THE EFFECTS OF A MATERNAL HIGH FAT DIET ON HEPATIC INNERVATION IN THE NONHUMAN PRIMATE

By

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CERTIFICATE OF APPROVAL

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PREFACE

Along with an increasing prevalence of adult obesity world wide, an increasing prevalence of obesity and its co-morbidities has also been documented in children. Diseases once only normally found in adults are occurring with greater frequency in pediatric populations. Type 2 diabetes mellitus as well as secondary co-morbidities such as hypertension, non-alcoholic fatty liver disease, hyperlipidemia, and metabolic syndrome are now becoming increasingly common in children. While associations between maternal obesity (without diabetes) or weight gain during pregnancy and childhood obesity have been suggested, the contributions of these conditions to fetal programming are not well understood. However, it is clear from studies in both humans and rodents that intra partum and early postnatal environments are critical to the development of systems that regulate body weight, energy and glucose homeostasis.

Seminal work in humans highlighted the effects that the intrauterine environment has on the development of adult cardiovascular disease, hypertension and bronchitis in cases of maternal undernutrition. While malnutrition is certainly still pervasive in our world today, modern industrialized societies are also suffering from overnutrition and obesity at epidemic rates. Our work is focused on understanding the effects that overnutrition and obesity have on developing children.

While work with rodents has provided much information, there are important species-specific differences between rodents and humans that preclude translation of rodent findings directly into human obesity research. The nonhuman primate model of maternal overnutrition utilized in this dissertation was chosen for the similarities that exist in development between the nonhuman primate and humans. We believe the

studies described in this dissertation will extend our current knowledge of hepatic nervous system development in the nonhuman primate, an area of research that has not been well characterized. In addition, our findings have important clinical implications for understanding how maternal nutrition impacts human development. Human studies based on our findings in the nonhuman primate are currently underway. Our model is a tremendous resource that will allow us, as well as other investigators, to implement rigorous explorations into the effects that obesity and/or a high fat diet have on the growing epidemic of human obesity; a disease whose prevention may have to begin even before conception.

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Chapter 1
Introduction

Obesity as an epidemic

The United States has the dubious distinction of having the highest prevalence of overweight and obesity worldwide. Nearly two-thirds of all adults are either overweight or obese (Baskin et al., 2005; Flegal et al., 2010). We are not alone however, as Mexico, the United Kingdom and Australia rank close behind (Loureiro, 2004). Other Eastern European countries such as Germany and Finland also have a high prevalence of overweight and obesity; more than half of adults aged 35-65 in Europe are either overweight or obese (Keil et al., 1989; J. C. Seidell et al., 1997). Once considered a problem only in high-income countries, overweight and obesity are now dramatically on the rise in countries undergoing rapid economic transition, particularly in urban settings (J. C. Seidell, Rissanen, A., 1997). One study in China showed that obesity in urban areas is most common in those with low socioeconomic status, whereas in rural areas, obesity is associated with high socioeconomic status (Popkin et al., 1993). In the U.S., obesity is highest in areas where low levels of education and low income predominate (Drewnowski et al., 2007; Flegal et al., 2002; Mokdad et al., 2003)

The prevalence of obesity is rapidly increasing and has characteristics that are comparable to a communicable disease epidemic (Mokdad et al., 1999). Indeed, the CDC itself has labeled the increasing prevalence of obesity in the United States as an epidemic. Globally, the World Health Organization (WHO) estimates for 2008 suggest that approximately 1.5 billion adults (age > 20) were overweight. Of these, nearly 500 million men and women were obese. In 2010, nearly 43 million children under the age of 5 years were overweight. Further, the WHO projects that by 2015, approximately 2.3

billion adults will be overweight and more than 700 million will be obese (" WHO factsheet no. 311,").

Identifying obesity in the United States

Since the passage of the 1956 National Health Survey Act, the National Center for Health Statistics, a component of the Centers for Disease Control and Prevention (CDC), has conducted seven separate examination surveys to provide current statistical data on the distribution, amount, and effects of illness and disability in the United States. The first three National Health Examination Surveys (NHES I, II, and III) were conducted between 1960 and 1970. In 1970, following a directive from the Secretary of the Department of Health, Education and Welfare, the National Center for Health Statistics initiated the National Nutrition Surveillance System. The aims of the National Nutrition Surveillance System were to support research efforts linking dietary habits with disease. Thus, the National Nutrition Surveillance System was combined with the National Health Examination Survey in 1970 to form the National Health and Nutrition Examination Survey (NHANES) . NHANES is a randomized, stratified survey that includes a personal interview and a standardized physical examination (Flegal et al., 2010).

The NHANES program conducted three separate surveys between 1971 and 1994 (NHANES I, II, and III). In 1982, a distinct survey was initiated aimed at documenting the health and nutritional status of Hispanic groups in the United States (HHANES). Beginning in 1999, the NHANES became a continuous national survey that releases annual data in two year cycles. While NHANES provides robust data on a national level, state health agencies were unable to utilize NHANES data in a state-specific manner. In 1984, the CDC established the Behavioral Risk Factor Surveillance System (BRFSS) to

collect state-level data. The BFRSS is a cross-sectional survey utilizing random-digit dialing to interview civilian state residents over 18 years old by telephone (Mokdad et al., 2003). Thus, the BRFSS and NHANES surveillance programs provide national and statewide data on a wide range of factors involved in illness and disability in the United States.

Data from NHANES and the BRFSS has documented that the prevalence of obesity has steadily increased in adult men and women since the 1960's (Ford et al., 2010; Ogden et al., 2007). In just seven years between 1991 and 1998, the prevalence of obesity increased nearly 50% in adult men and women of every race and educational level (Mokdad et al., 1999). By 2004, 33% of all U.S. adults were overweight with a body-mass index (BMI) of between 25 and 30. Additionally, 33% of adults were obese with BMI's higher than 30. Within this obese group, over 5% were extremely obese with BMI's higher than 40 (Ogden et al., 2007). The latest national estimates show that 68% of all American adults are overweight or obese (Flegal et al., 2010). While BMI data from NHANES is highly correlated to body fat percentage in adults (Flegal et al., 2009), BMI's calculated through the BRFSS may be underestimated due to the tendency of overweight respondents to underreport their weight and overestimate their height (Palta et al., 1982; Rowland, 1990). Thus, prevalence of obesity reported with the BFRSS program may be higher than the reported values.

As introduced above, the most common measure of obesity is the body-mass index (BMI) measured as weight in kg/ height in meters$^{\wedge 2}$. Other metrics such as direct skin-fold measurements, underwater weighing, and waist circumference divided by hip circumference as a measure of fat distribution, are not widely used in population based

studies (Maes et al., 1997; Visscher et al., 2001). A BMI below 18.5 kg/m^2 is defined as underweight, a BMI between 18.5 and 24.9 kg/m^2 is normal, BMI's between 25 and 29.9 kg/m^2 are regarded as overweight, and people with BMI's higher than 30 kg/m^2 are considered obese ("Physical status: the use and interpretation of anthropometry," 1995).

Public health consequences of obesity

It has been estimated in the United States that nearly 300,000 adults die every year from diseases directly attributable to obesity such as coronary heart disease, stroke and diabetes (Allison et al., 1999). Numerous studies have confirmed that all-cause-mortality risk is increased in overweight or obese individuals. In addition, the relative risk for incidence of type 2 diabetes, myocardial infarction, and ischemic stroke was shown to be even higher than for all-cause-mortality in these subjects (Baik et al., 2000; Manson et al., 1987; Manson et al., 1995; Sjostrom, 1992). The risk of endometrial cancer has a convincing association with obesity, while possible relationships with obesity exist for breast, kidney, colon and gall bladder cancer ("Food, Nutrition and the Prevention of Cancer: A Global Perspective," 1997). Obesity, particulary abdominal obesity, is a very strong risk factor for the development of cardiovascular disease (CVD) and type 2 diabetes (Carey et al., 1997; Chan et al., 1994; R. P. Donahue et al., 1987; Larsson et al., 1984; Rexrode et al., 1998). In fact, metabolic syndrome has been described as the convergence of an unfavorable lipid profile, abnormal glucose metabolism, hypertension and abdominal obesity (Bjorntorp, 1997; Ferrannini et al., 1991). Obesity is also an important risk factor in osteoarthritis, sleep apnea and respiratory disorders (Grunstein et al., 1995; Lean et al., 1999; Oliveria et al., 1999; Young et al., 1993). Thus, while obesity does play a role in all-cause mortality, its larger

impact on morbidity and disability will also force industrialized, as well as emerging nations, to address an increasing prevalence of obesity related disability.

From a financial perspective, overweight and obesity attributable medical spending accounted for 9.1% of total U.S. medical expenditures in 1998, with Medicaid and Medicare financing over half of these costs (Finkelstein et al., 2003). On an individual basis, inpatient and outpatient health care costs for overweight and obese people are an average of 37% higher than for people of normal weight. Medication costs also average 77% higher in overweight and obese individuals (Finkelstein et al., 2003; Loureiro, 2004; Sturm, 2002). Obesity has been shown to have a negative impact on the ability of a person to find a job and to remain employed. Once employed, obesity is associated with lower earnings and wages, particularly in women (Cawley, 2004). Employers who offer health insurance face higher costs to insure obese and overweight employees (Runge, 2007). Other workplace issues include increased absenteeism and reduced productivity. In 1998, it was estimated that obesity and obesity related conditions resulted in over $100 billion in doctors' visits and lost workdays each year (Wolf et al., 1998).

Genetic and environmental factors involved in obesity

The World Health Organization (WHO) has noted that the increase in obesity prevalence is occurring so quickly that it cannot be attributed to changes in genetic inheritance ("Obesity: preventing and managing the global epidemic," 1998). Given that only about 5% of obesity can be attributed to known single-locus mutations, Mendelian genetic shifts are unlikely to be the cause of the rapid increase in overweight and obesity prevalence (Farooqi et al., 2003). However, it is well established that polygenetic risk

factors have a strong effect on the development of obesity. There is a large body of evidence involving twin and adoption studies that support the heritability of individual differences in BMI rather than the effects of a shared environment (Allison, Heshka, Neale, & Heymsfield, 1994; Allison, Heshka, Neale, Lykken et al., 1994; Allison et al., 1996; Bodurtha et al., 1990; Fabsitz et al., 1992; Price et al., 1987; Sorensen et al., 1992; Sorensen et al., 1989; Stunkard, Foch et al., 1986; Stunkard et al., 1990; Stunkard, Sorensen et al., 1986). Moreover, the genetic etiology of obesity is not static and changes across an individuals lifespan (Hewitt, 1997).

While polygenetic effects are a primary force, environment has also been shown to play an important role in obesity. However, while a common environment is important in early life for its role in obesity, the importance of a shared familial environment diminishes with age. (Hewitt, 1997; Lajunen et al., 2009; Silventoinen et al., 2010). In adults it has been demonstrated however, that environment can alter the function of genes involved in obesity. The genetic predisposition towards obesity can be modulated by physical activity (Andreasen et al., 2008; Mustelin et al., 2009). Passive, active and reactive gene-environment mechanisms are additional gene-environment interactions that describe situations in which the environment is coupled to genotype (Jaffee et al., 2007).

Maternal effects related to the intrauterine environment can also effect the growth of the fetus leading to long lasting changes in the developmental program (Maes et al., 1997). Uteroplacental insufficiency and its associated in utero growth restriction have been shown in rodents to produce modifications in chromatin structure or DNA methylation leading to persistent changes in postnatal gene expression (Burdge et al., 2007; Fu et al., 2006; Fu et al., 2004; Gluckman et al., 2007; Ke et al., 2005; Lillycrop et

al., 2007; MacLennan et al., 2004). Recent work by others, with the nonhuman primate model of maternal overnutrition used in this dissertation research, has demonstrated that a maternal high-fat diet (HFD) is associated with covalent modifications of fetal chromatin structure and altered hepatic circadian gene expression (Aagaard-Tillery et al., 2008; Suter et al., 2011). Additional work with this nonhuman primate model has also shown that alterations in serum metabolite profiles are present in HFD fetuses (J. Cox et al., 2009).

Maternal effects are not the only link in the development of obesity. Obesity in adult males has been shown to promote hypogonadism, impaired spermatogenesis, decreased sperm concentration and motility, and increased sperm DNA damage (Amatruda et al., 1978; Fejes et al., 2005; Glass et al., 1977; Kodama et al., 1997; Kort et al., 2006; Magnusdottir et al., 2005; Schlesinger et al., 1994; Shafik et al., 1981; Strain et al., 1982; Twigg et al., 1998; Vermeulen et al., 1996). Recent work in rodents showed that paternal obesity and chronic consumption of a high-fat diet led to impaired glucose tolerance and insulin secretion in female offspring. Morphological changes were also observed in the female offspring's pancreas that included reduced large islet and β-cell areas as well as a postulated compensatory increase in small islets (Ng et al., 2010). These findings are in good agreement with previous work in humans that demonstrated the risk for adult obesity in offspring was significantly higher if either the mother or father was obese (Whitaker et al., 1997). The prevailing evidence suggests that obesity and overweight are complex phenotypes that develop through the convergence of multiple genetic and environmental risk factors experienced throughout an individual's lifetime.

Obesity in children

Within the adult obesity epidemic an increasing prevalence of obesity and its co-morbidities have also been documented in children (Ogden et al., 2002). Diseases once only normally found in adults are occurring with greater frequency in pediatric populations. Type-2 diabetes mellitus as well as secondary co-morbidities such as hypertension, non-alcoholic fatty liver disease, hyperlipidemia, and metabolic syndrome are now becoming increasingly common in children (Pinhas-Hamiel et al., 2005, 2007). It is clear from studies in both humans and rodents that intra partum and early postnatal environments are critical to the development of systems that regulate body weight, energy and glucose homeostasis in the offspring (Anguita et al., 1993; Levin et al., 1998; Ozanne et al., 1999; Ravelli et al., 1998; Ravelli et al., 1999; Roseboom et al., 2000; Srinivasan, Aalinkeel et al., 2006; Srinivasan, Katewa et al., 2006; Strauss, 1997; Thone-Reineke et al., 2006). However, the contributions that maternal obesity and maternal nutrition have on the development of childhood obesity and its co-morbidities are not well understood.

Fetal development is a critical period when exposure to myriad environmental perturbations can have lifelong effects on the structure and function of organs, tissues and body systems in the offspring through biological programming. For example, stress placed on the intrauterine environment by maternal smoking and Type-2 diabetes not only influences fetal growth, but also results in persistent alterations in the programming of childhood weight (Dabelea, Hanson et al., 2000; Oken et al., 2005). Maternal obesity and nutritional imbalances have profound effects on fetal development including neural tube defects, heart defects, shoulder dystocia and multiple congenital anomalies (Scialli,

2006). Mothers with gestational diabetes mellitus (GDM) frequently give birth to infants with macrosomia, impaired glucose tolerance, and a higher risk of developing obesity and diabetes as adults (Garcia Carrapato, 2003; Plagemann et al., 1997a; Silverman et al., 1993). In animal models, fetal nutritional deprivation programs adipocyte metabolism and fat mass towards a propensity for obesity, particularly when challenged post-natally with an energy dense diet (Bispham et al., 2005; Budge et al., 2005; Stocker et al., 2005).

Maternal nutrition

Seminal work in humans highlighted the effects that the intrauterine environment has on the development of adult cardiovascular disease, hypertension and bronchitis (Barker, Osmond, Golding et al., 1989; Barker, Osmond, & Law, 1989). Additional work demonstrated that reduced growth in early life was associated with altered adult glucose tolerance and type-2 diabetes (Hales et al., 1991). Evidence from the Dutch Famine Studies demonstrated that early nutritional deprivation in pregnant mothers led to small for gestational age babies (SGA), and long term health issues in the offspring (Ravelli et al., 1998). These findings led to the development of the *thrifty phenotype hypothesis*, which proposes that the development of type-2 diabetes and metabolic syndrome in adult life is related to the effects of poor nutrition in early life (Hales et al., 2001). While malnutrition is certainly still pervasive in our world today, modern industrialized societies are also suffering from the effects of overnutrition and obesity.

