peptide within the cell bodies without colchicine treatment, which could not be done in these NHP studies. But, while there was an abundance of double-label NPY/AgRP-*ir* fibers (shown as yellow) within the ARH of the adult monkey, it is worth noting that there is also an abundance of single-label NPY-*ir* fibers as well *(Figure 2-3)*. These results contrast with low levels of single-label NPY-*ir* in the ARH of the rat (Broberger et al., 1998b; Grove et al., 2003a), and suggest that another source of NPY significantly contributes to the peptide within the ARH of the NHP (see below).

Even though there was an abundance of NPY mRNA in the ARH at G100 (late second trimester), only lightly labeled NPY/AgRP-*ir* cell bodies were detected at this age. Furthermore, in contrast to older ages, only scattered NPY/AgRP-*ir* fibers were present, as well as scattered single-label NPY-*ir* fibers. This indicates that the ARH at G100 may not be fully developed, which is in agreement with the development of the ARH in the human fetus (Koutcherov et al., 2003).

By G130, an abundance of AgRP-*ir* and some NPY/AgRP-*ir* cells were evident throughout the ARH. The readily detectable cell bodies are likely due to increased expression and decreased transport of the peptide out of the ARH. There was also a moderate concentration of single-label NPY-*ir* fibers evident at this age, indicating that another source of NPY is innervating the ARH. By G170, the density of both double-label NPY/AgRP-*ir* and single-label NPY-*ir* fibers increased compared to earlier ages; however, they still had not reached the levels seen in the adult.

ARH-NPY Projections to PVH

In the adult hypothalamus there was an abundance of NPY/AgRP-*ir* and single-label NPY-*ir* fibers within all hypothalamic regions, with the highest concentration in the PVH *(Figure 2-4)* and DMH and lower levels in the LHA. However, qualitatively the relative concentration of NPY/AgRP fibers (originating from the ARH) to single-label NPY fibers (originating from other sources, i.e., PVH, DMH or brainstem) is low compared to that observed in the rat (Broberger et al., 1998b; Grove et al., 2003a). This indicates that in the NHP more of the NPY inputs into the PVH may come from sources other than the ARH.

As early as G100, NPY/AgRP-*ir* fibers were present in the PVH *(Figure 2-4)* and other hypothalamic areas, although they were relatively sparse. Single-label NPY fibers were also sparsely distributed throughout the PVH at this age. The density of NPY/AgRP-*ir* fibers greatly increased at G130 and G170. Again, the levels of both NPY/AgRP and single-label NPY fibers at G170 were less than the density in adults, particularly for NPY/AgRP. These data are shown schematically by computer assisted line drawing of AgRP-*ir* indicating the extent of ARH-derived NPY to hypothalamic nuclei at G130 and in the adult *(Figure 2-5)*. This indicates that ARH projections begin developing during the early third trimester of pregnancy in the NHP.

Brainstem NPY/Catecholamine Projections

As discussed above, an abundance of single-label NPY-*ir* fibers were observed within the ARH, PVH and other hypothalamic regions at all ages. It is well known that NPY/catecholamine neurons from the brainstem project throughout the hypothalamus. To determine if this is true in the NHP brainstem, we performed a preliminary study for double-label immunofluorescence for NPY and DBH in the brainstem. Within the solitary tract nucleus (NTS) and the ventral lateral medulla (VLM) there were many double-label fibers; however, single-label NPY-*ir* and DBH-*ir* fibers were readily evident throughout the brainstem *(Figure 2-6, A)*. The source of the single-label NPY-*ir* fibers in the brainstem are unknown and may originate from the ARH, PVH or even locally within the brainstem. NPY-*ir* neurons were readily detectable in the NTS and VLM; however, visualization of catecholamine cell bodies with this DBH antibody was very limited, thus we were unable to confirm that all NPY-*ir* cell bodies in the NTS and VLM were indeed catecholaminergic. These results indicate that it is not possible to completely

characterize all of the brainstem NPY projections simply by colocalization with DBH as is possible in the rodent. However, it remains true that NPY colocalized with DBH marks brainstem-derived NPY. Keeping this in mind, we characterized the development of NPY/DBH projections into the PVH and ARH in the NHP. Surprisingly, while there was a moderate scattering of NPY/DBH-*ir* fibers observed in the PVH of the adult NHP, there was an abundance of double-label fibers in the ARH *(Figure 2-6, C,E)*. Furthermore, the majority of DBH-*ir* in the ARH was colocalized with NPY, while the presence of single-label DBH-*ir* fibers was more abundant in the PVH.

Very few NPY/DBH fibers were observed in the hypothalamus at any gestational age, this included sparsely scattered NPY/DBH fibers in the ARH or PVH at near term (G170; *Figure 2-6, B,D*). This suggests that much of the NPY-*ir* present within the hypothalamus during fetal development likely comes from the brainstem. However, as mentioned above, it is possible that some brainstem NPY-*ir* fibers may not coexpress DBH.

αMSH and CART are not colocalized in the NHP

The primary source of αMSH is the ARH, while a small subpopulation of αMSH (POMC) neurons, resides in the solitary tract nucleus (NTS). In the rodent, nearly all αMSH neurons in the arcuate nucleus co-express cocaine and amphetamine related transcript (CART) (Vrang et al., 1999b). Additional CART neurons are present throughout the hypothalamus, including the PVH, LHA and DMH. Furthermore, while CART and αMSH neurons are both present in the NTS (Appleyard et al., 2005; Ellacott and Cone, 2004; Vrang et al., 1999b), these two neuropeptides are not colocalized in this nucleus. Therefore, in the rodent, colocalization of αMSH and CART peptides in

terminal fields is a marker of ARH-derived αMSH. In this study, we attempted to utilize this technique to characterize the development of ARH-αMSH projections through its colocalization with CART. However, while in the adult NHP we observed an abundance of both αMSH-*ir* and CART-*ir* fibers throughout the hypothalamus, these neuropeptides were never colocalized in fibers or cell bodies, at least within the sensitivity of our assay (*Figure 2-7*). Furthermore, single-label *in situ* hybridization showed an abundance of CART mRNA in the PVH, SON and LHA, with very low levels in the ARH (*data not shown*). There was, however, evidence of close appositions between αMSH-*ir* and CART-*ir* fibers within the PVH of the adult NHP, suggesting that these two populations of neurons may be interacting at efferent target sites. CART-*ir* fibers were sparse in the hypothalamus at G100 (*data not shown*), but were relatively abundant by G130 (*Figure 2-7*). In contrast, αMSH-*ir* neurons were evident in the ARH as early as G100 and G130 (*Figure 2-7* bottom panels); however, αMSH-*ir* fibers did not show significant innervation of other hypothalamic sites, such as the PVH at these times (*Figure 2-7* top panels). By G170, αMSH-*ir* fibers were evident throughout the hypothalamus but were moderate compared to the adult. These findings suggest that ARH-αMSH projections may not develop until after ARH-NPY/AgRP projections.

DISCUSSION

During the first 2 weeks in the developing rodent, there are striking developmental changes in the NPY system. Most notably, there is a transient expression of NPY mRNA in several hypothalamic areas, including the DMH, PFR, PVH and LHA in addition to the normal expression in the ARH (Grove et al., 2001a; Grove et al., 2005a; Grove and Smith, 2003; Singer et al., 2000b). Furthermore, we and others have

demonstrated that while NPY-*ir* fibers are present throughout the hypothalamus at birth, the ARH-NPY projections do not develop until the second and third postnatal week (Bouret et al., 2004a; Grove et al., 2003a). Finally, a surge of leptin that occurs in the early postnatal period (Ahima et al., 1998a) appears to be critical for the full development of the ARH circuits, including both NPY/AgRP and MSH in the rodent (Bouret et al., 2004b).

In contrast to the rodent, the present study demonstrated that there is an abundance of NPY/AgRP fibers throughout the hypothalamus at birth in the NHP, indicating that these projections develop prior to birth. Indeed, as early as the late second trimester (G100), NPY mRNA expression (*Figure 2-2*) was high in the ARH and scattered NPY/AgRP fibers (*Figure 2-3 to 2-5*) were evident throughout the hypothalamus. The density of these projections greatly increased during the third trimester leading up to birth (*Figure 2-3 to 2-5*). However, the density of projections at near term (G170) is less than that observed in the adult, suggesting that there is further development in the density of projections during the postnatal period, which is in agreement with the reports of others (El Majdoubi et al., 2000). These findings indicate that the late second and early third trimesters may be a critical period for the development of ARH circuits, which are key for homeostatic feedback regulation of body-weight (Saper et al., 2002a; Schwartz et al., 2000). This also indicates that maternal health (obesity and diabetes) and diet could have a critical impact on the development of these projections; this remains to be investigated. In contrast, the postnatal environment (postnatal nutrition) may have a greater impact in the rodent (Grove and Smith, 2003).

In the current study we found that fetal leptin levels were very low to undetectable during the late second (G100) and early third (G130) trimester and did not increase until right before birth (*Figure 2-1*), this low leptin is consistent with the near absence at G100 and G130 of white adipose tissue which develops during the 3rd trimester (unpublished observation). Therefore, in the NHP, there was no evidence of a leptin surge prior to the development of ARH projections. However, alternative explanations need to be considered. Foremost, even as early as G100, there were already low levels of ARH-NPY/AgRP-*ir* fibers in the PVH and DMH; therefore, the leptin surge that initiates the development of these projections may occur prior to G100.

Similar to the rodent, not all of the NPY-*ir* within the hypothalamus of the NHP comes from the ARH, as indicated by the significant concentration of single-label NPY-*ir* fibers (*Figures 2-3 and 2-4*). In fact, in the NHP hypothalamus, a qualitatively higher proportion of NPY fibers were not colocalized with AgRP than typically seen in rodents (Broberger et al., 1998b; Grove et al., 2003a). In the rodent, the other main source of NPY within the hypothalamus, including the PVH and ARH, are NPY projections from the brainstem, predominantly the A1/A2 regions (Chronwall et al., 1985; de Quidt and Emson, 1986; Everitt et al., 1984; Grove et al., 2003a). However, in the NHP, a limited proportion of the NPY fibers within the PVH at any of the ages investigated were colocalized with DBH (a marker of catecholamine fibers). In contrast, there was an abundance of NPY/DBH-*ir* fibers within the ARH in the adult NHP. While it is likely that not all brainstem NPY projections co-express DBH, their colocalization does identify NPY/catecholamine projections. These findings indicate that NPY/catecholaminergic neurons in the brainstem may preferentially innervate the ARH in the NHP, while in the

rodent there is a much broader projection pattern. Alternatively, in the NHP, it has not been determined whether all NPY produced in the brainstem strictly colocalizes with catecholamines, as has been shown in the rodent. The physiological importance of this species difference is unknown, but there are other examples of species differences in brainstem projections. Our group has also shown that glucagon-like protein (GLP) neuron projections from the NTS also preferentially innervate the ARH of the NHP, while in the rodent the primary projections are to the PVH and DMH (Tang-Christensen et al., 2001; Vrang and Grove, 2004). Therefore, primates may have a unique communication between brainstem neurons and ARH 1st order homeostatic sensory neurons, whereas in the rodent this interaction may occur in efferent target sites at the level of 2nd order neurons.

In the rodent, brainstem efferent projections to the hypothalamus develop in the early postnatal period (Rinaman, 2001; Rinaman, 2003), prior to the development of ARH projections; this includes the NPY/catecholamine projections, which are intact by P2 (Grove et al., 2003a). However, in the NHP, NPY/catecholamine fibers in the ARH were rare to sparse at G100 and G130. It is not until G170 (late third trimester), after the development of ARH projections that brainstem NPY fibers become common. Even at this late gestational age, the concentration of fibers is minimal compared to the adult (*Figure 2-6*). It is recognized that brainstem NPY/catecholamine neurons play several physiological roles, including stress responsivity, regulation of reproductive neuroendocrine axis and food intake. Thus, it is difficult to predict the physiological importance of the late gestational and early postnatal development of these projections. It

is also recognized that the catecholaminergic projections to the hypothalamus may actually be intact earlier in the third trimester, but that they do not express NPY.

While there was little NPY/AgRP or NPY/DBH fibers in the PVH at G100, there was an abundance of single-label NPY fibers. The source of these fibers is unknown but the expression in PVH neurons at this fetal age is consistent with local production. To date, nothing is known about the efferent targets of the PVH-NPY neurons. Our group has shown that these neurons are activated by fasting, indicating that they are responsive to changes in metabolic balance, or the stress of fasting, but there is no evidence that they express leptin receptors or directly respond to leptin. It is possible that these neurons are not responsive to peripheral metabolic signals, such as leptin, insulin and gut peptides, and thus are not part of the homeostatic feedback circuit.

One of the other significant species differences that became evident from this study is the lack of colocalization of αMSH with CART. In the rodent ARH-αMSH neurons coexpress CART (Vrang et al., 1999a), while αMSH neurons in the brainstem (NTS) do not. Our goal for these studies was to utilize CART as a marker of ARH-αMSH projections in the NHP (as was done with NPY/AgRP); however, this was not possible since CART and αMSH peptides were never colocalized in fiber terminals in the hypothalamus. We were able to detect scattered CART-*ir* neurons in the ARH of the adult NHP and these were never co-labeled with αMSH-*ir* (*Figure 2-7*). *In situ* hybridization showed a relatively low level of CART mRNA in this region as well (unpublished observation). This is in contrast to Elias et al. who reported a dense population of CART-*ir* neurons in the ARH of the human brain (Elias et al., 2001a). Unfortunately, this study did not investigate colocalization of CART with αMSH. Therefore, it was not possible

to determine the relative sources of αMSH fibers in the PVH during the different fetal ages. Interestingly, although ARH-NPY/AgRP-*ir* fibers were readily detectable in the PVH by G130, αMSH-*ir* fibers were very sparse at this age. By near term (G170), αMSH fibers in the PVH were abundant. Within the ARH, αMSH-*ir* neurons were easily detected by G130, indicating that the peptide is indeed produced but that projections to the PVH may not have developed yet. This raises the possibility that there may be a different ontogeny for the development of the orexigenic (NPY/AgRP) and anorexigenic (αMSH) projections out of the ARH.

In summary, the studies presented in this report demonstrate two key new findings. 1) The development of ARH-NPY occurs during the third trimester in the NHP, which is in contrast to the postnatal development in rodents. Therefore, health and dietary issues that arise during the third trimester may have long-term consequences on the development of these circuits. 2) There are several important species specific differences in neuronal projections between the NHP and rodent. The ARH is the major target of brainstem NPY/catecholaminergic projections in the NHP as opposed to the PVH and DMH in rodents. Furthermore, CART protein was undetectable by immunohistochemistry in αMSH neurons or fibers in the hypothalamus of the NHP, which is in contrast to the rodent. These species differences may be important in understanding the potential of therapeutics/interventions for the treatment of obesity in humans.

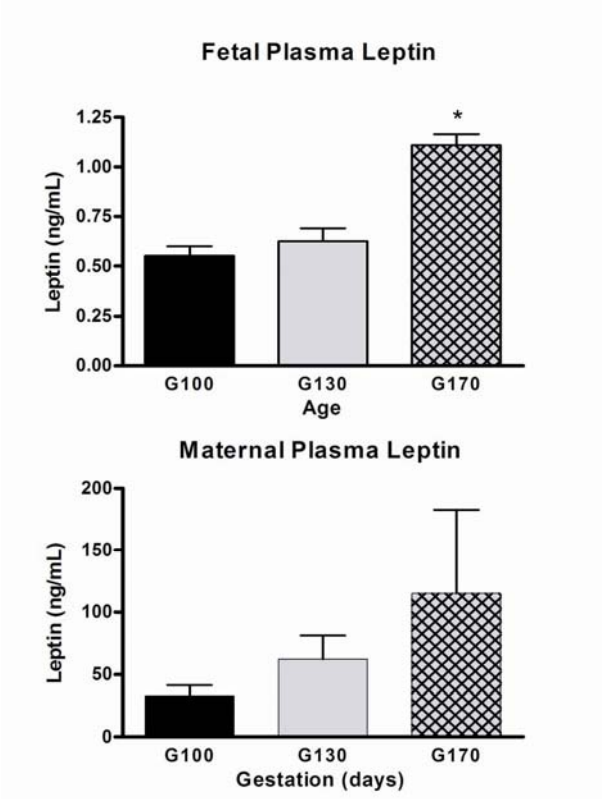


Figure 2-1. Leptin levels during development. Fetal and maternal leptin levels increase with prolonged gestational age. Values represent the mean ± SEM. Asterisk indicates significant difference by ANOVA. ($P<0.05$).

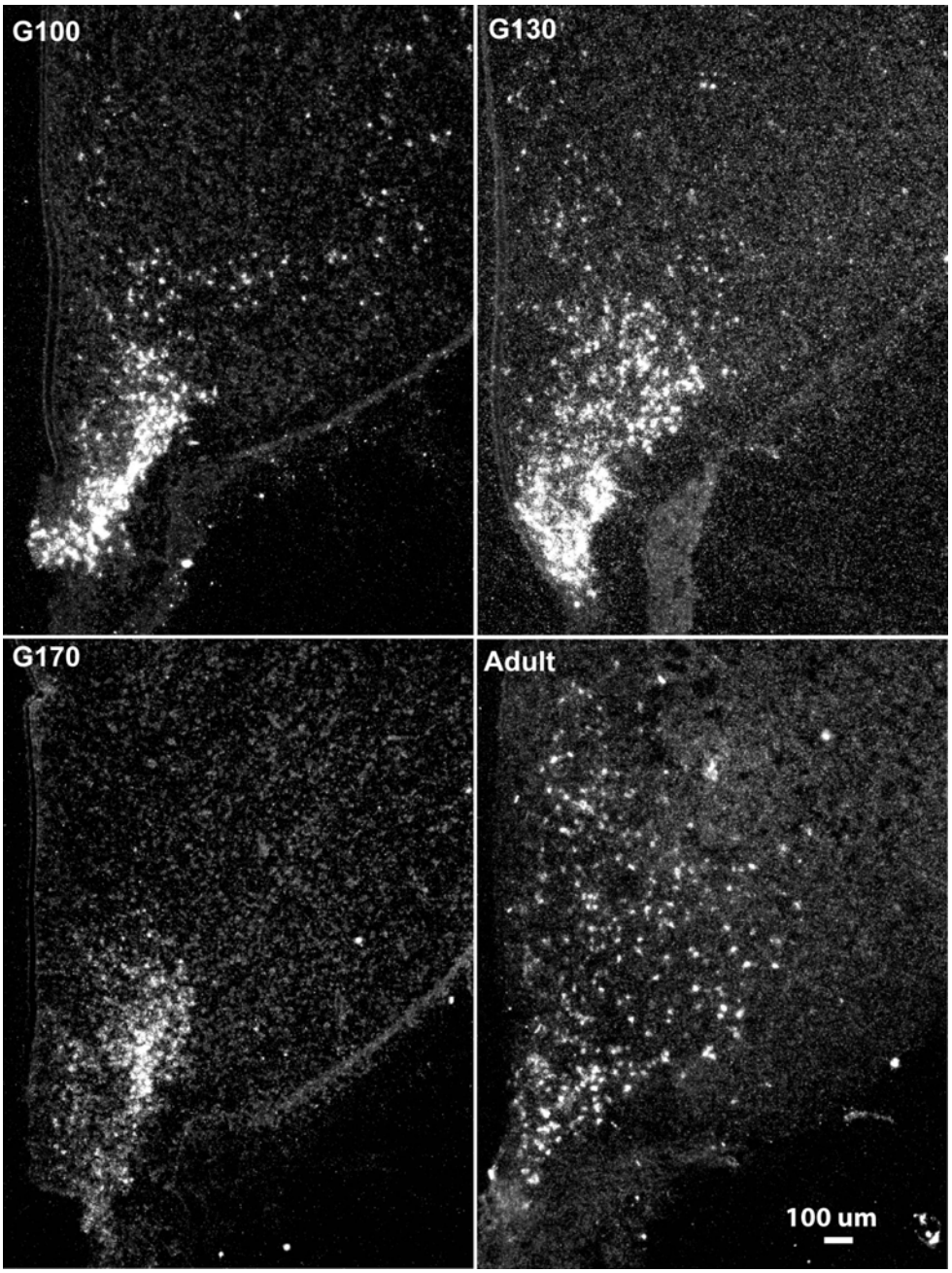


Figure 2-2. Prenatal development of NPY. Images are dark-field photomicrographs of silver grains representing human preproNPY cRNA probe labeling of ARH NPY. NPY mRNA appears to be highly expressed at all age groups investigated. These images correspond to -6.0 with respect to bregma according to Paxinos atlas. Images were captured at 4X magnification.

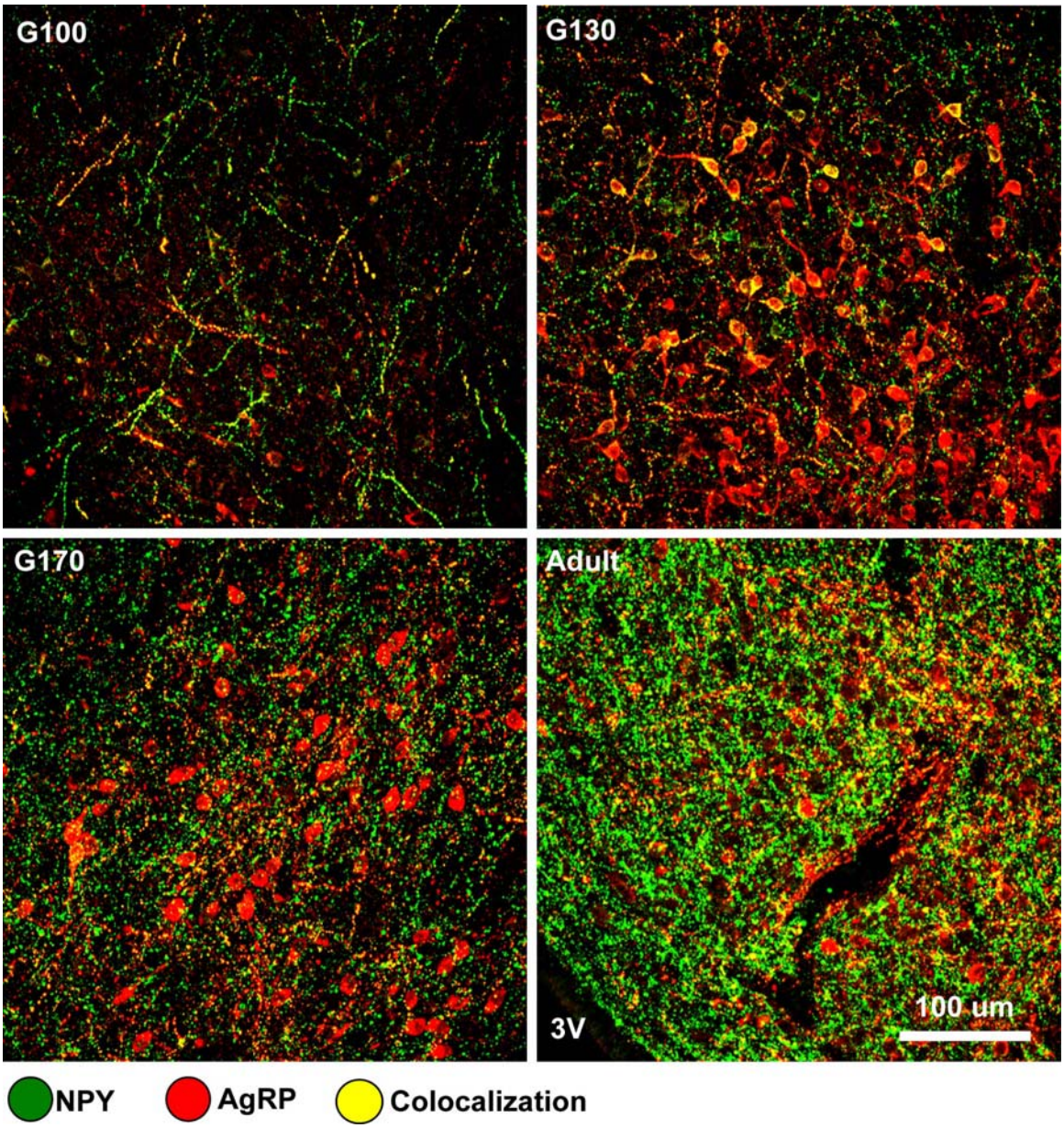


Figure 2-3. Development of NPY/AgRP neurons in the ARH. Figures represent color confocal digital images of double-label immunofluorescence for NPY (green) and AgRP (red), where colocalization is represented as yellow. AgRP immunoreactivity was always colocalized with NPY. NPY/AgRP-*ir* is detectable at G100 but sparse NPY/AgRP-*ir* cell bodies were visualized. Increased density of fibers and soma appear with increased animal age. Note that NPY-*ir* is not readily detectable in ARH cell bodies of the adult, while AGPR-*ir* in cell bodies is more evident. Images were captured with a 25X oil objective (0.75 NA) and represent an area of 400X400μm. They correspond to approximate level illustrated by area **A** in Figure 2-5.

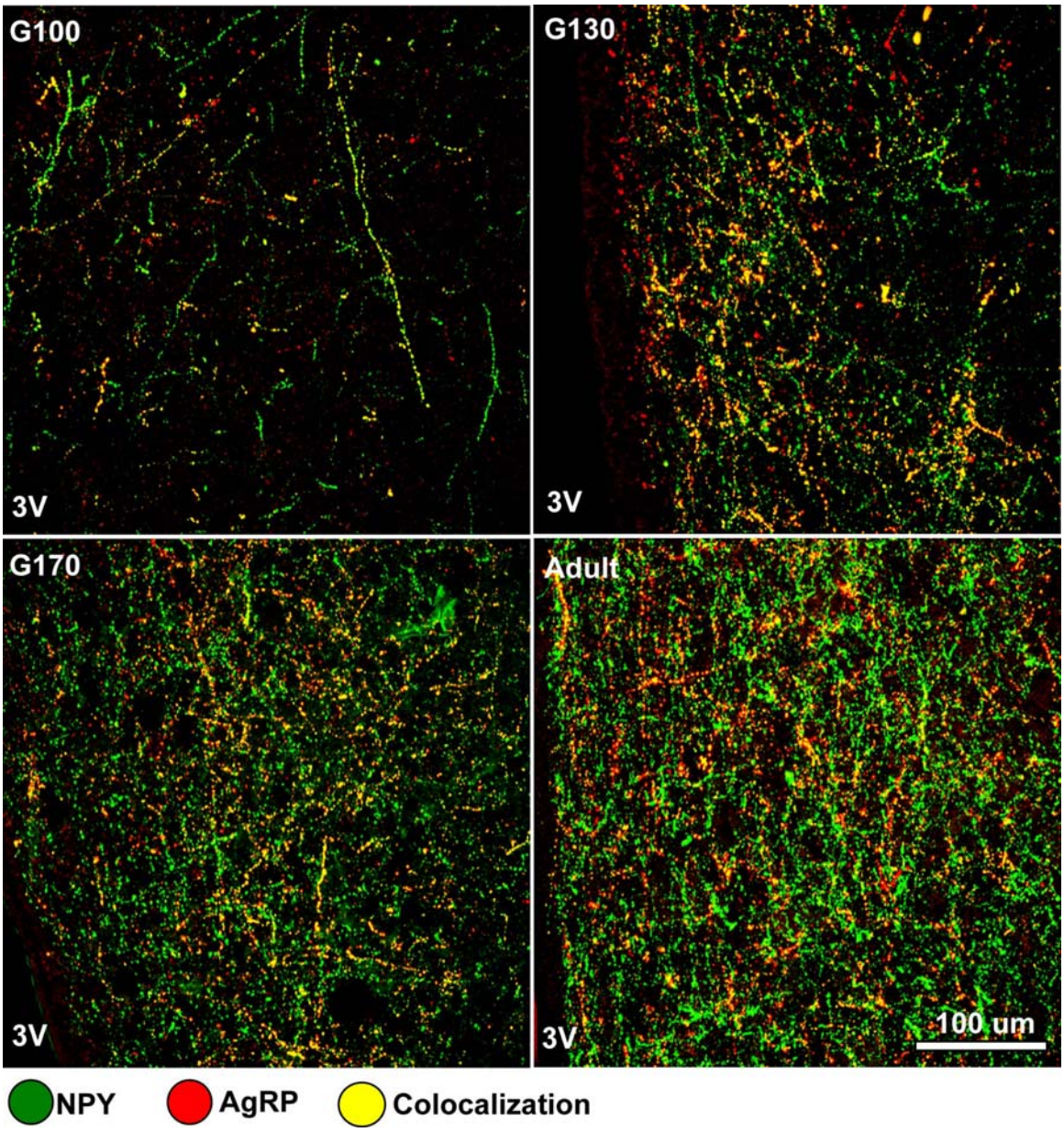


Figure 2-4. Development of NPY/AgRP neurons in the PVH. Figures represent color confocal digital images of double-label immunofluorescence for NPY and AgRP. Single-label NPY fibers are shown in green, AgRP fibers are shown in red and double-label NPY/AgRP fibers are shown in yellow. Sparse innervation was present at G100 with increases at G130 and G170. Overall levels were lower than the adult. Images were captured with a 25X oil objective (0.75 NA) and represents an area of 400 x 400μm. Images correspond to approximate level illustrated by area **B** in Figure 2-5.

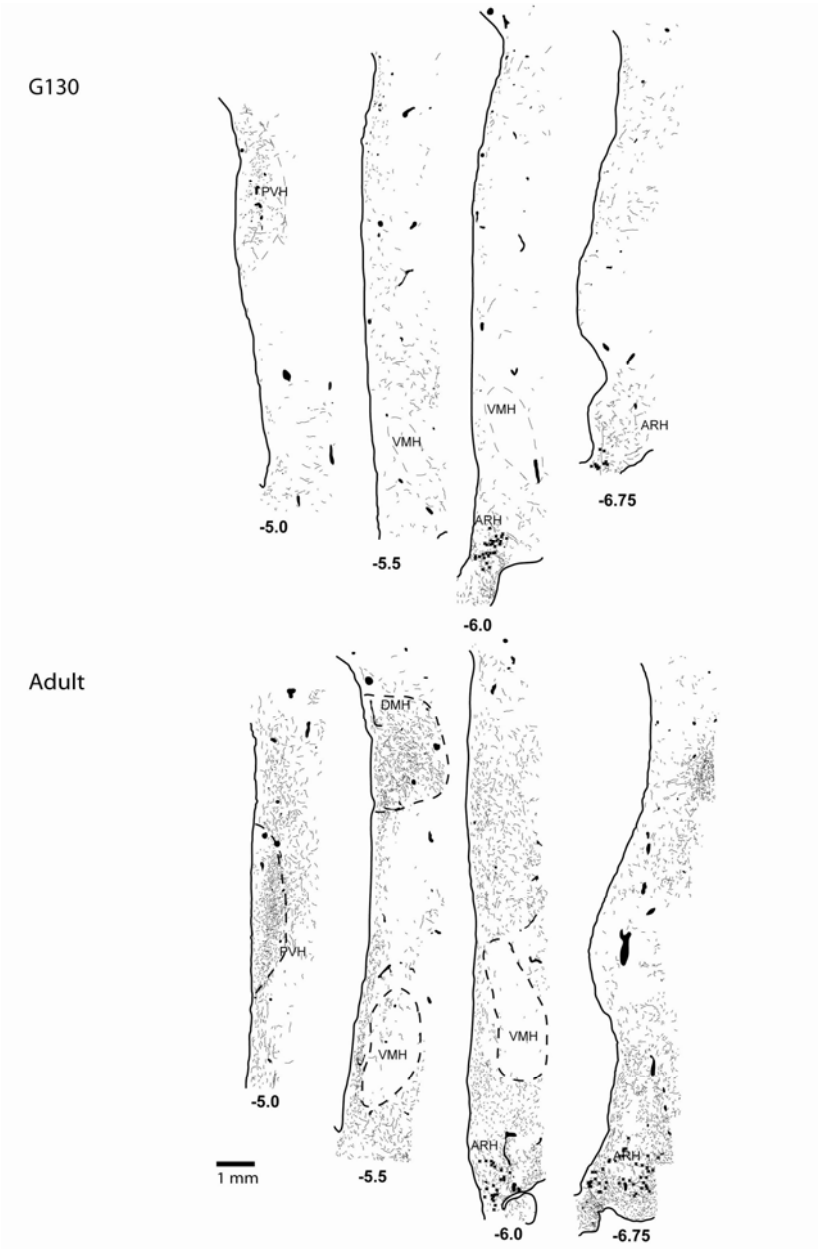


Figure 2-5. Schematic drawings of ARH-derived NPY projections in the hypothalamus of the G130 and adult Japanese macaque. Computer assisted line drawings illustrate the distribution of ARH derived hypothalamic NPY fibers and terminal fields using AgRP-*ir* as the marker at four levels in the adult corresponding approximately to -5.0. -5.5, -6.0 and -6.75 caudal to bregma according to Paxinos et al. Corresponding sections were chosen approximating similar levels by anatomical markers, though exact level is unknown. Terminal fields are demarcated with specks. Fiber projections are illustrated by lines. Darkened black shapes represent cross section of vasculature. Detectible cell bodies are demarcated with an "x". Box A and B in lower panel illustrate the approximate areas in color photomicrographs of ARH (**A**) and PVH (**B**).