Fuel mediated teratogenesis is a hypothesis which proposes that fetal exposure to an excess of fuels from pregnant mothers with diabetes can cause permanent metabolic changes leading to malformations, greater birth weight, and an increased risk of developing type-2 diabetes in later life (Freinkel, 1980). For example, women with

gestational diabetes mellitus (GDM) or poor glucose tolerance scores have heavier babies with decreased insulin sensitivity (Wilkin, 2002). Significantly, while maternal glucose can freely cross the placenta to the fetus, maternal insulin does not (Freinkel, 1980). The response to the increased glucose load by the developing fetal pancreas results in the production of additional insulin, which acts as a fetal growth hormone promoting growth and adiposity (Freinkel, 1980).

This environmental effect is independent of genetics as children born to mothers diagnosed with diabetes are at a significantly higher risk of future obesity and diabetes than siblings born prior to the development of maternal diabetes (Dabelea, Hanson et al., 2000; Dabelea, Knowler et al., 2000). In addition, breast milk from diabetic mothers has high insulin levels, which can cross the intestinal mucosa-blood barrier during the neonatal period. Infants born and breast fed by diabetic mothers (both type I and gestational) have a significantly increased risk of developing obesity and impaired glucose intolerance early in life, compared to babies born of diabetic mothers, but fed breast milk from nondiabetic women during the neonatal period (Plagemann et al., 2002). However, recent analysis found that breastfeeding, regardless of maternal diabetes or weight status, was inversely associated with childhood obesity (Mayer-Davis et al., 2006). These data point to both gestational and early postnatal nutritional cues as being critical to the development of systems that regulate body weight, energy and glucose homeostasis in offspring.

To date, the maternal influence over metabolic disorders in offspring is unknown, but could include high levels of maternal hormones (insulin and leptin) and/or nutrients (fatty acid/triglycerides and glucose) or changes in placental function (blood flow and

nutrient transport). Strict control of blood glucose, with diet and/or insulin therapy, significantly improves the overall health of the offspring, but fetal overgrowth still occurs in 10-20% of GDM women despite what appears to be satisfactory maternal glycemic control (Lepercq, 2003). Recent work has indicated that infant birth weight and macrosomia may have a stronger association with maternal triglycerides than maternal glucose tolerance (Di Cianni et al., 2005).

In the United States today, nearly 50% of all women of childbearing age are either overweight or obese (Vahratian, 2008). While there have been numerous studies on the diets of children, adults and women of childbearing age, there is a paucity of data on the diets of pregnant women in the United States. One of the few studies to investigate what pregnant women are eating found that low nutrient-dense foods consisting of refined carbohydrates and animal products high in saturated fat, were the major contributors to total energy, carbohydrate and fat intake (Siega-Riz et al., 2002). While this study was limited in scope, the consumption of a diet that is high in calories and saturated fats by these pregnant women is similar to findings from nationally representative studies of children and non-pregnant women (Block et al., 1985; Subar et al., 1998). Regrettably, the dietary analysis by Siega-Riz et al. did not examine the intake of essential fatty acids.

The essential fatty acids are polyunsaturated fatty acids with double bonds in the ω3 and ω6 positions, also referred to as N-3 and N-6 fatty acids respectively. These essential fats cannot be synthesized endogenously by mammals and must be provided in the diet (Heird et al., 2005). There are two parent fatty acids, linoleic (18:2, N-6) and linolenic acid (18:3, N-3), which are both metabolized by the same desaturases and

elongases to form longer-chain polyunsaturated fatty acids able to exert a full range of biological action (Burdge et al., 2005). Important metabolites for linolenic acid (18:3, N-3) are eicosapentanoic acid (EPA, 20:5, N-3) and docosahexaenoic acid (DHA, 22:6 N-3). An important metabolite of linoleic acid (18:2, N-6) is arachidonic acid (AA, 20:4 N-6). The essential fatty acids and long chain polyunsaturated fatty acid metabolites are critical for normal mammalian cell function and are involved in gene transcription, membrane fluidity and thickness which can in turn, affect receptor function (Cetin et al., 2009).

There is strong evidence that suggests that cellular membrane long chain polyunsaturated fatty acid (LC-PUFA) composition is largely determined by diet (Simopoulos, 1991). Once incorporated into cellular membranes, AA, EPA and DHA are competively cleaved by phospholipase A_2 enzymes to produce prostaglandins, leukotrienes and thromboxanes through the actions of cyclooxygenases, and lipoxygenases (Marszalek et al., 2005). Ingestion of N-6 fatty acids displaces N-3 fatty acids in cellular membranes, particularly platelets, neutrophils, erythrocytes, monocytes and hepatocytes. This in turn leads to increased production of prostaglandin E_2, thromboxane A_2, and leukotriene B_4. Thromboxane A_2 is a potent inducer of inflammation, platelet aggregation, vasoconstriction and leukocyte chemotaxis in the liver (Fisher et al., 1987; Katagiri et al., 2004; Nanji et al., 1993; Simopoulos, 1999). N-3 fatty acids are metabolized to thromboxane A_3, PGI_3, and leukotriene B_5. These molecules are involved in the inhibition of platelet aggregation, vasodialation and are weak inducers of inflammation and chemotaxis (Needleman et al., 1979; Simopoulos, 1991). In addition, N-3 fatty acids directly inhibit the ability of monocytes to produce

interleukin-1 (IL-1) and tumor necrosis factor (TNF) (Weber et al., 1991). Thus, N-6 metabolites tend to be pro-inflammatory, while N-3 metabolites tend to be anti-inflammatory.

The N-3 and N-6 LC-PUFAs, particulary docosahexaenoic acid (DHA, 22:6 N-3) and arachidonic acid (AA, 20:4 N-6) are essential dietary nutrients that are critical for proper infant growth and neurodevelopment. DHA and AA are both highly enriched in neural tissue, while DHA is the major component of retinal photoreceptor membranes, cerebral cortex, testis and sperm (Clandinin et al., 1980a, 1980b; M. Martinez, 1992; Neuringer et al., 1984). There is strong evidence that both plasma and cellular membrane LC-PUFA composition is largely determined by dietary ratios (Neuringer et al., 1984; Simopoulos, 1991). Importantly, over the last 100 years dietary N6/N3 ratios have gone from being close to 1:1 to approximately 20:1 (Simopoulos, 1999). A recent report in humans showed that a high N6:N3 ratio either in the maternal diet, maternal plasma or fetal plasma was associated with higher adiposity in children at 3 years of age. In addition, higher levels of EPA and DHA in umbilical cord plasma was associated with lower levels of childhood adiposity in the offspring (S. M. Donahue et al., 2011). Previous evidence suggests that exposure to either AA or DHA also regulates the differentiation of pre-adipocytes in a reciprocal manner *in-vitro* (Gaillard et al., 1989; Kim et al., 2006). Thus, emerging research further supports the importance of essential fatty acids and hints at new roles they may be playing in development.

Dietary Lipids and Development

In a fetus, there is limited capacity for *de novo* lipogenesis and the precursors for fetal fat accretion are primarily supplied trans-placentally and consist of maternal

substrates derived from lipids rather than from glucose (Haggarty, 2002; Herrera et al., 2000; Herrera et al., 2006). While only non-esterified fatty acids (NEFA) can be transferred from mother to fetus directly, other mechanisms including hydrolysis of triglycerides and receptor mediated transfer, allow fatty acids including DHA, AA, and EPA (20:5 N-3) to be transferred through the placenta to the fetus (Crabtree et al., 1998; Diamant et al., 1982; Herrera et al., 2006; Hudson et al., 1977; Ruyle et al., 1990). Furthermore, the placenta selectively transfers AA and DHA to the developing fetus (M. A. Crawford et al., 1997). The composition of the fatty acid supply to the fetus is mainly determined by the maternal lipid profile and suggests that modifications of maternal diet or metabolic homeostasis will affect delivery of lipid substrates to the fetus (Carlson et al., 1992; Olsen et al., 2000).

The brain growth spurt is a period in brain development when brain growth is accelerated above the growth of other organs. This period of development is a time when the brain is exquisitely sensitive to nutritional insult, which may lead to long lasting functional changes in the adult (Dobbing, 1972; Herschkowitz, 1988; Meisami, 1988a, 1988b). Although the developmental sequence of brain maturation and growth is very similar among mammalian species, the stage of brain development that is reached at the time of birth varies widely (Davison et al., 1966; Dobbing et al., 1979; Wiggins, 1982, 1986). The rhesus macaque reaches the peak of its brain growth spurt at roughly 40 days before birth, around gestational age day 130. Humans reach their peak brain growth spurt at birth and the rat reaches its peak brain growth spurt roughly 10 days after birth. While overall brain development is a continuum, the macaque, human and rat can be roughly categorized as prenatal, perinatal and postnatal brain developers respectively (Dobbing et

al., 1979). Thus, the timing of nutritional insults could have vastly different developmental effects in different species.

The central nervous system contains the second highest lipid concentration, outside of adipose tissue, of any organ in the body (Innis, 1991). Unlike adipose tissue, lipids in the brain are primarily structural glycerophospholipids that consist mainly of LC-PUFA's with very little triacylglycerol present (Eichenberg, 1969; Rissiter, 1970; Sastry, 1985). Within the visual system, the cells of the retina are also considered to be an integral part of the CNS and have a similar glycerophospholipid fatty acid composition as the cerebral grey matter (Bazan, 1982; Fliesler et al., 1983). The composition of fatty acids in the mammalian CNS and retina are also remarkably conserved across different species (R. E. Anderson, 1970; M. A. Crawford et al., 1976; M. A. Crawford, Doyle, W., Williams, G., Drury, P.J., 1989; M. A. Crawford et al., 1981; M. A. Crawford, Sinclair, S.J., 1972; Daemen, 1973; Fliesler et al., 1983).

While the distribution of N-6 and N-3 LC-PUFA's within the CNS is cell, membrane and lipid class specific, AA (20:4 N-6), adrenic acid (22:4 N-6) and DHA (22:6 N-3) are the predominant fatty acids found within the CNS (M. A. Crawford et al., 1976; Fliesler et al., 1983; Sastry, 1985). Interestlingly, adrenic acid is a naturally occurring polyunsaturated fatty acid formed through a 2-carbon chain elongation of AA (Ferretti et al., 1986; Horrocks, 1989). Thus DHA, AA and its metabolite, adrenic acid, are the primary species of structural fatty acids found in the CNS.

The N-6 and N-3 fatty acids, while present in low concentrations in the immature brain, are robustly and selectively incorporated into the brain during its development. Human and rodent data suggest that a preferential accumulation of N-6 fatty acids occurs

early in development followed by the accumulation of N-3 fatty acids later in CNS development (Alling et al., 1974; Clandinin et al., 1980a, 1980b; Galli et al., 1975; Hitzemann, 1981; Kishimoto et al., 1965; Samulski et al., 1982; Sinclair et al., 1972; Svennerholm, 1968; Walker, 1967; White et al., 1971). *In-utero*, the N-3 fatty acids necessary for fetal development, especially DHA, must be provided by placental transfer from the maternal circulation. Circulating levels of maternal DHA have been shown to be an important determinant of circulating fetal levels (G. J. Anderson et al., 2005; Elias et al., 2001; Helland et al., 2006; Innis, 2005; van Houwelingen et al., 1995). There is a positive linear relationship between CNS levels of DHA and maternal intake of linolenic acid (18:3, N-3) until a plateau is reached at about 250 mg 18:3/100 g diet. In addition, an inverse linear relationship exists for CNS levels of docosapentaenoic acid (22:5, N-6) with increasing maternal intake of linolenic acid (Bourre, Francois et al., 1989). Thus, there is a close association between tissue lipid composition and the type and ratio of lipids ingested.

It has been well established that deficiencies in N-3 fatty acids, particulary DHA, either through the maternal diet or breastmilk, can have profound effects on the visual and brain development in human and macaque offspring (Helland et al., 2003; Jensen et al., 2005; Neuringer et al., 1986; Neuringer et al., 1984). In humans, infants who were fed formula with no DHA had much lower levels of brain cortex DHA than infants who were breastfed. The decrease in cortex DHA was also accompanied by higher concentrations of adrenic (22:4 N-6) and docosapentaenoic (22:5 N-6) fatty acids (Farquharson et al., 1995; Makrides et al., 1994). Interestingly, changes in fetal brain DHA occur more readily when the maternal diet also contains a high N-6:N-3 ratio (Coti

Bertrand et al., 2006; Innis et al., 2001). Through the competitive inhibition of Δ6-desaturase, high levels of linoleic acid(18:2, N-6) inhibit the metabolism of linolenic acid (18:3, N-3) to DHA. Thus, "substantial and specific depletion of tissue DHA requires diets with both low levels of N-3 fatty acids and high levels of N-6 fatty acids" (Neuringer et al., 1988). Our studies demonstrate in the nonhuman primate, that maternal consumption of a HFD leads to significantly lower levels of DHA and total N3 fatty acids as well as a significantly increased N-6:N-3 ratio in maternal and fetal circulation. In addition, HFD breast milk contains significantly lowers levels of EPA and DHA and a slightly higher N-6:N-3 ratio (Grant et al., 2011)

Evidence has accumulated that detail the structural and functional effects that essential fatty acid deficiency has on the developing brain. Gangliosides are found in nerve endings and are believed to be involved with membrane receptor recognition and cell-cell communication (Fishman et al., 1976). Maternal essential fatty acid deficiency during gestation has been shown in the rodent to lead to a reduction in fetal brain gangliosides and irreversibly impaired learning behavior (Berra et al., 1980; Morgan et al., 1981). Undernutrition and consequent reduction in the availability of essential fatty acids also have important effects on myelination (Davison et al., 1966; Salvati et al., 2002; Wiggins, 1982; Yeh, 1988). Restrictions in maternal N-3 fatty acids alters neurogenesis in the rat cerebral cortex, leads to reduced DHA accretion with subsequent substitution by N-6 fatty acids, increased levels of dopamine, increased expression of D1 and D2 dopamine receptors and the retinoic acid receptor (Coti Bertrand et al., 2006; Innis et al., 2001; Kuperstein et al., 2005; Lim et al., 2005). Third generation offspring of rats fed a maternal diet deficient in linolenic acid (18:3, N-3) displayed decreased levels

of DHA (22:6, N-3) and increased docosapentaenoic acid (22:5, N-6) in the CNS, serum, heart, liver and other organs. Decreases in the activity of the CNS Na^+, K^+ ATPase was observed in synaptasomes, and visual and learning deficits were also observed (Bourre, Francois et al., 1989).

It is important to note that the temporal sequence of fatty acid deposition and functional timing of CNS membrane maturation has important implications for the timing of fatty acid deficiencies in determining the severity of impairment and potential for reverse (Innis, 1991). Under the best of situations, the turnover rate for structural fatty acids in the brain and the peripheral nervous system are very slow (Bourre, Durand et al., 1989; Bourre et al., 1987; Homayoun et al., 1988; Youyou et al., 1986). In cases where the essential fatty acid deficiency occurred *in-utero*, recovery of impaired learning behavior or avoidance behavior in rodents appears to be irreversible (Galli et al., 1975; Lamptey et al., 1978a; Morgan et al., 1981). An essential fatty acid deficiency, initiated *in-utero* and continued through postnatal life has also been shown in rodents to include delays in the development of motor skills such as negative geotaxis, cliff-drop aversion and audicular startle response in rodents (Lamptey et al., 1978b).

In the nonhuman primate fetus, essential CNS melanocortin circuitry involved in the regulation of metabolic homeostasis develops during the third trimester of pregnancy (B. E. Grayson et al., 2006). It was recently reported with the nonhuman primate model used in this dissertation, that chronic exposure to a maternal high-fat diet (HFD) led to increased expression of proopiomelanocortin (POMC) mRNA in the fetal arcuate nucleus and decreases in agouti-related peptide (AGRP) gene expression and AGRP immunoreactive nerve fibers throughout the hypothalamus in the context of hypothalamic

inflammation (B. E. Grayson, Levasseur et al., 2010). Earlier work with our model showed that significant increases of circulating inflammatory cytokines and chemokines were also present in fetal HFD plasma (McCurdy et al., 2009). Importantly, returning HFD mothers to a CTR diet during pregnancy normalized fetal hypothalamic POMC and AGRP expression as well as plasma inflammation to control levels (B. E. Grayson, Levasseur et al., 2010).

Additional work with our model revealed that HFD fetuses had significant perturbations of the serotonin system in the dorsal raphe. Increases in tryptophan hydroxylase 2 (TPH2), the rate-limiting enzyme in serotonin synthesis, and 5-HT1_AR, an inhibitory auto-receptor critical for regulation of serotonin synthesis were observed. When the offspring were brought to term, behavioral testing at postnatal day 130 revealed that female HFD offspring displayed increased latency to touch a potentially threatening novel object placed in their cage. Overall, 78% of the HFD offspring displayed anxious and/or aggressive behavior during the behavioral protocols. When these animals were sacrificed at 13 months, significant decreases in cerebral spinal fluid (CSF) serotonin levels were observed (Sullivan et al., 2010). While these studies do not directly address the impact of fatty acids on brain development, they do support the existing body of evidence describing the importance of maternal nutrition on fetal brain development and function during pregnancy and after birth.

In the liver, there is very limited data on the changes in N-6 and N-3 fatty acids that occur during development (Innis, 1991). However, a linear increase has been observed in AA (20:4, N-6), total N-6 and total N-3 fatty acid accretion that starts at about week 27 and continues to term in humans (Clandinin et al., 1981). Other work has

suggested that a linear increase in the percent of DHA and a decrease in the percent AA from week 30 to week 44 post-conception may indicate selective accretion of N-3 fatty acids into the developing liver (M Martinez, 1989). More detailed studies are needed in the liver at different stages of development to elucidate the contributions of maternal transfer versus endogenous synthesis for the deposition of LC-PUFA's into fetal liver.

NAFLD, lipotoxicity and apoptosis

Non-alcoholic fatty liver disease (NAFLD) represents a histological spectrum of disease that progresses from simple fatty liver (hepatic steatosis), to steatosis with inflammation (nonalcoholic steatohepatitis or NASH), steatohepatitis with fibrosis, and ultimately to cirrhosis and hepatocellular cancer (Day, 2005; Marion et al., 2004). Compared to hepatic steatosis, which is thought to have a largely benign clinical course (Dam-Larsen et al., 2004; Matteoni et al., 1999; Teli et al., 1995), NASH is a dangerous pathological condition whereby a significant percentage of patients progress to cirrhosis and ultimately a liver-related death (McCullough, 2006). Although NASH has been associated with obesity, diabetes, hyperlipidemia and insulin-resistance, little is known about its pathophysiology. It is generally accepted however, that the progression from hepatic steatosis to NASH involves multiple factors such as reactive oxygen species, gut-derived endotoxin, inflammatory cytokines, lipid peroxidation, and/or oxidative stress (Berson et al., 1998; Day et al., 1998; Reddy et al., 2006).

Our group has recently demonstrated with the nonhuman primate model used in this dissertation, that chronic consumption of a maternal HFD, independent of clinical obesity or diabetes, during pregnancy produced elevated fetal plasma triglyceride and glycerol levels that were highly correlated to maternal levels. In the

liver, HFD fetuses had premature gluconeogenic gene expression, hepatic steatosis, elevated triglyceride content, and evidence of oxidative stress. (McCurdy et al., 2009). These data indicate that hepatic lipotoxicity and NAFLD was present these HFD fetuses.

Lipotoxicity is the excess accumulation of lipid in nonadipose tissue. Usually occurring in the setting of high levels of plasma free fatty acids or triglycerides, excess lipid accumulation in the heart, skeletal muscle, pancreas and liver is involved in the pathogenesis of heart failure, obesity, metabolic syndrome and type-2 diabetes mellitus (Perez-Martinez et al., 2010). The adipocyte is the only cell that is specifically adapted to store large amounts of fatty acids as triglycerol. Once regarded as a passive storage compartment for excess triglycerides, the adipocyte has now been recognized as a sophisticated and complex endocrine gland with leptin and other polypeptide secretory products playing important roles in metabolic homeostasis (Ahima et al., 2000; Ailhaud, 2000). There is also strong evidence, primarily through the action of leptin, and perhaps adiponectin, that another role of the adipocyte is to protect nonadipose tissue from lipid overload (Moitra et al., 1998; Yamauchi et al., 2001).

While the triglyceride content of adipose tissue can range from a small fraction of lean-body mass to hundreds of pounds based on caloric intake, the triglyceride content of normal nonadipose tissue appears to be tightly regulated and remains within a narrow range regardless of caloric intake (Unger et al., 1999). In the presence of excess fatty acids, the fatty acid induced up-regulation of peroxisome proliferator-activated receptor α (PPARα) and its target enzymes; acyl CoA oxidase (ACO) and

carnitine palmitoyl transferase 1(CPT-1), shunt the fatty acids towards oxidative rather than lipogenic pathways (Zhou, Shimabukuro, Wang et al., 1998). The net result is that triglycerides are depleted in nonadipocytes by an increase in free fatty acid oxidation and a decrease in esterification. This is suggested to occur by a direct mechanism in peripheral tissues, including liver, and is regulated by intact leptin signaling (Shimabukuro et al., 1997; Unger, 2002; Unger et al., 1999).

In the absence of leptin signaling and high circulating lipid levels, increases in peroxisome proliferator-activated receptor γ (PPAR γ), sterol regulatory element binding protein (SREBP)-1c, acetyl CoA carboxylase (ACC), fatty acid synthetase (FAS) and glycerol phosphate acyl transferase (GPAT) and reduced expression of PPARα, ACO and CPT-1 together with increased ectopic accumulation of triglyceride were observed in the rodent pancreas (Y. Lee et al., 1997; Y. Lee et al., 2001; Zhou, Shimabukuro, Lee et al., 1998). Further, wild-type rodents fed a high-fat diet (60% of energy content) demonstrated a very large increase in body fat (150 fold) yet only developed mild hepatic steatosis (1-2.7 fold increase). When leptin-deficient (*ob/ob*) mice or leptin resistant (*db/db, fa/fa*) rats were fed a 6% fat diet, extremely high levels of hepatic triglycerides were observed (4-100 fold) (Unger et al., 2001). These changes suggest that a shift to enhanced lipogenesis and decreased oxidation mediated by leptin are important mechanisms regulating steatosis in nonadipose tissue.

Lipoapoptosis is a metabolic cause of programmed cell death. When fatty acid concentrations in a cell chronically exceed the limits of the oxidative pathway, the excess fatty acids enter pathways of nonoxidative metabolism. Esterification of fatty acids into triglycerides has been suggested to be the initial fate of excess fatty acids

within the cell (Unger et al., 2002). The liver appears to have a high tolerance for lipid surplus and also has the ability to export excess triglycerides in the form of very low density lipoproteins (VLDL) (Unger, 2003). However, when the hepatic capacity to oxidize, store and export fatty acids as triglycerides is overwhelmed by fatty acid influx, hepatic lipotoxicity and consequent lipoapoptosis are likely results (Trauner et al., 2010)

Hepatocyte apoptosis is a marker of disease severity in numerous hepatic disease states (Hatano, 2007; Ishii et al., 2003; Rudiger et al., 2002). In fact, the severity of hepatocyte apoptosis is significantly correlated to histopathological and biochemical markers of NASH and hepatic fibrosis (Feldstein et al., 2003). There is evidence that there are connections between fatty acids, hepatic steatosis and hepatic apoptosis. For example, incubation of HepG2 cells *in-vitro* with saturated and monounsaturated fatty acids produced steatosis and p-JNK-dependent apoptosis that was more pronounced with saturated fatty acids (Malhi et al., 2006). In primary rat hepatocytes, treatment with oleic and stearic acid induced steatosis and sensitized hepatocytes to tumor necrosis factor related apoptosis inducing ligand (TRAIL) mediated cytotoxity (Malhi et al., 2007). Our data show a significant increase in the numbers of apoptotic cells in fetal livers exposed to a maternal high-fat diet (Grant et al., 2011). Previous findings of severe hepatic steatosis, increases in p-JNK, and high circulating levels of inflammatory cytokines (McCurdy et al., 2009), as well as our current findings of increased fatty acids (particularly oleic acid, Chapter 3) in the fetal circulation are consistent with previously described mechanisms of hepatic apoptosis. To our knowledge, the increased apoptosis in the HFD fetal liver is a novel finding that reinforces the extent of damage occurring

within the developing fetal liver. However, the regenerative capacity of the liver is formidable and studies already in progress will determine whether permanent hepatic damage is evident in these animals.

Macroscopic Liver Architecture

The liver is the largest internal organ and is a central component of metabolic homeostasis. Beyond its critical role in glucose homeostasis, lipid metabolism and the production of bile acids, the liver is also involved in lipoprotein, amino acid, bilirubin, and nitrogen metabolism. Additional important functions are the synthesis and secretion of albumin and blood clotting factors, heme and porphyrin biosynthesis, retinoid and vitamin D processing, iron and copper metabolism, uptake and disposition of trace elements, drug and endo/xenobiotic metabolism, and important roles in immune function including lymph production (Crispe, 2009; Rodes, 2007). The anatomical structure of the liver provides it with a unique ability to survey and implement changes in the circulating milieu.

The anterior surface of the human liver is divided into right and left lobes by the falciform ligament. The liver hilus (porta hepatis) is located on the posterior surface and delimits the quadrate and caudate lobes. Thus, four lobes are traditionally used to describe the external structure of the human liver (Skandalakis et al., 2004). The structure of the macaque liver is very similar to human with the following exceptions. The right lobe is intimately attached to a central lobe with only a shallow cleft to demarcate the transition. The left lobe is clearly separated from the central lobe connected only by a fibrous attachment. The quadrate lobe is much narrower than in

humans and the caudate lobe is unique in that it nearly encircles the inferior vena cava (Hartman et al., 1933).

The liver is unique in that it has a dual blood supply from the common hepatic artery and the portal vein entering at the hilus. The portal vein is formed from the convergence of the splenic and superior mesenteric veins and delivers blood to the liver from the entire digestive tract. The portal vein provides approximately 70% of the total blood flow to the liver (Skandalakis et al., 2004). The common hepatic artery normally originates as a branch from the celiac trunk and ascends as the proper hepatic artery into the hepatoduodenal ligament. Within the ligament, the proper hepatic artery divides to form the right and left hepatic arteries entering the liver at the hilus. Distribution of arterial blood to the different functional regions of the liver is identical to the distribution of the portal vein (Rodes, 2007; Skandalakis et al., 2004). Blood leaving the liver does so at the superior liver hilus by the major and minor dorsal hepatic veins that are attached directly to the retrohepatic portion of the inferior vena cava (Gupta et al., 1981; Hartman et al., 1933; Mehran et al., 2000).

The liver has a unique pattern of vascularization in which the supplying (afferent) and the draining (efferent) blood vessels interdigitate uniformly, are separated by the hepatic parenchyma and are connected by capillary sized structures known as sinusoids (Arias, 2009). To provide afferent blood flow to every region of the liver, the portal vein and hepatic artery divide into progressively smaller branches to ensure consistent blood supply to the hepatic parenchyma while conforming to the shape of the liver (Saxena et al., 1999). Corrosion casts of the efferent hepatic veins reveal similar highly branched structures (Gupta et al., 1981; Mehran et al., 2000). In humans, portal veins and hepatic

arteries follow a strict pattern of branching. The terminal branches of the portal veins and hepatic arteries give rise to an orderly but directionally randomized series of first-order branches supplying a discrete region of hepatic parenchyma measuring approximately 1.5 mm^3. Each first-order branch gives rise to eleven second-order branches at roughly right angles. The hepatic veins follow the same branching pattern as portal veins except the hepatic veins do not follow the second-order branching pattern observed in the afferent vasculature. The net result in two dimensions, is the formation of the classic hexagon shaped hepatic lobule, in which one efferent central hepatic vein is surrounded by six afferent structures known as portal triads (Saxena et al., 1999).

Microarchitecural Elements of the Liver

The portal triad consists of a the terminal branches of the portal vein and hepatic artery as well as a bile duct densely interwoven within connective tissue sheaths. Portal and arterial blood enter the lobule through the portal triad and flow through parenchymal structures known as sinusoids on a path toward the hepatic vein (also known as the central vein) and ultimately out of the liver. Bile is produced by hepatocytes and biliary epithelial cells (also known as cholangiocytes) and flows in the opposite direction of blood flow from the hepatic vein towards bile ducts located near the portal vein and hepatic artery. The intrahepatic biliary tract starts near the hepatic vein with short tracts lined by hepatocytes, followed by a series of biliary canals of increasing diameter lined by biliary epithelial cells as the tract approaches the bile ducts (Rodes, 2007).

The parenchyma of the liver is largely composed of hepatocytes which account for 60% of the total liver mass (Daoust, 1958). The hepatocytes are irregularly shaped, but most closely resemble a polygon and are arranged in anastomosing cell plates

generally one to two cells thick that are oriented as if radiating from the central vein towards the portal triads (Motta, 1977; Sasse et al., 1992). Gaps are found between the anastomosing cell plates and are where the sinusoidal structures are located. The hepatocytes in the cell plate are polarized with an apical, or canalicular border facing other hepatocyes and form a bile canaliculus. The basolateral sides of the hepatocytes are orientated towards the sinusoids but are separated from the sinusoidal epithelium by the space of Disse (Motta, 1977). Importantly, the interlobular space of Disse is the anatomical location of autonomic nerve fibers in higher-order primates, such as humans and macaques (Bioulac-Sage et al., 1990; Burt et al., 1989; Forssmann et al., 1977; McCuskey, 2004; Rodes, 2007; D. G. Tiniakos et al., 2008). To date, no evidence of innervation has been demonstrated within the hepatic lobule of rodents (Ueno et al., 2004). These findings highlight that important differences in hepatic neuroanatomy exist between higher-order primates and rodents and that these differences must be kept in mind when interpreting data from rodent studies.

The sinusoids are unique capillary structures formed by highly fenestrated liver sinusoidal endothelial cells (LSEC) (McCuskey et al., 1993; Smedsrod et al., 1994). Separated from the hepatocytes by the space of Disse, the LSEC contain fenestrae that are organized in sieve plates. The fenestrae facilitate the transport and exchange of fluid, solutes and particles between the sinusoidal lumen and the space of Disse (Rodes, 2007). The LSEC have a very high endocytotic capacity and play an important role in clearing the blood of macromolecular waste products and in antigen uptake (Rodes, 2007; Smedsrod et al., 1994). The LSEC express both MHC class I and II molecules, and facilitate the adhesion of leukocytes and lymphocytes by secretion of chemokines and

expression of adhesion molecules (Lalor et al., 1999; Limmer et al., 2000; Lohse et al., 1996). Following stimulation, the LSEC release prostanoids that can have profound effects on hepatocyte metabolism and liver hemodynamics (Smedsrod et al., 1994). Interestingly, the size of the fenestrae are not fixed and change in diameter following exposure to serotonin, alcohol, nicotine and pressure (Arias, 2009).