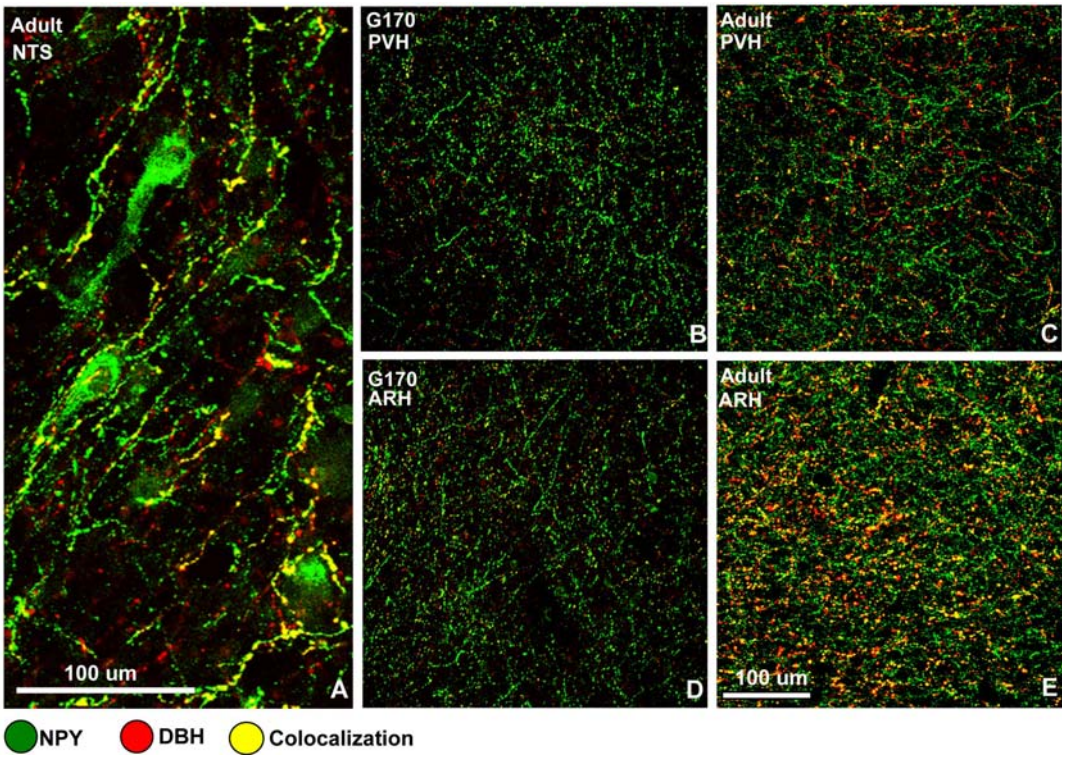


Figure 2-6. Presence of NPY/DBH fibers in the brainstem. Figures represent color confocal digital images of double-label immunofluorescence for NPY and DBH. Single-label NPY and DβH fibers are shown in green and red, respectively, and double-label NPY/DBH fibers are shown in yellow. NPY cell bodies are present in the NTS with single- and double-label NPY/DBH fibers (A). The G170 animal expresses few catecholamine fibers in both the PVH and ARH (B and D). Both single-label DBH and co-labeled NPY/DBH are present in the adult PVH and ARH (C and E). Images represent a 10-μm thick collection of optical slices taken at 0.5-μm intervals. Images were captured with a 25X oil objective (0.75 NA) and represent an area of 400X400μm. Images correspond to approximate level illustrated in Figure 2-5 by area **B** in the PVH panel and area **A** in the ARH panel.

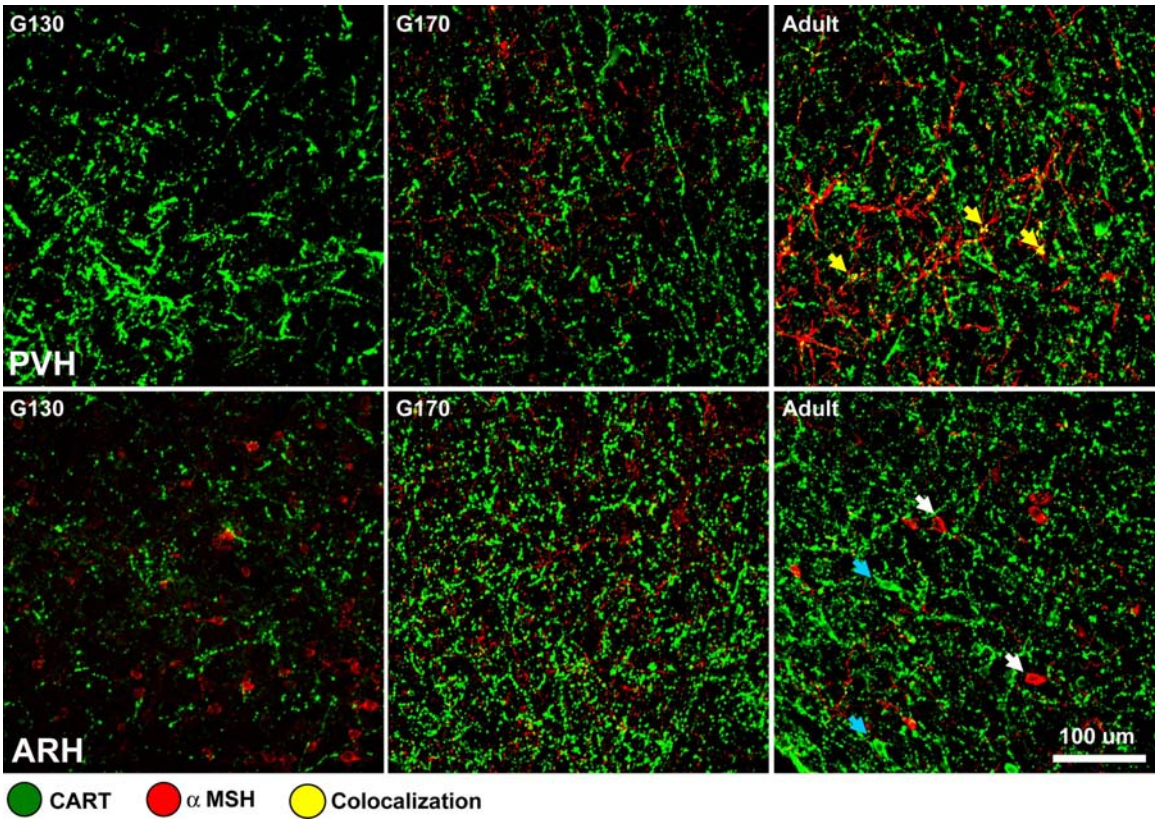


Figure 2-7. Prenatal development of CART and αMSH in the PVH and ARH. Figures represent color confocal digital images of double-label immunofluorescence for CART (green) and αMSH (red). Evidence of αMSH initially manifests as early as G130 in the ARH and G170 in the PVH. CART and αMSH do not colocalize in the NHP though close appositions are evident occasionally. Yellow arrows indicate close apposition of fibers present in the PVH. Blue and white arrows indicate the presence of singly labeled CART and αMSH soma respectively in the ARH. Images represent a 10-µm thick collection of optical slices taken at 0.5-µm intervals. Images were captured with a 25X oil objective (0.75 NA) and represent an area of 400 X 400µm.

Chapter 3
NHP Model of Maternal Obesity

A nonhuman primate model to mimic human obesity during pregnancy

Many animal models are used to determine the impact of maternal obesity on long-term body-weight homeostasis as described in Chapter 1, though each has its benefits and limitations. Although the rodent model has advantages such as the flexibility in use, extensive molecular characterization and transgenic capabilities, there are many caveats with respect to brain development that make what we learn challenging to translate to the human. The rodent has large litters each with their own placenta. Therefore, the number of offspring, the placement within the uterus and the birthing order potentially has an impact on birth weight and overall health. In the rodent, parturition occurs prior to full brain development, including hypothalamic feeding circuits, and thus the environment of the postnatal weeks following birth impacts long-term body-weight. Though maternal metabolic status during gestation has been shown in many rodent models to impact the offspring, early neonatal environment which includes competition for milk, composition of the milk and maternal rearing characteristics have a profound influence on the long-term metabolic status of these offspring as well. In light of the obesity epidemic in humans, a nonhuman primate model is essential to understand the impact of maternal diet on *in utero* development.

The primary goal of our model of maternal obesity was to investigate the effects of maternal metabolic health and diet on the development of metabolic systems in the fetal offspring. In this model we placed young adult female Japanese macaques on either a high-fat/calorically-dense diet (HFD) similar to the typical American diet or a low fat diet (CTR) for 2-4 years. Our initial focus has been their fetuses (referred to as F-CTR or F-HFD) at gestational day 130 (G130, early third trimester). During the fifth year of the study we switched the mothers on the HFD to a CTR diet prior to the onset of pregnancy.

The diet-reversal manipulation (DR) mimics the decision of a mother who resolves to eat a healthy low-fat diet during pregnancy despite maternal obesity. We also studied these fetuses at G130 (F-DR).

This project is a collaborative study encompassing many interest groups that focus on the liver, pancreas, immune system and brain. The maternal and fetal phenotype as well as extensive characterization of the alterations seen in fetal hepatic physiology has been recently reported (McCurdy et al., 2009). The primary focus of this dissertation is the characterization of the brain effects at G130 though this chapter summarizes some of the overall phenotype to place this work in the overall context of the model (McCurdy et al., 2009).

General description of the maternal phenotype in the non-pregnant state

In this model, we utilize age- and weight-matched young Japanese macaques that are housed in a harem (social setting) with a ratio of male:female of 2:9. Animals were placed on either a control (CTR) monkey chow diet consisting of 15% calories from fat or a high-fat/calorically-dense diet (HFD) consisting of 32% calories from fat, (a combination of HF monkey chow and highly palatable peanut butter treats). Table 3-1 refers to monkey chow specifications.

	CTR Diet	HF Diet	CTR Diet	HF Diet
	By composition		*By calories*	
Protein (%)	*20.6*	*17.1*	*26.8*	*16.1*
Carbohydrate (%)	*70.8*	*55.5*	*58.5*	*52.3*
Fiber (%)	*14*	*8*		
Fat (%)	*5.5*	*14.9*	*14.7*	*31.6*
Saturated	*1.69*	*2.46*		
Monounsaturated	*1.65*	*2.53*		
Polyunsaturated	*2.58*	*3.05*		
Cholesterol (ppm)	*83*	*946*		
Metabolizable Energy (kcal/gm)			*2.87*	*3.8*

Table 3-1: Nutrient specification for the CTR and HF diet as reported by the manufacturer.

Animals were allowed to remain on their respective diets year-round for four consecutive years (Table 3-2). Pre-pregnancy weights and blood draws occurred in September/October of each year, prior to commencement of the breeding season which lasted until March (Table 3-2). During this time, a glucose tolerance test (GTT) was also performed. During subsequent monthly health checks, animals were examined for pregnancy by palpation and then later dated by ultrasound examination. During the early third trimester, another GTT was performed. Fetuses were collected by caesarean section on G130 in the spring and early summer months (Table 3-2). This time point had been determined to be important for not only hypothalamic development but was also for pancreatic islet reorganization.

Just prior to the breeding season of the fifth year, HFD animals were transitioned to CTR diet for another pregnancy and referred to as DR. These offspring were also sacrificed at G130.

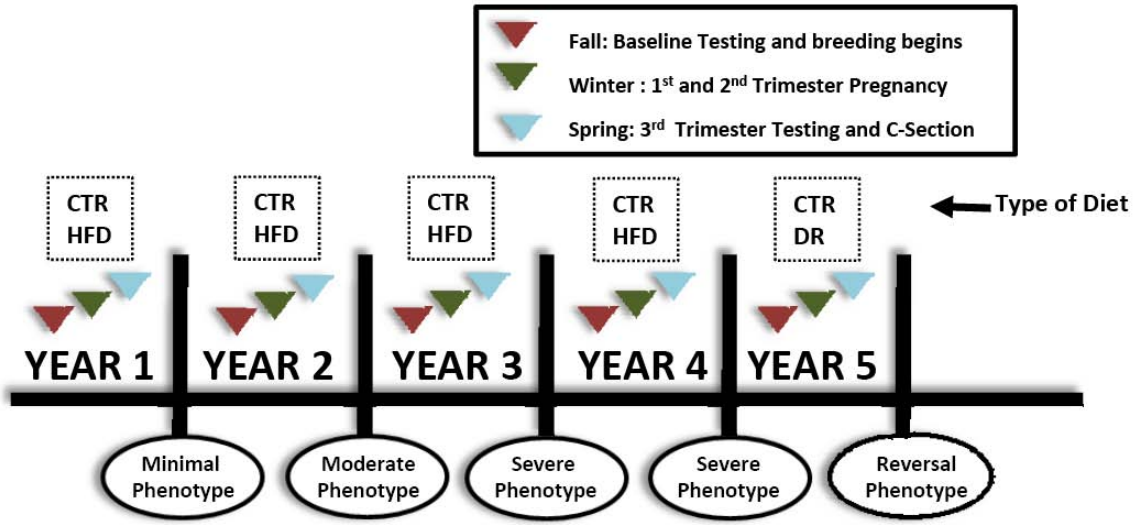


Table 3-2: Schematic representation of the 5 year protocol to procure G130 fetal tissues for CTR, HFD and DR studies.

Non-pregnant female monkeys on HFD segregate into two metabolic phenotypes

Throughout the four years on HFD, young adult female Japanese macaques exhibited increased responsiveness to the diet. During the first year, animals placed on a HFD defend a healthy phenotype. Few measurable changes appear to emerge until the second year on the diet, in which the animals segregate into two groups based on body-weight gain as well as response during GTT: 1) a resistant phenotype that defends against body-weight gain and remains sensitive to insulin (HFD-resistant, HFD-R) and 2) a sensitive phenotype that exhibits body-weight gain and loses the ability to respond to glucose appropriately (HFD-sensitive, HFD-S). Because measurable changes appear during year 2 and persist through year 3 and 4, animals are pooled into their respective groups from year 2-4.

Prior to pregnancy, HFD-S animals have a significant increase in body-weight due to consumption of the HFD (*Figure 3-1, A*). This is predominantly due to increased adiposity (McCurdy et al., 2009). Only the HFD-S animals exhibit an increase in body fat % whereas CTR and HFD-R animals are similar in their body composition during the 4 years on the diet.

In the non-pregnant state, HFD-S animals do have elevated leptin levels that are not statistically significant due to a high degree of variability because these animals are pooled from years 2-4 on the diet (*Figure 3-1, B*). However, we reported that indeed HFD-S from year 4 on the diet do have significantly increased leptin levels in comparison to CTR and HFD-R animals (McCurdy et al., 2009). In addition, though triglycerides are not altered significantly in the HFD-S animals in comparison to CTR, the glycerol levels are increased in these animals (*Figure 3-1, D*). These data indicate that the HFD does

pose a significant metabolic challenge to approximately 60% of the animals placed on the diet.

DR non-pregnant animals show no initial metabolic changes

DR animals are barely on the diet for a month prior to the baseline weight measurement and tests shown in *Figure 3-1*. Therefore, we are not afforded an adequate picture of the impact of the switch to CTR diet in the non-pregnant female prior to the next breeding season (*Figure 3-1, A-D*). DR animals continue to be characterized as either resistant or sensitive based on their designation during years 2-4 on the diet.

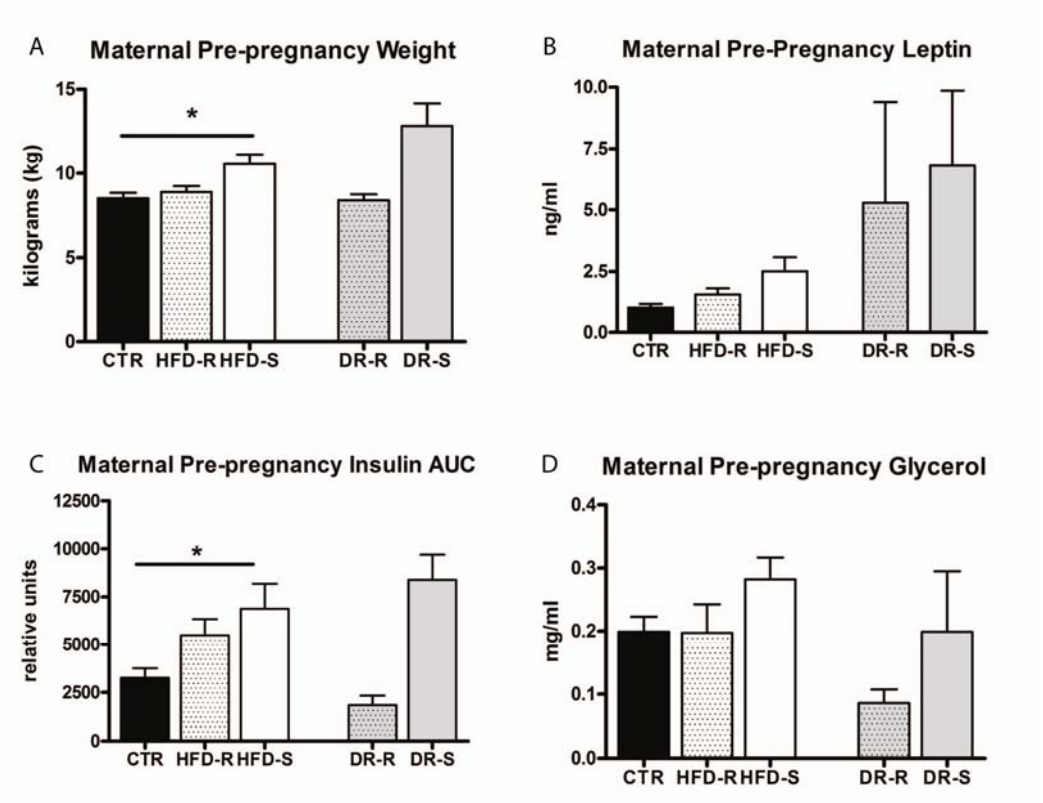


Figure 3-1: Maternal consumption of HFD results in increased adiposity, hyperinsulinemia and hyperglycerolemia. A. Body-weights. B. Leptin levels. C. Insulin area under the curve (AUC) during glucose tolerance test. D. Glycerol levels. * signifies $P < .05$. Data were analyzed using 1-way ANOVA with Bonferroni post-hoc analysis. DR-R and DR-S are shown for comparison and not included in analysis. (*n*, CTR=16, HFD-R=8, HFD-S=10, DR-R=3, DR-S=4)

HFD further exacerbates the normal metabolic challenges of pregnancy

Although we have performed extensive phenotypic analysis of the pregnant monkeys (McCurdy et al., 2009), only the data pertinent to this dissertation are presented here.

In general, pregnancy is accompanied by many physiologic challenges to the maternal metabolism even in the absence of dietary manipulation. Body-weight increases are normal during pregnancy. Surprisingly, increase in body-weight among CTR, HFD-R and HFD-S animals due to pregnancy was similar (*data not shown*). However, HFD-S animals are heavier than CTR and HFD-R at the 3rd trimester since they started gestation heavier due to increased overall adiposity (*Figure 3-2, A*).

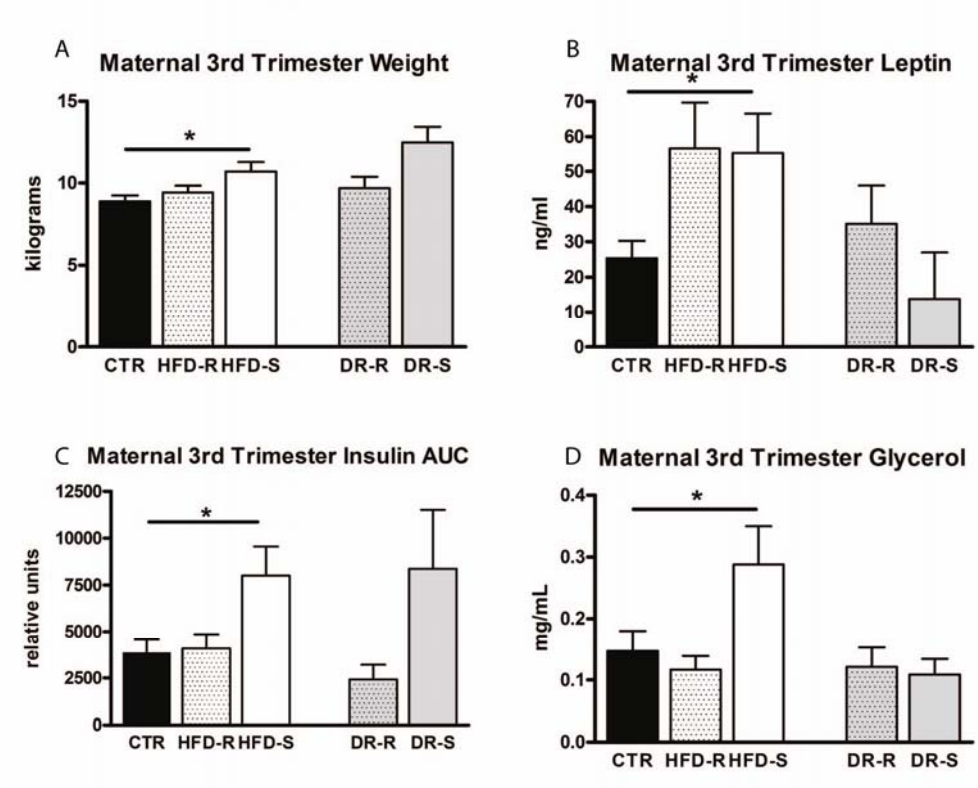


Figure 3-2. Adiposity, hyperinsulinemia and hyperglycerolemia are further exacerbated in pregnant females consuming HFD. A. Body-weights. B. Leptin levels. C. Insulin area under the curve (AUC) following during glucose tolerance test. D. Glycerol levels. * signifies $P < .05$. Data were analyzed using 1-way ANOVA with Bonferroni post-hoc analysis. DR-R and DR-S are shown for comparison and not included in analysis. (*n*, CTR=18, HFD-R=7, HFD-S=9, DR-R=3, DR-S=4)

Leptin levels during pregnancy typically rise greater than 20-fold over non-pregnant states. This holds true for our NHP model whose levels increase dramatically in all groups over pre-pregnancy levels (*Figure 3-2, B*). Though HFD-S animals have increased levels of adiposity not present in HFD-R animals, leptin levels in both of these phenotypes is elevated. This, presumably, is a result of the impact of diet on placental production of leptin.

GTT performed during the third trimester of pregnancy results in very similar circulating glucose levels in CTR, HFD-R, HFD-S and DR animals during the 60 minutes following administration of intravenous dextrose (*Figure 3-3, A*). However, insulin responses to GTT continue to be most exaggerated in HFD-S animals (*Figure 3-3, B*) with HFD-S animals exhibiting significantly different insulin AUC in comparison to CTR and HFD-R animals (*Figure 3-2, C*). Finally glycerol levels in HFD-S animals are nearly double the levels found in CTR and any other group (*Figure 3-2, D*).

In general, though HFD-S and HFD-R animals are exposed to the same diet, their metabolic response is very different. The HFD-R animals exhibit almost no detectible metabolic difference from CTR animals in the parameters we tested. Either by their genetic make-up or their hard-wired control systems, the HFD-R animals defend a "normal phenotype" throughout these studies, even during pregnancy.

DR pregnant animals show minimal metabolic improvements to HFD phenotype

During pregnancy, the DR-S animals do not benefit dramatically in their overall metabolic phenotype from the switch to the low-fat diet. They do not experience a significant loss in body-weight or improved insulin response during GTT (*Figure 3-2, A*,

C). However, they do experience a drop in their levels of leptin and glycerol which approximates that of CTR animals (*Figure 3-2, B, D*).

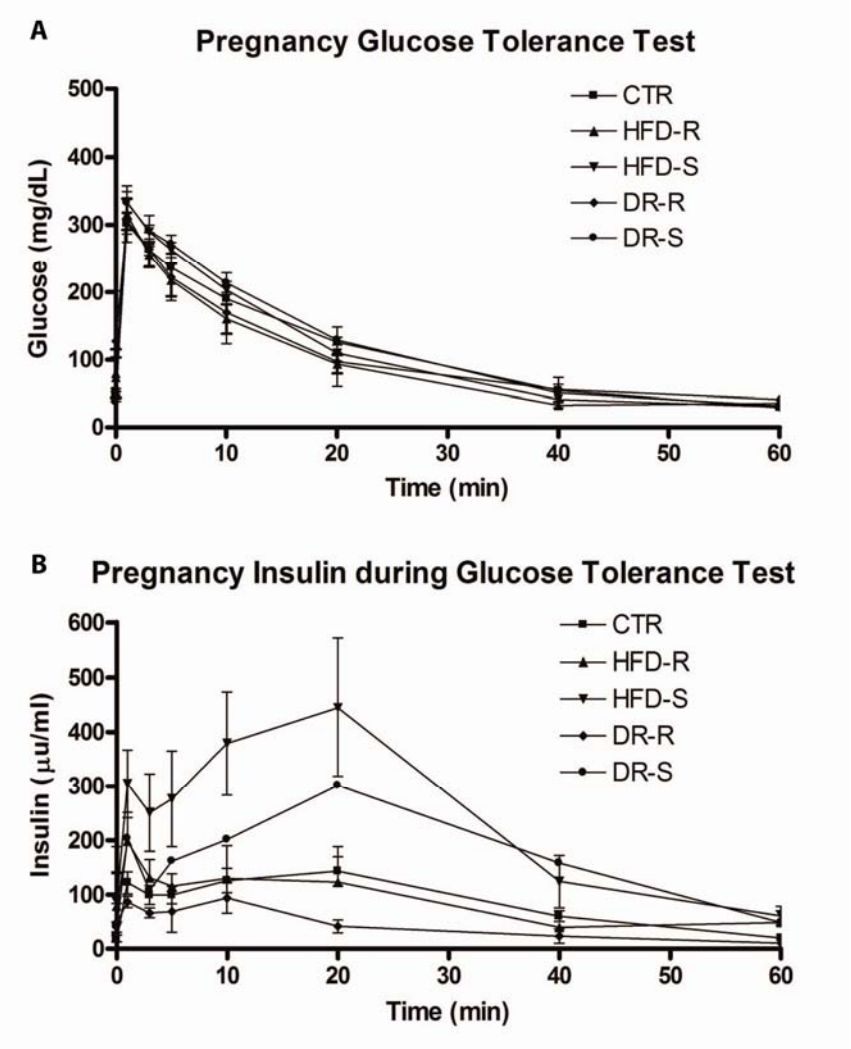


Figure 3-3. Pregnancy results in insulin resistance in HFD-sensitive animals. Glucose (A) and Insulin (B) levels during 60 minute time course of glucose tolerance test. Groups are marked as follows: CTR (■), HFD-R (▲), HFD-S (▼), DR-R (♦) and DR-S (●).

F- HFD exhibit hyperlipdemia and hyperglycerolemia

The F-HFD exhibited widespread changes in every organ and physiological system we have investigated to date, irrespective of the maternal response to the diet (HFD-R or -S). Overall, F-HFD have decreased body-weight in comparison to F-CTR

(*Figure 3-4, A*). This reduced body-weight is predominantly due to a loss of lean body mass since the 3rd trimester NHP fetus has little to no fat depots. Insulin, glucose and non-esterified fatty acids (*data not shown*) (McCurdy et al., 2009) are unaltered in all groups and leptin levels are barely above the limit of detectability for this assay (*Figure 3-4, B*). This is not surprising since leptin is produced by adipose tissue and during this early gestational time point, adipose tissue is not present. In contrast, triglycerides and glycerol are elevated in both F-HFD-R and F-HFD-S in comparison to F-CTR presumably coming from the mother (*Figure 3-4, C-D*). These increased levels of triglycerides and glycerol point to lipolysis in the mother irrespective of the maternal phenotype on HFD.

DR does ameliorate the metabolic parameters of the fetus

F-DR-S body-weight appears to have returned to F-CTR levels (*Figure 3-4, A*). Also F-DR-S triglycerides and glycerol levels approximate those of the F-CTR and reflect the marked reduction of maternal circulating fats (*Figure 3-4, C-D*). These parameters accentuate general normalization of F-DR with maternal consumption of CTR diet despite continued maternal obesity.