Hepatic stellate cells (HSC), also known as fat storing cells or Ito cells, are located in the space of Disse between the basolateral surfaces of the hepatocytes and the anti-luminal side of the LSEC. The HSC have spindle shaped cell bodies and several long branching cytoplasmic processes (S. L. Friedman, 2008; Rodes, 2007). The long branching processes of the HSC encircle the sinusoids in the space of Disse between the LSEC's and the basolateral sides of the hepatocytes (S. L. Friedman, 2008). The processes of the HSC can also penetrate between hepatocytes, and into adjacent hepatic cell plates to extend into nearby sinusoids (Cassiman et al., 2002; K Wake, 1988). On each of these cytoplasmic processes there are numerous pointed spines known as *hepatocyte-contacting processes* that face and establish close intercellular contacts between the microvillous structures of hepatocytes (see below) and the HSC (K Wake, 1995). While the role of these *hepatocyte-contacting processes* is currently unknown, it has been suggested that they are involved cell differentiation processes as well as in a contractile mechanism that inhibits contact between the HSC and the hepatocyte (Gressner et al., 1992; Melton et al., 2007; K. Wake, Motomatsu, K., Ekataksin, W, 1991).

The best-known function of the HSC itself is the key role it plays in retinoid storage and metabolism. The HSC's contain most of the vitamin A found in the body

(Blomhoff et al., 1991; Cassiman et al., 2002). Cellular retinol-binding protein-1 (CRBP-1) is involved in retinoid metabolism and is a reliable HSC marker in human liver (Van Rossen et al., 2009). In addition, some of the long sinusoidal encircling HSC cytoplasmic processes contain thick (5 nm) actin-like filaments that may be involved in structural reinforcement of the endothelial lining or in the regulation of hepatic hemodynamics by contraction of the sinusoids (K. Wake, 1980).

Like the LSEC, hepatic stellate cells are involved in hepatic immunoregulation (Crispe, 2009; Maher, 2001). The HSC express toll-like receptors (TLRs) and respond to innate immune signals (Paik et al., 2003; Seki et al., 2007). These multifunctional cells are also capable of producing an array of chemokines, cytokines and growth factors (Bonacchi et al., 2003; S. L. Friedman, 2008; Marra et al., 2002; Schwabe et al., 2003). Recent studies defined the HSC as an antigen presenting cell (APC). They express MHC class I and II molecules as well as CD1d and activate classical T-cells and natural killer T- cells (Crispe, 2009; Winau et al., 2007).

In addition to retinoid storage and their role in immunoregulation, the HSC's are one of several distinct intrinsic myofibroblast-like cell types involved in liver repair and injury (Cassiman et al., 2002). When activated by liver injury, the HSC phenotype changes from a quiescent state to a proliferative state that includes synthesis of fibrogenic matrix proteins involved in cirrhosis (S. L. Friedman, 2000). Emerging evidence supports the idea that the sympathetic nervous system (SNS) is involved in the pathogenesis of cirrhosis (Hsu, 1992; Iwai et al., 1996). Further, it has been proposed that HSC's are " hepatic neuroglia that are regulated directly by the SNS and that provide a local source of cathecholamines" (Oben & Diehl, 2004). This hypothesis is based on

findings that HSC's synthesize and release norepinephrine (NE), prostaglandins and other neurotransmitters, express adrenoceptors, and that NE and neuropeptide Y (NPY), a neuropeptide that is released with NE from sympathetic nerve terminals, regulates HSC growth (Athari et al., 1994; Oben, Roskams et al., 2004; Oben et al., 2003). It must be noted that these studies were all performed in mice and there is very little available evidence that similar mechanisms are found in the nonhuman primate. However, recent work in humans has demonstrated that human HSC's express α_{1A} adrenoceptors, and in response to NE stimulation, produce α_{1A} dependent calcium spikes and secrete proinflammatory cytokines in a dose-dependent manner (Sancho-Bru et al., 2006). As will be discussed in more detail below, rodents, in contrast to humans and nonhuman primates, are nearly devoid of innervation within the hepatic parenchyma (Ueno et al., 2004). Thus, important differences in innervation exist in the liver between the rodent and the nonhuman primate that may have important implications for the autonomic mechanisms involved in regulation of HSC function.

The hepatocytes are the main parenchymal cell of the liver, comprising 87% of the volume of the intralobular tissue, and are responsible for a wide range of specialized functions. They are large polarized multifaceted cells arranged in cell plates in the hepatic parenchyma so that they are accessible to portal and arterial blood from adjacent sinusoids (Rodes, 2007). Approximately 40% of the hepatocyte cell surface is exposed to sinusoid blood flow (Schaffner, 1985). The basal membrane of the hepatocyte facing the sinusoidal space is populated by numerous microvilli, which in addition to defining the parenchymal boundary of the space of Disse, greatly enhances hepatocyte surface area and capacity for receptor and transport functions. The fenestrated endothelium of the

LSEC's define the sinusoidal boundary of the space of Disse and along with the hepatic stellate cell, are intimately associated with basal membrane of the hepatocyte (Rodes, 2007).

The lateral membranes of the hepatocyte, the membrane between adjacent hepatocytes, do not contain microvilli, are relatively straight and contain membane specializations consisting of tight junctions, adherent junctions, gap junctions and desmosomes (Phillips, 1987). The lateral membrane is also the location of the bile canaliculus, a specialized intercellular junction formed from the plasma membranes of adjoining hepatocytes. The gap junctions consist mainly of connexins and are involved in intercellular communication between hepatocytes (Rodes, 2007). In the rodent, gap junctions have been suggested as the primary mechanism for propagation of autonomic nervous system signals within the hepatic parenchyma (Iwai et al., 1991; Seseke et al., 1992).

The Kupffer cells are resident macrophages in the liver and are located in the sinusoids. They are essential for normal functioning of the liver and the innate immune response. Their main function is to remove foreign material from the blood by either phagocytosis or pinocytosis and process it by lysosomal degradation (Rodes, 2007). They are anchored in the sinusoid through cytoplasmic processes that are in contact with LSEC's. However, it has been shown that some of the cytoplasmic processes of the Kupffer cells penetrate the fenestrae of the LSEC and make contact with hepatic stellate cells and hepatocytes (Arias, 2009; McCuskey et al., 1990).

Many other diverse cell types including natural killer T-cells, liver dendritic cells and hepatic stem cells are also found in the liver (Crispe, 2009; Rodes, 2007). While all

are necessary for the effective functioning of the liver, a description of every hepatic cell-type is outside of the scope of this introduction. The focus of this dissertation is on the effects that a maternal high-fat diet has on the development of the hepatic nervous system in the nonhuman primate. Thus, a brief description of the specific aforementioned cell types, their anatomical relationship to each other in the parenchyma, as well as the autonomic nervous system, provides some background on the complex and integrated anatomy that may underlie basic autonomic control of liver function. However, an incomplete understanding of the neuroanatomy of the liver persists even today, impeding the progress of studies designed to elucidate the regulation of liver function by the autonomic nervous system (Yi, la Fleur et al., 2010).

Hepatic Innervation

The liver is innervated by both the sympathetic and parasympathetic nervous systems. These autonomic nerve fibers are primarily associated with the afferent vasculature and enter the liver at the hepatic hilus (Shimazu, 1996). The autonomic nerves form two communicating plexuses within the liver. An anterior plexus is associated with the hepatic artery and a posterior plexus is associated with the portal vein and the bile duct. Additional innervation may also be associated with the hepatic vein, but this has not been well described (M. I. Friedman, 1988).

Afferent as well as efferent nerves containing adrenergic, cholinergic, and peptidergic components are all found in the liver (Shimazu, 1996). Postganglionic sympathetic nerves are part of the splanchnic system originating in the celiac and superior mesenteric ganglia. The preganglionic parasympathetic nerves are part of the vagus, while postganglionic parasympathetic nerves are derived from ganglia presumed

to be located at the hepatic hilus and within the portal regions (H. R. Berthoud, Neuhuber, W.L., 1998; M. I. Friedman, 1988).

Adrenergic and cholinergic nerves of the autonomic nervous system are found in distinct populations within and adjacent to the portal triads. Portal tract innervation by adrenergic nerves is well characterized and very consistent across mammalian species (Amenta et al., 1981; Burt et al., 1989; Feher et al., 1992; Feher et al., 1991; Forssmann et al., 1977; Goehler et al., 1991; Goehler et al., 1988; McCuskey, 2004; Metz et al., 1980; Nobin et al., 1978; Reilly et al., 1978; Skaaring et al., 1976). Interlobular innervation of autonomic nerve fibers however, is subject to much variation between species. In higher-order primates, such as humans and macaques, the parenchymal sympathetic nerve fibers involved in regulation of hepatic function are found within the space of Disse and are in close contact with hepatocyes HSC's, LSEC's and Kupffer cells (Bioulac-Sage et al., 1990; Burt et al., 1989; Forssmann et al., 1977; McCuskey, 2004; D. G. Tiniakos et al., 2008; Tsuneki et al., 1981). In the rhesus macaque, electron microscopy has revealed nerve fibers, containing large dense-cored transmitter vesicles, embedded into grooves in parenchymal hepatocytes (Forssmann et al., 1977). No evidence of intralobular innervation by sympathetic fibers has been demonstrated to date in the rodent (Reilly et al., 1978; Skaaring et al., 1976; Ueno et al., 2004).

Neuropeptide-Y fibers have been shown to have a similar distribution and display co-localization with tyrosine hydroxylase (TH) immunoreactivity in the liver of humans and other mammals (Burt et al., 1989; Feher et al., 1991; Goehler et al., 1991). NPY positive fibers have also been found to be present in the hepatic lobule in close apposition with LSEC's and hepatocytes in the Japanese macaque (Ding et al., 1994). In addition,

following hepatic denervation of sympathetic fibers with the neurotoxin, 6-hydroxydopamine, NPY-positive fibers were undetectable in the rat liver (Goehler et al., 1991). Taken together, these results suggest that NPY is involved with sympathetic function in the mammalian liver.

The extent of cholinergic innervation of the hepatic lobule is unclear in any species studied. While some reports suggest that intralobular innervation by parasympathetic fibers is present in the rat, results from other investigators could not confirm these findings (Metz et al., 1980; Reilly et al., 1978; Satler et al., 1974; Skaaring et al., 1976). More recent work examined the intralobular localization of acetylcholinesterase (AChE) in rats, hamsters, guinea pig, dogs and humans by the histochemical method of Karnovsky & Roots. The authors found no evidence of AChE-positive fibers within the lobule of any species examined, although fine fibers were observed along the borders of the portal triad (Akiyoshi et al., 1998; Karnovsky et al., 1964).

The neuroanatomy of the intrahepatic nerves as well as the role that these nerves play in regulating hepatic hemodynamics, hepatocyte function and metabolism, Kupffer cell activity, HSC function, and LSEC permeability are not well understood (Shimazu, 1996; Yi, la Fleur et al., 2010). Mechanisms involving direct neural stimulation of these cells as well as indirect mechanisms mediated by innervation of non-parenchymal cells have been proposed (Anil et al., 1987; M. I. Friedman, 1988; Gardemann et al., 1992; Lautt, 1983; Lautt et al., 1987; McCuskey, 1966, 1967). In the rodent, it has been suggested that nervous control of hepatic metabolism is regulated by diffusion of released transmitters or indirectly through changes in blood flow or tissue oxygenation (Shimazu,

1996). Alternatively, neural signals might be propagated within the lobule through the large numbers of gap junctions present in the rat liver (Gilula, 1982; Seseke et al., 1992). In support of this idea, an inverse relationship between intralobular nerves and gap junctions has been reported (Forssmann et al., 1977; Reilly et al., 1978). The presence of intralobular nerves in species such as humans and macaques, and their close association with parenchymal and sinusoid cell types, suggest that nervous signals may be regulating hepatic function by directly acting on these cells (Anil et al., 1987; M. I. Friedman, 1988; Gardemann et al., 1992; Niijima, 1989a; Nobin et al., 1978).

Thus far, this discussion has focused on the efferent component of hepatic autonomic innervation. Communication between the liver and the central nervous system is bi-directional and in contrast to the better characterized efferent sympathetic systems identified in the liver, the sensory functions of the liver have not been thoroughly studied (H. R. Berthoud, 2004; Shimazu, 1996). It has been reported that substance P (SP), vasoactive intestinal peptide (VIP), and calcitonin gene related peptide (CGRP) neuropeptides are associated with sensory nerves in the liver (Barja et al., 1982, 1984; Goehler et al., 1988; Ito et al., 1990; Sasaki et al., 1986; Sasaki et al., 1984; Shimazu, 1996). Substance P immunoreactive fibers are observed in portal regions and the hepatic parenchyma of guinea pig, dogs and humans (Akiyoshi et al., 1998). CGRP and VIP immunoreactive fibers are reported to innervate portal veins and hepatic arteries within the portal stroma. No evidence for intralobular innervation of VIP or CGRP has been reported in any species studied (Akiyoshi et al., 1998; H. R. Berthoud, 2004; Goehler et al., 1988; Ito et al., 1990; Sasaki et al., 1986; Sasaki et al., 1984; Shimazu, 1996).

Early histological studies in both dog and human identified three types of sensory nerve endings in the liver. Pacinian corpuscles were found in connective tissue, bare endings were localized to the lobules, central veins and bile ducts, and glomerular type endings were found near the bile ducts (Tsai, 1958). Functional studies in the liver and portal vein have shown that afferent sensory mechanisms are in place for the detection of glucose, amino acids, lipids and fatty acids and hormones (Niijima, 1989b; Nishizawa et al., 2000; Randich et al., 2001; Torii et al., 2001; Uneyama et al., 2002). Additional studies have revealed the existence of hepatic osmoreceptors, baroreceptors and receptors sensitive to changes in ion concentration (Sawchenko et al., 1979).

Mechanisms underlying glucose sensing by the liver have been the subject of focused investigation. It was demonstrated that vagal afferent fibers in the common hepatic branch are responsive to hepatic glucose stimulation in the portal vein both in isolated liver preparations and *in vivo* (H. R. Berthoud et al., 1992; Niijima, 1983). This work was extended by showing that the vagal glucose sensing system in the portal vein is dependent on the glucose transporter GLUT-2 and glucagon-like peptide-1 (GLP-1) (Burcelin et al., 2000; Burcelin et al., 2001). Glucose sensing is suspected to occur within the liver itself, however identification of the proteins and neural mediators involved remain elusive (Cherrington, 1999). Given the critical role that glucose homeostasis plays in the maintenance of life itself, it seems unlikely that the extra-hepatic portal vein is the sole glucose sensing system in the liver.

Hepatic Nervous System Development

Few studies have specifically examined the ontogeny of hepatic innervation. Those that have are very limited in the species examined and the developmental

timepoints assesed. In the mouse, NPY immunoreactive fibers are present in low density in some portal regions in 19-day-old embryos. Following birth, NPY fibers are found in every portal region examined, reaching a peak density at one week after birth before decreasing to adult levels at two weeks after birth (Ding et al., 1997). In the same study, NPY innervation was found in the hepatic parenchyma of the guinea pig as well as in the adult dog, monkey and human. The authors also compared the NPY innervation present in neonatal guinea pigs (3-7 days old) with adults and found no differences in the distribution or density between the two groups (Ding et al., 1997).

The most thorough study to date was performed in human liver and evaluated the development of hepatic innervation in normal fetal tissue, from gestational ages of 8-40 weeks and in normal adult tissue with a pan-neuronal marker (D. G. Tiniakos et al., 2008). Hepatic innervation in the portal triads generally began at about 20 weeks in gestation and increases in density reaching adult levels near term. Many fibers are found in close association with the hepatic artery, less surrounding the portal vein and only occasionally associated with the bile ducts. In the hepatic parenchyma, innervation is observed at very low density in isolated cases at 40 weeks, proceeding to moderate density in the adult (D. G. Tiniakos et al., 2008).

In humans, peptidergic innervation by galanin, CGRP and somatostatin in the portal regions generally follow the same developmental and distributional patterns described above, with one major exception. At or near term, galanin, CGRP and somatostatin immunoreactivity are no longer observed in the portal triad. This trend was observed in the adult samples as well. In addition, galanin, CGRP and somatostatin positive nerves were not observed in either the hepatic parenchyma or near the central

vein in any age studied- including adult tissue. In contrast, NPY innervation was not observed in any region of the liver, including the portal triad or the parenchyma, in any of the human fetal samples studied. NPY innervation was present only in the adult livers, where it was found in moderate density in the portal regions, in lower density in the hepatic parenchyma and in sparse association with the central vein (D. G. Tiniakos et al., 2008).

Development of human hepatic innervation first appears in the portal regions in the second trimester and increases to adult levels by term. Innervation of the lobule is not observed until term and increases to a higher density in the adult. It also appears that a temporal regulation of peptidergic neurotransmitter expression exists in the developing human liver. These findings support the idea that development of portal innervation occurs *in-utero*, while parenchymal innervation and maturity of liver innervation occurs after birth in humans (Delalande et al., 2004).

In the adult macaque the organization of sympathetic innervation is similar to that found in the adult human (Nobin et al., 1978). To our knowledge, the ontogeny of hepatic innervation has never been explored in the macaque. While this dissertation does provide fundamental information regarding the development of hepatic innervation in the macaque, a more extensive study involving fetal, neonatal, juvenile and adult tissue is needed.

Nervous Regulation of Hepatic Glucose Metabolism

Hepatic function is regulated by complex interactions between circulating factors, and the hepatic branches of the splanchnic and vagus nerves. Pioneering work by Shimazu et al., introduced the role that the hypothalamus and the peripheral nervous

system play in the regulation of hepatic metabolism (Shimazu, 1962; Shimazu et al., 1965). Further work by this group delineated a reciprocal regulation of hepatic glycogen metabolism by the sympathetic and parasympathetic nervous systems in both rabbits and rats. Electrical stimulation of the sympathetic nervous system via the ventromedial hypothalamic nucleus (VMH) caused a rapid increase in blood glucose, activation of hepatic glycogen phosphorylase-*a*, a decrease in liver glycogen, increased hepatic gluconeogenesis and reduced glycolysis. Conversely, stimulation of the parasympathetic nervous system via the lateral hypothalamic nucleus (LHA) resulted in lower blood glucose, activation of hepatic glycogen synthetase, an increase in liver glycogen, and decreased gluconeogenesis (Shimazu et al., 1966; Shimazu et al., 1978; Shimazu et al., 1975).

These studies were extended when selective activation of the VMH with physiological levels of norepinephrine produced a robust activation of hepatic glycogen phosphorylase-*a* within one minute after treatment. This effect was completely abolished by hypothalamic pretreatment with propranolol (a β-adrenergic antagonist) or systemic application of hexamethonium bromide (ganglionic blocker). Microinjection of acetylcholine, serotonin and γ-aminobutyric acid (GABA) into the VMH did not effect glycogen phosphorylase activity in the liver (Matsushita et al., 1980). Further, microinjection of the LHA with acetylcholine or carbachol (cholinergic agonist) significantly increased the activity of hepatic glycogen synthetase *I* within an hour after treament with no effects on hepatic phosphorylase-*a* activity. The increase in hepatic glycogen synthetase *I* was blocked following intrahypothalamic microinjection of

atropine or scopolamine, or peripheral cholinergic blockade with hexamethonium or *N*-methylatropine (Matsushita et al., 1979).

Recent investigations into the circadian regulation of hepatic glucose metabolism demonstrated in both humans and rats that a rise in plasma glucose concentrations occurs prior to wakening and that this rise is caused by a suprachiasmatic nucleus (SCN) stimulated increase in hepatic glucose production (Boden et al., 1996; Bolli et al., 1984; La Fleur, 2003; La Fleur et al., 1999). Further, regulation of sympathetic preautonomic neurons in the paraventricular nucleus (PVN) by the SCN has been shown to control hepatic glucose production in the rat (Cailotto et al., 2005; Cailotto et al., 2008; Kalsbeek et al., 2004; Kalsbeek et al., 2006). SCN regulation of parasympathetic preautonomic neurons in the PVN is also involved in the regulation of insulin release from the endocrine pancreas during night-time feeding (Kalsbeek et al., 2008). Additional input from the hypothalamic NPY, orexin, VIP, and pituitary adenylate cyclase activating peptide (PACAP) systems also play important roles in regulation of hepatic glucose production and hepatic insulin sensitivity (Marks et al., 1997; van den Hoek et al., 2008; van den Hoek et al., 2004; Yi et al., 2009; Yi, Sun et al., 2010).

Tract tracing from the liver has revealed some of the complex neurocircuitry involved in SCN regulation of hepatic glucose production in the rat. Following either a hepatic sympathectomy or hepatic parasympathectomy, pseudo-rabies virus (PRV) retrograde tract tracing revealed that the SCN is connected to the liver through both the sympathetic and parasympathetic branches of the autonomic nervous system. First-order, second-order and third-order neurons were determined by extending survival times of the rodents. The sympathetic circuitry included the intermediolateral column (IML) of the

thoracic spinal cord (first order), ventrolateral medulla, raphe, locus ceruleus, PVN, LHA (second order), NTS, medial preoptic area (MPO), dorsal medial hypothalamus (DMH), arcuate nucleus, VMH and the SCN (third order). The parasympathetic circuitry included the dorsal motor nucleus of the vagus (DMV, first order), PVN, DMH, MPO (second order), VMH and the SCN (third order) (Kalsbeek et al., 2004).

It has been reported that activation of the PVN resulted in a significant increase in plasma glucose concentrations as well as increases in plasma glucagon and corticosterone (Kalsbeek et al., 2004). Previous studies demonstrated that PVN activation led to significant increases in plasma catecholamines (Cole et al., 2002; Martin et al., 1991). In addition, glucagon, catecholamines and corticosterone can all stimulate hepatic glucose production (Cherrington, 1999; Corssmit et al., 2001; Nonogaki, 2000). Importantly, selective denervation of only the sympathetic input to the liver completely prevented the hyperglycemia observed with PVN stimulation (Kalsbeek et al., 2004). These results suggest that central stimulation of the PVN and consequent sympathetic signaling to the liver, not the concomitant increases in plasma glucagon and corticosterone, were responsible for regulating glucose production by the liver in the rat.

Electrical stimulation of the exterior hepatic nerves around the hepatic artery and portal vein in isolated rat liver was reported to activate noradrenergic, cholinergic and peptidergic fibers. Within the liver itself, sympathetic output dominated the response which was marked by an overflow of noradrenaline into the hepatic vein, increased glucose output, a shift to lactate output and decreased oxygen uptake (Shimazu, 1996). Additional work in this model, demonstrated that vascular resistance was increased and a redistribution of blood flow was regulated by sinusoid contraction (Gardemann et al.,

1992; Ji et al., 1984). Following hepatic sympathetic nerve stimulation, in addition to noradrenaline release, NPY and galanin are also released with evidence that suggests hepatic production of both neuropeptides takes place in the rat liver (Kowalyk et al., 1992; Shimazu, 1996; Taborsky et al., 1994). NPY has been shown in other tissues to potentiate the vasoconstriction action of noradrenaline (Pernow et al., 1989). Galanin is localized to hepatic sympathetic nerve fibers and functions as a robust inhibitor of insulin release in the canine (McDonald et al., 1985; Mundinger et al., 1997).

Electrical stimulation of the exterior hepatic nerves in isolated rat liver in the presence of α and β blockers activated parasympathetic fibers (Shimazu, 1996). Parasympathetic stimulation of isolated rat liver under hyperglycemic conditions slightly enhanced insulin-dependent glucose uptake while antagonizing glucose output in an atropine dependent manner (Gardemann et al., 1986; Gardemann et al., 1992). In addition, increased plasma protein synthesis and secretion was observed following direct vagal stimulation in the *in-situ* perfused rat liver (Watanabe et al., 1990).

While these data point strongly towards direct autonomic regulation of hepatic glucose metabolism, other studies provide a window into the complex neuroendocrine and autonomic processes that work as a single integrated system in the control of hepatic glucose homeostasis. For example, direct electrical stimulation of the splanchnic nerve increases adrenal catecholamine release (Damase-Michel et al., 1993; Takeuchi et al., 1993), and impacts glucagon and insulin release from the pancreas (Bloom et al., 1975, 1978; Kaneto et al., 1975). Injection of bombesin into the VMH or LHA causes hyperglycemia with increased glucagon secretion (Iguchi et al., 1984; Iguchi et al., 1983). In contrast, injection of insulin into the VMH or LHA causes hypoglycemia (Iguchi et al.,

1981). Intravenous or intraventricular injection of pharmacological doses of leptin increases hepatic glucose production associated with reductions in hepatic glycogen content in mice (Kamohara et al., 1997). Further, reflex induced hyperglycemia, following surgical trauma or hemorrhage in rodents or felines, is not prevented by hepatic denervation or adrenalectomy alone, rather the combination of adrenalectomy and denervation is necessary to abolish the acute hyperglycemic response (Lautt et al., 1977; Lautt et al., 1982). Species-specific differences in hepatic innervation also exist and may make the translation of findings from one species to another very complex (Forssmann et al., 1977; Metz et al., 1980; Nonogaki, 2000). Thus, indirect mechanisms, neural regulation by glucoregulatory neuropeptides, redundant independent systems and species-specific considerations must be carefully addressed in functional studies of the liver.

Orthotopic liver transplantation provides a model of denervated liver that permits the investigation of nervous control of glucose homeostasis in humans.
(Luzi et al., 1997; Perseghin et al., 1997). Studies designed to assess the level of hepatic reinnervation that occurs in humans following a liver transplant found that parenchymal fibers permanently disappeared one week after transplantation. In addition, portal innervation was completely gone between six and sixty weeks after transplantation with the rare reappearence of isolated portal innervation occurring after sixty weeks (Boon et al., 1992). The concentration of norepinephrine present in hepatic tissue decreased 100 fold one month after transplantation when compared to healthy control livers. A significant reduction in hepatic epinephrine was also observed in the transplant patients and persisted, along with norepinephrine out to 30 months, when follow-up was

terminated (Kjaer et al., 1994). Taken together these findings indicate that a near-permanent loss of hepatic innervation occurs rapidly after liver transplantation in humans.

In the absence of direct neural signals from the autonomic nervous system but with neuro-humoral factors present, reduced fasting glucose concentrations and reduced endogenous glucose production were observed in transplanted liver patients despite increased circulating glucagon. In addition, a rebound in endogenous glucose production following hypoglycemic challenge was significantly reduced in the transplant group. This study examined glucose function at a range of between 2-28 months after transplantation (Perseghin et al., 1997). An earlier study by this group found that 26 months after transplantation glucose homestasis was normalized but defects in leucine and free-fatty acid metabolism were present (Luzi et al., 1997).

The relatively subtle changes that occur in glucose metabolism following liver transplantation and the evidence of near complete loss of innervation within the organ itself, presents a clear paradox when faced with the abundant evidence demonstrating the importance of autonomic innervation in the regulation of liver function. It has been proposed that the hormonal/humoral milieu reaches a new functionally relevant balance that compensates for the loss of neuroregulation of metabolic functions in the liver (Perseghin et al., 1997). It must also be noted that patients undergoing orthotopic liver transplantation most likely have end-stage liver disease. It is very hard to separate the metabolic adaptations the body has made leading up to the point where a liver transplant is necessary, from the metabolic outcomes observed when tested after transplantation (Shangraw, 2006). Importantly, the effects that these "subtle" changes following liver transplantation produce over a long period of time have yet to be tested (Perseghin et al.,

1997). Intrinsic autoregulatory mechanisms inside the liver itself may also be able to control hepatic metabolism in the basal state (Yi, la Fleur et al., 2010). It has also been suggested that it is easier for humoral and autoregulatory mechanisms to maintain balanced hepatic glucose output without any autonomic innervation, than it is with the loss of either sympathetic or parasympathetic input (Cailotto et al., 2008).

The Cholinergic Anti-inflammatory Pathway

Early work in immune organs demonstrated that nerve fibers are localized in close apposition to the cellular mediators of innate and adaptive immunity (Felten et al., 1985). In the thymus and the spleen, robust sympathetic innervation in the absence of parasympathetic or sensory innervation, suggests that regulation of the immune function in these organs is provided by the efferent sympathetic nervous system (Bellinger et al., 1993; Cano et al., 2001; Nance et al., 1989; Nance et al., 1987; Schafer et al., 1998; Trotter et al., 2007; Wan et al., 1994). However, recent work has suggested that the role that the parasympathetic nervous system plays in the immune system is not so clearly defined (Huston et al., 2006; Nance et al., 2007).

During endotoxemia, the liver and the spleen are the primary sources of the inflammatory cytokine TNF-α found in the serum (Gregory et al., 1998; Huston et al., 2006; Kumins et al., 1996). Electrical stimulation of the peripheral vagus, in rats exposed to a lethal dose of lipopolysaccaride (LPS), significantly inhibited hepatic production of TNF-α and significantly attenuated the development of hypotension. These responses were observed in the absence of any measurable increases in corticosterone or IL-10 (Borovikova et al., 2000). These findings were extended and refined by demonstration that the cholinergic nicotinic α7 receptor (CHRNA7) and Kupffer cells are critically

involved in vagally mediated protection from Fas-induced hepatic apoptosis in mice (Hiramoto et al., 2008). Further, electrical stimulation of the vagus in CHRNA7 knockout mice failed to attenuate the cytokine release observed in wild-type littermates (Wang et al., 2003). Additional studies solidified the role that vagus nerve signaling plays in inhibiting cytokine effects and improving outcomes in models of sepsis, hemorrhagic shock, ischemia, ileus, arthritis and pancreatitis (Altavilla et al., 2006; Bernik et al., 2002; de Jonge et al., 2005; Guarini et al., 2003; Mioni et al., 2005; Saeed et al., 2005; van Westerloo et al., 2006).

The cholinergic anti-inflammatory pathway has been defined as the efferent arm of the inflammatory reflex. The inflammatory reflex is initiated by vagal afferent signals that are relayed to the nucleus of the solitary tract (NTS). The afferent signals have classically been assumed to be secondary to peripheral cytokine sensing by the vagus (Watkins et al., 1995). However, recent work demonstrated that dietary fat, acting via cholecystokinin (CCK) receptors in the gut, also activates the afferent vagal arm of the cholinergic anti-inflammatory pathway (Luyer et al., 2005). Afferent signals are relayed to the rostral ventrolateral medullary (RVLM), nucleus ambiguous (NA) and the dorsal vagal motor nucleus (DVM). Information is also sent to the hypothalamic-pituitary axis (HPA), activating humoral anti-inflammatory responses by the adrenal release of glucocorticoids via ACTH (Tracey, 2002, 2009).

Efferent signals are sent to the celiac ganglion via pre-ganglionic efferents originating in the sympathetic trunk of the spinal cord or through the vagus nerve (Tracey, 2009). Vagal efferents also travel through the subdiaphragmatic trunk, the celiac ganglion and the splenic nerve (Huston et al., 2006; Rosas-Ballina et al., 2008). At

the level of the target tissue macrophage, acetylcholine release by the vagus binds CHRNA7. Ligand binding enables sodium and calcium influx, however signal transduction in immune cells does not require ion channel activity or membrane depolarization (Tracey, 2009). Signal transduction in macrophages involves pleiotropic cascades that regulate the activation of NF-κB through the inhibition of phosphorylation of IκB (Yoshikawa et al., 2006). In addition, the JAK2/STAT3 pathway is activated, inhibiting NF-κB binding to DNA and increasing the expression of suppressor of cytokine signaling (SOCS3) (de Jonge et al., 2005). The net result is selective inhibition of inflammatory cytokines in macrophages while preserving production of anti-inflammatory cytokines (Borovikova et al., 2000; Parrish et al., 2008; Pavlov et al., 2007).