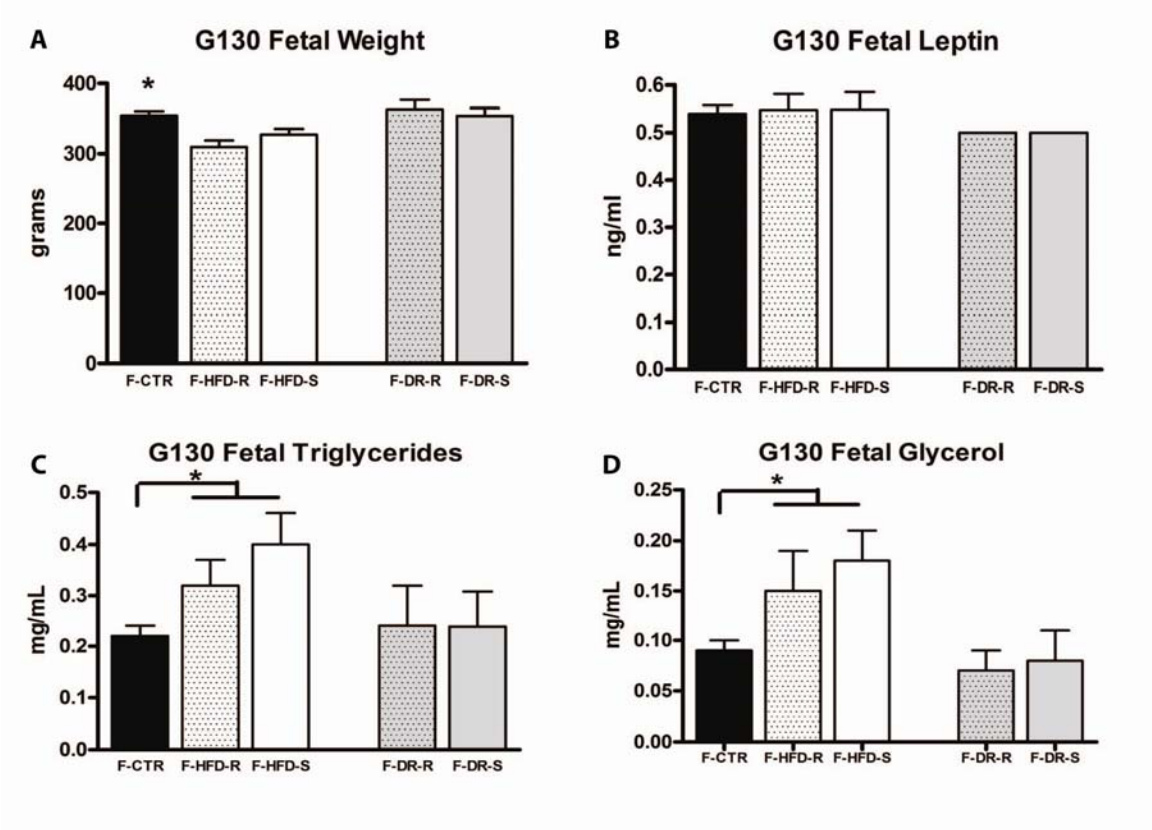


Figure 3-4. F-HFD have hyperlipidemia and hyperglycerolemia in the presence of reduced G130 body-weight. A. G130 fetal body-weights. B. Leptin levels. C. Triglycerides. D. Glycerol levels. * signifies $P < .05$. Data were analyzed using 1-way ANOVA with Bonferroni post-hoc analysis. F-DR-R and F-DR-S are not included in analysis. (*n*, F-CTR = 18, F-HFD-R = 8, F-HFD-S =14, F-DR-R=3, F-DR-S=4)

Circulating Cytokines are up-regulated in HFD animals

Irrespective of maternal sensitivity to diet, F-HFD have elevated circulating cytokines. Of the 36 cytokines probed, 13 cytokines were significantly up-regulated in F-HFD plasma in comparison to F-CTR (Table 3-3). Though circulating inflammatory cytokines (IL-6, TNFα, CRP and IL-10) have been shown to be up-regulated in obese mothers in comparison to lean mothers (Stewart et al., 2007), we believe these cytokines are to a large degree of placental in origin though they could also be of a maternal or fetal source. The placenta is known to produce a host of stage-specific cytokines (Hauguel-de

Mouzon and Guerre-Millo, 2006). In particular, the third trimester placenta produces a high level of cytokines that are important to the progression of normal gestation. These cytokines have been shown to be produced by every cell type in the placenta which includes the resident macrophages (Hofbauer cells), endothelial cells, syncytiotrophoblast and cytotrophoblast cells. Recent studies from our laboratory on third trimester placental blood show that indeed higher levels of cytokines are produced in the placenta of our model than in the mother or fetus. In our model, the consumption of the HFD does in fact increase the overall magnitude of cytokine production within the placenta that then spills over to the fetal circulation. It is suggested that these cytokines are part of the pathway that imparts the normal state of insulin resistance of pregnancy. Thus, this increase in cytokines may be part of the greatly increased insulin resistant state of the HFD-S animals.

	Target	Alternate	F-CTR	F-HFD	*p value*		Target	Alternate	F-CTR	F-HFD	*p value*
	C5a		0.14 ± 0.01	0.43 ± 0.15	*0.198*		IL-16	LCF	0.17 ± 0.03	0.41 ± 0.12	*0.113*
*	CD40 Ligand	CD154	0.11 ± 0.02	0.30 ± 0.06	*0.031*		IL-17		0.13 ± 0.03	0.28 ± 0.07	*0.117*
*	G-CSF	CSFβ	0.14 ± 0.01	0.28 ± 0.05	*0.045*	*	IL-17E		0.09 ± 0.02	0.31 ± 0.06	*0.023*
	GM-CSF	CSFα	0.14 ± 0.02	0.27 ± 0.05	*0.079*		IL-23		0.09 ± 0.02	0.27 ± 0.07	*0.141*
	GROα	CXCL1	0.16 ± 0.02	0.47 ± 0.18	*0.222*	*	IL-27		0.11 ± 0.01	0.28 ± 0.05	*0.033*
	I-309	CCL1	0.11 ± 0.01	0.28 ± 0.07	*0.132*	*	IL-32α		0.09 ± 0.02	0.28 ± 0.05	*0.022*
	IFNγ		0.11 ± 0.02	0.28 ± 0.07	*0.153*	*	IP-10	CXCL10	0.12 ± 0.03	0.30 ± 0.03	*0.013*
*	IL-1α	IL-1F1	0.10 ± 0.02	0.29 ± 0.04	*0.014*		I-TAC	CXCL11	0.17 ± 0.03	0.39 ± 0.07	*0.051*
	IL-1β	IL-1F2	0.08 ± 0.02	0.25 ± 0.06	*0.051*		MCP-1	CCL2	0.15 ± 0.02	0.30 ± 0.05	*0.069*
	IL-1ra	IL-1F3	0.36 ± 0.13	0.68 ± 0.13	*0.161*		MIF	GIF	2.63 ± 0.51	4.85 ± 0.86	*0.091*
	IL-2		0.14 ± 0.03	0.25 ± 0.06	*0.173*	*	MIP-1α	CCL3	0.26 ± 0.06	1.00 ± 0.20	*0.024*
	IL-4		0.13 ± 0.03	0.25 ± 0.08	*0.219*		MIP-1β	CCL4	0.11 ± 0.00	0.27 ± 0.06	*0.123*
*	IL-5		0.09 ± 0.02	0.27 ± 0.05	*0.027*		RANTES	CCL5	0.50 ± 0.08	3.92 ± 1.02	*0.079*
	IL-6		0.07 ± 0.01	0.27 ± 0.07	*0.106*		SDF-1	CXCL12	0.13 ± 0.02	0.30 ± 0.06	*0.053*
	IL-8	CXCL8	0.09 ± 0.01	0.50 ± 0.21	*0.186*		Serpin E1	PAI-1	1.36 ± 0.22	3.44 ± 0.77	*0.059*
*	IL-10		0.07 ± 0.02	0.22 ± 0.03	*0.017*		sICAM-1	CD54	0.09 ± 0.01	0.25 ± 0.06	*0.108*
*	IL-12 p70		0.11 ± 0.03	0.25 ± 0.03	*0.019*	*	sTREM-1		0.12 ± 0.02	0.30 ± 0.06	*0.036*
	IL-13		0.30 ± 0.07	0.31 ± 0.06	*0.857*	*	TNFα	TNFSF1A	0.12 ± 0.02	0.27 ± 0.05	*0.042*

Table 3-3. Plasma levels of cytokines using the Proteome Profiler Array in G130 F-CTR and F-HFD. Expressed as mean±SEM, Exact *P* value listed. Data were analyzed using Student's *t* test (*n*, F-CTR=3, F-HFD=4)

Maternal HFD causes premature activation of fetal hepatic activity and early reprogramming

After exiting the placenta, 50% of the blood passes directly to the liver. Therefore of all of the fetal organs, the liver experiences the maternal milieu first. This makes the liver very susceptible to blood-borne nutrients as well as hormones which diffuse or are transported trans-placentally. Considering both the mother and fetus have increased circulating glycerol levels, we focused initially on characterizing hepatic alterations in our model. Huge droplets of triglycerides and lipids were present in F-HFD animals in comparison to F-CTR as determined by Oil Red O staining (*Figure 3-5, A-B*). Likewise, direct measurements of liver triglycerides show significantly elevated levels in F-HFD in comparison to F-CTR (McCurdy et al., 2009). These changes suggest the presence of nonalcoholic fatty liver disease (NAFLD). In the absence of fat depots in the G130 fetus for lipid storage, the liver becomes the lipid repository. Overwhelmed with the presence of lipid, the liver appears to suffer additionally from oxidative damage as illustrated through increased 4-hydroxy-2-nonenal (HNE), a marker of lipid peroxidation and oxidative stress (*Figure 3-5, C-D*). In addition, heat shock proteins (HSP) 27, 40, 70 and 90 were also up-regulated (microarray data, *data not shown*). This was confirmed by RT-PCR and western blot analysis (Aagaard-Tillery et al., 2008). These HSPs are ubiquitous markers of oxidative stress as well as cytokine activation. These proteins are also involved in modulation of chromatin structure and signal long-term dysregulation of the liver. This illustrates the stress and toxicity experienced by the fetal liver with lipid accumulation and/or oxidative stress.

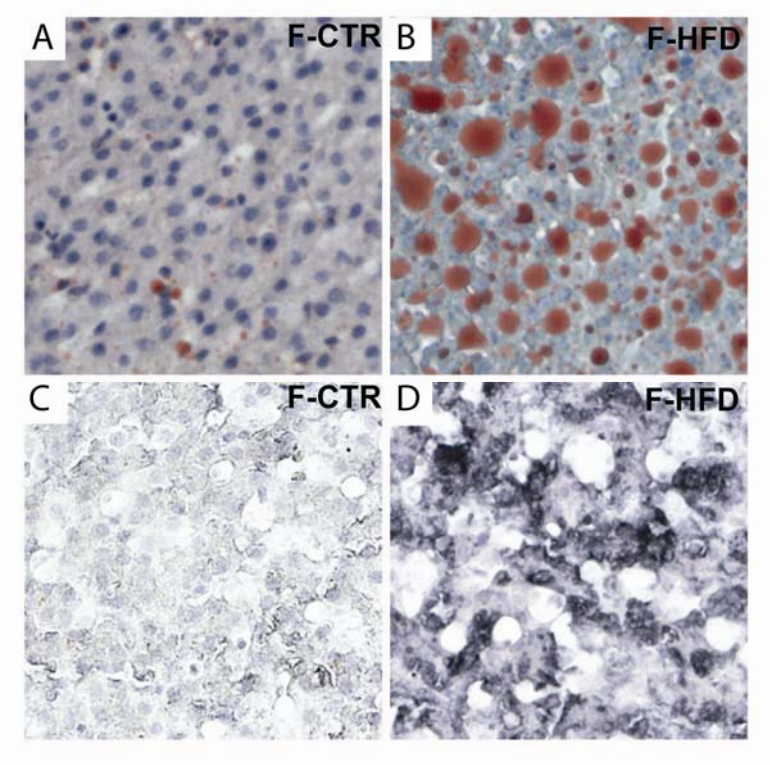


Figure 3-5. Maternal consumption of HFD leads to accumulation of lipids and activation of oxidative stress pathways within the fetal liver. (A-B) Oil Red O in F-CTR and F-HFD livers (C-D) HNE staining (HNE is a marker of lipid peroxidation and oxidative stress.) 20X magnification (McCurdy et al., 2009).

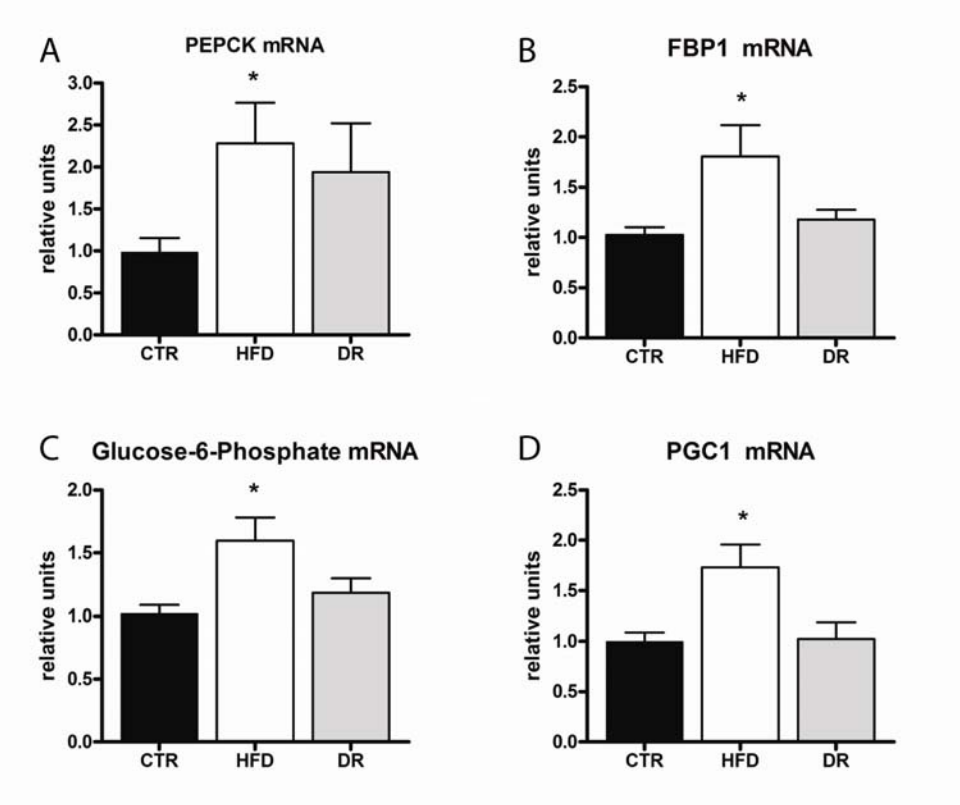


Figure 3-6: Partial block of developmental fatty liver disease in offspring of mothers switched to CTR diet after four years of HFD consumption. Quantitative real-time PCR of (A) Phosphoenolpyruate carboxykinase (PEPCK). (B) Fructose-bisphosphatase (C) Glucose-6-phosphatase (D) PPARγ coactivator-1α (PGC-1α) * signifies $P < .05$. Data were analyzed using 1-way ANOVA with Bonferroni post-hoc analysis. (*n*, F-CTR = 9, F-HFD-R = 6, F-HFD-S = 9)

Next, we sought to determine the state of the glucose metabolism pathway in the fetus. At this stage of development hepatic gluconeogenesis is normally low due to absence of the rate-limiting enzyme phosphoenolpyruvate carboxykinase (PEPCK). However, in our model, F-HFD have increased levels of PEPCK as well as fructose-bisphosphatase 1 (FBP1) and glucose-6-phosphatase (*Figure 3-6, A-C*). PPARγ coactivator-1α (PGC-1α), the broad transcriptional regulator for gluconeogenesis is also

upregulated (*Figure 3-6, D*). These alterations in gene expression point to a premature programming of the liver to accommodate the influx of lipids.

In F-DR animals, the hallmarks of NAFLD are greatly improved and liver function appears similar to the F-CTR (*Figure 3-6, A-D*). Therefore, consumption of a diet low in fat by the mother during pregnancy appears to protect the fetus.

Concluding Remarks

Considering the marked phenotypic difference between adult HFD-R and HFD-S animals, we expected marked differences in the fetal outcomes based on whether they were F-HFD-R or -S. Both of these groups of adult animals are consuming a high-fat, calorically-dense diet. However the HFD-R mothers are relatively healthy. However, the F-HFD-R do not appear to be protected and experience the detrimental effects of the HFD. The F-HFD-S also experience the detrimental effects of the HFD but this is less surprising since their mothers have overt insulin resistance and marked increases in adiposity. As will be reported in Chapters 4 and 5, the changes I see in the fetal brain also exist regardless of the maternal phenotype. These data provide evidence of effects of consumption of a HFD on fetal development, regardless of the effects on the mother. Therefore, a maternal phenotype of insulin resistance or obesity is not necessary to have profound effects on the developing fetus. These discoveries have obvious clinical implications in human populations because women with a normal pre-gravid BMI consuming a HFD are at a risk of harming their offspring.

Chapter 4
Changes in Melanocortin Expression and Inflammatory Pathways in Fetal Offspring in Nonhuman Primates Fed a High-Fat Diet

Grayson, B. E., LeVasseur, P., Williams, S. M., Smith, M. S., Marks, D. L., and Grove, K. L. (2009). Changes in melanocortin expression and inflammatory pathways in fetal offspring of nonhuman primates fed a high-fat diet. *Endocrinology* **Submitted**.

ABSTRACT

The hypothalamic melanocortin system, which controls appetite and energy expenditure, develops during the 3rd trimester in primates. Thus, maternal nutrition and health may have a profound influence on the development of this system. In order to study the effects of chronic maternal high-fat diet (HFD) on the development of the melanocortin system in the fetal nonhuman primate, we placed adult female macaques on either a control (CTR) diet or a HFD for up to 4 years. A subgroup of HFD animals were also switched back to CTR diet during the 5th year of the study (Diet-Reversal, DR). Third trimester fetuses from mothers on HFD (F-HFD) showed increases in pro-opiomelanocortin and melanocortin 4 receptor mRNA expression, while agouti-related protein mRNA and peptide levels were decreased in comparison to CTR fetuses (F-CTR). Pro-inflammatory cytokines, including IL1β and IL1R1, were elevated in the hypothalamus and may be responsible for the changes in the fetal melanocortin system. Fetuses of DR animals (F-DR) had normal melanocortin levels. These results raise the concern that chronic consumption of a HFD during pregnancy, independent of maternal obesity and diabetes, can lead to widespread activation of pro-inflammatory cytokines that may alter the development of the melanocortin system. These abnormalities could impair long-term nutrient sensing and lead to early-onset obesity. These abnormalities may be prevented by healthful nutrient consumption during pregnancy even in obese and severely insulin-resistant individuals.

INTRODUCTION

The melanocortin system in the hypothalamus is pivotal in the regulation of energy balance and body-weight homeostasis. The pro-opiomelanocortin (POMC) gene has several peptide products (adrenocorticotropic hormone (ACTH), α-, β- and γ melanocyte-stimulating hormone (MSH)) which serve as feeding signals through interactions with their respective G-protein coupled receptors (MCR1-5), but primarily through the MC3 and MC4 receptors which are expressed in several key hypothalamic and brainstem sites (Cone, 2005). In addition, the endogenous antagonist of this system, agouti-related peptide (AgRP), is a potent orexigenic signal (Broberger et al., 1998a; Ollmann et al., 1997). AgRP mRNA is exclusively located in the arcuate nucleus (ARH) where it is colocalized with another orexigenic peptide, neuropeptide Y (NPY). Highlighting the importance of the melanocortin system, mutations in this system cause hyperphagia and obesity in mouse models as well as humans.

In the developing rodent, the maturation of the feeding-related circuits occurs postnatally (Bouret et al., 2004a; Grove et al., 2003a). More specifically the ARH-NPY/melanocortin projections start developing soon after birth and are not completely developed and functional until the end of the third postnatal week (Grove et al., 2003a; Grove and Smith, 2003). Furthermore, leptin has been shown to be a critical neurotrophic factor for the development of ARH circuits (Bouret et al., 2004a; Bouret et al., 2004b). Increasing the quantity and quality of nutrition ingested postnatally by pups induces an overweight phenotype with hyperleptinemia that persists into adulthood (Gorski et al., 2006; Grove et al., 2003a; Grove et al., 2005b; Morris et al., 2005; Plagemann et al., 1999c). Moreover, maternal high-fat feeding specifically during pregnancy can also have

long-term consequences on metabolic systems in rodent offspring (Guo and Jen, 1995a; Srinivasan et al., 2006). These long-term effects are likely mediated at least in part, through alterations in hypothalamic feeding circuitry.

In nonhuman primates (NHP), however, melanocortin neurocircuitry develops during the third trimester of pregnancy (Grayson et al., 2006). Therefore, the critical window of this neurocircuitry development in primates exists *in utero* and may therefore be susceptible to perturbations from maternal nutrition and health (Dietz, 1994). A variety of factors, such as overall fat and calorie load, glucose levels and insulin resistance, hyperlipidemia and lipotoxicity can affect the growing fetus. Pregnancies with poor glycemic control and/or gestational diabetes have a high risk of macrosomia at birth and obesity and insulin resistance later in life (Gillman et al., 2003; Lindsay et al., 2000; Plagemann et al., 1997). Presently, greater than 50% of women of child-bearing age are either overweight or obese (Baskin et al., 2005). It is likely that the increased rate of childhood obesity in recent years is at least partly due to maternal diet and metabolic disease.

Previous studies from our group demonstrated that chronic maternal HFD can cause lipotoxicity in the liver of the fetal and neonatal offspring (McCurdy et al., 2009). Furthermore, these animals display elevated levels of circulating inflammatory cytokines. Rodent studies have also shown that both HFD and cytokines can affect melanocortin neurons (Enriori et al., 2007; Scarlett et al., 2006). In the present study, we hypothesized that chronic consumption of high calorie/fat diet (HFD) during pregnancy could alter the hypothalamic melanocortin system during this critical period of development. For these studies, we utilized a NHP model of chronic maternal consumption of high calorie/fat

diet (HFD) to investigate the effects of maternal diet and metabolic phenotype. We report alterations in both POMC and AgRP and activation of pro-inflammatory cytokines in the hypothalamus in the gestational day 130 (G130) fetuses exposed to maternal HFD (F-HFD) in comparison to CTR fetuses (F-CTR). We also demonstrate that developmental abnormalities in these fetuses can be prevented by reversing HFD animals to CTR diet (F-DR) right before pregnancy. This data suggests that maternal diet during fetal development may compromise the development of the melanocortin system.

MATERIALS AND METHODS

All animal procedures were approved by the Oregon National Primate Research Center (ONPRC) Institutional Animal Care and Use Committee and conformed to NIH guidelines on the ethical use of animals. All animals were maintained in outdoor/indoor group harem housing (male: female ratio of 2:9) on a natural light schedule.

Part 1: In these studies F-CTR and F-HFD tissues were harvested, processed and analyzed first. Age and weight matched adult *Macaca fuscata,* Japanese macaques, were fed a control (CTR) or high- fat/calorie diet (HFD) for up to 4 years. The CTR diet (#5000, Purina Mills Co., St Louis, MO) provided 13% calories from fat and the HFD (Test Diet, 5A1F, Purina Mills Co., St Louis, MO) supplied 35% of calories from fat and included calorically-dense (peanut butter) treats. During monthly health checks, animals were examined for pregnancy by palpation and then later dated by ultrasound examination. Both groups consisted of primiparous and multiparous pregnant animals. All pregnancies were singleton. A maximum of five C-sections is allowed per animal. Fetuses were collected by caesarean section on G130 (early 3rd trimester; full term is 175 days) from each pregnancy in CTR and HFD groups during years 2, 3 and 4 on the diets

by the trained surgical staff at ONPRC. The adult animals were allowed to recover under veterinary supervision before they were returned to the outdoor group housing. The fetuses were taken directly to necropsy and were deeply anesthetized with sodium pentobarbital (>30 mg/kg IV). The chest cavity was opened, the ascending aorta was bisected and the cardiovascular system was flushed with 0.9% saline containing 5,000 units of heparin/L to inhibit clotting. Fetal brains were taken either fixative-perfused or fresh (F-CTR, *n*=4 fresh, *n*=4 fixed) (F-HFD years 3/4, *n*=6 fixed, F-HFD years 2/4, *n*=7 fresh). In the case of fixation, trans-carotid perfusion of the head with 4% paraformaldehyde in buffered sodium phosphate (pH 7.4) was performed. The brain was removed, blocked and post-fixed overnight at 4°C in the same fixative and subsequently transferred for 24 hours to 10% glycerol buffered with $NaPO^4$ and for 48 hours into 20% glycerol buffered with $NaPO^4$. Tissue was frozen by submersion in < -50°C methyl butane and then stored in -80°C until sectioning. When tissues were harvested fresh, the brain was removed and blocked. The hypothalamus was divided into three parts, anterior, dorsal and ventral, and then stored in –80°C until RNA extraction.

***Part 2*:** In year 5 of the study a subset of HFD adults were switched to the CTR diet before the ensuing pregnancy (diet-reversal, DR). Subsequently, F-DR tissues were harvested and processed with F-CTR tissues from that same year. All animals were fixative-perfused as described above (F-CTR, *n*=4) (F-DR, *n*=5).

ARH NPY, POMC and AgRP mRNA

Coronal hypothalamic sections (35μm) were collected in 1:6 series using a freezing microtome. Sections were stored in ethylene glycol cryoprotectant at –20°C until time of use. Sections were wet mounted in RNAse free KPBS for each experiment.

Human NPY, POMC and AgRP cRNA probes were transcribed from 500, 1115 and 400bp cDNAs, respectively, and labeled with 100% ^{33}P-UTP. *In situ* hybridization for NPY, POMC and AgRP mRNA was performed using methods previously described (Grayson et al., 2006; Grove et al., 2001b). For visualization of the probe, labeled sections were exposed to Kodak BioMax film overnight. Images were captured using a Nikon Eclipse E800 Microscope coupled with a CoolSnap HQ camera (Photometrics, Westchester, PA) along with Metamorph Software (Universal Imaging Corp). Integrated morphometry analysis was used to measure total density by multiplying total area by optical density of an area approximating the ARH (reported in relative units, r.u.). An average of 6 matched mid-ARH sections were analyzed for each animal.

Neuropeptide immunoreactivity

To characterize the development of AgRP- and NPY-*ir*, a cocktail of goat anti-NPY (a kind gift of P. Larsen; 1:50,000), and guinea pig anti-AgRP (1:5000; Antibodies Australia, Melbourne, Australia) was used. To characterize the relative contribution of the POMC gene products, a cocktail of sheep anti-αMSH (#AB5087, 1:3,000; Chemicon, Temecula, CA) and rabbit anti-β-endorphin (#AB5028, 1:3,000; Chemicon, Temecula, CA) or rabbit anti-adrenocorticotrophic hormone (ACTH) (#20070, 1:5000, Immunostar, Hudson, WI) antibodies was used. To characterize the expression of IL1 receptor, goat anti-mIL1RI (#AF771, 1:2000; R&D Systems, Minneapolis, MN) antibody was used. ILIR1, αMSH and AgRP specificity were tested using peptide blocking assays and found to be specific (*Supplemental Figure 4-1*). Standard immunohistochemical methods were used as previously described (Grayson et al., 2006). Briefly, sections were washed in KPBS and then blocked in 2% donkey serum in 0.4% triton-X/KPBS. During

immunostaining control experiments, antibodies for αMSH, AgRP and IL1R1 was preabsorbed overnight with 50X excess of peptide and brought to final volume of 50µl with water (αMSH, #043-01, Phoenix Pharmaceuticals, Inc., Burlingame, CA; AgRP, kind gift of Neurocrine Biosciences Inc., San Diego, CA; ILIR1 #771-MR, R&D Systems, Minneapolis, MN) and then used for standard IHC. Primary or reabsorbed antibodies were diluted to their working concentrations in 2% donkey serum in 0.4% triton-X/KPBS and incubated with the tissue for 48 hours at 4°C. Tissue was then washed in KPBS and fluorescent secondary antibodies applied for 1 hour at room temperature at dilution of 1:200 (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA).

Image Analysis

Confocal laser microscopy, as previously described (Grove et al., 2000), was used to capture the NPY/AgRP, αMSH and IL1R1 immunofluorescent images. All images were captured with 25x oil objective (NA = 0.75). For fluorescence intensity measurements of AgRP in the PVH, a series of optical planes at 0.5 µm intervals along the z-axis of the section were scanned for each fluorescent signal and stored as a stack of 1024 x 1024 pixel images, processed with MetaMorph and presented as maximum projections totaling 5 µm. Four fields of view per section (upper/lower, right/left) (illustrated in *Figure 4-2, B*) in four equally spaced sections per animal were imaged and analyzed. Total immunoreactive fluorescent intensity was measured using the same conditions and same threshold for all images. Total gray value was measured for each image. Sections were blinded with respect to group. For qualitative illustration of αMSH and IL1R1, anatomically-matched sections were chosen (*n*=3 animals/group). Captured images are 10 µm thick projections at either 10X or 25X. A standardized adjustment of

all the images was made for brightness and contrast for illustrative purposes using Photoshop (Adobe Systems Inc., San Jose, CA).

Cortisol Assays

ACTH and cortisol assays were performed by the ONPRC Endocrine Services Core. Briefly, hormones was measured in 10µl of monkey serum using an Immulite® 2000 automated assay machine (Diagnostics Systems Laboratory, Inc., Los Angeles, CA). Inter- and intra-assay CV's were 6.7% and 2.0%, respectively.

Real-time PCR of feeding-related targets

Standard quantitative real-time PCR (qPCR) protocol was followed as previously described (Xiao et al., 2005). The following inventoried human primers/probe sets (Applied Biosystems, Inc., Foster City, CA) were used: agouti related protein (AgRP) #Hs00361403_g1, (BDNF) #Hs00538277_m1, cocaine- and amphetamine- regulated transcript (CART) #Hs00182861_m1, insulin like growth factor 1 (IGF1) #Hs00609566_m1, insulin receptor (INSR) #Hs00169631_m1, insulin receptor substrate 1(IRS1) #Hs00178563_m1, insulin receptor substrate 2 (IRS2) #Hs00275843_s1, leptin receptor (LEPR) #Hs00174497_m1, µ opioid receptor, (MOR) #Hs0016850_m1, neuropeptide Y (NPY) #Hs00173470_m1, NPY1R #Hs00197884_m1, suppressor of cytokine signaling-3 (SOCS3) #Hs00269575_s1, vascular endothelial growth factor (VEGF) # Rh02621759_m1. All sequences had greater than 95% homology with the macaque genome according to published sequences. Macaque-specific probe and primer sets were designed using Primer Express Software due to poor regional homology with human primers for MC4R: forward (AGGCTTCACATTAAGAGGATTGCT), reverse (ACGCCAATCAGGATGGTCAA) and probe (CGCTCCCTTCATATTGGCGCCTTG)

and UCP2: forward (GGCAGAGTTCCTCTATCTCGTCTT), reverse (CCCAACCATGATGCTGATTTC) and probe (TGCCCCATCTCGGTTTTTCTCCATCT). All qPCR data is expressed as a ratio of target to 18SRNA expression. Beforehand, 18sRNa levels were determined to not be affected with respect to diet.