Initial work with this anti-inflammatory pathway demonstrated that incubation of human macrophages with acetylcholine, muscarine or α-bungarotoxin in the presence of LPS stimulation significantly reduced the release of pro-inflammatory cytokines *in-vitro* (Borovikova et al., 2000). Interestingly, it has been reported that following LPS exposure, norepinephrine, acting via β-adrenergic receptors, can also inhibit the production of TNF-α in macrophages (Ignatowski et al., 1996; Meltzer et al., 2004). Thus, in addition to the vagally-mediated responses requiring CHRNA7 in target tissue, a synergistic relationship with sympathetic signaling may also exist in innate macrophage immune responses (Huston et al., 2006; Rosas-Ballina et al., 2008; Tracey, 2002, 2009).

Conclusion

While numerous rodent models have examined the effects that maternal nutrition has on the development of the offspring, important species-specific

differences exist that preclude translation of rodent findings directly into human obesity research. The nonhuman primate model of maternal high-fat feeding utilized in this dissertation was chosen for the similarities that exist in development between nonhuman primates and humans. Our model is a tremendous resource that will allow us, as well as other investigators, to implement rigorous explorations into the effects that obesity and/or a HFD have on development that are not possible in human subjects. For example, postnatal studies currently underway will address how animals exposed to maternal HFD *in-utero* respond to re-challenge with a HFD as adults. This dissertation also includes studies addressing post-natal effects, with an emphasis on preventing the effects that a maternal HFD has on breastfeeding infants.

Despite the clear evidence of sympathetic and parasympathetic innervation of the liver, the role that the autonomic nervous system plays in the control of hepatic energy metabolism remains unclear. There are still many details regarding the neuroanatomy of the liver that remain to be addressed (Yi, la Fleur et al., 2010). To date, there is a paucity of data on the autonomic innervation of the nonhuman primate liver. While similarities exist in the hepatic innervation between man and the adult nonhuman primate (Forssmann et al., 1977; Nobin et al., 1978), the lack of more detailed knowledge in hepatic neuroanatomy or hepatic autonomic development in the nonhuman primate is a major obstacle for functional studies of liver metabolism in these animals. Our work begins to address gaps in current knowledge of the hepatic nervous system by describing the neuroanatomy of sympathetic innervation in the fetal and juvenile macaque. In addition, we examine the effects that high-fat diet exposure has on hepatic sympathetic innervation in the juvenile macaque.

We believe the studies described in this dissertation have important clinical implications for understanding how maternal nutrition impacts liver development. In addition, these studies may help extend our current knowledge of the systems that regulate body weight, and illuminate new avenues for treatment of the growing epidemic of obesity.

AIMS OF DISSERTATION AND APPROACH

Over the last twenty years, the alarmingly rapid demographic shift towards a more obese phenotype in children, coupled with an increasing prevalence of maternal obesity, suggest that in addition to an underlying genetic predisposition to obesity, both epigenetic and environmental mechanisms are providing robust contributions to this epidemic. Fetal development is a critical period when exposure to insult may have lifelong effects on biological programming. While much work has been done to highlight the detrimental effects that maternal nutrient deprivation has on fetal development, relatively few studies have been undertaken to examine the impact that nutrient excess and obesity has on the developing fetus. Given that nearly one-third of American women of childbearing age are obese with body mass indexes (BMI) of 30 or higher, there is a *critical need* to address the immediate and long-term effects that maternal obesity and nutrient excess has on metabolic homeostasis in the offspring.

The ***goal of this dissertation*** is to identify in the non-human primate how chronic maternal nutrient excess and obesity during pregnancy, as well as diet reversal during pregnancy alone, effects hepatic development (Chapters 3 and 4) and the innervation of fetal and post-natal non-human primate livers (Chapter 4). The ***central hypothesis of this dissertation*** is *that intra partum exposure to maternal nutrient excess and obesity will have a permanent impact on the innervation of the non-human primate fetal and post-natal liver.*

Specific Aim 1. ***Characterize the fatty acid profile of the diet and identify changes in plasma fatty acids between CTR, HFD, and REV dams and fetuses.*** (Chapter 3)

To adequately address how chronic maternal nutrient excess and obesity during pregnancy effects hepatic development, it is first necessary to understand the diet itself. Given that our group is focused on understanding the effects that chronic consumption of a maternal high-fat diet has on development in the offspring, we will begin our analysis with identifying the fatty acids present in the diet, maternal plasma and breastmilk, and fetal plasma.

Hypothesis: We hypothesize that circulating fatty acid profiles will be significantly different between CTR and HFD diet groups and that these profiles will resemble the fatty acid profile found in the diet. In addition, we hypothesize that maternal diet reversal (REV) will normalize circulating fatty acids to CTR levels in both dams and fetuses.

***Approach*:** Use gas chromatography-mass spectroscopy (GC-MS) to identify and quantify the fatty acids present in each maternal diet, fasting maternal plasma and breastmilk and fetal plasma.

Specific Aim 2. ***Identify changes in hepatic innervation between control diet, HFD, and diet reversal fetal and post-natal liver.*** (Chapter 4)

Our preliminary data supports that inflammation is present in fetal livers exposed to a chronic high fat diet. Furthermore, recent work with this model has demonstrated that

evidence of NAFLD and lipotoxicity are present in the high fat diet fetal livers (McCurdy et al., 2009). Within the confines of this hepatic pathology, we reason that normal development of hepatic innervation will be perturbed.

***Hypothesis*:** We hypothesize that chronic maternal consumption of a high fat diet will generate changes in the innervation of fetal and post-natal high fat diet liver. Furthermore, we hypothesize that changes in innervation will be returned to control levels with diet reversal.

***Approach*:** Use pathway specific RT^2 PCR arrays, confirmatory real-time PCR, and immunohistochemistry (IHC) to examine the hepatic neuroanatomy of autonomic fibers, neurotransmitters, neuropeptides and neural receptors known to modulate hepatic function.

Chapter 2

Japanese Macaque (*Macaca fuscata*) Model of Maternal High-Fat Diet Induced Obesity and Insulin Resistance

INTRODUCTION

Extensive evidence in both humans and rodents have made it clear that the gestational and postnatal environments are critical for the proper development of the systems responsible for the regulation of metabolic homeostasis in the offspring (Anguita et al., 1993; Levin et al., 1998; Ozanne et al., 1999; Ravelli et al., 1998; Ravelli et al., 1999; Roseboom et al., 2000; Srinivasan, Aalinkeel et al., 2006; Srinivasan, Katewa et al., 2006; Strauss, 1997; Thone-Reineke et al., 2006). However, important species-specific differences exist between humans and rodents that make translation of rodent findings into human studies difficult. Our group has developed a unique nonhuman primate model to examine the effects that a maternal high-fat diet and maternal obesity has on offspring development. This model was created to capitalize on the many similarities that are present between nonhuman primates and humans in both the development and function of systems integral to metabolic homeostasis. This chapter includes a detailed description of our model, its strengths and weaknesses and a summary of relevant findings so that this dissertation can be properly positioned within the overall context of the model.

Hepatic function is regulated by complex interactions between circulating factors and the autonomic nervous system. The hypothalamus and the brainstem have been shown to be important CNS regions involved in hepatic function (Marks et al., 1997; Shimazu, 1962; Shimazu et al., 1965; van den Hoek et al., 2008; van den Hoek et al., 2004; Yi, la Fleur et al., 2010). While the primary focus of this dissertation is the effects that a HFD has on hepatic innervation, the important role that the CNS plays in the regulation of hepatic function via the autonomic nervous system requires a model that

closely resembles human systems in both the brain and liver. A brief discussion of important species-specific differences that exist between humans, nonhuman primates and rodents in the neuropeptide Y (NPY) systems of CNS and the liver provides ample justification for the use of the nonhuman primate in our studies.

There is a paucity of data regarding the development of CNS circuits that regulate energy homeostasis in nonhuman primates and humans (B. E. Grayson, Kievit et al., 2010). However, what is known about NPY development in the hypothalamus highlights critical differences between rodents and nonhuman primates. For example, NPY is expressed in the arcuate nucleus (ARH) of the rodent brain before birth and projections from these neurons develop during the postnatal period (Grove et al., 2001; Singer et al., 2000). In the nonhuman primate and human brain, ARH neurons express NPY during the 2nd trimester with ARH-NPY projections beginning to develop late in the 2nd trimester and continuing throughout the 3rd trimester and into the early postnatal period (B. E. Grayson et al., 2006). In addition, transient expression of NPY in multiple hypothalamic nuclei, including the paraventricular nucleus (PVN) and the lateral hypothalamic nucleus (LHA), hypothalamic regions important for hepatic function, has been observed in the rodent during the early postnatal period. In the nonhuman primate, NPY expression in many of these same nuclei is present beginning in the 2nd trimester and persists through adult life (B. E. Grayson, Kievit et al., 2010).

In the rodent liver, NPY innervation of portal triads is present at low density before birth, reaches a peak density in the triads one week after birth before decreasing to adult levels in the portal triads two weeks after birth (Ding et al., 1997). No evidence of innervation of the hepatic parenchyma, by any nervous system marker, has been

demonstrated to date in the rodent liver (Reilly et al., 1978; Skaaring et al., 1976; Ueno et al., 2004). By contrast, our data demonstrates that NPY innervation of the portal triads is present in the 3rd trimester fetal macaque liver and by one year of age robust NPY innervation of the portal triad and hepatic parenchyma is observed. The innervation of the macaque hepatic parenchyma by NPY after the 3rd trimester is in good agreement with previous reports of NPY parenchymal innervation observed in human and macaque adult liver (Ding et al., 1994; D. G. Tiniakos et al., 2008).

Within the neuropeptide Y system alone, significant developmental and neuroanatomical differences exist in both the brain and liver between nonhuman primates and rodents. While it is still unclear whether the rodent can be utilized to model complex human neurocircuitry and function, the similarities that exist between humans and nonhuman primates make it likely that higher-order mammalian models are the surest path to understanding human metabolic development and function. Thus, this chapter describes our nonhuman primate model of maternal high-fat diet induced obesity in detail and summarizes important relevant findings to date. Importantly, while a number of manuscripts have recently been published with our model, a detailed description of each is outside of the scope of this chapter. Thus, only data overtly relevant to this dissertation are presented here.

MATERIALS AND METHODS

All animal procedures have undergone an extensive review process and were in accordance with the guidelines of Institutional Animal Care and Use Committee of the Oregon National Primate Research Center (ONPRC) and Oregon Health & Science University. Protocols involved in this study were developed to ameliorate suffering and have been approved under IACUC ID number: IS00000224 (0622 for internal purposes). The Animal Care and Use Program at the ONPRC abides by the Animal Welfare Act and Regulations (CFR 9, Ch 1, Subchapter A) enforced by the USDA, the Public Health Service Policy on Humane Care and Use of Laboratory Animals, in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health, and the recommendations of the Weatherall report; *The Use of Non-human Primates in Research*.

Japanese macaques matched for age (5-7 years at start) and weight (7-9 Kg) were randomly assigned to two dietary groups in the fall of 2002: 1: Control diet (CTR; 13% of calories from fat; Monkey Diet no. 5052, Lab Diet, Richmond, IN, USA) or 2: High-fat diet (HFD; 35.2% of calories from fat; Custom Diet 5A1F, Test Diet, Richmond, IN, USA). The HFD also included calorically dense treats made with peanut butter. Both diets are sufficient in vitamin, mineral, and protein content for normal growth. Prior to this study, all animals were maintained on standard monkey chow in large outdoor enclosures and were naive to any experimental protocols.

Manufacturers specifications provided for both diets show that the total metabolizable energy content of the CTR chow was 2.87 kcal/g and was apportioned at 26.8% energy from protein, 58.5% energy from carbohydrate, and 14.7% energy from

fat. The main source of fat in the CTR diet was soybean oil. The total energy content of the maternal HFD chow was 4.2 kcal/g and was apportioned at 16.7% energy from protein, 51.5% energy from carbohydrate, and 31.8% energy from fat. The main sources of fat in the HFD were lard, animal fat, butter and safflower oil.

The animals were group housed and had *ad libitum* access to food and water. The group housing is important as it provides for normal social behavior and exercise, which contribute to the psychological well being of the animals and more closely resembles the human condition. However, because the animals are group housed it is not possible to determine individual food/calorie intake.

Each maternal group was housed with two males so that pregnancies would occur during the yearly breeding season (November – February). The females were checked each successive year for pregnancies starting in November by ultrasound, which allows an estimate of gestational age ± 5 days. Twice a year the animals underwent IV glucose tolerance tests (IVGTT) (**Methods 3-S1**), once during the late summer (nonpregnant state) and once during the early 3rd trimester of pregnancy. All of the above procedures were done under ketamine sedation (5–10 mg/kg).

For our fetal studies, ONPRC veterinarians terminated singleton pregnancies from dams by cesarean section at gestational day 130 (G130), as determined by ultrasound. Normal full-term pregnancies for Japanese macaques is 175 days, thus G130 is in the early 3rd trimester. G130 was chosen after preliminary studies determined that this gestational age represented a critical period for the development of several metabolic systems: 1) hypothalamic circuits have started to develop, 2) there is widespread

pancreatic β-cell development, 3) there is a full functioning placenta that is not at near term, and 4) there is very little white adipose tissue (WAT).

Before the cesarean section, pregnant dams were fasted overnight for approximately 16 hours prior to surgical procedure. Females were initially sedated with ketamine hydrochloride (100 mg/ml) at a dose of 10-15 mg/kg. Once animals were sedated they were delivered to the surgical area and placed on isoflurane gas; induced at 3%, then maintained at 1.0-1.5%. Cesarean sections were performed by trained ONPRC veterinarians and their staff, and occurred on scheduled days between 10:00 and 10:30 am.

Pre and post-operative care was maintained by the ONPRC veterinary staff. Immediately prior to the cesarean section animals received an intravenous dose of hydromorphone (0.5mg if under 10kg, 1.0mg if over 10kg). An additional intravenous dose of hydromorphone was administered post-operatively, usually within an hour after the start of the procedure. For the remainder of the day following the cesarean section, intravenous hydromorphone was given at 4:00 pm and again at 8:00 pm in combination with buprenorphine (0.3 mg IM). The following day, hydromorphone was administered at 8:00 am, 12:00 pm, 4:00 pm and then again with buprenorphine at 8:00 pm. Animals remained in the surgical ICU area for approximately 7 days under close veterinary observation and were then released back into their group.

After cesarean section, fetuses were deeply anesthetized with sodium pentobarbital (> 30 mg/kg i.v.) and exsanguinated. All peripheral tissues and brain were removed, weighed and stored for subsequent protein and RNA extractions or for histological analyses. All surgical procedures used in this study, were performed each

scheduled day in an identical manner, following an *a priori* defined protocol in both technique and timing. Thus for plasma analyses, blood draws were taken at approximately the same time of day for dams and fetuses. Maternal blood was taken during c-section from the femoral artery, and fetal blood samples were taken from the abdominal aorta during necropsy.

Maternal breast milk for fatty acid analysis was obtained at postnatal day 30 from CTR and HFD dams giving birth to full term infants. The breast milk was collected during routine postnatal day 30 dual-emission X-ray absorptiometry (DEXA) procedures for the offspring. The DEXA procedure followed an *a priori* defined protocol and breast milk was obtained at approximately the same time of day for each animal. At 9:00 am, the mother was sedated with ketamine (15 mg/kg) or telazol, if ketamine resistant. The baby was separated from the mother for DEXA scanning. Two hours later, 0.5mL oxytocin was injected intravenously into the mother to stimulate milk let down. The breast and nipple were massaged and milk was collected into a 15mL conical vial. The milk sample was immediately stored on ice until centrifugation to separate aqueous milk and cream layers.

Previous work with this model has demonstrated significant increases in maternal leptin levels, decreases in maternal insulin sensitivity, and increases in maternal weight gain starting with the second year of maternal HFD exposure, and these changes persist and become greater through year four (McCurdy et al., 2009). For our fetal studies, we are reporting differences in HFD fetuses whose mothers were exposed to the maternal diet for at least four consecutive years. In the fifth year of our studies, a diet-reversal protocol (REV) was initiated to assess dietary impact independent of maternal obesity.

This protocol entailed switching a subgroup of adult females that had been exposed to a high-fat diet for four consecutive years, to a control diet 1-3 months before becoming pregnant and throughout the pregnancy.

This dissertation also contains two juvenile cohorts to examine the postnatal effects of HFD exposure. Full-term offspring were exposed to their mother's diets until weaning. At postnatal day 180, the CTR and HFD offspring were weaned to create diet cohorts with the first dietary designation indicating the maternal diet *in-utero* and before weaning, and the second designation indicating the offspring's diet after weaning.

Juvenile necropsy was carried out by an *a-priori* protocol by the veterinary staff at the ONPRC. Briefly, the juvenile offspring were deeply anesthetized with sodium pentobarbital (> 30 mg/kg i.v.) and exsanguinated. All peripheral tissues and brain were removed, weighed and stored for subsequent protein and RNA extractions or for histological analyses. Head circumference and width as well as crown rump length were also reported as a standard procedure in the necropsy protocol.

REPORTED FINDINGS WITH OUR MODEL

Our model of maternal high-fat induced obesity is a sophisticated and powerful tool in the battle to understand the causes of human obesity and metabolic disorders. The results detailed in this chapter and this dissertation are the product of nearly nine years of investigation within the ongoing evolution our model. Findings reported with this model have already provided fundamental information related to metabolism and also offered tantalizing glimpses into unexplored territory in our understanding of the underlying causes of obesity.

Maternal phenotype

One of the first manuscripts published with our model described maternal phenotypes, as well as findings in early third trimester fetuses from dams that had been exposed to the HFD for 2-4 years (McCurdy et al., 2009). An important yet unexpected finding in the adult HFD dams was the development of HFD resistant (HFD-R) and HFD sensitive (HFD-S) phenotypes after 2 years exposure to the HFD. The discovery of the HFD-S phenotype began with the observation that in contrast to the HFD-R group, a significant increase in the insulin area under the curve (IAUC) following i.v glucose tolerance tests (i.v.GTT) was observed in the HFD-S group at the end of one year of exposure to the HFD. The HFD-R dams had normal insulin secretion during i.v. GTT's at this time point. Within the HFD-S dams, significant weight gain began after 2 years on the HFD. Following 4 years on the HFD diet, the HFD-S dams displayed a near doubling of body-fat, a greater than 3-fold increase in IAUC, a 5-fold increase in circulating leptin levels and a 35% increase in body weight. The HFD-R group had no significant weight gain and had normal insulin secretion during i.v. GTT's throughout 4 years of HFD exposure. The segregation into HFD-R and HFD-S maternal phenotypes provided the unexpected advantage of examining the effects of the HFD independent of maternal obesity and insulin resistance (McCurdy et al., 2009).

In the pregnant state, each dietary group consisted of dams undergoing either their first, second or third pregnancy. Animals from each group displayed similar weight gain during pregnancy had similar fasting glucose levels, and serum free fatty acids and triglycerides. However, the HFD-S dams had significantly higher fasting leptin levels, IAUC's, and serum glycerol (McCurdy et al., 2009). Additional analysis of the maternal

serum revealed that elevated levels of cholesterol were present in both HFD-S and HFD-R circulation. Within the HFD-S dams other serum metabolites including α-tocopherol, γ-tocopherol and glycine were significantly elevated when compared to CTR serum (J. Cox et al., 2009).

With diet-reversal (REV), pregnant dams had higher body weights when compared to HFD dams. However, REV dams as a group had significantly lower IAUC's and slightly lower fasting insulin, leptin and glycerol levels than HFD dams. In addition high-fat sensitive dams that were subject to diet reversal (REV-S) had significantly lower IAUC's than HFD-S dams. Thus, diet reversal significantly reduced the insulin response to a glucose challenge in both diet-resistant and diet-sensitive dams.

Fetal phenotype

In the gestational day 130 fetuses, very little WAT was observed concurrently with nearly undetectable levels of circulating leptin. HFD fetuses had lower bodyweights, and significantly higher circulating glycerol and total triglyceride levels than CTR fetuses. Fetal glycerol levels were also significantly correlated to maternal levels (McCurdy et al., 2009). Surprisingly, fetal levels of serum cholesterol and α-tocopherol were significantly lower in the HFD circulation when compared to CTR fetuses (J. Cox et al., 2009). Additionally, within the HFD fetal plasma, significant upregulation of proinflammatory cytokines and chemokines were observed when compared to CTR (McCurdy et al., 2009).

Maternal diet-reversal normalized fetal body weight and circulating glycerol and triglyceride levels to CTR levels. However, no significant differences were observed in any of the other phenotype parameters examined between CTR and REV fetuses

(McCurdy et al., 2009). While the majority of fetal outcomes are independent of maternal diet sensitivity, length of maternal exposure to the HFD and fetal gender, other recent work with this model reported that fetal serum metabolite profiles could only be distinguished between REV and HFD diet groups by maternal obesity and not by maternal diet (J. Cox et al., 2009; McCurdy et al., 2009).

Fetal Brain

It was recently reported with our model that exposure to a maternal HFD is associated with significant changes in the melanocortin system and increased inflammation in the fetal hypothalamus (B. E. Grayson, Levasseur et al., 2010). This report demonstrated that hypothalamic expression of proopiomelanocortin (POMC) was significantly upregulated in the arcuate nucleus (ARH) of HFD fetuses. In addition, expression of the melanocortin 4 receptor (MC4R) was significantly upregulated while agouti-related peptide (AgRP) expression was significantly reduced in a qPCR analysis of the hypothalamus. No changes were observed in the hypothalamic expression of neuropeptide-Y (NPY) between CTR and HFD diet groups. It had been previously demonstrated in the macaque that ARH AgRP/NPY projections to the paraventricular nucleus of the hypothalamus (PVH) develop during the third trimester (B. E. Grayson et al., 2006). Thus, this developmental process may be sensitive to HFD exposure during the third trimester. Using immunohistochemistry, Grayson and colleagues reported that AgRP fibers were significantly reduced in the PVH of HFD fetal brains. Further, they noted on a qualitative level that AgRP immunoreactive fibers were reduced throughout the entire HFD fetal hypothalamus (B. E. Grayson, Levasseur et al., 2010).

Inflammation was also present in the HFD fetal hypothalamus and this group reported that expression of eight inflammatory markers, including IL-1β, were upregulated when compared to CTR fetuses. Significant increases in activated microglia were also observed in the HFD fetal ARH (B. E. Grayson, Levasseur et al., 2010).

While increased circulating cortisol levels were reported in both HFD dams and HFD fetuses, cortisol levels were returned to normal in both maternal and fetal circulation following diet-reversal. In addition, POMC and AgRP mRNA expression, as well as microglial activation were returned to CTR levels in the REV hypothalamus. Importantly, AgRP immunoreactive fiber density in the PVH was also returned to CTR levels with maternal diet-reversal (B. E. Grayson, Levasseur et al., 2010).

Fetal Liver

Within the HFD fetal liver, evidence of hepatic steatosis, oxidative damage, elevated phosphorylation of c-Jun N-terminal protein kinase (JNK1) along with increased levels of liver triglycerides, suggested that nonalcoholic fatty liver disease (NAFLD) was present in the HFD fetal liver. In addition, evidence of premature gluconeogenic gene expression in the HFD liver was demonstrated by the increased expression of important enzymes in the gluconeogenic pathway. Partial return to CTR levels of liver triglycerides and the expression of hepatic gluconeogenic genes were also reported in the REV fetal liver (McCurdy et al., 2009).

Alterations in chromatin structure have also been observed in HFD fetal liver with our model. An early report described site-specific lysine modifications, rather than global changes, to HFD fetal hepatic chromatin structure. In addition, alterations in the downstream expression of a number of genes transcriptionally responsive to the site-

specific histone modifications were also observed (Aagaard-Tillery et al., 2008). These findings were extended by the demonstration that circadian gene expression was also altered in the HFD fetal liver (Suter et al., 2011).

Postnatal effects of the HFD

When fetuses were brought to term and examined in the postnatal period, increased liver triglycerides were observed in the HFD offspring at postnatal day 30 and postnatal day 180. In addition, increased body-fat in the HFD offspring was observed beginning at postnatal day 90 and at postnatal day 180 (McCurdy et al., 2009). Persistent changes in hepatic circadian gene expression were also observed in 1 year-old HFD offspring (Suter et al., 2011).

Recent work with our model reported changes in the brainstem serotonergic system and behavioral differences in 130 day-old HFD offspring. In the rostral dorsal raphe (rDR) increased expression of genes important for serotonin synthesis (TPH2 and 5-HT_{1A}R) were observed. These data were independent of maternal phenotype and were not sex dependent. Behavioral studies involving these animals revealed that female offspring exhibited an increased latency to touch a potentially threatening novel object. This effect was not observed with non-threatening novel objects or with experiments involving food. In addition, over 70% of all the HFD offspring, males and females alike, displayed aggressive or anxious behavior compared with 11% of the CTR offspring. Cerebral spinal fluid obtained at the time of behavioral testing showed decreased levels of serotonin in the HFD offspring (Sullivan et al., 2010).

Summary

Adaptations of developing organs to the maternal nutritional environment were first described by Barker and colleagues and are referred to as fetal metabolic programming (Hales et al., 1991). This idea proposes that long-lasting changes in important metabolic organs may occur during critical developmental time points as a result of maternal nutritional insults. In the HFD fetal liver, observations of NAFLD, premature expression of hepatic gluconeogenic genes, specific changes in hepatic chromatin structure, and altered expression of hepatic circadian genes that persist into postnatal life suggest that persistent changes are occurring in this organ (Aagaard-Tillery et al., 2008; McCurdy et al., 2009; Suter et al., 2011). Chapter 3 extends these results by demonstrating that the HFD is associated with increased apoptosis in the fetal liver. Chapter 4 supports the idea of that long-lasting changes are occurring in the liver by demonstrating that alterations in hepatic sympathetic innervation are taking place in the HFD/HFD juvenile liver. Outside of the liver, our model has provided evidence that long-lasting changes are also taking place in the central nervous systems of HFD offspring (B. E. Grayson, Levasseur et al., 2010; Sullivan et al., 2010).

Ultimately, the primary goal of our work is to understand the causes of human obesity. Our nonhuman primate model has important strengths that make it uniquely suited to pursue this goal. The similarities between central and hepatic NPY systems in humans and nonhuman primates have been discussed. Much work has also been done highlighting similarities in brain development between humans and macaques (B. E. Grayson et al., 2006; B. E. Grayson, Kievit et al., 2010; Grove et al., 2005). Singleton pregnancies and similarities to human placental structure and function are additional

qualities of this macaque model that have important implications for maternal nutrient transfer to the developing fetus (Carter, 2007; Frias et al., 2011).

Some weaknesses are present as well in our model. The colony of Japanese macaques used in our studies originated from nine founding animals (K.L. Grove, personal communication). Thus, our model utilizes an in-bred population with limited genetic diversity. The clear segregation of HFD-S and HFD-R dams might be a subtler maternal phenotype in a more genetically diverse study population. In addition, the animals in our studies are group housed. While this condition nurtures their social needs, it eliminates the possibility of monitoring individual food intake (McCurdy et al., 2009).

When viewed as an entire body of work, the overwhelming majority of our findings thus far demonstrate that the diet itself is paramount in our model. While maternal phenotype and sex play an important role in some outcomes, more work is needed to untangle the contributions that they provide in development. Given the importance of the maternal diet, Chapter 3 is an in-depth analysis of the fatty acid profiles of the diet, maternal plasma, maternal breastmilk and fetal plasma. Fundamental work of this nature is absolutely necessary before the development of effective clinical interventions, aimed at reducing the human obesity epidemic can begin.

Chapter 3

Maternal High Fat Diet is Associated With Decreased Plasma N–3 Fatty Acids and Fetal Hepatic Apoptosis in Nonhuman Primates

Wilmon F. Grant, Melanie B. Gillingham, Ayesha K. Batra, Natasha M. Fewkes, Sarah M. Comstock, Diana Takahashi, Theodore P. Braun, Kevin L. Grove, Jacob E. Friedman, Daniel L. Marks. (2001). Maternal High Fat Diet is Associated With Decreased Plasma n–3 Fatty Acids and Fetal Hepatic Apoptosis in Nonhuman Primates. *PLoS One* Feb 25;6(2):e17261.

ABSTRACT

To begin to understand the contributions of maternal obesity and over-nutrition to human development and the early origins of obesity, we utilized a non-human primate model to investigate the effects of maternal high-fat feeding and obesity on breast milk, maternal and fetal plasma fatty acid composition and fetal hepatic development. While the high-fat diet (HFD) contained equivalent levels of n-3 fatty acids (FA's) and higher levels of N-6 FA's than the control diet (CTR), we found significant decreases in docosahexaenoic acid (DHA) and total N-3 FA's in HFD maternal and fetal plasma. Furthermore, the HFD fetal plasma N-6:N-3 ratio was elevated and was significantly correlated to the maternal plasma N-6:N-3 ratio and maternal hyperinsulinemia. Hepatic apoptosis was also increased in the HFD fetal liver. Switching HFD females to a CTR diet during a subsequent pregnancy normalized fetal DHA, N-3 FA's and fetal hepatic apoptosis to CTR levels. Breast milk from HFD dams contained lower levels of eicosopentanoic acid (EPA) and DHA and lower levels of total protein than CTR breast milk. This study links chronic maternal consumption of a HFD with fetal hepatic apoptosis and suggests that a potentially pathological maternal fatty acid milieu is replicated in the developing fetal circulation in the nonhuman primate.

INTRODUCTION

Over the last twenty years, obesity has dramatically increased in the United States across every ethnic group studied (Mokdad et al., 2003; Mokdad et al., 1999). Women of childbearing age have not been spared from this upsurge, as nearly 50% of all women of childbearing age are either overweight or obese and one-third have body mass indexes (BMI) of 30 or higher (Hedley et al., 2004; Vahratian, 2008). A particularly concerning part of the emerging epidemic of obesity is the increasing rise in the percentage of children and adolescents that are either overweight or at risk for overweight (Mokdad et al., 2003; Mokdad et al., 1999; Ogden et al., 2006). In addition, diseases once only found in adults are occurring with greater frequency in pediatric populations. Type 2 diabetes mellitus as well as secondary co-morbidities such as hypertension, non-alcoholic fatty liver disease, hyperlipidemia, and metabolic syndrome are now becoming increasingly common in children (Pinhas-Hamiel et al., 2005, 2007).

The increasing prevalence of metabolic diseases and obesity in children is most often attributed to a combination of an energy conserving, or 'thrifty' genotype, with a prevalent imbalance of nutrient intake and expenditure in the developed world. An emerging body of evidence also suggests that our ability to respond to metabolic challenges during postnatal life is modified by environmental influences during fetal development. Fetal development is a critical period when exposure to environmental insults *in-utero* has lifelong effects on the structure and function of organs, tissues and body systems in the offspring.

There is strong evidence in humans that maternal nutrient deprivation during pregnancy can program adipocyte metabolism and fat mass towards a propensity for

obesity, and lead to a wide range of developmental effects in the offspring (Bispham et al., 2005; Budge et al., 2005; Oken et al., 2007; Painter et al., 2007; A. D. Stein et al., 1995; A. D. Stein et al., 2004; Z. Stein et al., 1975; Stocker et al., 2005). In addition, maternal obesity and gestational diabetes mellitus (GDM) during pregnancy has also been implicated in the development of metabolic disorders in offspring, including macrosomia, impaired glucose tolerance, and a higher risk of developing obesity and diabetes as adults (Boney et al., 2005; Dabelea, Hanson et al., 2000; Dabelea et al., 1999; Garcia Carrapato, 2003; Plagemann et al., 1997a, 1997b; Silverman et al., 1993; Van Assche et al., 2001; Whitaker, 2004; Yogev et al., 2008). While epidemiological evidence has shown that the intrauterine environment has profound effects on fetal growth and the programming of childhood weight, the mechanisms underlying metabolic programming are poorly understood, particularly in humans.