Inflammatory Cytokines and Receptors Superarray

Reverse transcription was performed on 1µg of RNA using TaqMan Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA). For each reaction, cDNA synthesis was performed using 1µg of RNA in a reaction containing 2µl 10X RT Buffer, 4µl 25mM $MgCl_2$, 3µl 10mM dNTPs, 1µl 50µM Random Hexamers, 0.5µl RNase Inhibitor, 1µl MultiScribe Reverse Transcriptase, q.s. to 20µl with nuclease-free water. RT reactions were performed on an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) programmed for 25°C for 10 min, 37°C for 1 hr, and 95°C for 5 min. Samples were diluted with 80µl nuclease-free water and stored at 4°C until qPCR was performed. qPCR was performed according to the manufacturer's directions (RT^2Profiler PCR Array User Manual, v1.5) using RT^2 Profiler™ PCR Array Human Inflammatory Cytokines & Receptors array (Cat # APH-011A) on an ABI 7300 Real-Time PCR System (Applied Biosystems, Inc.). For each 96-well plate, a master mix was made containing: 1225µl Applied Biosystems Power SYBR Green PCR Master Mix, 98µl diluted cDNA synthesis reaction, and 1127µl ddH_2O. The master mix was vortexed and 21µl added to each well. qPCR reactions were 95°C for 10min, and 40 cycles of 95°C for 15sec followed by 60°C for 1min. A dissociation (melting) curve was performed per manufacturer's instructions and only samples with one peak at temperatures greater than 80°C were accepted. Auto

Ct values were calculated using 7300 RQ Study Software v.1.3, verified and adjusted as necessary. Gene expression values are expressed as ΔC_t for each set of duplicates. The fold-change for each gene was calculated as $2^{(-\Delta\Delta Ct)}$, where $\Delta\Delta C_t = \Delta C_t$ (F-HFD group) - ΔC_t (F-CTR group). The wells from all significantly regulated genes were analyzed for specificity by demonstrating a single amplified band on an agarose gel, and by DNA sequence analysis.

Statistical Analysis

Comparisons were conducted using Student's t test using Prism Biostatistical Software (v.4) (San Diego, CA). Differences were considered significant if $p < 0.05$. Data is presented as mean ±SE.

RESULTS

A complete characterization of our NHP maternal HFD model has been previously described (McCurdy et al., 2009). Briefly, adult pregnant monkeys on a HFD, as a group, have significantly increased body-weight, adiposity, and fasting blood insulin and leptin levels during the third trimester. We previously reported that adult pregnant monkeys on a HFD segregate into diet-sensitive and diet-resistant phenotypes (McCurdy et al., 2009). The diet-sensitive animals have a 14% increase in body-weight, with a near doubling of body fat, and three-fold increase in insulin secretion during IV glucose tolerance tests, but have normal glucose clearance indicating they are insulin-resistant but not diabetic. In contrast, diet-resistant animals have no significant changes in any of these parameters even after four years on the HFD.

In the data presented here, it should be noted that we see no fetal segregation of effects based on maternal phenotype/response to the HFD. Previously, we demonstrated

the F-HFD animals had slightly lower (but statistically significant) body-weights at G130, but leptin levels were not different from F-CTR (McCurdy et al., 2009). Overall, the most notable phenotype of all the F-HFD, irrespective of maternal adiposity or insulin sensitivity, was evidence of nonalcoholic fatty liver disease, as indicated by increased liver triglycerides, oxidative stress/damage and reprogramming of hepatic gluconeogenesis pathways. These offspring also have increased circulating levels of cytokines.

The present study focused on effects of fetal exposure to HFD on the development of ARH circuitry. In part 1, the main focus was differences in hypothalamic neuropeptide/receptor targets in F-CTR and F-HFD. In the F-CTR animal, POMC mRNA was present throughout the rostral/caudal extent of the ARH (*Figure. 4-1, B*); however, levels were low, consistent with this system being immature at this gestational age as previously shown (Grayson et al., 2006). In F-HFD animals, POMC expression was two fold up-regulated ($p < 0.05$) (*Figure 4-1, C,D*). Consistent with previous studies (Grayson et al., 2006), αMSH immunoreactivity (-*ir*) was low to undetectable in F-CTR group and qualitatively there was no apparent difference in the F-HFD group (*Figure 4-1, E, F*). In addition, levels of ACTH- and β-endorphin-*ir* were also low in these fetal animals (*data not shown*). In contrast, robust levels of αMSH- (*Figure 4-1, G*), ACTH- and β-endorphin-*ir* (*data not shown*) were present in the adult indicating that these antibodies worked in this species. Thus, the increased POMC expression does not appear to be translated into increased peptide levels.

In the fetal hypothalamus, NPY mRNA as determined by *in situ* hybridization, was present in the ARH and paraventricular nucleus (PVH), but there was no difference

in the expression between the two groups (F-CTR, *n*=5, 3.78 r.u. ± 1.40; F-HFD, *n*=3, 3.29 r.u. ± 1.32) (*data not shown*). NPY mRNA was also quantified in separate samples by qPCR and no difference was observed. In contrast, as determined by qPCR, F-HFD exhibited a four-fold reduction in AgRP mRNA (F-CTR, *n*=4, 0.94 ± 0.22; F-HFD, *n*=7, 0.38 ± 0.11; $p < 0.05$) as well as a twofold up-regulation of MC4R mRNA (F-CTR, *n*=4, 0.93 ± 0.07; F-HFD, *n*=7, 1.80 ± 0.24; $p < 0.05$) (*Figure 4-2, A*). A number of other relevant genes were also analyzed (Table *1*). While BDNF and SOCS3 were elevated in F-HFD, none of these changes were statistically significant.

Since ARH AgRP/NPY projections to the PVH develop during the third trimester (Grayson et al., 2006), NPY- and AgRP-*ir* were quantified in the PVH. No measurable difference in NPY-*ir* was detected in the PVH between F-CTR and F-HFD animals (F-CTR, *n*=4, 3.00 ± 0.62; F-HFD, *n*=7, 3.05 ± 0.56). In contrast immunoreactive fluorescence was decreased six-fold in F-HFD in the PVH (*Figure, 4-2, C*). While AgRP-*ir* fibers were readily detectable in F-CTR (*Figure 4-2, D*), they were sparse in the F-HFD (*Figure 4-2, E*). AgRP-*ir* in the adult is shown for comparison (*Figure 4-2, F*).

Cortisol is known to be elevated in response to HFD, readily crosses the placenta and regulates hypothalamic neuropeptide expression (Brooks et al., 1992; Power and Schulkin, 2006). We therefore measured cortisol levels in both maternal and fetal serum. Indeed, there was a small but significant increase in both maternal and fetal cortisol levels in the F-HFD compared to F-CTR (*Figure 4-3*). Circulating ACTH levels were also measured but no differences were found (F-CTR, *n*=8, 81.24 ± 16.74; F-HFD, *n*=18, 94.71 ± 14.28).

Since inflammatory cytokines are known to influence directly hypothalamic POMC and AgRP neurons (Deboer et al., 2008; Scarlett et al., 2007a; Scarlett et al., 2008), we used a qPCR based cytokine directed Superarray©. Of 84 targets investigated, eight distinct inflammatory factors and receptors were up-regulated in F-HFD compared to F-CTR, including IL1β and IL1R1 (Table 2). To identify cellular localization of ILIR1, immunohistochemistry was used. In both F-CTR and F-HFD the vast majority of IL1R1-*ir* was localized to endothelial cells of the microvasculature (*Figure 4-4, A,B*), with some immunoreactivity localized to neurons (*Figure 4-4, B*) in the lateral aspects of the ARH (*Figure 4-4, C*). However, no qualitative difference could be determined.

In part 2, we sought to determine the effect of the diet-reversal manipulation in the fifth year of the study in comparison to F-CTR. To specifically determine whether the dysregulation caused by HFD could be reversed by switching back to a CTR diet, we assessed POMC and AgRP mRNA using *in situ* hybridization (*Figure 4-5, A,B*) and AgRP protein expression using immunohistochemistry (*Figure 4-5, C*) in F-CTR and F-DR hypothalami. We also measured serum cortisol levels in F-CTR and F-DR animals (*Figure 4-5, D*). In each of these experiments, the consumption of the CTR diet by mothers who had consumed the HFD for the 4 previous years appeared to normalize expression of these targeted parameters.

DISCUSSION

While population-based human studies reveal alarming correlations between fetal HFD exposure and adult weight regulation (Hedley et al., 2004; Ong, 2006; Schaefer-Graf et al., 2005), information pertaining to the brain effects is minimal. Rodent studies point to causative factors contributing to the dramatic rise in childhood obesity. However,

the development of the circuits controlling body-weight occurs *in utero* in the primate as opposed to postnatally in the rodent. This developmental difference in primates makes the fetus particularly sensitive to maternal environment. We hypothesize that the fetal primate is particularly vulnerable to abnormalities in brain development caused by maternal diet and metabolic health during pregnancy.

In the present study, we report that the hypothalamic melanocortin system of 3rd trimester offspring is altered by prolonged maternal exposure to HFD, exhibited by up-regulation of POMC and MC4R mRNA and down-regulation of AgRP mRNA in the ARH. In adult rodent models of HFD exposure, the short-term hypothalamic response is an increase in POMC to evoke satiety (Bergen et al., 1999; Torri et al., 2002; Ziotopoulou et al., 2000). However, in the long-term, normalization or even decreased POMC expression has been reported in rodents (Huang et al., 2003; Ziotopoulou et al., 2000). In addition, in some models, permanently suppressed POMC tone has been attributed to the obese state (Kim et al., 2000). In our model, the fetus experiences the constant influx of lipids over the course of 130 days of gestation. Though the increase in POMC we observed may be an appropriate adaptation to high circulating nutrient levels under certain circumstances, the projections of these neurons have not fully developed at this fetal age. The physiological impact of this alteration is thus unclear but may have long-term effects on development.

Based on the increased POMC mRNA observed, we initially hypothesized that the F-HFD might accelerate development and/or increase projections of POMC neurons to downstream hypothalamic targets. Surprisingly, αMSH-*ir* fibers in the PVH and ARH were sparse in both F-CTR and F-HFD, similar to that previously described in the CTR

G130 fetus (Grayson et al., 2006). β-endorphin and ACTH, other POMC gene products, were similarly low in both the F-CTR and F-HFD despite the presence of prohormone convertases 1 and 2 (*data not shown*). Therefore, it is possible that the increased ARH POMC mRNA expression is not being translated into changes in peptide levels. On the other hand, it is also possible that the fibers simply have not yet developed and thus the peptide cannot travel. However, if this was the case, we would expect an accumulation in the cell body which was not evident in these studies. It is more likely that peptide production and secretion have both increased and therefore net immunoreactivity for αMSH appears unchanged. Nonetheless, these neurons are driven to significantly elevated levels of mRNA expression during a critical period of development where they cannot physiologically impact food intake directly.

At G130, NPY and AgRP fibers are just beginning to develop (Grayson et al., 2006). In this model, no significant difference in NPY mRNA or protein was observed between F-CTR and F-HFD. However, AgRP mRNA and protein were dramatically suppressed in F-HFD. Canonically, AGRP is up-regulated during fasting while exogenous injection of AgRP leads to increased food consumption (Stutz et al., 2005). Transgenic overexpression of AGRP results in hyperphagia and obesity. On the other hand, AgRP has also previously been shown to be transiently reduced in the early phase of HFD consumption in adult mice (Bergen et al., 1999; Hansen et al., 2004; Wang et al., 2002). However, in long-term models of overnutrition, AgRP expression is typically elevated (Dunbar et al., 2005; Huang et al., 2003). Therefore as with the alterations observed in POMC mRNA, AgRP mRNA changes may reflect an attempt to adapt to HFD which cannot be translated into an appropriate downstream effect.

From these studies it is not possible to determine what is driving the changes in the melanocortins (i.e. POMC and AgRP), whether it is a direct effect of the lipids or indirect via activation of other systems. Melanocortin expression has been shown to be affected by central and peripheral pro-inflammatory cytokine administration and can function as an anti-inflammatory agent. Specifically, IL1β has been shown to act directly on POMC neurons in the ARH via the IL1 receptor to stimulate αMSH release (Scarlett et al., 2007b). In addition, IL-1 acts directly on AgRP neurons, and AgRP release is inhibited by IL-1 (Deboer et al., 2008). Conversely αMSH is known to antagonize many actions of IL1 and other cytokines, including fever, anorexia, anxiety and hypothalamic-pituitary-adrenal (HPA) axis activation (Catania and Lipton, 1993; Catania et al., 1992; Hiltz and Lipton, 1990; Macaluso et al., 1994). The neuroendocrine effects of cytokine exposure are known to be inhibited by αMSH and augmented by AgRP infusion via melanocortin receptors in both the rodent and primate (Huang et al., 1998; Weiss et al., 1991; Xiao et al., 2003). These studies reflect an important relationship between the melanocortin and cytokine systems centrally.

In Superarray© PCR analyses of the medial basal hypothalamus, two main categories of cytokines were affected: 1) the interleukin-1 family was up-regulated and 2) cytokines expressly involved with trafficking across the blood-brain barrier. Taken as a whole, these data suggest that the maternal HFD is driving the pro-inflammatory pathway which in turn may be driving POMC gene expression in the fetus and may potentially impact the development of the blood-brain barrier. One potential outcome of this local inflammation is an increase in the secretion of anti-inflammatory αMSH. However, as we

have demonstrated, in this early gestational time point, αMSH production and projections likely are not sufficiently mature to mount a response to the HFD.

In light of these targeted changes to the melanocortin system, we postulate several long-term effects may occur. Since there does not appear to be downstream consequences in terms of fiber densities, the majority of the effects may be maintained at the cell body level. Ultimately, these neurons may have altered responsivity to incoming signals. Altered sensitivity to peripheral feedback signals may result leading to a change in overall set point later on. Ultimately these cells could suffer from premature cell death that may alter the population overall.

High-fat feeding and pro-inflammatory cytokines are known to increase cortisol levels in adults (Tannenbaum et al., 1997). In support of this, we have demonstrated significantly increased maternal cortisol levels in our HFD model. Importantly, we have demonstrated that maternal HFD also significantly increased fetal cortisol levels. It is unclear whether this fetal increase is due to a direct effect of the HFD or inflammatory cytokines on the fetal HPA axis, or due to passive flux of maternal cortisol. However, elevated cortisol levels during fetal development have been shown to prematurely activate the HPA axis and expedite the maturation of fetal organs (Challis et al., 2001). Excessive amounts of cortisol can cause intrauterine growth retardation which in many models results in adult-onset obesity and diabetes (Challis et al., 2001). In our previous studies in these same animals, fetal weight was significantly reduced and may reflect intrauterine effects. The implication of elevated cortisol in this model requires further future investigation.

A crucial unanswered question remains as to whether these changes in the fetus persist into postnatal life and further on into adulthood and consequently impact long-term body weight regulation. Future studies in the primate are necessary to determine the persistence of fetal changes. Nevertheless, in the rodent, it is established that *in utero* exposure to impaired glucose tolerance and gestational diabetes results in long-term alterations in hypothalamic architecture and physiology (Franke et al., 2005; Plagemann et al., 1999a; Plagemann et al., 1999b; Plagemann et al., 1999c). Furthermore, postnatal overfeeding in rodents also has long-lasting effects on hypothalamic function and hormone responsivity (Davidowa and Plagemann, 2000a; Davidowa and Plagemann, 2000b; Heidel et al., 1999; Plagemann et al., 1999c). These observations support our assumption that the hypothalamic changes seen in HFD fetal primates contributes to long-term challenges to body-weight regulation.

In rodent models of adult diet-induced obesity, restoration to a normal fat diet for approximately 20 weeks after high-fat diet consumption can help reverse responses to glucose, insulin sensitivity and central leptin responsivity (Enriori et al., 2007). In humans, modest changes in body-weight with diet intervention are known to improve metabolic parameters associated with obesity/diabetes. Thus, generalized clinical practice is to encourage limited weight gain (<15 lbs., normal 25-30 lbs.) for obese women during pregnancy. This is accomplished by a combination of limiting calorie quantity as well as altering the quality. Human studies have shown when weight gain is limited there is a decreased incidence in macrosomia, ceaserean delivery and preeclampsia; however, small-for-gestational age (SGA) babies were more prevalent in these mothers (Kiel et al., 2007). SGA is also associated with an obesity phenotype in children and adults. In this

study, DR animals were placed on an *ad libitum* low fat CTR diet specifically during pregnancy. While this did not dramatically change the metabolic phenotype of these animals, the fetal hypothalamic melanocortin expression and circulating cortisol was normalized in this cohort. Thus healthful, low fat eating may be the best method to protect offspring of obese mothers from *in utero* induced long-term body-weight dysregulation.

Though only approximately 5% of the prevalent cases of obesity are due to single-locus gene mutations, a large majority of these are a result of MC4R and POMC mutations which result in varying levels of obesity, hyperphagia, hyperinsulinemia and hyperglycemia which often begin during childhood (Farooqi, 2007). This attests to the importance and susceptibility of melanocortin system to body-weight maintenance. Taken together, increased POMC and decreased AgRP expression implies targeted action of HFD on the melanocortin system. This action is in the absence of effects on other feeding-related pathways, notably NPY. Furthermore, there is a dramatic hypothalamic up-regulation of pro-inflammatory cytokines in offspring from mothers which consumed HFD during pregnancy. Although it is undetermined whether this environment will have lasting effects on the offspring, we hypothesize that this early challenge to the melanocortin pathway may be a critical factor in the development of early-onset obesity in juvenile offspring.

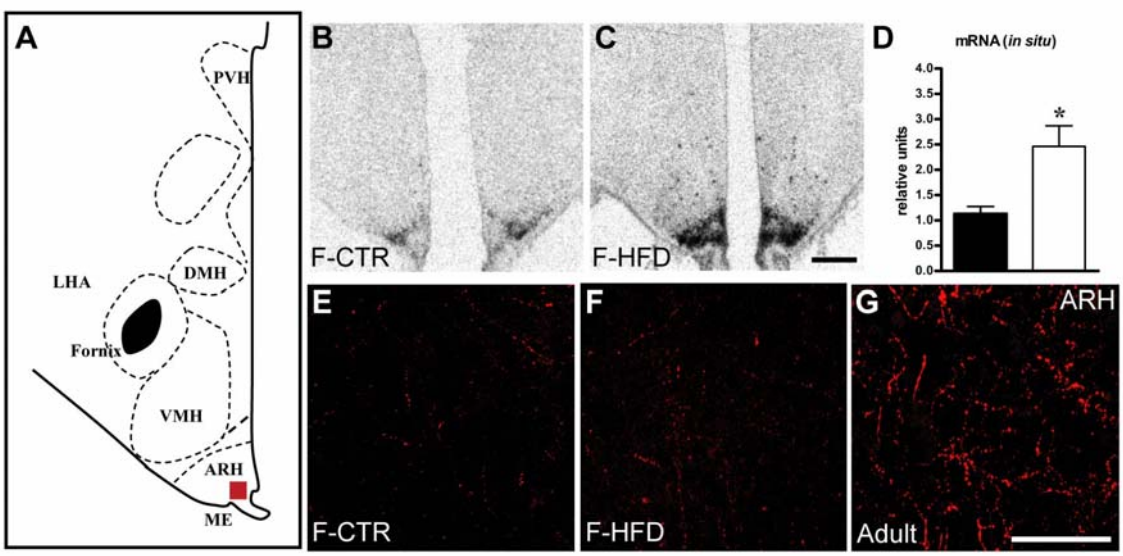


Figure 4-1. Effects of Maternal HFD on fetal POMC and αMSH expression. (**A**) Schematic of hypothalamic area. (**B,C**) Representative low-power autoradiographic images of POMC *in situ* hybridization signal in the ARH in F-CTR **B** and F-HFD **C** G130 hypothalamus. Images correspond to bregma -06.75 mm according to brain atlas (Paxinos, 1999). Scale bar represents 1mm. (**D**) Maternal consumption of HFD caused 2-fold up-regulation of POMC mRNA in fetal ARH (n=4 – 5, *p < 0.05) (black=F-CTR, white=F-HFD). (**E-G**) Representative fluorescent confocal micrographs of αMSH immunoreactivity in ARH in approximate location of red square on schematic in **a**. αMSH immunoreactivity is low to undetectable (F-CTR, **E**; F-HFD, **F)** in the ARH and not evident in other projection areas (*data not shown*). Note adult αMSH immunoreactivity is robust in **G**. Scale bar represents 100 microns.

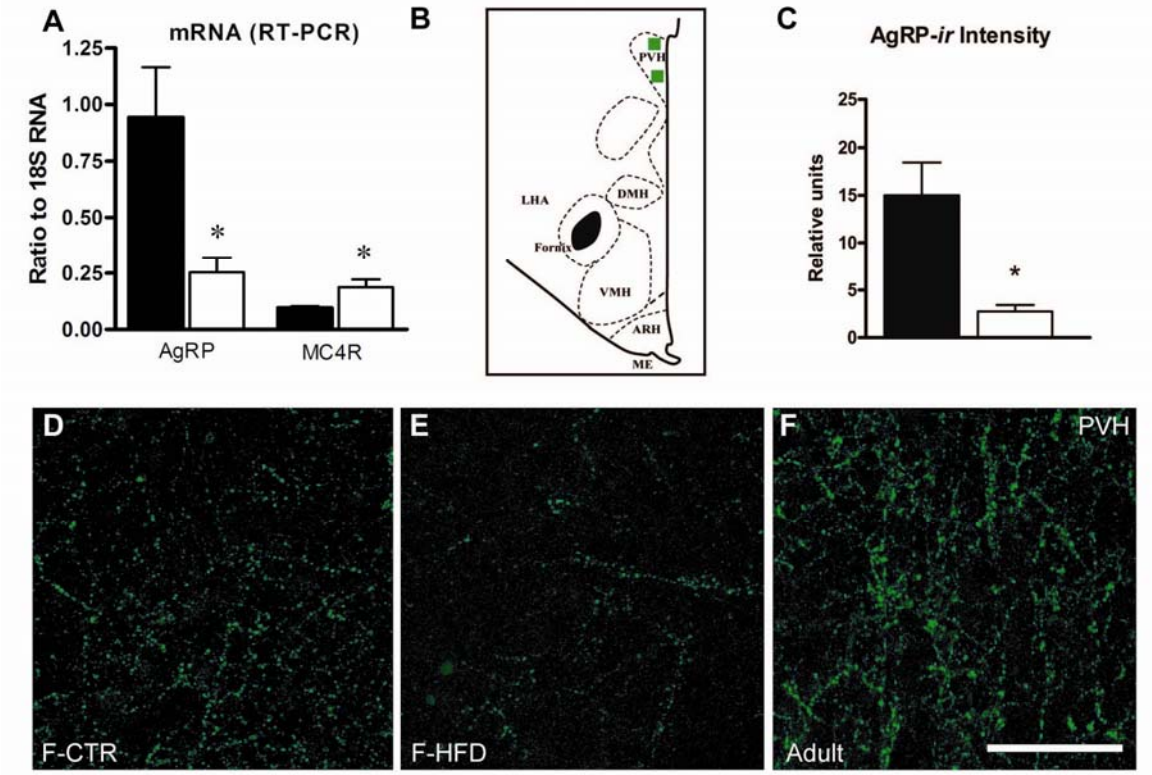


Figure 4-2. Effects of Maternal HFD on fetal AgRP and MC4R expression. (**A**) Maternal consumption of HFD caused a decrease in AgRP mRNA and increase in MC4R mRNA expression in G130 offspring by real-time PCR of medial basal hypothalamic blocks (n=4-7, *$p < 0.05$) (black=F-CTR, white=F-HFD). (**B)** Schematic of hypothalamic area. Green squares represent approximate location of confocal images in **D-F**. (**c**) Overall AgRP immunoreactivity in PVH is 6-fold higher in F-CTR than F-HFD (n=4-5, $p < 0.05$) (black=F-CTR, white=F-HFD). (**D-F**) Representative fluorescent confocal micrographs of AgRP immunoreactivity in the PVH in general area denoted by green square in **B**. PVH is a terminal projection field for AgRP fibers from ARH. F-CTR AgRP immunoreactivity, **D**, is greater than F-HFD in **E**. Adult immunoreactivity, **F**, is shown for comparison. Scale bar represents 100 microns.

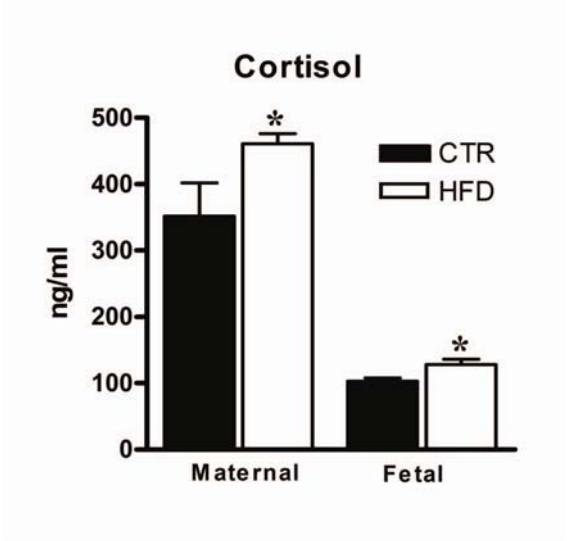


Figure 4-3. Serum maternal and fetal cortisol measurements. Both maternal and fetal F-CTR levels differed significantly from levels of animals on F-HFD (n=6-8, *p < 0.05).

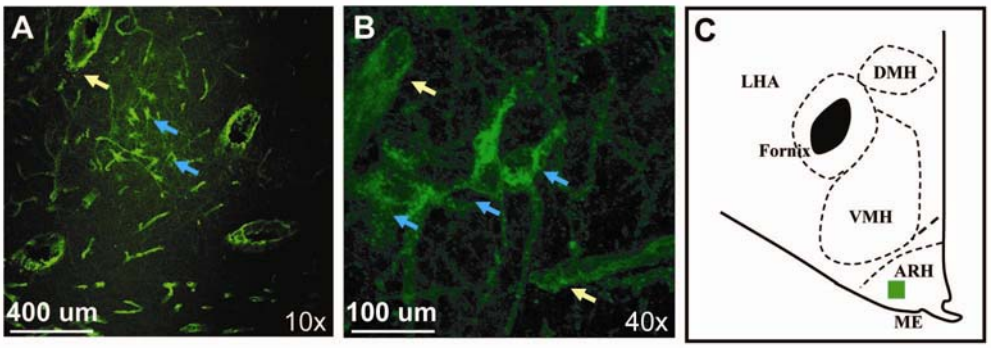


Figure 4-4. IL1RI immunoreactivity in the ventromedial hypothalamus. (**A**) Representative 10x confocal fluorescent micrograph of IL1R1 immunoreactivity shown in region demarcated by the green square in schematic in **C**. (yellow arrows, vascular endothelial cells) (blue arrows, lateral ARH neurons). (**B**) Representative 40x confocal fluorescent micrograph of IL1R1 immunoreactivity.

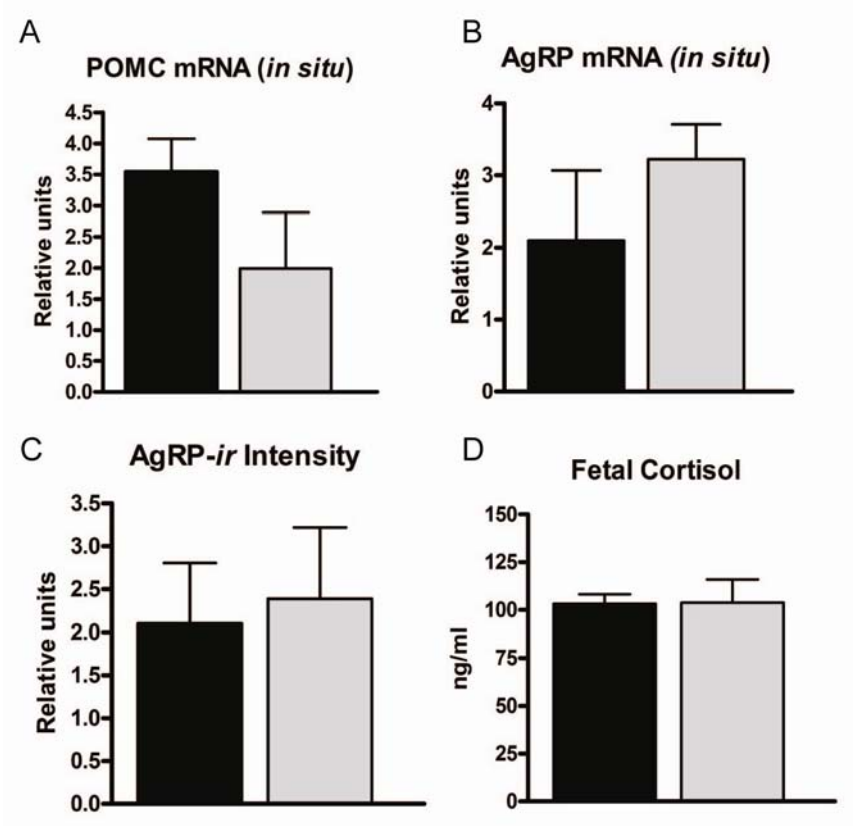


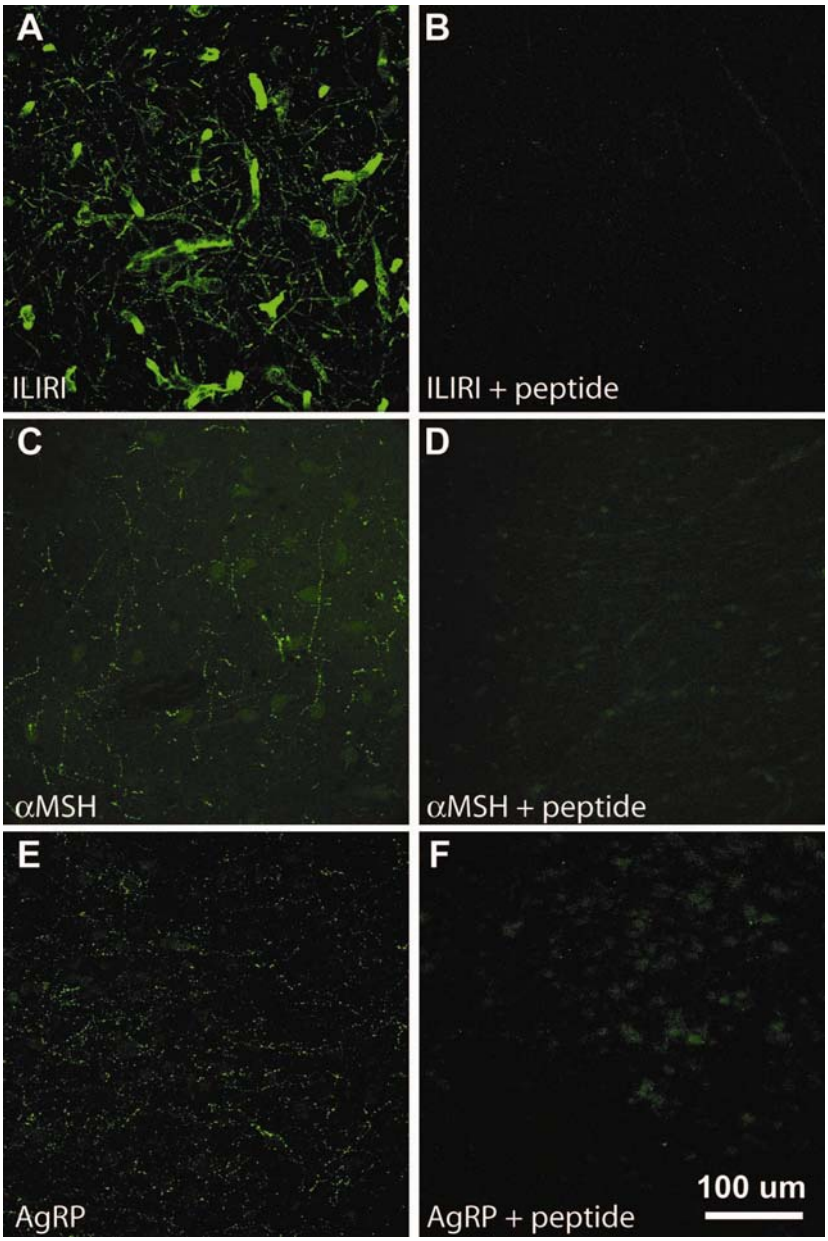
Figure 4-5. Effects of maternal diet-reversal (DR) on fetal POMC, AgRP and cortisol. **(A,B)** Quantification of fetal *in situ* hybridization of POMC and AgRP mRNA **(C)** Comparison of AgRP-*ir* fiber fluorescence **(D)** Comparison of fetal cortisol measurements expressed in ng/ml (CTR=black, DR=gray)

	F-CTR	F-HFD	*p* value
BDNF	1.90 ± 0.35	4.27 ± 1.13	0.10
CART	0.22 ± 0.05	0.32 ± 0.07	0.33
GLUT1	0.98 ± 0.31	1.45 ± 0.11	0.13
IGF1R	0.99 ± 0.09	1.04 ± 0.03	0.20
INSR	1.11 ± 0.13	1.90 ± 0.33	0.08
IRS1	0.48 ± 0.12	0.42 ± 0.05	0.60
IRS2	1.19 ± 0.08	1.51 ± 0.11	0.22
LEPR	1.54 ± 0.40	1.48 ± 0.16	0.86
MOR	0.58 ± 0.08	0.90 ± 0.22	0.24
NPY1R	0.21 ± 0.021	0.19 ± 0.034	0.67
SOCS3	0.65 ± 0.09	1.14 ± 0.30	0.25
UCP2	0.84 ± 0.11	1.23 ± 0.14	0.07
VEGF	3.27 ± 0.19	5.37 ± 1.16	0.13

Table 4-1. Hypothalamic mRNA expression by RT-PCR in G130 fetuses. Values are mean ±SEM. (*n*=4-7).

Gene	Fold Increase
Interleukin-1 Family	
IL1β	7.39**
IL1R1	1.76**
IL1F6	22.48*
IL1F7	2.35*
BBB Trafficking	
CCL26 (Eotaxin)	4.02*
CCR3 (Eotaxin-R)	7.53*
CCL19 (MIP-3)	3.01**
CCL2 (MCP-1)	3.09*

Table 4-2. Brain cytokines are up-regulated in G130 F-HFD compared to F-CTR. The fold-change for each gene was calculated as $2^{(-\Delta\Delta Ct)}$, where $\Delta\Delta C_t = \Delta C_t$ (F-HFD group) - ΔC_t (F-CTR group). (n=4-7, *$p < 0.05$ or **$p < 0.01$).



Supplemental Figure 4-1. Preabsorption of antisera with control peptide. **(A)** Confocal fluorescent image of ILIRI-*ir* at 25x. **(B)** Confocal image of ILIR1-*ir* preabsorbed with control peptide **(C)** Confocal fluorescent image of αMSH-*ir* at 25x. **(D)** Confocal image of αMSH-*ir* preabsorbed with control peptide **(E)** Confocal fluorescent image of αMSH-*ir* at 25x **(F)** Confocal image of AgRP-*ir* preabsorbed with control peptide

Chapter 5
Central Changes in the Serotonergic System in Response to Maternal High-Fat Diet in the Fetal Nonhuman Primate

ABSTRACT

The central serotonin system (5-hydroxytryptamine, 5-HT) has long been associated with body-weight regulation and obesity, in addition to psychological diseases such as stress and anxiety disorders and depression. Increased 5-HT activity has been implicated in hypophagia and weight reduction. Furthermore, 5-HT agonists and 5-HT reuptake inhibitors stimulate weight loss in rodents as well as humans. On the other hand, depletion of 5-HT leads to increased food intake and obesity in rodents. The purpose of these studies was to use our nonhuman primate model (NHP) to investigate the contribution of chronic maternal high-fat diet (HFD) consumption on the central 5-HT system in the fetus. Japanese macaques were placed on one of two diets for 4 years; 1) control diet – 15% fat calories or 2) high calorie and fat diet – 32% fat calories. During each year of the diet, fetuses were obtained at gestational day 130 (full term = 175 days), a critical period for the development of feeding-related circuits in the central nervous system. Fetuses from mothers maintained on HFD for 2-4 years exhibited significant up-regulation of TPH2 mRNA in the midbrain and hypothalamus. In addition, 5-HT immunoreactivity was specifically decreased in the arcuate nucleus of the hypothalamus (ARH). In addition we see increased level of 5-HT1_A mRNA in the raphe of the midbrain. We interpret these changes as a significant up-regulation and turnover of 5-HT transmission as a result of exposure to maternal HFD. Early developmental 5-HT dysregulation may have wide ranging long-term effects including behavior, alterations in stress responsiveness, and body-weight regulation, with respect to food preference/palatability as well as energy expenditure.

INTRODUCTION

The serotonin (5-hydroxytryptamine, 5-HT) system has a well established role in modulating appetitive systems predominantly through actions on the melanocortin system. In addition, 5-HT has a robust role in stress and anxiety pathways and ultimately psychological well-being. As a result, the 5-HT system provides a strong link between obesity and emotionally-motivated food intake. In light of the epidemic occurrence of childhood obesity, the impact of overnutrition on the early development of the serotonin system is important to understand.

Generally, 5-HT modulates food intake through action as a satiety factor (*see reviews* (Heisler et al., 2003; Lam and Heisler, 2007)). The 5-HT system in the rodent modulates food intake through widespread reciprocal innervation of many orexigenic and anorexigenic systems including the neuropeptide Y (NPY) and melanocortin systems. Chemical depletion of 5-HT in the brain has long been known to increase food intake and adiposity in rodents (Breisch et al., 1976; Samanin et al., 1977). In states of positive energy balance, such as the diet-induced obese rat, a decreased central turnover of 5-HT has been observed (Hassanain and Levin, 2002). Obese humans are reported to have lower plasma tryptophan and 5-hydroxyindole acetic acid (5-HIAA) levels than lean humans indicating a depressed serotonergic tone (Ashley et al., 1985; Breum et al., 2003). Additionally, a study of monoamine levels in obese women reports significantly lower levels of the 5-HT metabolite 5-HIAA in cerebrospinal fluid (CSF) in comparison to lean women (Strombom et al., 1996). Furthermore, pharmacological administration of 5-HT agonists and selective serotonin reuptake inhibitors (SSRIs) demonstrate that stimulation of 5-HT activity results in weight reduction in rodents as well as humans

(Lamberto et al., 1993; Smedegaard et al., 1981). Finally, variations in the promoter region of the 5-HT transporter (5-HTT) are associated with higher levels of anxiety and is a risk factor for obesity

The principal source of central 5-HT are the nine raphe nuclei located in the midbrain (Lam and Heisler, 2007). 5-HT synthesis from tryptophan is regulated by the rate-limiting enzyme tryptophan hydroxylase (TPH). *TPH2* is the predominant isoform of this enzyme in the brain, though *TPH1* is expressed developmentally within the rodent hypothalamus and the periphery (Nakamura et al., 2006). After its release at the synapse, 5-HT is shuttled back into neurons via the 5-HT transporter (5-HTT) and is eventually degraded through the action of monoamine oxidase (MAO) to 5-HIAA. The 5-HT system is complex, regulated by an extensive array of receptors with numerous pre- and post-synaptic receptor subtypes. Within the raphe, 5-$HT1_A$ autoreceptors modulate feedback of 5-HT stimulation from various inputs. Specifically, these somatodendritic receptors inhibit 5-HT neuronal cell firing and 5-HT release onto postsynaptic sites (Hamon et al., 1990). In the rat hypothalamus, 5-HT_{1A} receptors have been localized postsynaptically with NPY, AgRP, CART, POMC, MCH and orexin cell bodies (Collin et al., 2002). Within the hypothalamus of rodents, 5-HT effects on food intake are mediated predominantly by presynaptic stimulation of anorexigenic pro-opiomelanocortin (POMC) neurons via 5-$HT2_C$ receptors (Heisler et al., 2002) and a reduced inhibitory action through 5-$HT1_B$ receptors on NPY/AgRP neurons. In addition, postsynaptic 5-$HT1_A$ receptors, present in the ventromedial nucleus of the hypothalamus (VMH) also appear to have a role in the suppression of food intake and increased energy expenditure.

In the human, 5-HT neurons are born by the 5th week after conception and organized into the nine raphe nuclei by the end of the first trimester (Sundstrom et al., 1993; Takahashi et al., 1986). 5-HT projections continue to mature during the first years of life and then 5-HT levels begin to decline to adult levels by age 5 (Hedner et al., 1986). Within the rat, 5-HT neurons appear in the raphe on the 13th fetal day (Lidov and Molliver, 1982). 5-HT terminals are pivotal to target differentiation processes such as neurogenesis, dendritic refinement, synaptic remodeling and cell migration. This is demonstrated by studies in which prenatal depletion of 5-HT delays onset of neurogenesis in target tissues (Lauder and Krebs, 1976; Lauder et al., 1982). Conversely, when 5-HT production is stimulated, neurite outgrowth is enhanced (Shemer et al., 1988; Shemer et al., 1991). Furthermore, terminal density of 5-HT innervation may be important for regulation of other neurotransmitter and peptide systems, in particular dopamine (Benes et al., 2000) and glutamate (Dooley et al., 1997; Lavdas et al., 1997). Because of the ubiquitous expression of 5-HT in the brain, nearly every neuropeptide and neurotransmitter system is impacted by 5-HT terminal differentiation (Whitaker-Azmitia, 2001). Consequently, any alterations in 5-HT development can potentially have widespread neuronal effects.

Previously we reported that fetuses exposed to maternal HFD (F-HFD) *in utero* exhibited a targeted disruption of the melanocortin system during the third trimester in comparison to fetus exposed to maternal CTR diet (F-CTR)(Grayson et al., 2009) . These findings include two-fold up-regulation of POMC mRNA, decreased AgRP mRNA and fiber immunoreactivity (-*ir*) as well as increased levels of MC4R mRNA (Grayson et al., 2009). Since the 5-HT system modulates food intake partially through its innervation of

the melanocortin system, it is possible that altered melanocortin system development may be due, in part, to 5-HT alterations.

The purpose of this study was to determine whether the third trimester F-HFD exhibited changes in their 5-HT system, in comparison F-CTR. This was accomplished by quantitative analysis of TPH2, 5-HTT and 5-HT_{1A} mRNA expression in the raphe using *in situ* hybridization. To further investigate the downstream targets of this change in 5-HT tone, the mRNA expression of hypothalamic 5-HT_{1A}, $_{1B}$, $_{2A}$, $_{2B}$ and $_{2C}$ receptors as well as TPH2, MAO_A, and 5-HTT was also investigated. As compared to F-CTR, maternal consumption of HFD caused disruption of the fetal 5-HT system. The long-term effects of this disruption on postnatal outcome are crucial to understand, since 5-HT abnormalities are well-recognized to have a vast array of detrimental effects.

MATERIALS AND METHODS

Animals

All animal procedures were approved by the Oregon National Primate Research Center (ONPRC) Institutional Animal Care and Use Committee and conformed to NIH guidelines on the ethical use of animals.

A complete characterization of the maternal and fetal phenotype has been reported (McCurdy et al., 2009). Briefly, animals were maintained in outdoor/indoor group harem housing (male: female ratio of 2:9) on a natural light schedule. Age and weight matched adult *Macaca fuscata,* Japanese macaques, were fed a control (CTR) or high fat/calorie diet (HFD) for up to 4 years. The CTR diet (#5037, Purina Mills Co., St Louis, MO) provided 15% calories from fat and the HFD (Test Diet, 5A1F, Purina Mills Co., St Louis, MO) supplied 32% of calories from fat and included calorically-dense treats.

During monthly health checks, animals were examined for pregnancy by palpation and then later dated by ultrasound examination

Experimental Design

Fetuses were collected by caesarean section on gestational day 130 (G130) (early 3rd trimester; full term is 175 days) from each pregnancy in CTR and HFD groups on years 2-4 of the diets. Fetal brains were harvested from each year and taken either fresh or fixative-perfused (F-CTR, *n*=4 fresh, *n*=5 fixed) (F-HFD 2/3/4, *n*=6 fixed, F-HFD 3/4, *n*=4 fresh). The fetuses were perfused with 4% paraformaldehyde fixative (pH 7.4) and harvested as previously described (Grayson et al., 2006). When tissues were harvested fresh, the brain was blocked and then stored in –80°C until RNA extraction. The hypothalamus, midbrain and brainstem were blocked into separate pieces and frozen rapidly in liquid nitrogen and then stored at –80°C until RNA was extracted.

***In situ* Hybridization**

Fetal hypothalamic and midbrain blocks were sectioned at 35μm using a freezing microtome and were collected in 1:24 series. Sections were stored in ethylene glycol cryoprotectant at –20°C until time of use. For *in situ* hybridization, 1:8 series of sections were slide-mounted in RNAse free potassium phosphate buffer saline (KPBS) (pH 7.4) and vacuum-dessicated overnight. The cRNA probes were transcribed from cDNA clones (kindly provided by CL Bethea) (Pecins-Thompson et al., 1998), TPH2 – 300 base pairs (bp), 5-HTT – 253bp, and 5-$HT1_A$ – 431bp. The cRNA probes were transcribed in the presence of 100% P^{33} labeled UTP (Perkin Elmer, Waltham MA; Table 1). Standard *in situ* hybridization methods were used as previously described (Grayson et al., 2006; Grove et al., 2001b). For visualization, the probe-labeled sections were exposed to sheet

film (Biomax MR, Kodak) for an appropriate period of time (TPH2 –5 days; 5-HTT – 2 days; 5-HT1_A – 5days). Autoradiographic images were captured using a CoolSnap HQ camera (Photometrics, Westchester, PA) along with Metamorph Software (Universal Imaging Corp). Integrated morphometry analysis was used to measure total density by multiplying total area by optical density of the three levels of the midbrain, rostral (approximately bregma -17.78 ±1 mm), medial (bregma -19.75 ±1 mm) and caudal (bregma -23.40 ±1mm) (reported in relative units (r.u.)). An average of 3-4 matched sections were analyzed for each level in each animal.

Fluorescent Immunohistochemistry

Two experiments were performed using fluorescent immunohistochemistry: 1) 5-HT-*ir* projections in ARH-containing sections to determine the terminal field density and 2) TPH2-*ir* in raphe-containing sections to determine the relative numbers of TPH2-containing neurons. Standard immunohistochemical methods were used as previously described (Grayson et al., 2009). Briefly, sections were washed in KPBS and then blocked in 2% donkey serum in 0.4% triton X-100/KPBS. The following antibodies were obtained: rabbit anti-5-HT (#S5545, 1:5000, Sigma-Aldrich) and rabbit anti-TPH2 (#NB100-74555, 1:2500, Novus Biologicals). Primary antibodies were diluted to their working concentrations in 2% donkey serum in 0.4% triton-X-100/KPBS and incubated with the tissue overnight at 4°C. After incubation, the tissue was rinsed in KPBS, incubated in FITC or rhodamine-labeled donkey anti-rabbit or mouse IgG (Jackson Immunoresearch, West Grove, PA; 1:200) in 0.4% triton-X-100/KPBS for 1 hr. Sections were wet mounted and coverslipped with glycerol-based mountant and stored at 4°C prior to analysis.

Image Analysis

Confocal laser microscopy, as previously described (Grove et al., 2000), was used to capture immunofluorescent images. All images were captured with 25x oil objective (NA = 0.75). For fluorescence intensity measurements of 5-HT in the ARH, a series of optical planes at 0.5 μm intervals along the z-axis of the section were scanned for each fluorescent signal and stored as a stack of 1024 x 1024 pixel images, processed with MetaMorph and presented as maximum projections totaling 5 μm. Two fields of view per section (right/left) in three equally spaced anatomically-matched sections per animal were imaged and analyzed (CTR/HFD *n*=3). Total immunoreactive fluorescent intensity was measured using the same conditions and threshold parameters for all images. Total gray value was measured for each field. Analysis was performed by individuals blinded with respect to group. The Metamorph Imaging System was used to process the images, and brightness and contrast levels of the digital images were adjusted with Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Cell counting

Immunohistochemical images were captured under fluorescent illumination using a Photometrics CoolSNAP HQ camera (Roper Scientific, Tucson, AZ) connected to a Nikon microscope (E800) with a Plan Apo 4X objective. Total numbers of immunoreactive cells were counted in all raphe-containing sections.

Real-time PCR

Real-time RT-PCR was performed on both medial basal hypothalamic blocks and a single 3mm midbrain slice. Medial basal hypothalamic blocks covered 3mm x 3mm area surrounding the third ventricle. Fetal midbrain blocks were sliced using a brain mold and

external landmarks to encompass a 3mm region around the dorsal raphe. Briefly, tissue was homogenized in 1 ml Trizol reagent (Invitrogen, Carlsbad, CA) and total cellular RNA was isolated according to the manufacturer's specifications. Total RNA was further purified using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA). The concentration of RNA was determined by spectrophotometry (Nanodrop ND-1000, NanoDrop Technologies, Wilmington, DE), and RNA integrity was confirmed by bioanalysis (Agilent 2100 Bioanalyzer, Agilent Technologies, Inc., Santa Clara, CA). RNA samples (1μg) were DNAse-treated and reverse transcribed using random hexamer primers (Promega, Madison, WI). Genes of interest were investigated using inventoried macaque-specific real-time PCR primers/probe sets when available (Applied Biosystems, Foster City, CA). The inventoried sets used were as follows: 5-HT_{1A} (#Hs00265014_s1), TPH1 (#Rh02847747_m), TPH2 (#Rh02788840_m1) and MAO_A (#Rh02793876_m1). Otherwise, macaque-specific Taqman primer and probe sets were designed using Primer Express Software and purchased separately (primers: Invitrogen, Carlsbad, CA; probes: Operon Biotechnologies, Inc., Huntsville, AL). All sequences used are summarized in Table 1. Gene targets were normalized to asparagine-linked glycosylation 9 (ALG9) as an internal control of RNA quantity.

Cerebrospinal Fluid Assays

Cerebrospinal fluid (CSF) was collected at time of necropsy prior to exsanguinations. Samples were snap frozen in liquid nitrogen and stored in -80°C until time of assay. 5-HT and 5-HIAA ELISAs were performed by the ONPRC Endocrine Services Core. Briefly, commercially available ELISA kits were used to measure 5-HT (#RE59121) and 5-HIAA

(#RE59131) (IBL Transatlantic, Toronto, Ontario, CAN) in 50µl of monkey CSF according to the manufacturer's specifications (CTR *n*=5, HFD *n*=8).

Statistical Analysis

Comparisons were conducted using two-tailed Student's t test using Prism Biostatistical Software (v.4) (San Diego, CA). Differences were considered significant if $p < 0.05$. Data is presented as mean ±SE.

RESULTS

Our initial focus was to determine the density and intensity of 5-HT projections to the ARH, where the 5-HT system modulates the melanocortin system. Within the ARH, 5-HT-*ir* was dramatically lower in F-HFD in comparison to F-CTR (*Figure 5-1*). The decrease was due largely to fluorescent intensity with very little change in terminal density (immunoreactive area). In general, overall density was slightly decreased but overall immunoreactive brightness per fiber was diminished. Immunohistochemistry of 5-HTT in the hypothalamus confirmed the presence of fibers innervating the ARH (*data not shown*). We also determined if differences existed in the prefrontal cortex and did not see any qualitative changes (*data not shown*).

In order to determine whether production levels of 5-HT are changed at the level of the mRNA, we performed *in situ* hybridization for TPH2. In the fetal monkey, TPH2 mRNA was localized throughout the raphe region similar to that previously reported in the adult (Sanchez et al., 2005). Relative levels of TPH2 mRNA were elevated greater than two-fold in the rostral raphe but was not changed in the medial or caudal raphe in F-HFD (*Figure 5-2, A-C*) However, there was no difference in the total number of TPH-*ir* cell bodies between these two groups (*Figure 5-3*). Therefore, it is assumed that the 5-

HT-producing neuronal population in the raphe have increased levels of TPH2 mRNA per cell without an increase in cell number, possibly reflecting increased synthesis and release of 5-HT. Since the 5-HT transporter is important for resequestration of 5-HT, alterations in 5-HTT could indicate increased activity at the synapse. However, 5-HTT mRNA levels by *in situ* hybridization were unchanged in the three midbrain levels investigated in F-HFD in comparison to F-CTR (*Figure 5-4, A-C*).

5-HT_{1A} autoreceptor activity is critical for the feedback mechanism controlling 5-HT production at the level of the midbrain. In F-HFD, 5-HT_{1A} autoreceptor expression was up-regulated in the rostral and medial raphe but not in the caudal 5-HT-producing regions (*Figure 5-5, A-C*).

In order to perform a high throughput analysis of other receptors and enzymes within the raphe, we used quantitative real-time PCR on single 3mm slices from the raphe-containing region of the midbrain. No differences were determined between F-CTR and F-HFD in these experiments (*data not shown*). The high degree of variability within the mRNA expression levels of TPH2, MAOA, 5-HTT, 5-HT_{1A}, $_{1B}$, $_{2A}$, $_{2B}$ and $_{2C}$ receptors may have been reduced by microdissection of raphe regions, which may have revealed some regional differences. Similarly, enzyme, transporter and receptor experiments were performed using tissue from medial basal hypothalamic blocks (*Figure 5-6, C,D*). TPH2 mRNA levels in the medial basal hypothalamus were significantly increased in F-HFD animals concomitant with reported differences assessed by *in situ* in the midbrain (*Figure 5-2*). No further differences in expression were detected in receptor or transporter mRNA levels (*Figure 5-6, C,D*). We also determined whether TPH1 was detectible in the NHP fetal brain because of reported activity of this enzyme during development. TPH1

expression was detectible in the fetal hypothalamus but values were far below that of TPH2 in both F-CTR and F-HFD (*data not shown*). Therefore, we report that TPH2 is the predominantly active isoform in the G130 NHP at an mRNA level.

To further determine if 5-HT-*ir* changes in F-HFD are due to overall increases in 5-HT turnover at the level of the hypothalamus, which could potentially be released into the CSF, 5-HT and metabolite 5-HIAA levels were assessed by ELISA within the CSF of G130 fetuses. No changes were determined in 5-HT. A trend toward decreased 5-HIAA was determined (*Figure 5-7, A,B*).

DISCUSSION

The present study focused on the impact of maternal consumption of HFD on the fetal 5-HT system by investigating the expression of serotonergic-specific enzymes, transporter and receptor mRNA expression in the raphe as well as their 5-HT-containing projections to the ARH in G130 fetal monkeys.

Within the ARH, 5-HT-*ir* is diminished without a change in fiber density. We interpret this as an increased turnover of 5-HT within the ARH. Taken together with the increased levels of TPH2 mRNA in both the midbrain and hypothalamus, we interpret these changes as an up-regulation of 5-HT production to produce a satiety response (*Figure 5-8*). Though the fetus is unable to govern its own nutrient intake, the fetal appetitive pathways are reactive to the abundance of maternal nutrient intake. In this model, maternal consumption of HFD also leads to altered expression of the fetal melanocortin system, with elevated POMC and decreased levels of AgRP within the ARH (Grayson et al., 2009). In an adult animal, this increased production and transmission of

5-HT would modulate the melanocortin system, resulting in a hypophagic response to the calorie-rich diet.

In the F-HFD we also see an increase in 5-HT_{1A} receptors within the dorsal raphe. This up-regulation in the dorsal raphe signifies an attempt to exert a brake on neuronal 5-HT release and decrease firing in the efferent targets of these neurons (Sprouse and Aghajanian, 1987). This upregulation of 5-HT_{1A} receptors is therefore possibly a compensatory response to the increased level of release in the 5-HT system.

In this study we do not see 5-HT changes within the CSF. This is probably due to the fact that changes within the 5-HT system are specific to the hypothalamus. For example, we did not see qualitative changes in 5-HT-*ir* within the prefrontal cortex (*data not shown*) nor were there changes in the more caudal regions of the raphe that project to brainstem nuclei. The CSF sample used in the 5-HT and 5-HIAA assay was a spinal extract and therefore likely do not reflect local changes in the region of the hypothalamus.

Though we do believe that 5-HT production and release is up-regulated in F-HFD, an alternative interpretation to these data is that the 5-HT system is suppressed. The data could be interpreted that there is decreased production of 5-HT as evidenced by the lower 5-HT-*ir* in the hypothalamus and the high levels of 5-HT_{1A} receptor mRNA. In this interpretation, the increase of rate-limiting enzyme TPH2 in both the raphe and hypothalamus is interpreted as an attempt to compensate for an inhibition of 5-HT production exerted by elevated 5-HT_{1A} receptors within the dorsal raphe. This compensation may be generated by the increased glucocorticoids in these animals (Grayson et al., 2009). This interpretation is less favorable in light of the overall changes

we see in the melanocortin system which are supported by increased 5-HT production and release to effect a satiety signal.

In the present studies, no expression differences were detected within hypothalamic 5-HT receptor population. This is not surprising since the medial basal hypothalamus is a heterogeneous mix of neuronal populations that express a variety of pre- and postsynaptic 5-HT receptors that have both competing and reciprocal actions. Without determining the specific phenotype of these cells, conclusions concerning their expression would be difficult.

In our model of maternal HFD consumption, a diet of 32% fat results in the development of a diet-resistant and diet-sensitive maternal phenotype (McCurdy et al., 2009). The diet resistant mothers experience no weight gain on HFD and their glucose tolerance and insulin sensitivity are similar to that of CTR mothers. In contrast, diet-sensitive mothers experience considerable weight gain in addition to glucose intolerance. In the present studies, the effects on the fetal 5-HT system occur irrespective of maternal sensitivity to diet. These fetuses have neither elevated insulin nor leptin levels. However they experience the transfer of hypercaloric, fat-laden diet. And the fetal 5-HT system exhibits the same overall response independent of maternal sensitivity to the diet. Overall, we conclude from these observations that consumption of a high-fat diet during pregnancy has many broad implications on the metabolic health of the offspring irrespective of the mother's ability to defend her own body-weight during the consumption of a diet high in fat.

This conclusion also holds true for the immune response of the F-HFD. We have previously reported that inflammatory pathways are activated both centrally and

peripherally in F-HFD (McCurdy et al., 2009). The pro-inflammatory cytokines, in particular IL1β and TNFα, are known to affect the 5-HT system (Gemma et al., 2003; Ishikawa et al., 2007; Merali et al., 1997). Interferonα has been shown to reduce the density of both 5-HT and noradrenergic terminal fields in the cortex (Ishikawa et al., 2007). Additionally, NFκB activation is present within 5-HT neurons exposed to steroids (Bethea et al., 2006). *In vitro* models have shown that IL1β can inhibit 5-HT neuron firing (Manfridi et al., 2003); furthermore IL1β administration does acutely increase 5-HIAA levels in the nucleus accumbens and hippocampus (Merali et al., 1997). Though we were unable to find IL1β or NFκB antibodies that worked appropriately in the fetal brain, we hypothesize that altered inflammatory expression induced by maternal HFD consumption may potentially have effects on the 5-HT system.

Not only do our studies suggest that maternal HFD can impact the fetal 5-HT system, but they suggest the need for further studies to investigate the impact of maternal SSRI intake during pregnancy and the effects on fetal appetitive circuits. Recent reports indicate that as many as 15% of pregnant women are prescribed SSRIs during pregnancy (Cooper et al., 2007). Though SSRIs are prescribed during gestation, adverse fetal conditions are increasingly being reported which include neonatal withdrawal syndrome and abnormal crying (Sanz et al., 2005). Neonates from mothers prescribed SSRIs during pregnancy experienced suppressed levels of 5-HIAA in cord blood and in addition, exhibited a 4-fold increase in scores for serotonergic symptoms in comparison to controls (Laine et al., 2003). These symptoms included increased tremors, restlessness and rigidity and are a marker of 5-HT hyperstimulation. SSRI use during pregnancy and its effects on

the metabolic phenotype of offspring has not been studied and has many implications for the long-term psychological as well as physical health of the offspring.

In conclusion, the regulatory role of 5-HT in food intake and energy balance is well established in rodents and is likely even more pivotal in primate species, where higher behavioral functions (i.e., mood and hedonic motivation) can affect food intake beyond the need to meet basic energy requirements. Though the impact of diminished 5-HT neurotransmission is not understood in the fetal nonhuman primate, it is needless to say that the impact on both energy balance as well as stress and mood disorders is important. Further work in the juvenile animals is underway to determine the effect of HFD on temperament and anxiety and whether these correlate with neuroanatomical measures.

	Probe	Forward Primer	Reverse Primer
5-HT1$_B$	CTCATCACCTTGGCCACCACGCTC	ATCGCCCTACCCTGGAAAGTA	GTCCGGTACACTGTGGCAATC
5-HT2$_A$	CCAGGGTCCTACACAGGCAGAAGGACTATG	GTTCCAGCGGTCGATCCA	CGATACCCAGCACCTTGCAT
5-HT2$_B$	TCAAAAACAAGCCACCTCGACGCCT	CCATGCTTTACAGAAGAAGGCTTAC	AAGGTGTTTCATCCCTTTGGAA
5-HT2$_C$	CATTGGGTTTTGATCTCAGCA	ACTGGGAAATGAAATCCCAGAGT	CACCTTGTGTGAAGCACACAAA
5-HTT	TGTCATCTTCACGGTGCTCGGTTACATG	GGTGAACTGCATGACGAGCTT	CTTTGGCCACCTCAGACACA
ALG9	ACTGTCTTCCTGTTCGGG	CGATACCGCCTGGAGCACTA	AACAGTGCCACAGAGCGAGAA

Table 5-1. Probe/primer sets for RT-PCR.

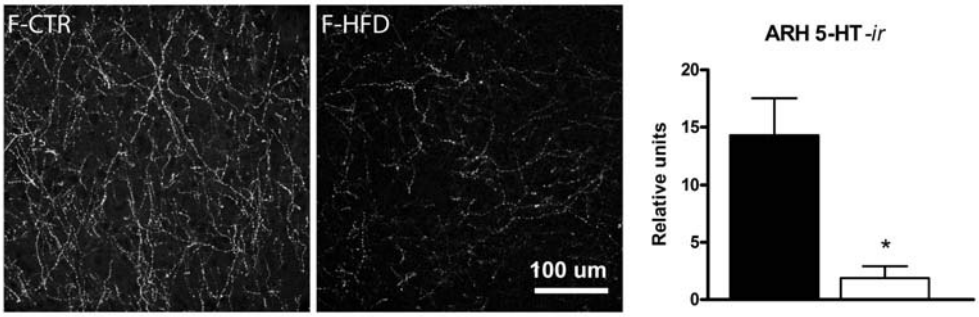


Figure 5-1. 5-HT-*ir* is reduced in F-HFD. Fluorescent confocal micrographs of 5-HT immunoreactivity in the ARH in F-CTR and F-HFD in the G130 hypothalamus. Images correspond to a distance of -06.75 mm according to the brain atlas (Paxinos, 1999). Scale bar represents 100 microns. Maternal consumption of HFD resulted in down-regulation of 5-HT-*ir* in fetal ARH (n=3, $p < 0.05$) (F-CTR=black, F-HFD=white).

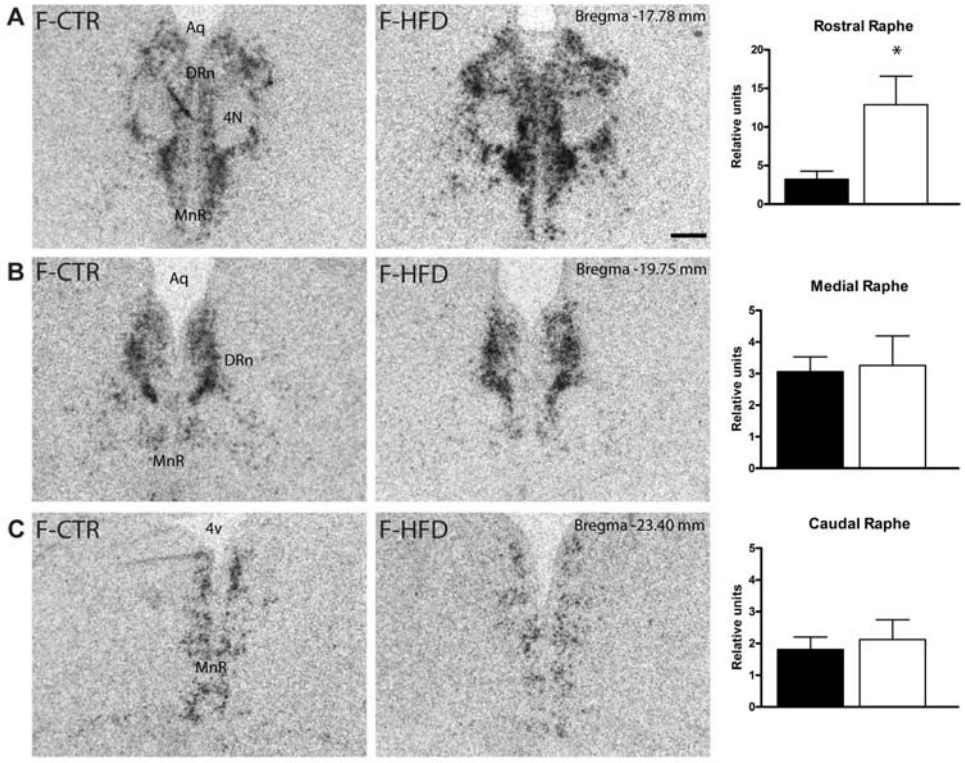


Figure 5-2. TPH2 mRNA is up-regulated with maternal consumption of HFD. Representative low-power autoradiographic images of TPH2 in situ hybridization signal in the G130 midbrain of CTR in (**A**) rostral, (**B**) medial and (**C**) caudal areas. Images correspond to a distance of -17.78, -19.75 and -23.40 mm with respect to bregma according to brain atlas (Paxinos, 1999). Scale bar represents 1mm. Maternal consumption of HFD caused up-regulation of TPH2 mRNA in fetal raphe in the rostral areas (**A**) but not medial (**B**) and caudal (**C**) areas. ($n=4 – 5$, $p < 0.05$) (CTR=black, HFD=white).

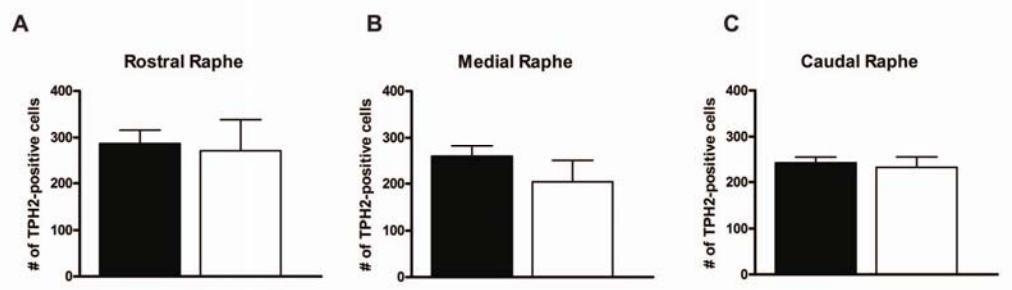


Figure 5-3. Similar numbers of TPH2 labeled neurons are present in rostral, medial and caudal raphe regions. (n=4 – 5, $p < 0.05$) (F-CTR=black, F-HFD=white).

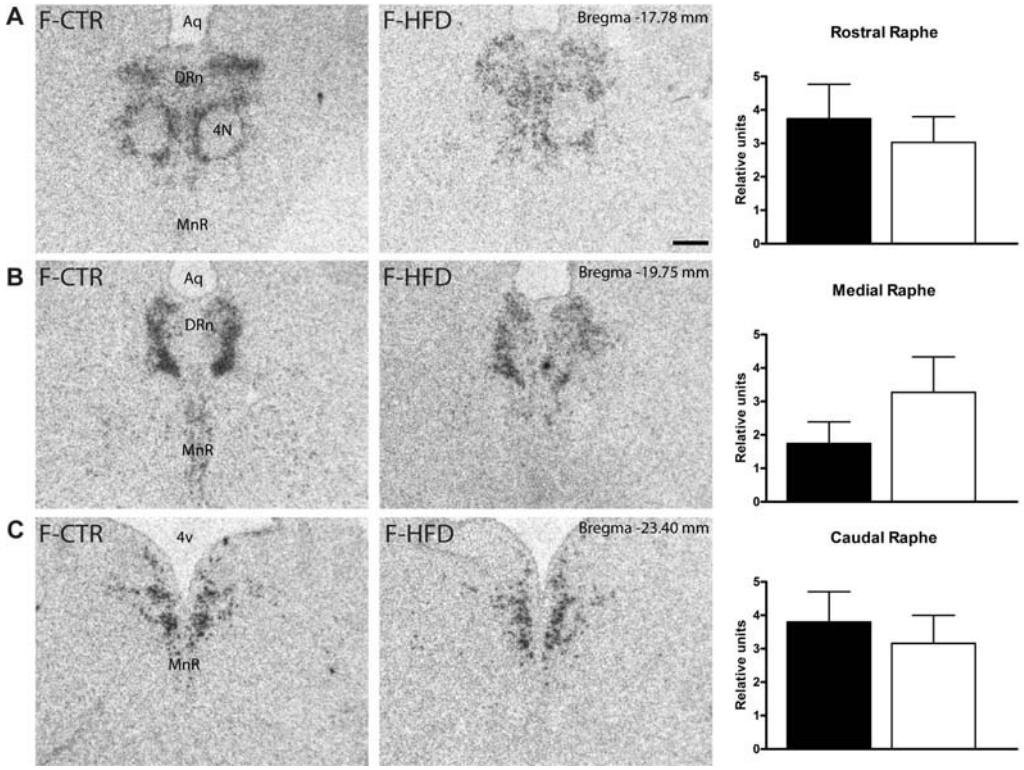


Figure 5-4. 5-HTT levels are not altered by maternal consumption of HFD. Representative low-power autoradiographic images of 5-HTT *in situ* hybridization signal in the G130 midbrain of CTR in (**A**) rostral, (**B**) medial and (**C**) caudal areas. Images correspond to a distance of -17.78, -19.75 and -23.40 mm with respect to bregma according to brain atlas (Paxinos, 1999). Scale bar represents 1mm. (n=4 – 5, $p < 0.05$) (F-CTR=black, F-HFD=white).

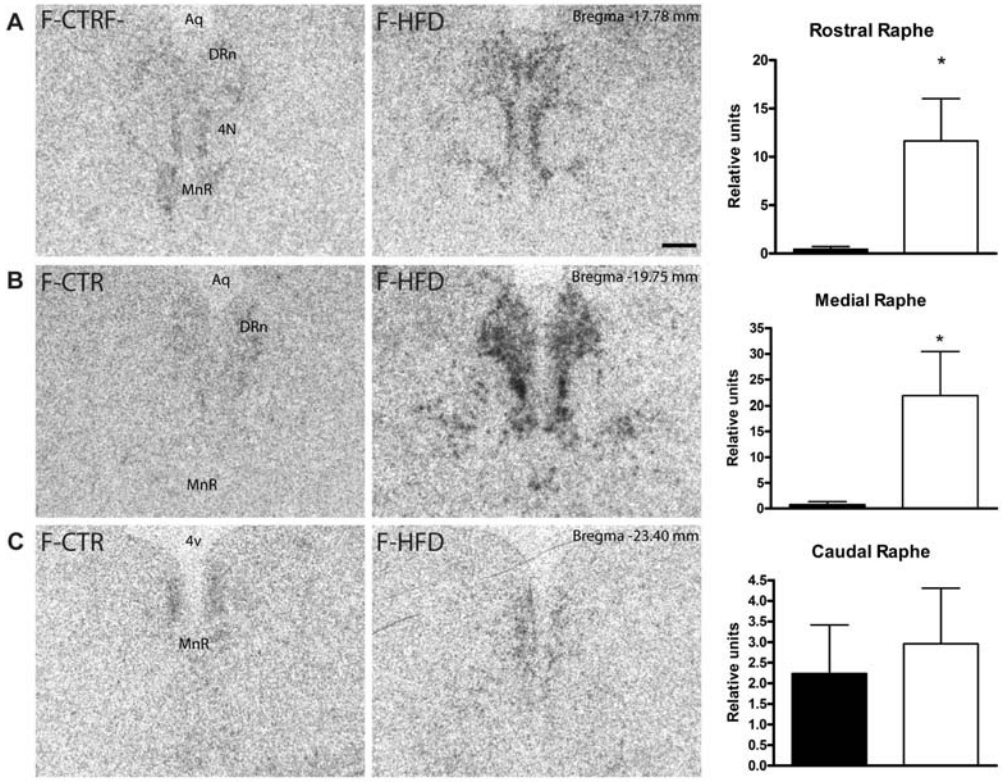


Figure 5-5. 5-HT$_{1A}$ mRNA levels are elevated in F-HFD. Representative low-power autoradiographic images of 5-HT$_{1A}$ *in situ* hybridization signal in the G130 midbrain of CTR in (**A**) rostral, (**B**) medial and (**C**) caudal areas. Images correspond to a distance of -17.78, -19.75 and -23.40 mm with respect to bregma according to brain atlas (Paxinos, 1999). Scale bar represents 1mm. Maternal consumption of HFD caused up-regulation of 5-HT$_{1A}$ mRNA in fetal raphe in the rostral areas (**A**) and medial (**B**) but not caudal (**C**) areas. (n=4 – 5, $p < 0.05$) (F-CTR=black, F-HFD=white).

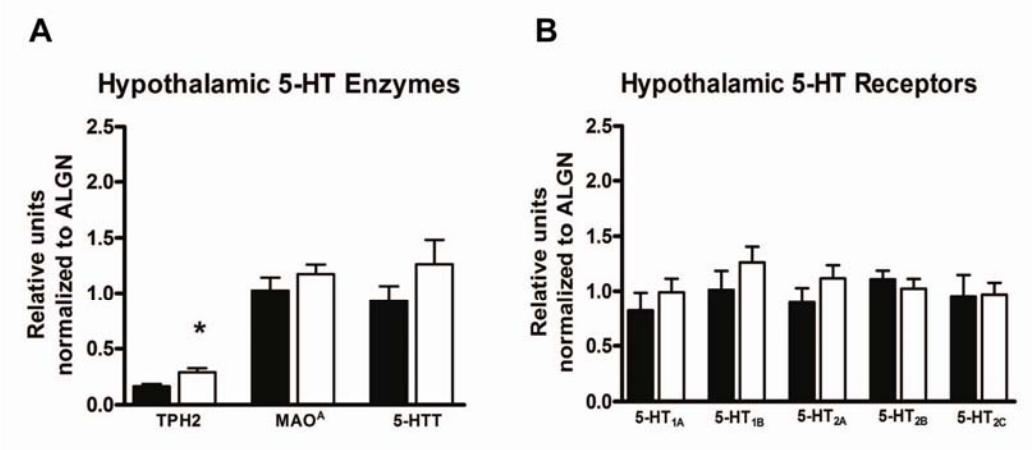


Figure 5-6. Enzyme, transporter and receptor mRNA expression in the hypothalamus. Relative expression of TPH2, MAOA and 5-HTT (**A**) and 5-HT receptors in medial basal hypothalamic blocks using real-time PCR (**B**). (n=4 – 7, $p < 0.05$) (F-CTR=black, F-HFD=white).

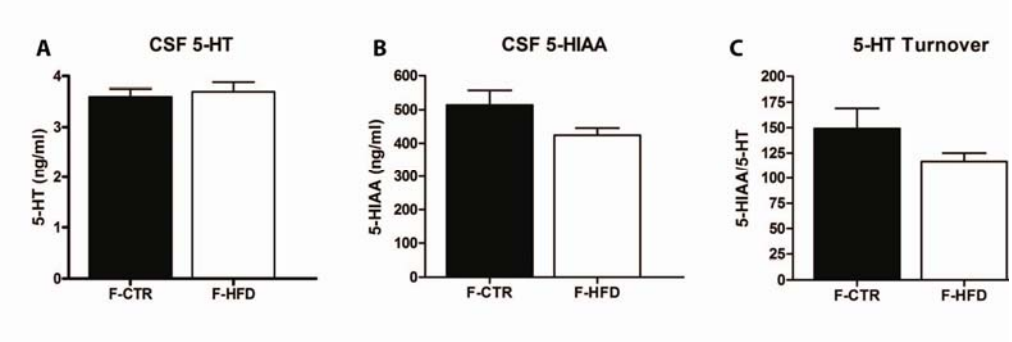


Figure 5-7. 5-HT and 5-HIAA levels and 5-HT turnover in cerebrospinal fluid from F-CTR (black bar), or F-HFD (white bar). (*n*, F-CTR=6, F-HFD=8) (F-CTR=black, F-HFD=white).

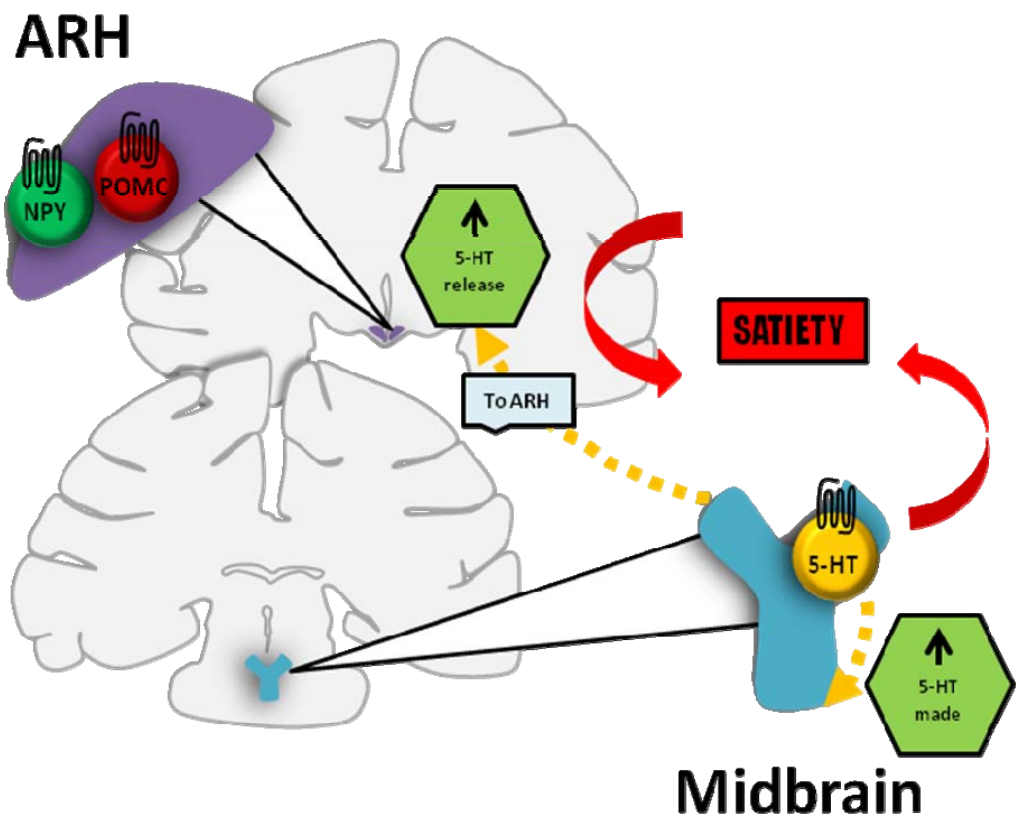


Figure 5-8. Schematic representation of 5-HT action within the hypothalamus and midbrain in the G130 NHP. 5-HT-*ir* is low and TPH2 levels are high. These are interpreted as increased 5-HT production and release to supply an overall satiety signal in the midst of maternal-HFD consumption during gestation.

Chapter 6
Overall Discussion and Significance

DISCUSSION AND SIGNIFICANCE

With the increased prevalence of childhood obesity, the contribution of maternal health and diet on fetal metabolic systems has become an important issue. The work presented in this dissertation demonstrates some early findings concerning the effect of maternal obesity on feeding-related neurocircuitry in the NHP fetus. The primary focus of this work was to first determine the normal ontogeny of the major feeding-related neuropeptides and their projection patterns to associated nuclei during the latter part of gestation of the NHP. Having established the timing of the projections, I then used our model of maternal diet-induced obesity to determine the impact of maternal consumption of HFD on the development of feeding-related neurocircuitry within the G130 fetus. Though I surveyed a variety of peptide systems I found relatively selective alterations within the melanocortin and serotonin systems. Considering the importance of these two systems to metabolic as well as emotional health, the early disruption of these systems may have far-reaching consequences for the long-term health of the offspring.

Importance of the third trimester for feeding-related peptides in the NHP

One of the major findings reported in Chapter 2 was that the projections of ARH-derived feeding-related neuropeptides develop prior to parturition during the third trimester similar to that in humans. Specifically, early in the third trimester, NPY/AgRP neurons exhibit robust projections to the PVH whereas αMSH fibers have a slightly delayed maturation just prior to parturition. This is in direct contrast to the rodent in which these projections mature during the first three postnatal weeks. The relative ontogeny of the orexigenic NPY/AgRP in the rodent and NHP develop prior to those of anorexigenic αMSH and thus raises the question of the importance of the NPY system on

many levels. With birth being imminent, it is important for survival of the neonate to have a robust feeding circuitry to control and direct its early nutrient needs. With the switch from dependence on the mother for nutrition to ingestion of nutrition, it becomes crucial that appetitive mechanisms are intact. It is therefore logical that the orexigenic pathways develop first to assure that the needs of the growing neonate are met. In the NHP, with the αMSH projections developing after the NPY projections, the anorexigenic controls are placed in the feeding network subsequent to the orexigenic drive, perhaps tempering the activity of the orexigenic system.

While it may seem obvious that the timing of the circuit is purely to ready the fetus for postnatal life, these circuits may also be important for energy/fluid homeostasis prior to birth. NPY has been shown to be important to the swallowing mechanism in fetal sheep (El-Haddad et al., 2003; Roberts et al., 2000). Fetal swallowing has an essential role in amniotic fluid volume control as well as development of the gastrointestinal and respiratory tract. Centrally administered NPY significantly increases the rate of swallowing in sheep illustrating that this circuit is intact and functional during the third trimester (Ross et al., 2003). Therefore, an intact and functional NPY system in the control of food intake is crucial prior to birth.

If NPY's effects supersede αMSH and maintain an unopposed orexigenic drive, what is the role of leptin, a potent signal that opposes the orexigenic drive of NPY? Surprisingly, leptin also has been shown to have similar effects to NPY when administered centrally in the fetal sheep (Roberts et al., 2001). This is a paradoxical "developmental switch" since leptin is canonically considered a potent anorexigenic hormone in the adult. Some have hypothesized this role of leptin results in an "unopposed

orexigenic" drive existing in the fetus to cause rapid growth and increase in size. This orexigenic role of leptin has not been described in the rodent. However, the anorexigenic role for leptin really is not established either in the postnatal rodent until well after the second postnatal week. Exogenous administration of a variety of peripheral hormones such as leptin, ghrelin and PYY do result in *c-fos* and *pSTAT* activation in the ARH but this signal is not tranduced down-stream since there is no hormone-mediated *c-fos* activation in efferent target neurons (Grove et al., 2005b). In addition, the pups do not exhibit hypophagia as measured by decreased milk in their gut contents. It is not until the third postnatal week that these effects are demonstrated corresponding to the completion of development of these circuits (Grove et al., 2005b).

Within the developing rodent, the importance of leptin continues to be at the forefront of early feeding circuit development. The leptin surge that occurs during the first two postnatal weeks has been shown to be important to the normal development of hypothalamic feeding circuitry in models of obesity such as the ob/ob mouse, the polygenic obese rat and chronic postnatally overfed rodent. In these models leptin has established neurotrophic actions on the development of hypothalamic feeding circuitry and promotes neurite outgrowth.

Despite these data in the rodent, in our studies in the NHP we were unable to verify the importance of leptin to the maturation of the hypothalamic circuits. In contrast, in the NHP, leptin levels are very low just prior to and during the development of hypothalamic circuits during the third trimester. This is not surprising since throughout gestation, the fetal NHP has little to no fat depots. An alternate source of leptin could be local production (hypothalamic or pituitary in origin) which could act in a paracrine

fashion to promote hypothalamic development. Work in this vein demonstrated leptin to be undetectable *(B. Grayson, unpublished data*). Therefore, leptin is not produced within the hypothalamus during gestation. Additionally, we did consider other sources rather than fetal production. Though maternal and placental levels of leptin are high, it does not appear to pass through to the fetus at this time point, as we would have been able to detect them by RIA in the fetal circulation. Therefore, since a leptin surge is not associated with the maturation of these circuits, we do not believe leptin plays the crucial role in the NHP that it does in the rodent during early development.

The major caveat to our interpretation of this data is that we may have missed the leptin surge because our ontogeny studies are separated by just over thirty gestational days. Without looking at any time points prior to G100 and between G100 and G130, we cannot be sure we did not miss this surge. However, we would expect levels to be at least somewhat higher at G130 than G100; this is not the case. In addition, though at G170 there is a minimal increase, the NPY/AgRP projections are already robust at this time. A different as of yet undescribed cue may exist in a primate that is important for hypothalamic development.

The maternal metabolic environment during pregnancy

Considering primate hypothalamic feeding neurocircuitry develops *in utero*, maternal health status potentially can impact the fetus greatly. Maternal health encompasses many different aspects which include food and beverage consumption, vitamin and mineral intake, emotional well-being, stress responsiveness and exercise as well as the interplay between these individual aspects. But even within the boundaries of a normal pregnancy, the associated metabolic changes to the mother are enormous.

Normal pregnancy is considered to be a diabetogenic state. This diabetogenic state intensifies during the final trimester of pregnancy and is the combined metabolic effect of increasing levels of human placental lactogen (hPL), progesterone, prolactin, and cortisol. It is characterized by increased postprandial blood glucose levels as a result of decreased sensitivity to insulin. This acquired resistance to insulin protects the fetus from fluctuations of glucose during intermeal fasts. In particular, during the final trimester, hPL acts to mobilize free fatty acids through lipolysis to maintain a steady fuel source. Additionally, pregnancy has also been called a state of facilitated anabolism with the intention of conserving energy for fetal development and milk production following parturition. These changes result in an altered metabolic state in the pregnant woman.

The need to address the effects of maternal obesity

Because the pregnancy results in a specialized metabolism within the mother to foster the growth and development of the fetus, certain conditions can exacerbate the vulnerable physiology of pregnancy. Among the most common are preeclampsia, gestational hypertension, gestational diabetes and maternal obesity. Each of these diseases has clinical implications on the quality and quantity of nutrients transmitted to the fetus. However, maternal obesity is by far the most common. Our model of maternal obesity in the NHP endeavors to determine the profound impact that maternal obesity has on fetal metabolic systems.

Anorexigenic and not orexigenic regulation is most impacted by HFD

In the remainder of this dissertation, I used our NHP model of maternal obesity to determine the effect of HFD on the feeding neurocircuitry in the G130 fetus. The results demonstrate that maternal HFD consumption affects the anorexigenic regulation of food

intake more readily then the orexigenic component. In chapter 4, I report targeted changes within the melanocortin system. Specifically we demonstrate increased POMC mRNA with no change in protein, a suppression of both AgRP mRNA and protein and elevated MC4 receptor mRNA in F-HFD in comparison to F-CTR. In chapter 5, we report that the 5-HT system is similarly driven in the F-HFD. We observed increases in TPH2 mRNA as well as decreased 5-HT-ir within the ARH. Both of these systems provide potent anorexigenic inputs to the hypothalamus

There are many potential effects of targeting specifically the anorexigenic component of food-intake regulation early in development. As these neurocircuits continue to develop into postnatal life there could be alternative pruning of the neurite projections into the target nuclei. A decreased anorexigenic innervation would potentially suggest a diminished ability to effect a satiety response. Also higher expression of neurotransmitter concentrations at target sites (as in the case of 5-HT) may also result in retrograde signals to decrease the overall levels of production and/or expression of the cell or by activating apoptotic pathways to prune the neuron in the nucleus of origin. The long-term effect of this would be a dampened anorexigenic drive within the hypothalamus represented by fewer neurons and less axonal arborization. Functionally this could translate into less tolerance of fluctuations in caloric density and a relaxation in control on counterregulatory mechanisms to correct the mismatch between energy demands and appetitive drive, resulting in an increased body weight.

Inflammatory cytokines drive the hypothalamic changes

In considering what may be responsible for the changes in melanocortin and serotonin expression, my hypothesis is that increased circulating levels of inflammatory

cytokines, as well as increased local production within the brain parenchyma, are partially responsible for catalyzing these events. In particular, IL1β mRNA is increased in the hypothalamus. In rodents, IL1β administration results in *c-fos* activation specifically within POMC neurons in the ARH (Scarlett et al., 2007b). We believe a similar induction of POMC mRNA is occurring in the fetal NHP. In addition, several reports exist that the Nur response element/signal transducer and activatory of transcription (NRE/STAT) site which activates the POMC gene promoter region is cytokine sensitive. Specifically, leukemia inhibitory factor (LIF) can drive this NRE/STAT region (Latchoumanin et al., 2007). We were unable to detect LIF by real-time PCR in the hypothalamus in either the F-CTR or F-HFD as well as LIF protein by immunohistochemistry (*B. Grayson, unpublished data*). Nonetheless, we expect that continued work in this vein will reveal that the POMC gene is indeed very sensitive to a host of cytokines.

Potential effects of pro-inflammatory cytokines on brain cells

We can only speculate concerning the impact of early cytokine activation on the cellular function of the hypothalamus (*Figure 6-1*). Within the brain an intimate connection exists within the neurovascular unit composed of the microvascular endothelium, the neuron and the glial cell. The capillaries in this unit supply glucose, oxygen and exogenous hormonal signals. The glial cells provide support to the neuron modulating local levels of nutrients as well as access to the microvasculature through activity of their foot processes. In our F-HFD animals, we report an increase in inflammatory cytokine mRNA within the hypothalamus presumably from the increased saturated fat consumption (*Figure 6-1, A,C*). The source of these inflammatory cytokines is unknown. They could potentially emanate from the neurons. However, the cytokines

are more likely produced by the other components of the neurovascular unit or by resident and infiltrating microglia (*Figure 6-1, B*). Furthermore, fetal life is marked by a decreased blood brain barrier suggesting that the circulating cytokines may in fact penetrate the brain quite easily (*Figure 6-1, D*). When exposed to pro-inflammatory cytokines *in vitro*, neonatal microglia and glial cells have been shown to undergo transformation to their activated state (*Figure 6-1, C*). They also produce reactive oxygen species (ROS), nitric oxide (NO) and chemokines that can be toxic to neurons (Jonakait, 2007). Production of ROS and free radicals can directly cause lipid peroxidation resulting in cell membrane damage. This in turn could potentially lead to abnormal electrophysiological properties in these neurons and aberrant intracellular calcium responses (*Figure 6-1, E*). In the rodent, prenatal activation of microglia through inflammatory processes results in impaired cognitive function at the level of the glutamatergic synapse (Roumier et al., 2008). In addition, as discussed earlier, neuropeptide levels can be altered at the level of transcription (*Figure 6-1, E*). And finally, in particular during development these changes could result in pruning of infiltrating neurites and potentially lead to cell death (*Figure 6-1, E*). Again, within our model, we were not able to localize the hypothalamic cell source of the cytokines and did not pursue whether any proliferation or activation occurred in hypothalamic glia and microglia populations. However, evidence from the literature does support putative impairment to the function of the neurovascular unit through cytokine activation.

Detrimental effects of diets high in saturated fats, low in polyunsaturated fats

In addition, to the detrimental effects of cytokines on hypothalamic feeding neurocircuitry, there are other factors associated with out maternal HFD model that also

may play critical roles in developmental changes in the fetus. One factor is the HFD itself. I have not conclusively determined whether the HFD is directly causing the changes in the fetal brain or if they are secondary to other altered metabolic factors within the mother or fetus. I hypothesize that the increased level and quality of lipids consumed by the mother is causative of the alterations we see. In the introduction of the model in chapter 3, it is reported that mothers consuming HFD have increased circulating glycerol levels. Additionally, trans-saturated fats were elevated and omega-3 fatty acids were reduced in plasma samples of F-HFD (*D. Marks, personal communication*). In the rodent, ingestion of saturated fats during pregnancy and lactation directly effects the percentage of fatty acids in the parenchyma of the brain shifting to increased trans-saturated fats and decreased polyunsaturated fats (Albuquerque et al., 2006). Specifically, the omega-3 fatty acids that are essential to cell membrane structure are docosahexaenoic acid (DHA) and arachadonic acid (AA). These cannot be synthesized *de novo* and therefore must be acquired from the diet. In rodents fed a diet low in n-3 fatty acids, analysis of brain tissue revealed large reductions in omega-3 fatty acids reductions 16-fold in oligodendrocytes, 12-fold in myelin, 2-fold in neurons, 6-fold in synaptosomes, and 3-fold in astrocytes (Bourre et al., 1984). Therefore ingestion of a diet low in n-3 fatty acids has demonstrable effects on brain morphology. DHA and its derivatives have also been shown to have anti-inflammatory properties (Orr and Bazinet, 2008). In another rodent studies where the source of dietary fatty acids are altered, neurite growth cone membrane composition and monoamine concentrations are directly affected (Innis and de la Presa Owens, 2001). Taken together, the data provide further evidence of possible

long-term changes on brain neurocircuitry due to altered composition and type of fatty acid ingested during the gestational period.

The long-term effects of early 5-HT dysregulation

Another factor that may be important to the developmental changes in the fetus is the up-regulation of the 5-HT system. Though our major focus has been to characterize the short-range effects of maternal HFD on fetal metabolic systems, 5-HT dysregulation in the fetal NHP may have long term implications for psychological and cognitive function. In our model, we report early increased drive and release of 5-HT within the ARH. We do not know whether this early up-regulation will result in early pruning of highly expressed neurites due to the high level of 5-HT release in target areas. In addition, the overall 5-HT tone may be dampened as compensation to early augmented levels. Early changes to the 5-HT system during this developmentally vulnerable time could potentially explain the comorbidity within children and adults alike of obesity and attention deficit disorder, impulsivity disorder and depression (Anat Agranat-Meged, 2008; Anderson et al., 2007; Sjoberg et al., 2005; Wurtman, 1993). In addition, decreased cognitive function has been associated with obesity in children (Li et al., 2008). Though we did survey other monoaminergic systems (see Appendix C), our major focus was the serotonin system. The combined effect of alterations in each of these systems could have long-term implications for body-weight regulation as well as psychological health.

Putative impact of diet composition on the epigenetic mechanism in neurons

Another factor associated with our model that could have developmental significance is the epigenetic modification. In general imprinting refers to the inheritance of genes in which either the maternal or paternal allele is expressed preferentially due to

the silenced expression of the other allele. On an epigenetic level, histone modification or DNA methylation could be responsible for this inactivation. In the case of histone modifications, activators and repressors of transcription can associate with coactivators and corepressors that have activities that weaken the ability of histones to bind to DNA through either histone acetyl transferase (HAT) or histone deacetylase (HDAC) activity. In the case of DNA methylation, the presence of methylated DNA can result in all transcriptional activity being suppressed. To date, quite a few gene loci have been identified as imprinted as a result of parent of origin allelic alterations. Prader-Willi and Angelman syndromes are well-characterized disorders due to parent of origin imprinting (Davies et al., 2008). Table 6-1 summarizes other gene loci that have been described specifically in the area of body-weight regulation. The possibility that over/underconsumption of nutrients or even possibly environmental or chemical exposures may be driving the stimulus for epigenetic changes requires much more work to provide conclusive evidence. Burgeoning work in this vein illustrates that diets low in protein for merely four days during the peri-implantation period can result in low birth weight babies that have cardiovascular abnormalities that are gender-specific (Kwong et al., 2006; Kwong et al., 2000). Furthermore, this is poignantly illustrated in recent work in which *Mecp2* deletion resulted in overt hyperphagia and obesity presumably through removal of suppression of some unknown gene within *Sim1*-expressing neurons in the VMH (Fyffe et al., 2008). In the liver, we report that maternal HFD results in hyperacetylation in fetal histones as well as altered levels of both HDAC mRNA and protein (Aagaard-Tillery et al., 2008). We are presently pursuing whether similar changes exist in the F-HFD hypothalami. The potential impact of epigenetic modifications due to

alterations in nutrient composition is disturbing considering the constant changes in food additives as well as sources for nutrients in our diet by the food industry.

Future directions: do these changes persist?

Though extensive effort has focused on characterizing the alterations in the fetus, the overriding question is whether these changes persist in the juvenile and how do they modify the metabolism of the adult. The corollary of our fetal studies are juvenile studies in which we determined the effects on yearlings remaining on their mother's respective diet through and post weaning (J-CTR and J-HFD). Initially we reported that the F-HFD during G130 were lighter than the F-CTR (McCurdy et al., 2009). We have further evidence that this may be due to placental insufficiency because of the toxicity of the HFD. We would expect that during their first postnatal year, J-HFD may experience periods of catch-up growth. Indeed, though J-HFD offspring maintain this underweight phenotype, by six months of age they undergo catch-up growth. We have preliminary evidence that J-HFD yearlings have significantly increased body-weight (*Figure 6-2, A*) as well as increased adiposity as suggested by heavier retroperitoneal fat pads (*Figure 6-2, B*) at necropsy. By this point they have been weaned from their mothers and are ingesting independently the diet fed their mother during gestation.

Considering the F-HFD has a disrupted melanocortin system, we would predict that these animals would have an altered drive to eat. The juvenile animals provide an excellent model for testing various food palatability preferences and linking them to alterations in antatomical substrates. Since these animals are freely-feeding, they can express preference for foods of varying compositions i.e. varying fat and carbohydrate as well as the palatability of foods (*E. Sullivan, unpublished data*). We have preliminary

data that J-HFD do have significantly different preferences for food and consume increased levels of these foods when given the choice. We are also able to measure their motor activity. Linking these types of data to the various neuropeptide and neurocircuits described in this dissertation, a functional output can be determined in an NHP model of HFD consumption.

Considering the serotonergic changes we demonstrated in the F-HFD, we would expect alterations in feeding behavior as well as stress tolerance. We are also pursuing behavioral testing to determine whether any psychological perturbations exist as a result of HFD consumption. These include tests that introduce novel objects that are both food and nonfood items and determining the reaction of the juveniles in terms of latency to approach the objects. Preliminary work indicates that J-HFD have an increased latency to interact with a novel threatening object in comparison to J-CTR (*Figure 6-3, A*) (*E. Sullivan, unpublished data*). We interpret these perturbed responses in the J-HFD to novel objects as continued disruption of the serotonergic system. We have also replicated the studies of 5-HT in the CSF and we have determined the J-HFD have significantly lower levels in comparison to J-CTR (*Figure 6-3, A*) (*B. Grayson, unpublished data*). Further anatomical work needs to be done to characterize the changes in the 5-HT system. Taken together, these data do suggest that exposure to HFD during gestation and postnatally results in changes in body-weight regulation that result in overweight juveniles.

Taken together, preliminary evidence in the juvenile animals suggests that exposure to HFD pre- and postnatally results in a variety of metabolic changes as well as alterations in stress responsiveness. These alterations in metabolism and psychological

well-being may be part of the developmental wiring that leads to body-weight dysregulation later in life.

The NHP Model and Early Origins of Obesity in Humans

In summary, the work of this dissertation has broadly determined the impact of maternal HFD consumption on the feeding-related neurocircuitry in the fetus. The changes described in this feeding-related neurocircuits have apparent metabolic ramifications in the juvenile and probably implications for long-term body-weight maintenance. Historically, research involving the negative impact of maternal smoking and alcohol consumption has resulted in altered behavior in mothers during pregnancy. We show that indeed the developing fetus is vulnerable to the insult of maternal HFD consumption. These long-term implications for health in the offspring will hopefully motivate mothers to maintain a health, balanced dietary fat intake during pregnancy.

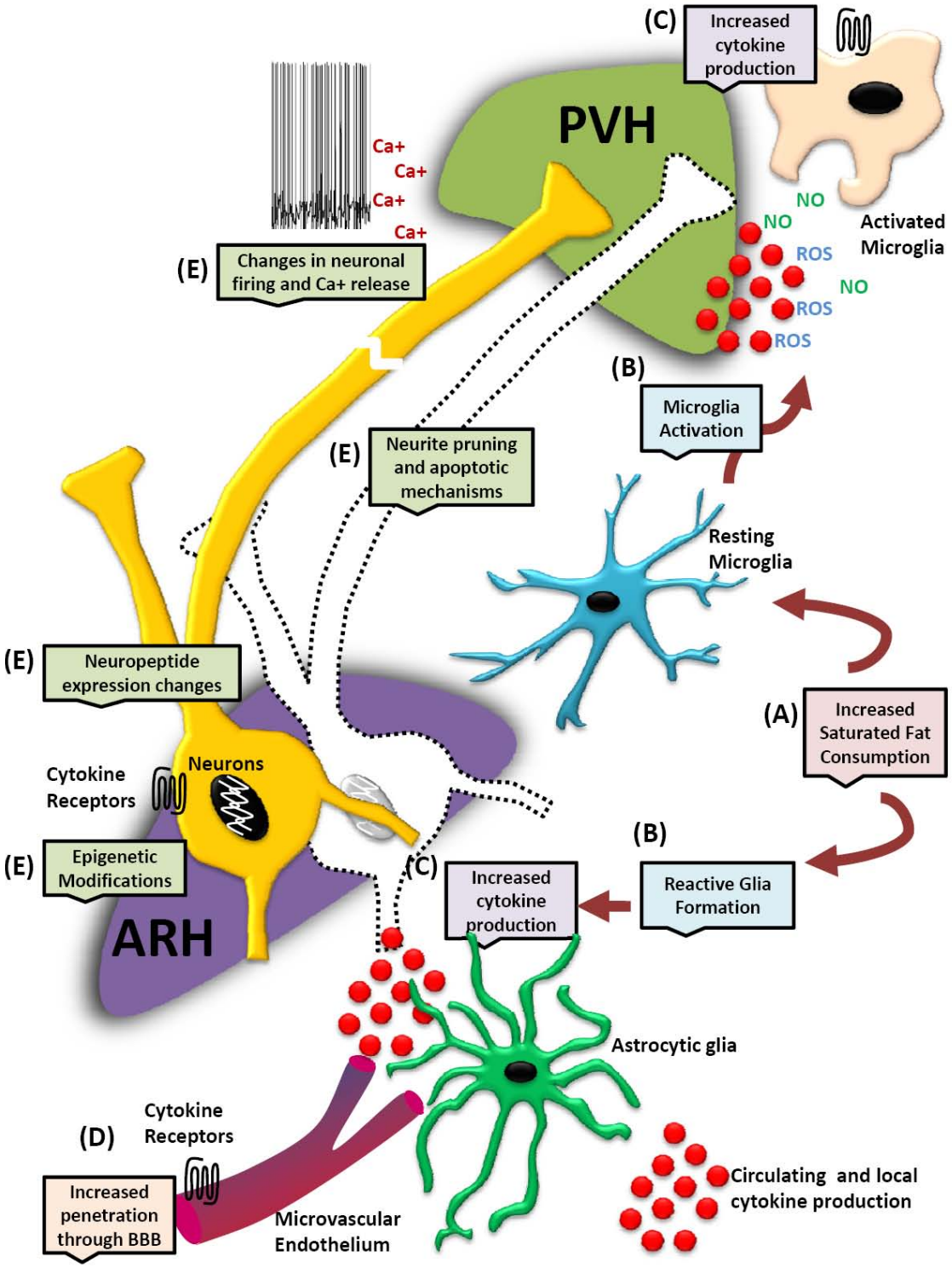


Figure 6-1. Schematic of putative changes in the hypothalamus as a consequence of maternal consumption of HFD during pregnancy.

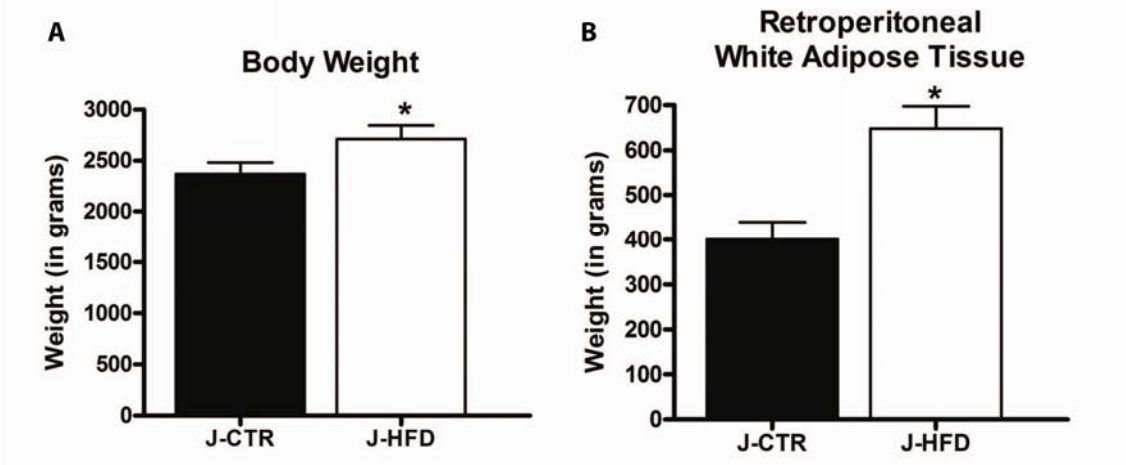


Figure 6-2. Juvenile animals continued on respective diets of mother during pregnancy have increased body-weight and adiposity. Body-weight measurements were made after at age 14 months, having remained with mother prior to weaning and then weaned on to maternal diet during pregnancy (**A**) Retroperitoneal fat pad measurement (**B**). (*n*, J-CTR=8, J-HFD=8) ($p < 0.05$)

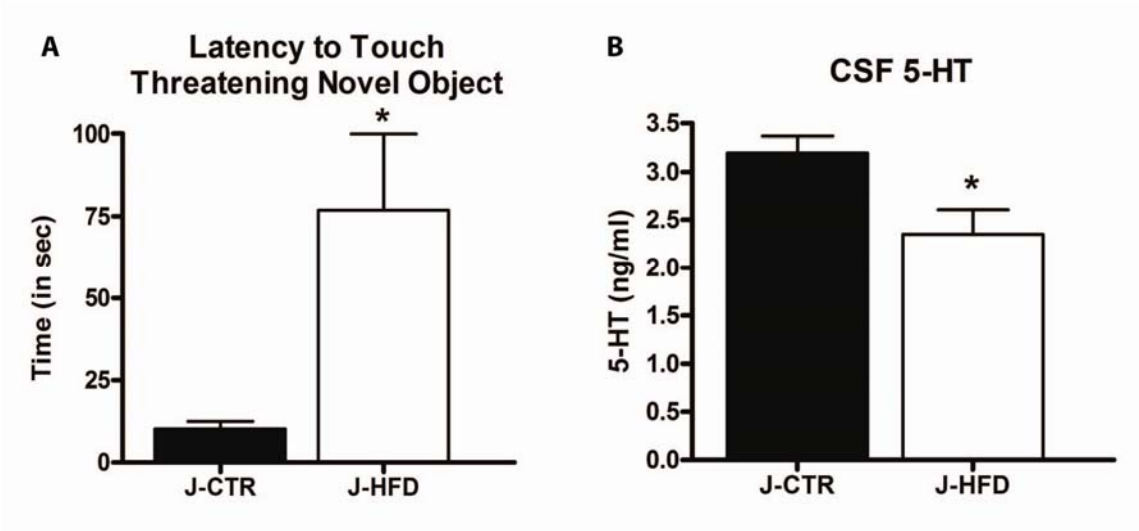


Figure 6-3. Juvenile animals on HFD exhibit psychological and physiologic changes reflecting increased sensitivity to stress. When a novel object is introduced, J-HFD have an increased latency to explore (**A**) (*n*, J-CTR=5, J-HFD=21). Measurement of CSF 5-HT indicates overall lower 5-HT levels (**B**). (*n*, J-CTR=6, J-HFD=7) ($p < 0.05$)

Chromosome	Location	Gene
5	16.7 - 23.3 cM	Type II diabetes susceptibility locus
5	16.7 cM	Type II diabetes susceptibility locus
5	56.2 - 59.7 cM	Type II diabetes susceptibility locus
5	67.4 - 75.4 cM	Body mass index (BMI), obesity
6	6p21.3	HLA-DQ and type 1 diabetes susceptibility
6	6q24	Transient neonatal diabetes
6	90.9 - 97.7 cM	Type II diabetes susceptibility locus
10	10p12, 52-61 cM	Obesity, BMI
11	11p15.5 (02)	IGF2
12	127.5 - 136.6 cM	Body mass index (BMI), obesity
12	12q	Type II diabetes susceptibility locus
12	12q24	Type II diabetes susceptibility locus
13	13q32	Obesity, BMI
15	15q11-q13 (0)	Prader-Willi syndrome
15	15q11-q13 (00)	Angelman syndrome
16	16q	Type 1 diabetes
17	137.6 cM - qter	Type II diabetes susceptibility locus
18	18p11.31	Type II diabetes susceptibility locus

Table 6-1 Established imprinted genes involved in energy balance. Adapted from Catalogue of parent of Origin Effects http://igc.otago.ac.nz/home.html.

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Appendix A
Prenatal Development of Hypothalamic MCH and Orexin in the NHP

INTRODUCTION

The lateral hypothalamic area (LHA) is an important "feeding" center that encompasses a large diffuse area of neurons with two very separate sub-populations of feeding-related neurons, orexin and melanin-concentrating hormone (MCH). Despite their distinct phenotypes, they share many similar sites of projection (Bittencourt et al., 1992; Elias et al., 1998b). MCH and orexin neurons exhibit complex efferent projections throughout the hypothalamus and provide potent orexigenic stimuli to sites of feeding modulation such as the paraventricular hypothalamic nucleus (PVH), dorsomedial hypothalamic nucleus (DMH) and the arcuate nucleus of the hypothalamus (ARH). Stimulation of the LHA results in hyperphagia, and destruction of this nucleus results in decreased food intake and weight loss. Orexin, involved primarily in arousal (the sleep/wake cycle) and autonomic control of glucose homeostasis (Cai et al., 1999; Huang et al., 2001), appears to develop later in the rodent with cell bodies visible at P5. However, orexin projections are not fully developed until the 3rd postnatal week (Yamamoto et al., 2000). MCH and its receptors have been implicated not only in energy balance but also thermogenesis(Ludwig et al., 1998). Within the LHA of the rodent, MCH expression is detectible as early as P2 with fibers being readily detectable throughout the brain as early as P5 (Presse et al., 1992).

Many reciprocal projections also exist between the LHA and ARH. This includes interaction with both NPY/AgRP and POMC/CART neurons. While these circuits have been extensively studied in adult rodents, and more recently in the developing rodent, little has been done to elucidate development of these important energy homeostatic circuits in primates. In the NHP, the period of maturation of these neuropeptides occurs

during the final trimester of fetal development. The goal of the present study was to determine the ontogeny of the MCH/Orexin projections in the fetal Japanese Macaque during gestational days 100, 130 and 165.

MATERIALS AND METHODS

Animals and tissue

Coronal hypothalamic sections (25μm) were collected in 1:12 series using a freezing microtome as previously described (Grayson et al., 2006). Animals were studied at approximate gestational days 100, 130 and 170 and as adults (5-7 years of age) (n = 3/group).

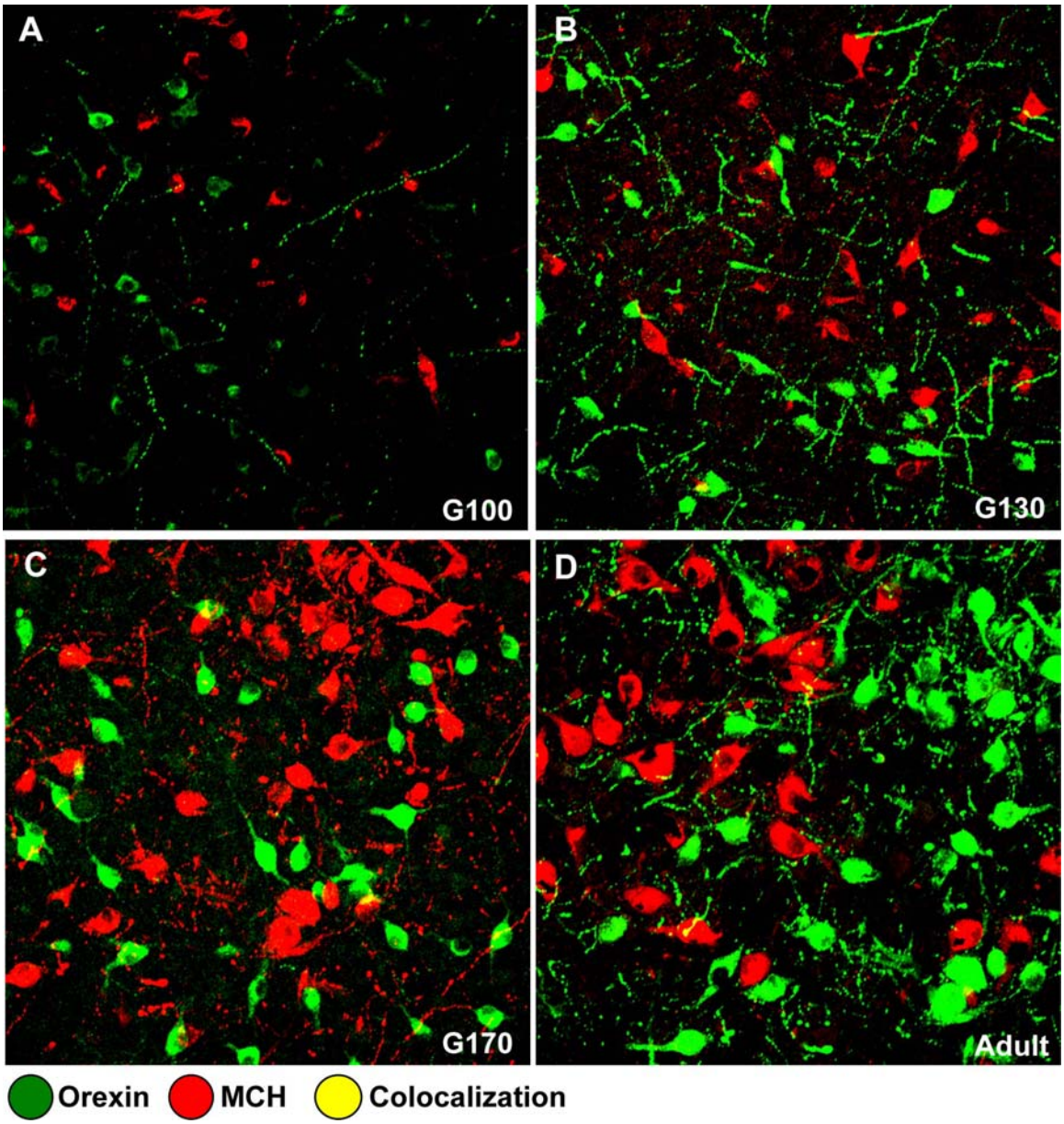
Immunohistochemistry

To characterize the development of orexin and MCH neurons in the LHA and efferent projections to the ARH, a cocktail of goat anti-orexin (#SC-8070, Santa Cruz Biotechology, Inc., Santa Cruz, CA, 1:5,000) and a rabbit anti-MCH (#H-070-47, Phoenix Pharmaceuticals, Inc., Burlingame, CA, 1:3000) was used. Standard immunohistochemical methods were used as previously described (Grayson et al., 2006). Briefly, sections were washed in KPBS and then blocked in 2% donkey serum in 0.4% triton-X/KPBS. Primary or reabsorbed antibodies were diluted to their working concentrations in 2% donkey serum in 0.4% triton-X/KPBS and incubated with the tissue for 48 hours at 4°C. Tissue was then washed in KPBS and fluorescent secondary antibodies applied for 1 hour at room temperature at dilution of 1:200 (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA). Confocal images were captured as previously described (Grayson et al., 2006).

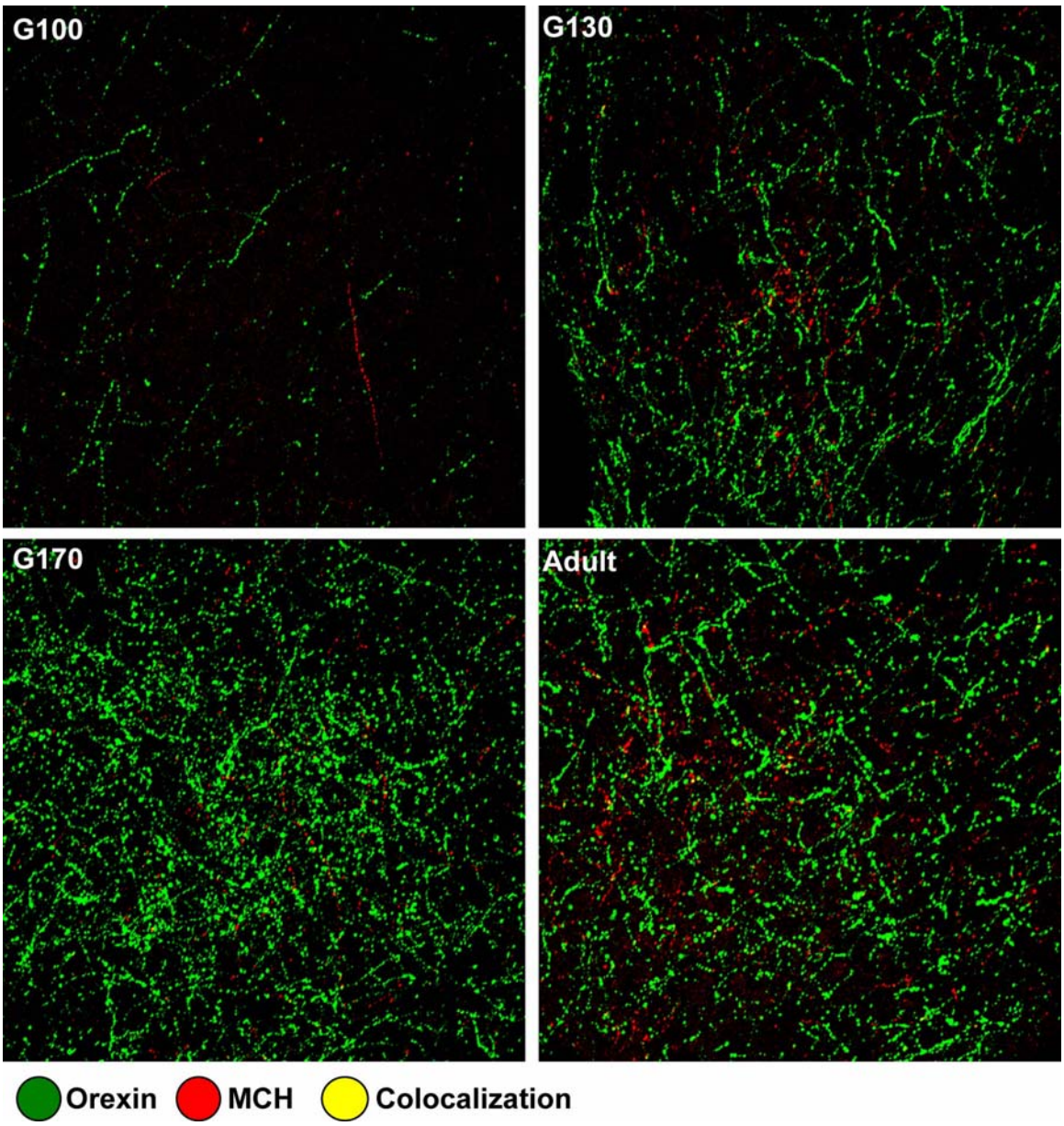
RESULTS AND DISCUSSION

As reported in rodents, orexin and MCH-*ir* neurons were present throughout the LHA in the adult NHP *(Figure A-1, D*) with dense projections throughout the hypothalamus, including the PVH (*data not shown*) and ARH *(Figure A-2, D)*. As early as G100 both orexin- and MCH-*ir* neurons were readably detectable in the LHA *(Figure A-1, A)*. However, while orexin-*ir* fibers were abundant throughout the hypothalamus, MCH-*ir* fibers were sparse *(Figure A-2, A)*. By G130 orexin and MCH-*ir* fibers were abundant throughout the hypothalamus *(Figure A-2, B)*.

Since these neuropeptides develop prenatally in the NHP, the maternal environment may critically influence the formation of these circuits. Additionally, because the relative ontogeny MCH/orexin is different between rodents and the NHP, it is plausible that functional differences in the maturation of these brain feeding circuits exist. Given the alterations we report in the ARH between F-CTR and F-HFD during G130 discussed in Chapter 4, changes in the MCH/orexin system may also increase susceptibility to obesity due to *in utero* programming.



Appendix Figure A-1. Development of orexin and MCH neurons in the LHA. Figures represent color confocal digital images of double-label immunofluorescence for orexin (green) and MCH (red). Orexin and MCH are detectable at G100 (**A**) but sparse -*ir* cell bodies were visualized. Increased density of fibers and soma appear with increased animal age (**B-C**). Adult images are shown for comparison (**D**). Images were captured with a 25X oil objective (0.75 NA) and represent an area of 400X400μm.



Appendix Figure A-2. Development of orexin and MCH projections to the ARH. Figures represent color confocal digital images of double-label immunofluorescence for orexin (green) and MCH (red). Orexin is detectible at G100 in the ARH whereas MCH-ir if very low (**A**) Increasing immunoreactivity is present with increasing age (**B-C**). Adult images are shown for comparison (**D**). Images were captured with a 25X oil objective (0.75 NA) and represent an area of 400X400μm.

Appendix B
Heteronuclear RNA and Pro-opiomelanocortin Gene

INTRODUCTION

Many neuropeptides require extensive cleavage and post-translational modification in order to yield a mature peptide that can exert its biological function. Hypothalamic neuropeptides, including pro-opiomelanocortin require cleavage (Pritchard et al., 2002; Zhou et al., 1993). In the case of POMC prohormone convertase 1/3 (PC1/3) are important for the initial cleavage to Pro-ACTH and β-lipotropin and prohormone convertase 2 (PC2) is important for the futher clipping of ACTH to ACTH 1-17 and CLIP. ACTH 1-17 further leads to the production of mature forms of αMSH with the addition of other post-translational modifications by enzymes such as carboxypeptidase E and *n*-acetyltransferase. In the case of POMC, the products of this cleavage are sorted through the *trans* Golgi network and trafficked through the neuron via the regulated secretory pathway for final release from secretory granules in response to a stimulus.

In our studies of POMC within the NHP hypothalamus, we demonstrated an up-regulation of POMC mRNA in fetal offspring from mothers on HFD (Grayson et al., 2009). However, αMSH peptide levels remain unchanged. There are several processing points which may account for this disparity. Initially we considered alterations in PC2 mRNA and protein levels due to HFD. Using quantitative PCR and immunohistochemistry, we report no differences in PC2 levels. Therefore we considered a point upstream at the level of transcription. We hypothesized that perhaps in the F-HFD, levels of heteronuclear RNA (hRNA), an intermediate RNA prior to mRNA transcription, were greater than in the F-CTR. Indeed if our *in situ* hybridization probe was in fact detecting hRNA, this would explain the high levels of POMC by *in situ*

hybridization in the absence of peptide expression. To answer this question, we designed conventional PCR primers within the 3 exons present in the POMC gene, to determine whether alternative products were produced.

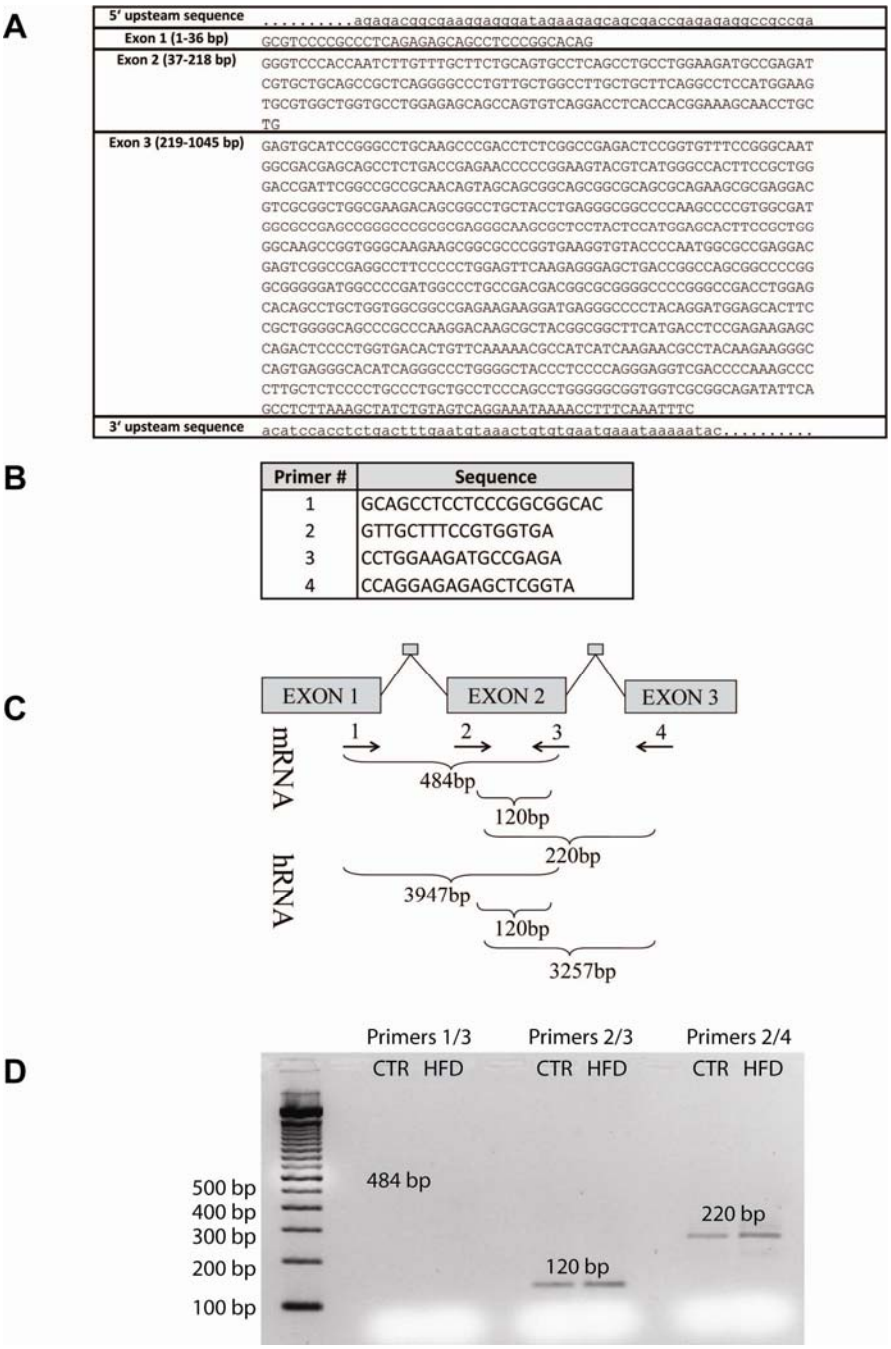
MATERIALS AND METHODS

RNA samples were prepared for conventional PCR by random-primer reverse transcription reaction using random hexamer primers (Promega Corp., Madison, WI) and 1 μg RNA on a MJ Research Thermal Cycler (Global Medical Instrumentation, Ramsey, Minnesota) programmed for 37°C for 1 hr, and 95°C for 5 min. Conventional PCR was performed using a cocktail of 45μl of Platinum PCR Supermix (#11306-016, Invitrogen Corporation, Carlsbad, CA), 1μl each of forward and reverse primers, 1μl of cDNA from either a CTR or HFD animal and 1μl of water on a BioRad iCycler Thermal Cycler (BioRad Laboratories, Hercules, CA). The sequences of primers used are summarized in *Figure B-1, B*. The amplification was performed as follows: 94°C for 11min, and 40 cycles of 94°C for 30sec, 55°C for 30sec followed by 72°C for 4min. In this protocol, the extending time was increased to accommodate the approximately 4 kb product that was expected if indeed hRNA was present. These products were then run on a 1.5% agarose gel and viewed with the GelDoc system.

RESULTS AND DISCUSSION

POMC is a 1045bp gene composed of 3 exons and 2 intervening introns, 3703bp and 2763bp in size respectively (*Figure B-1, A*). We designed forward and reverse PCR primers to span the two introns (*Figure B-1, B*). These primers would produce products of the following three sizes if only mRNA sequences were present: 1) primers 1 and 3 would produce a product of 484bp 2) primers 2 and 3 would produce a product of 120bp,

and 3) primers 2 and 4 would produce a product of 220bp. If indeed hRNA were also present then possible PCR products would be: 3947bp, 120bp and 3257bp (*Figure B-1, C*). PCR gel shows that primers 2/3 and primers 2/4 each yield only one PCR product indicating the presence of only mRNA and no hRNA (*Figure B-1, D*). Primers 1/3 failed presumably because of the presence of a variable region in the area of forward primer 1 which in other contexts, also has failed to successfully produce a product *(data not shown).* These data negate the hypothesis that our *in situ* probe is sensitive to hRNA in our fetal samples. These data support the presence of up-regulated POMC at the mRNA level without concomitant increases in peptide possibly due to immaturity of the neuronal projections.



Appendix Figure B-1. Heteronuclear RNA is not detectible in POMC cDNA. POMC gene sequence as reported in ***ENSEMBLE*** (**A**). Primers designed to test presence of hRNA within the sample (**B**). Schematic representing the resulting product length if only mRNA is present versus the product length of hRNA is present with each primer combination spanning the three exons (**C**). PCR gel showing the presence of 120 and 220bp products without the presence of the longer 3257bp product (**D**).

Appendix C
Alterations in Neurotransmitter Expression in Fetal NHP Exposed to Maternal High-Fat Diet

INTRODUCTION

Clusters of nuclei in the hypothalamus are generally described by the major hormone or peptide which they secrete. However, these neurons often co-express other neuropeptides and neurotransmitters. These neuro-regulators fall into several categories including amino acids, monoamines, peptides, ions and gases and often have a number of receptors which enact a wide variety of downstream actions depending on the specificity of interaction. Recently more of these neurotransmitters have been implicated in the neuronal control of food intake. Variations in quantity of the neurotransmitter and its receptor may underlie generalized neuronal dysfunction that may lead to obesity.

In these studies, our goal was to determine differences in neurotransmitter expression between F-CTR and F-HFD ventral basal hypothalamic tissue. We utilized the Superarray platform to determine changes in 84 different gene targets. Because fetal brain development is underway in the third trimester, we expected a variety of alterations in family groups of neurotransmitters.

MATERIALS AND METHODS

Experiment 1: RNA used in these experiments was extracted using ventral-medial basal hypothalamic tissues collected as previously described (Grayson et al., 2009) Reverse transcription was performed on 1μg of RNA using RT² First Strand Kit (#C-03, SABiosciences, Fredrick, MD). For each reaction, cDNA synthesis was performed using 1μg of RNA on a MJ Research Thermal Cycler (Global Medical Instrumentation, Ramsey, Minnesota) programmed for 37°C for 1 hr, and 95°C for 5 min. qPCR was performed according to the manufacturer's directions using the Human Neurotransmitter Receptors and Regulators RT² Profiler™ 96 well PCR Array (#PAHS-060,

SABiosciences, Fredrick, MD) on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA). For each 96-well plate, a master mix was made containing: 1225µl Applied Biosystems Power SYBR Green PCR Master Mix (#PA-012), 98µl diluted cDNA synthesis reaction, and 1127µl ddH_2O. The master mix was vortexed and 21µl added to each well. qPCR reactions were 95°C for 10min, and 40 cycles of 95°C for 15sec followed by 60°C for 1min. A dissociation (melting) curve was performed per manufacturer's instructions and only samples with one peak at temperatures greater than 80°C were accepted. The fold-change for each gene was calculated as $2^{(-\Delta\Delta Ct)}$, where $\Delta\Delta C_t = \Delta C_t$ (F-HFD group) - ΔC_t (F-CTR group).

Experiment 2: For follow-up and confirmation of the targets, qPCR was performed on single gene targets based on either *P* value or fold change differences. For these follow-up studies, the following inventoried human primers/probe sets (Applied Biosystems, Inc., Foster City, CA) were used: cholinergic receptor α4 (CHRNA4) #Hs00181247_m1, cholinergic receptor β2 (CHRNB2) # Hs00181267_m1, cholinergic receptor δ (CHRND) # Hs00181284_m1,γ-aminobutyric acid, subunit α1 (GABRA1) #Hs00168058_m1, γ-aminobutyric acid, subunit α2 (GABRA2) # Hs00168069_m1, γ-aminobutyric acid, subunit Q (GABRQ) #Hs00610921_m1, glutamate receptor 1, ionotropic (GRIA1) #Hs00246956_m1 and somatostatin 3 receptor, (SSTR3) # Hs00265633_s1.

RESULTS AND DISCUSSION

Using the Superarray assays, a great amount of variability existed in the assay and samples and as a result, none of the differences were statistically significant (*Figure C-1 and 2*). However, using a criterion of greater than ±50% change in expression, we determined 20 of the 84 targets, representing 12 distinct families of neurotransmitters to

be promising leads (blue highlight, *Figure C-1 and 2*). Using single target real-time PCR, we chose to confirm a subset of these chosen targets. The serotonin family receptor expression is reported in Chapter 5. In addition, we performed the assays for 5 other targets. Amongst them, changes in inhibitory transmitter receptor for GABA, subunit A1 was significantly down-regulated in HFD animals. These data show that the developing fetal brain is sensitive to perturbations in maternal dietary fat consumption. In particular, the changes in dopamine receptor expression, which has recently been prominently distinguished in motivated feeding irregularities, may be promising to follow up on.

Neurotransmitter Superarray				T-TEST	Fold Up- or Down-Regulation
Symbol	RefSeq	Gene Name	Description	p value	Test Sample / Control Sample
ABAT	NM_000663	GABA-AT/GABAT	4-aminobutyrate aminotransferase	0.69	-1.14
ACHE	NM_000665	YT	Acetylcholinesterase (YT blood group)	0.90	-1.05
ANXA9	NM_003568	ANX31	Annexin A9	0.81	-1.09
BRS3	NM_001727	BRS3	Bombesin-like receptor 3	0.67	-1.15
BZRP	NM_000714	MBR/PBR	Benzodiazapine receptor (peripheral)	0.77	-1.10
CCKAR	NM_000730	CCK-A/CCK1-R	Cholecystokinin A receptor	0.56	-1.23
CCKBR	NM_176875	CCK-B/GASR	Cholecystokinin B receptor	0.74	-1.11
CHAT	NM_020985	CMS1A/CMS1A2	Choline acetyltransferase	0.70	-1.14
CHRM1	NM_000738	HM1/M1	Cholinergic receptor, muscarinic 1	0.76	-1.08
CHRM2	NM_000739	HM2	Cholinergic receptor, muscarinic 2	0.57	-1.34
CHRM3	NM_000740	HM3	Cholinergic receptor, muscarinic 3	0.67	-1.16
CHRNA1	NM_000079	ACHRA/ACHRD	Cholinergic receptor, nicotinic, alpha 1 (muscle)	0.98	-1.01
CHRNA2	NM_000742	CHRNA2	Cholinergic receptor, nicotinic, alpha 2 (neuronal)	0.88	1.13
CHRNA3	NM_000743	MGC104879	Cholinergic receptor, nicotinic, alpha 3	0.95	1.03
CHRNA4	NM_000744	BFNC/EBN	Cholinergic receptor, nicotinic, alpha 4	0.05	-1.97
CHRNA5	NM_000745	CHRNA5	Cholinergic receptor, nicotinic, alpha 5	0.82	1.11
CHRNA6	NM_004198	CHNRA6	Cholinergic receptor, nicotinic, alpha 6	0.96	1.03
CHRNA7	NM_000746	NACHRA7	Cholinergic receptor, nicotinic, alpha 7	0.87	-1.05
CHRNB1	NM_000747	ACHRB/CHRNB	Cholinergic receptor, nicotinic, beta 1 (muscle)	0.25	-1.49
CHRNB2	NM_000748	EFNL3	Cholinergic receptor, nicotinic, beta 2 (neuronal)	0.96	-1.03
CHRNB4	NM_000750	CHRNB4	Cholinergic receptor, nicotinic, beta 4	0.96	-1.01
CHRND	NM_000751	ACHRD/CMS2A	Cholinergic receptor, nicotinic, delta	0.07	-1.91
CHRNE	NM_000080	ACHRE/CMS1D	Cholinergic receptor, nicotinic, epsilon	0.35	-1.30
CHRNG	NM_005199	ACHRG	Cholinergic receptor, nicotinic, gamma	0.11	-1.47
COMT	NM_000754	COMT	Catechol-O-methyltransferase	0.95	-1.02
DRD1	NM_000794	DADR/DRD1A	Dopamine receptor D1	0.23	-1.64
DRD2	NM_000795	D2DR/D2R	Dopamine receptor D2	0.14	-1.60
DRD3	NM_000796	D3DR	Dopamine receptor D3	0.14	-1.23
GABRA1	NM_000806	EJM	Gamma-aminobutyric acid (GABA) A receptor, alpha 1	0.52	1.51
GABRA2	NM_000807	GABRA2	Gamma-aminobutyric acid (GABA) A receptor, alpha 2	0.32	-1.59
GABRA3	NM_000808	MGC33793	Gamma-aminobutyric acid (GABA) A receptor, alpha 3	0.25	-1.37
GABRB1	NM_000812	GABRB1	Gamma-aminobutyric acid (GABA) A receptor, beta 1	0.95	-1.02
GABRB2	NM_000813	MGC119386	Gamma-aminobutyric acid (GABA) A receptor, beta 2	0.52	-1.76
GABRD	NM_000815	MGC45284	Gamma-aminobutyric acid (GABA) A receptor, delta	0.92	-1.03
GABRE	NM_021990	GABRE	Gamma-aminobutyric acid (GABA) A receptor, epsilon	0.43	-1.35
GABRG1	NM_173536	DKFZp686H2042	Gamma-aminobutyric acid (GABA) A receptor, gamma 1	0.41	-1.18
GABRG2	NM_000816	CAE2/ECA2	Gamma-aminobutyric acid (GABA) A receptor, gamma 2	0.98	1.01
GABRP	NM_014211	MGC126386	Gamma-aminobutyric acid (GABA) A receptor, pi	0.69	-1.13
GABRQ	NM_018558	THETA	Gamma-aminobutyric acid (GABA) receptor, theta	0.10	-1.61
GABRR1	NM_002042	GABA-A rho-1	Gamma-aminobutyric acid (GABA) receptor, rho 1	0.80	-1.08
GABRR2	NM_002043	GABRR2	Gamma-aminobutyric acid (GABA) receptor, rho 2	0.03	-1.84
GAD1	NM_000817	GAD	Glutamate decarboxylase 1 (brain, 67kDa)	0.80	-1.05

Neurotransmitter Superarray (cont'd)				T-TEST	Fold Up- or Down-Regulation
Symbol	RefSeq	Gene Name	Description	p value	Test Sample / Control Sample
GALR1	NM_001480	GALNR/GALNR1	Galanin receptor 1	0.39	-1.32
GALR2	NM_003857	GALNR2	GALANIN RECEPTOR 2	0.62	1.18
GALR3	NM_003614	GALR3	Galanin receptor 3	0.26	-1.22
GCH1	NM_000161	DYT5/GCH	GTP cyclohydrolase 1 (dopa-responsive dystonia)	0.95	-1.02
GCHFR	NM_005258	GFRP/HsT16933	GTP cyclohydrolase I feedback regulator	0.11	-1.52
GLRA1	NM_000171	STHE	Glycine receptor, alpha 1	0.22	-1.21
GLRA2	NM_002063	GLR	Glycine receptor, alpha 2	0.93	-1.02
GLRA3	NM_006529	GLRA3	Glycine receptor, alpha 3	0.05	-2.75
GPR103	NM_198179	AQ27/SP9155	G protein-coupled receptor 103	0.84	-1.08
GPR147	NM_022146	NPFF1/NPFF1R1	G protein-coupled receptor 147	0.65	-1.14
GPR24	NM_005297	MCHR1/SLC1	G protein-coupled receptor 24	0.24	-1.61
GPR73	NM_138964	GPR73a/PKR1	G protein-coupled receptor 73	0.09	-1.72
GPR73L1	NM_144773	GPR73b/GPRg2	G protein-coupled receptor 73-like 1	0.13	-1.68
GPR74	NM_053036	NPFF2/NPGPR	G protein-coupled receptor 74	0.94	-1.05
GPR83	NM_016540	GIR/GPR72	G protein-coupled receptor 83	0.95	-1.02
GRIA1	NM_000827	GLUH1/GLUR1	Glutamate receptor, ionotropic, AMPA 1	0.10	-2.00
GRIN1	NM_007327	NMDA1/NMDAR1	Glutamate receptor, ionotropic, N-methyl D-aspartate 1	0.44	-1.80
GRPR	NM_005314	GRPR	Gastrin-releasing peptide receptor	0.66	-1.14
HCRTR2	NM_001526	OX2R	Hypocretin (orexin) receptor 2	0.17	-1.55
HTR1B	NM_000863	5-HT1B/5-HT1DB	5-hydroxytryptamine (serotonin) receptor 1B	0.30	-1.88
HTR2A	NM_000621	5-HT2A/HTR2	5-hydroxytryptamine (serotonin) receptor 2A	0.53	-1.69
HTR3A	NM_000869	5-HT-3/5-HT3A	5-hydroxytryptamine (serotonin) receptor 3A	0.78	-1.07
HTR3B	NM_006028	5-HT3B	5-hydroxytryptamine (serotonin) receptor 3B	0.32	-1.22
MAOA	NM_000240	MAOA	Monoamine oxidase A	0.19	-1.37
NMBR	NM_002511	NMBR	Neuromedin B receptor	0.30	-1.38
NMUR1	NM_006056	(FM-3)/FM-3	Neuromedin U receptor 1	0.34	1.39
NMUR2	NM_020167	FM4/NMU2R	Neuromedin U receptor 2	0.95	-1.02
NPY1R	NM_000909	NPYR	Neuropeptide Y receptor Y1	0.16	2.29
NPY2R	NM_000910	NPY2R	Neuropeptide Y receptor Y2	0.09	-1.81
PHOX2A	NM_005169	ARIX/CFEOM2	Paired-like (aristaless) homeobox 2a	0.76	-1.19
PPYR1	NM_005972	NPY4-R/NPY4R	Pancreatic polypeptide receptor 1	0.95	-1.02
PRLHR	NM_004248	GPR10/GR3	Prolactin releasing hormone receptor	0.52	-1.15
SLC5A7	NM_021815	CHT/CHT1	Solute carrier family 5 (choline transporter), member 7	0.60	-1.17
SORCS1	NM_052918	SORCS1	Sortilin-related VPS10 domain containing receptor 1	0.95	-1.02
SORCS2	NM_020777	SORCS2	Sortilin-related VPS10 domain containing receptor 2	0.29	-1.15
SSTR1	NM_001049	SRIF-2	Somatostatin receptor 1	0.28	-1.31
SSTR2	NM_001050	SSTR2	Somatostatin receptor 2	0.79	-1.16
SSTR3	NM_001051	SSTR3	Somatostatin receptor 3	0.28	3.64
SSTR4	NM_001052	SSTR4	Somatostatin receptor 4	0.88	1.06
TACR1	NM_001058	NK1R/NKIR	Tachykinin receptor 1	0.45	1.36
TACR2	NM_001057	NK2R/NKNAR	Tachykinin receptor 2	0.99	-1.00
TPH1	NM_004179	TPH/TPRH	Tryptophan hydroxylase 1 (tryptophan 5-monooxygenase)	0.81	-1.10

Appendix Figure C-1. Neurotransmitter and Regulator PCR Superarray.
Highlighted targets illustrate genes of interest that warrant future study (F-CTR, n=4, F-HFD, n=7).

Appendix D
Double-label of AgRP and CART in the Adult NHP

INTRODUCTION

CART is abundantly expressed in the brain, including hypothalamic regions such as the VMH, PVH, ARH, but also the nucleus accumbens, VTA, thalamic nuclei, dorsal raphe, amygdala, olfactory bulbs, spinal cord and NTS (Ersin O. Koylu, 1998; Smith et al., 1997). In the rodent, CART has been found to co-localize with other prominent neuropeptides and neurotransmitters such as POMC, tyrosine hydroxylase and MCH in the hypothalamus, GABA in the nucleus accumbens and acetylcholine in the gut myenteric system (Couceyro et al., 1998; Elias et al., 2001b; F. Brischoux, 2002; Koylu et al., 1999). Though in the adult rodent, CART is known to colocalize with αMSH in the ARH and MCH in the LHA, this does not appear to be phylogenetically conserved. We found that indeed during all developmental time points investigated as well as in the adult NHP, CART and αMSH fibers did not colocalize (Grayson et al., 2006). Furthermore in the human, CART is colocalized with NPY and AgRP and does not colocalize with αMSH (Menyhert et al., 2007).

The goal of this study was to determine whether in the adult NHP, CART also colocalized with AgRP as it did in the human. If indeed CART is colocalized with AgRP, we could use this colabel technique to track CART neuronal projections specifically from the ARH.

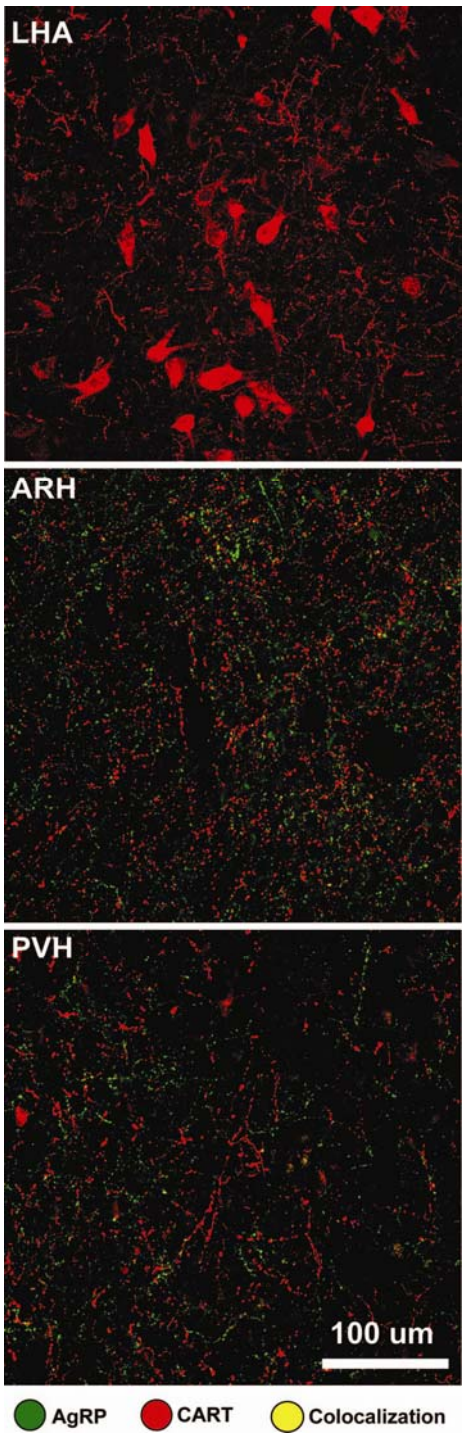
MATERIALS AND METHODS

To determine whether CART and AgRP are coexpressed in the same neurons we used a cocktail of rabbit anti-CART (1:2000, #H-003-63, Phoenix Pharmaceuticals, Inc, Burlingame, CA) and guinea pig anti-AgRP (1:5000; Antibodies Australia, Melbourne,

Australia). Standard immunohistochemical and microscopy procedures were used as described in Chapter 2.

RESULTS AND DISCUSSION

In the LHA, ARH and PVH, we did not find any evidence of colocalization between CART and AgRP (*Figure D-1*). This is a significant difference from the report in the human of colocalization between CART and AgRP in the ARH. We previously reported that in the NHP, CART also does not colocalize with αMSH as has been reported in the rodent. CART appears to deviate significantly from the rodent and the human in this regard. We have previously reported that a 48 hour fast in the NHP does indeed suppress the expression of CART mRNA within the ARH similar to the rodent (Van Vugt et al., 2006). Therefore, CART in the NHP is in fact affected by fluctuations in energy homeostasis. This significant species difference accents the yet undescribed role of CART in the primate regarding food intake.



Appendix Figure D-1. CART and AgRP are not colocalized in the NHP. Double-label fluorescent immunohistochemistry for AgRP (green) and CART (red) in the LHA, ARH and PVH. No overlap is visualized though close appositions are evident.

Appendix E
Reciprocal Innervation of CRH and 5-HT in the Fetal NHP

INTRODUCTION

The hypothalamic-pituitary adrenal (HPA) axis is important in the regulation and feedback control of a variety of stress responses. In this axis, corticotropin-releasing hormone (CRH) is produced primarily by neurons in the PVH and released into the hypothalamic-hypophyseal portal system. CRH acts on corticotrophs to release ACTH from the pituitary in response to stress. ACTH modulates production and release of glucocorticoids from the adrenal gland. The 5-HT system is also known for its importance in modulating the response to stress. Robust reciprocal innervation of the CRH and 5-HT system is known to modulate anxiety and stress behavior as well as psychological well-being.

The goal of this study was to determine the degree of reciprocal innervation between CRH and 5-HT in the G130 fetal macaque and to determine whether qualitative differences existed between F-CTR and F-HFD.

MATERIALS AND METHODS

Fluorescent Immunohistochemistry

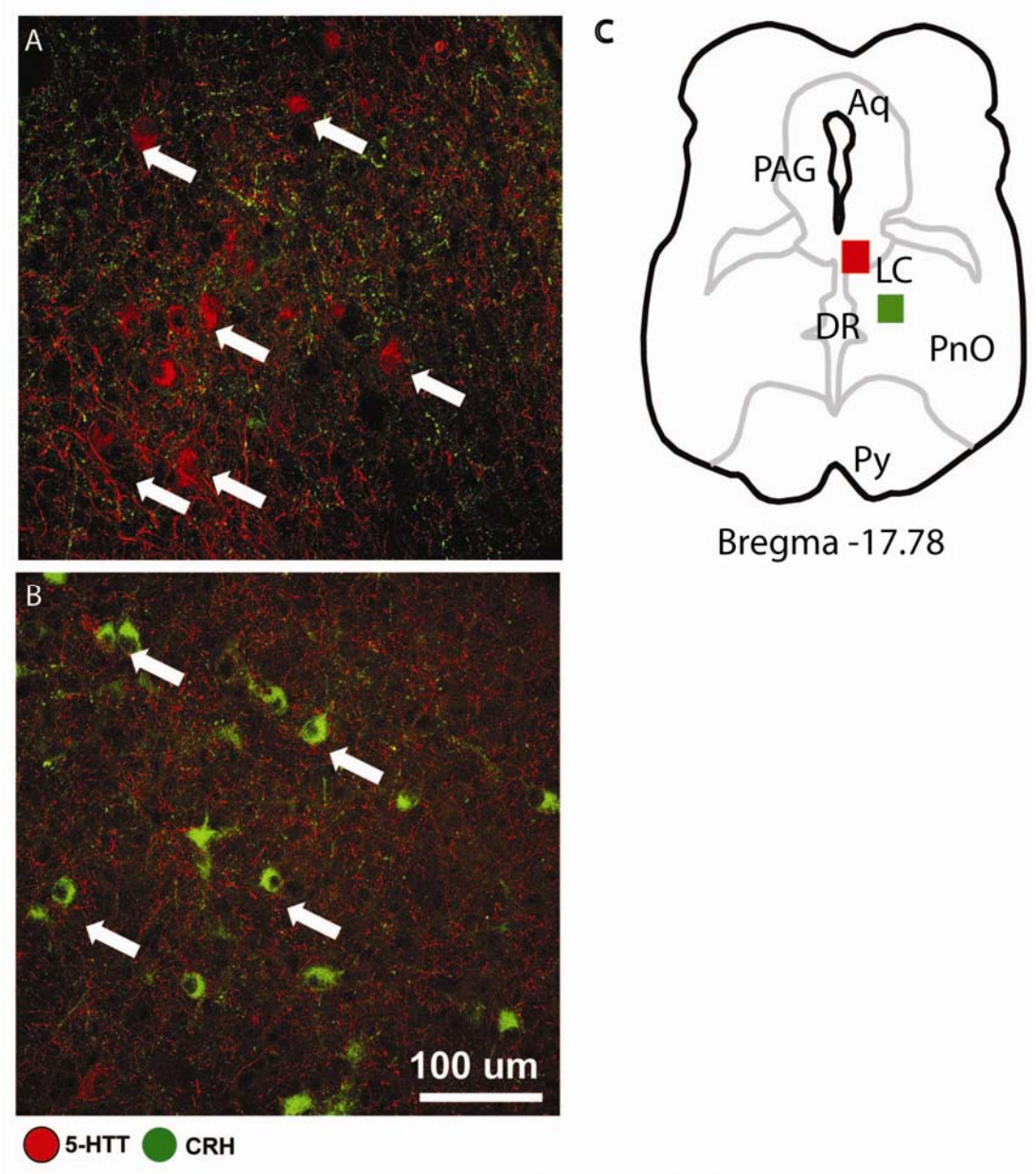
Hypothalamic and raphe-containing sections were obtained as previously described in Chapter 5. 5-HTT and CRH double-label immunohistochemistry was performed. (5-HTT was used as a surrogate for 5-HT due to the need for an alternate antibody species for co-labeling experiment). Standard immunohistochemical methods were used as previously described (Grayson et al., 2009). Briefly, sections were washed in KPBS and then blocked in 2% donkey serum in 0.4% triton X-100/KPBS. The following antibodies were used: anti-5-HTT (#ST51-2, 1:1000, mAB Technologies) and rabbit anti-CRH (1:2500, a kind gift of W. Vale). Primary antibodies were diluted to their working concentrations in 2% donkey serum in 0.4% triton-X-100/KPBS and incubated

with the tissue overnight at 4°C. After incubation, the tissue was rinsed in KPBS, incubated in Rhodamine-labeled donkey anti-mouse or FITC-labeled donkey anti-rabbit IgGs (Jackson Immunoresearch, West Grove, PA; 1:200) in 0.4% triton-X-100/KPBS for 1 hr. Sections were wet mounted and coverslipped with glycerol-based mountant and stored at 4°C prior to analysis. As previously described in Chapter 5, images of 5-HTT- and CRH-*ir* were captured at 25X and represent a 10 μm thick maximum projection.

RESULTS AND DISCUSSION

Among the many roles of the 5-HT system, its impact on stress pathways is well established. We have previously demonstrated that F-HFD exhibit changes in circulating cortisol levels (Grayson et al., 2009), suggesting that maternal HFD consumption affects fetal stress pathways . To investigate whether these CRH alterations are related to the 5-HT system, we examined the level of reciprocal innervation by corticotropin-releasing hormone (CRH) and 5-HT in both the PVH and raphe. We performed double-label immunohistochemistry for 5-HTT and CRH in both the hypothalamus and raphe. Very low CRH-*ir* was present in the PVH and CRH cell bodies were largely undetectable (*data not shown).* 5-HT-*ir* was robust in the PVH. On the other hand, CRH innervation of 5-HT neurons in the raphe was high in the G130 fetus (*Appendix Figure F-1, A*). Furthermore, extrahypothalamic CRH neurons were easily visualized in the area of the locus coeruleus and pontine nucleus in the midbrain (*Appendix Figure F-1, B*). These extrahypothalamic CRH neurons also had many close appositions by 5-HT containing fibers (*Appendix Figure F-1, B*). No qualitative differences were observed between F-CTR and F-HFD regarding CRH innervation (*data not shown*). These studies suggest that reciprocal

innervation of CRH and 5-HT is far more robust in the midbrain than at the level of the PVH at this developmental time point.



Appendix Figure E-1. Fluorescent micrographs 5-HTT and CRH in the midbrain. (**A**) 5-HTT labeled neurons (red) in the dorsal raphe with CRH terminal projections (green) (**B**) extrahypothalamic CRH neurons (green) in the pontine nucleus with 5-HT close appositions (red) (**C**) Diagrammatic representation of localization of photomicrographs (**A**) denoted with red square and (**B**) denoted with green square. Images taken at approximate distance of -17.78 with respect to bregma. (**Abbreviations:** Aq aqueduct, PAG periaqueductal grey, LC locus coeruleus, DR dorsal raphe, PnO pontine nucleus, Py pyramids).