Rodent studies have demonstrated that a maternal high fat diet during gestation and lactation, or overfeeding during the postnatal period, alters the development of the pancreas and liver as well as central and peripheral nervous systems involved in energy homeostasis (Anguita et al., 1993; Chang et al., 2008; Davidowa, Heidel et al., 2002; Davidowa, Li et al., 2002; Davidowa et al., 2003; Davidowa et al., 2000a, 2000b, 2001, 2004; Levin et al., 1998; Ozanne et al., 1999; Srinivasan, Aalinkeel et al., 2006; Srinivasan, Katewa et al., 2006; Thone-Reineke et al., 2006; Xiao et al., 2007). While extremely valuable, these rodent studies do not address the fact that there are critical developmental differences between rodents and primates, including both humans and nonhuman primates (NHP). For example, the development of important central circuits regulating appetite and metabolism occurs prenatally in humans and NHP, while rodent

maturation of these systems primarily occurs postnatally (Bouret et al., 2004; B. E. Grayson et al., 2006; Grove, Allen et al., 2003; Grove & Smith, 2003; Koutcherov et al., 2002). Furthermore, the macro- and micro-architecture of the placenta is markedly different between rodents and humans, and this has important implications for fetal nutrient transfer during development (reviewed in (Maltepe et al., 2010)). Therefore, studies designed to provide mechanistic links between the maternal gestational metabolic environment and fetal metabolic programming, in support of previous human epidemiological observations, requires animal models that closely resemble human development.

To this end, we have utilized a unique non-human primate model of maternal high fat/calorie diet-induced obesity (in the absence of gestational diabetes) to address the impact that *chronic* maternal consumption of a high-fat diet (HFD) may have on metabolic programming (McCurdy et al., 2009). We acknowledge that 'high-fat' is a phrase that simplifies the complex nature of our dietary intervention and therefore have supplied a detailed analysis of the dietary constituents. Nonetheless, this model utilizes chronic high fat feeding, and the level and composition of dietary fat are not outside the norms for a modern western diet (Peters, 2003).

Our group has previously shown that a maternal HFD alters fetal development and expression of hypothalamic neuropeptides in the context of hypothalamic inflammation, as well as inducing changes in the central serotonergic system (B.E. Grayson et al., 2007; Sullivan et al., 2010). In the liver, HFD fetuses had premature gluconeogenic gene expression, steatosis, elevated triglyceride content, and oxidative stress. In addition, epigenetic changes and altered circadian gene expression has been

shown in the HFD fetal liver (Aagaard-Tillery et al., 2008; Suter et al., 2011). HFD fetal plasma contained elevated levels of inflammatory cytokines, and elevated triglycerides and glycerol that were highly correlated to maternal levels. Other work with this model has shown that alterations in serum metabolite profiles are present in HFD fetuses (J. Cox et al., 2009). The majority of these changes were observed irrespective of maternal obesity or maternal insulin resistance status and persisted into the early postnatal period. Importantly, switching HFD mothers to a control diet during pregnancy alone (diet-reversal; REV) reversed a number of the observed fetal hepatic pathologies towards control levels (McCurdy et al., 2009; Suter et al., 2011).

In the present study, we further characterize our NHP model of maternal HFD induced obesity (McCurdy et al., 2009), by a detailed analysis of fatty acids in the diet, maternal plasma, breastmilk, and fetal plasma. In addition, we extend previous findings by evaluating the effects that a maternal HFD has on inflammation and apoptosis in the fetal liver. Our results show that the experimental maternal HFD leads to increased apoptosis in the developing fetal liver. In addition, maternal and fetal HFD plasma have reduced levels of circulating N-3 fatty acids when compared to CTR. Importantly, we demonstrate that a maternal dietary intervention during pregnancy (REV) normalized fetal hepatic apoptosis and returned plasma N-3 fatty acids to CTR levels in dams and fetuses. These data support the idea that the placenta does not protect the developing fetus from a pro-inflammatory maternal lipid milieu. Because these effects are associated with maternal diet during gestation and lactation, and some are reversed with dietary manipulation limited to these intervals, these data have critical public health implications.

MATERIALS AND METHODS

Macaque model of maternal overnutrition

All animal procedures have undergone an extensive review process and were in accordance with the guidelines of Institutional Animal Care and Use Committee of the Oregon National Primate Research Center (ONPRC) and Oregon Health & Science University. Protocols involved in this study were developed to ameliorate suffering and have been approved under IACUC ID number: IS00000224 (0622 for internal purposes). The Animal Care and Use Program at the ONPRC abides by the Animal Welfare Act and Regulations (CFR 9, Ch 1, Subchapter A) enforced by the USDA, the Public Health Service Policy on Humane Care and Use of Laboratory Animals, in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health, and the recommendations of the Weatherall report; *The Use of Non-human Primates in Research*.

Japanese macaques matched for age (5-7 years at start) and weight (7-9 Kg) were randomly assigned to two dietary groups in the fall of 2002: 1: Control diet (CTR; 13% of calories from fat; Monkey Diet no. 5052, Lab Diet, Richmond, IN, USA) or 2: High-fat diet (HFD; 35.2% of calories from fat; Custom Diet 5A1F, Test Diet, Richmond, IN, USA). The HFD also included calorically dense treats made with peanut butter. Both diets are sufficient in vitamin, mineral, and protein content for normal growth. Prior to this study, all animals were maintained on standard monkey chow in large outdoor enclosures and were naive to any experimental protocols.

Manufacturers specifications provided for both diets show that the total metabolizable energy content of the CTR chow was 2.87 kcal/g and was apportioned at

26.8% energy from protein, 58.5% energy from carbohydrate, and 14.7% energy from fat. The main source of fat in the CTR diet was soybean oil. The total energy content of the maternal HFD chow was 4.2 kcal/g and was apportioned at 16.7% energy from protein, 51.5% energy from carbohydrate, and 31.8% energy from fat. The main sources of fat in the HFD were lard, animal fat, butter and safflower oil.

The animals were group housed and had *ad libitum* access to food and water. The group housing is important as it provides for normal social behavior and exercise, which contribute to the psychological well being of the animals and more closely resembles the human condition. However, because the animals are group housed it is not possible to determine individual food/calorie intake. For maternal plasma studies, 11 CTR, 6 HFD, and 7 REV dams were used.

Each maternal group was housed with two males so that pregnancies would occur during the yearly breeding season (November – February). The females were checked each successive year for pregnancies starting in November by ultrasound, which allows an estimate of gestational age ± 5 days. Twice a year the animals underwent IV glucose tolerance tests (IVGTT) (**Methods 3-S1**), once during the late summer (nonpregnant state) and once during the early 3rd trimester of pregnancy. All of the above procedures were done under ketamine sedation (5–10 mg/kg).

For our studies, ONPRC veterinarians terminated singleton pregnancies from dams by cesarean section at gestational day 130 (G130), as determined by ultrasound. Pregnant dams were fasted overnight for approximately 16 hours prior to surgical procedure. Females were initially sedated with ketamine hydrochloride (100 mg/ml) at a dose of 10-15 mg/kg. Once animals were sedated they were delivered to the surgical area

and placed on isoflurane gas; induced at 3%, then maintained at 1.0-1.5%. Cesarean sections were performed by trained ONPRC veterinarians and their staff, and occurred on scheduled days between 10:00 and 10:30 am.

Pre and post-operative care was maintained by the ONPRC veterinary staff. Immediately prior to the cesarean section animals received an intravenous dose of hydromorphone (0.5mg if under 10kg, 1.0mg if over 10kg). An additional intravenous dose of hydromorphone was administered post-operatively, usually within an hour after the start of the procedure. For the remainder of the day following the cesarean section, intravenous hydromorphone was given at 4:00 pm and again at 8:00 pm in combination with buprenorphine (0.3 mg IM). The following day, hydromorphone was administered at 8:00 am, 12:00 pm, 4:00 pm and then again with buprenorphine at 8:00 pm. Animals remained in the surgical ICU area for approximately 7 days under close veterinary observation and were then released back into their group.

After cesarean section, fetuses were deeply anesthetized with sodium pentobarbital (> 30 mg/kg i.v.) and exsanguinated. All peripheral tissues and brain were removed, weighed and stored for subsequent protein and RNA extractions or for histological analyses. All surgical procedures used in this study, were performed each scheduled day in an identical manner, following an *a priori* defined protocol in both technique and timing. Thus for plasma analyses, blood draws were taken at approximately the same time of day for dams and fetuses. Maternal blood was taken during c-section from the femoral artery, and fetal blood samples were taken from the abdominal aorta during necropsy.

Fetal studies were performed with 11 CTR, 7 HFD and 6 REV animals. Normal full-term pregnancies for Japanese macaques is 175 days, thus G130 is in the early 3rd trimester. G130 was chosen after preliminary studies determined that this gestational age represented a critical period for the development of several metabolic systems: 1) hypothalamic circuits have started to develop, 2) there is widespread pancreatic β-cell development, 3) there is a full functioning placenta that is not at near term, and 4) there is very little white adipose tissue (WAT).

Previous work with this model has demonstrated significant increases in maternal leptin levels, decreases in maternal insulin sensitivity, and increases in maternal weight gain starting with the second year of maternal HFD exposure, and these changes persist and become greater through year four (McCurdy et al., 2009). For our fetal studies, we are reporting differences in HFD fetuses whose mothers were exposed to the maternal diet for at least four consecutive years. In the fifth year of our studies, a diet-reversal protocol (REV) was initiated to assess dietary impact independent of maternal obesity. This protocol entailed switching a subgroup of adult females that had been exposed to a high-fat diet for four consecutive years, to a control diet 1-3 months before becoming pregnant and throughout the pregnancy.

Maternal breast milk for fatty acid analysis was obtained at postnatal day 30 (postnatal day 29.5 ± 3 days, (mean ± SD)) from 6 CTR and 16 HFD dams giving birth to full term infants. The breast milk was collected during routine postnatal day 30 dual-emission X-ray absorptiometry (DEXA) procedures for the offspring. The DEXA procedure followed an *a priori* defined protocol and breast milk was obtained at approximately the same time of day for each animal. At 9:00 am, the mother was sedated

with ketamine (15 mg/kg) or telazol, if ketamine resistant. The baby was separated from the mother for DEXA scanning. Two hours later, 0.5mL oxytocin was injected intravenously into the mother to stimulate milk let down. The breast and nipple were massaged and milk was collected into a 15mL conical vial. The milk sample was immediately stored on ice until centrifugation to separate aqueous milk and cream layers.

Due to study design parameters in which the focus of the REV group was on fetal effects, breast milk from the REV dams was not available. In addition, during the two-year period that the breast milk study encompassed, there were fewer pregnancies in CTR dams (15) than in the HFD dams (23). The volume of milk collected was highly variable and dependent on whether nursing had occurred immediately prior to our procedures. Visual inspection of the breast-milk was also used to identify and exclude samples that were discolored or contaminated with maternal blood. Thus, to obtain enough breast milk for the insulin, total protein, leptin and cytokine assays we performed, and to provide sufficient power for analysis, we sampled additional lactating CTR Japanese macaque dams. Milking of these additional CTR dams was performed during bi-annual colony health examinations and included milk that was older than 30 days post-partum (post-natal day 160 ± 34 (mean ± SEM)). No differences were observed in the CTR group between the older milk and the 30 days post-partum milk for the insulin (12 CTR, 18 HFD dams) total protein (11 CTR, 8 HFD dams), leptin (20 CTR, 13 HFD dams) and cytokine assays (14 CTR, 18 HFD dams), so they were grouped for analysis. Detailed protocols for the insulin, total protein, leptin and cytokine assays are described in **Methods 3-S2**.

Fatty acid profiles

The fatty acids present in each maternal diet, fasting maternal plasma breast milk and fetal plasma were analyzed by a modification of the methods described by Langerstedt et al. (Lagerstedt et al., 2001). Deuterated fatty acids including d3C10:0, d3C14:0, d3C16:0, d3C18:0, d3C20:0 and d4C22:0 were added to samples prior to extraction as internal standards. Following hydrolysis and extraction, fatty acids were derivatized to the pentafluorobenzyl (PFB)-esters. The fatty acid-esters were analyzed by gas chromatography-mass spectroscopy (GC-MS) on a Trace DSQ (Thermoelectron) operating in the negative ion chemical ionization mode with methane as the reagent gas. The fatty acid-PFB esters were separated on a DB-5ms capillary column (30 m x 0.25 mm x 0.25 μm) with helium as the carrier gas at a flow rate of 1 ml/min. Individual fatty acids were monitored with selected ion monitoring and a dwell time of 50 ms for each ion species. Each fatty acid was matched to the deuterated internal standard closest in length and retention time. Peak area ratios of known amounts of standard fatty acids and the internal standards were used to generate calibration curves to quantify unknowns using Xcalibur software.

Dynamic range and efficiency curves for Real-Time PCR

Macaque specific primer sets were evaluated to determine the efficiency of our primer sets within a working range of cDNA concentrations and identify an optimum concentration of cDNA to use in Real-time PCR assays. A cDNA dilution series was made from four random samples from each dietary group. The cDNA was diluted based on initial RNA concentration and the assumption of 100% reverse transcription efficiency. The dilution series was designed so that each primer set started at an upper

limit of 50ng total cDNA/reaction and decreased in 10 ng increments to a minimum of 1ng/reaction (Pfaffl, 2001).

Primer validation Real-Time PCR reactions were run on an Applied Biosystems 7300 as relative quantification plates with SYBR master mix used at a 2x dilution. Following automatic thresholding and standard baseline adjustments after each run, Ct values were plotted as a function of the log (10) of the cDNA concentration and a linear slope was calculated (ABI, 2006). Efficiency was calculated as 10^(-1/slope) and was used for our experimental quantification (Pfaffl, 2001). For experimental assays we chose a cDNA concentration that gave us Ct threshold values across all dietary groups of between 20 and 32 cycles. In cases where we could not detect the target of interest in any diet group; threshold Ct values >35, or SYBR fluorescence not rising above background at all, we confirmed the presence of the target and the specificity of our target primers on fetal spleen processed in an identical manner as our liver samples. Additional protocols describing liver tissue RNA extractions (**Methods 3-S3**) and primer design (**Methods 3-S4**) are included as supporting information.

Real-Time PCR

Experimental Real-Time PCR reactions were run on an Applied Biosystems 7300 as relative quantification plates. Target and endogenous control primers were used at a final concentration of 471nM in a 21 μl reaction. SYBR master mix was used at the manufacturer's recommended 2x dilution. Dissociation curves were produced for every well to monitor primer amplification of a single target. Alg9 was used as an endogenous control for all our experiments. The Alg9 primer set was designed by core facilities at the Oregon National Regional Primate Center as an endogenous control for macaque

gene expression analysis, and subjected to extensive gene stability validation in our model by use of the geNorm VBA applet (Kidd et al., 2007; Vandesompele et al., 2002). Primer sequences used for Real-Time PCR are described in **Table 3-S1**.

Relative quantification of target gene expression was calculated across each dietary group using empirically derived efficiency values for each primer set and calculating an efficiency-corrected fold by the following formula:

$$\text{Fold} = (E_{target})^{\Delta Ct(\text{target control- target sample})} / (E_{endo})^{\Delta Ct(\text{endo control- endo sample})}$$

where E is the respective primer efficiency. The ΔCt_{target} was calculated by choosing one calibrator sample from the control diet group and subtracting subsequent target Ct values from that calibrator across all groups. In addition, ΔCt_{endo} was calculated by using endogenous control Ct values for the same calibrator sample as above, and subtracting subsequent endo Ct values from that calibrator (Pfaffl, 2001). In situations where the optimum cDNA concentration of our endogenous control differed from our target, we produced cDNA dilutions for the endogenous control and target from the same reverse-transcriptase reaction. Following the Real-Time PCR reaction random target well reactions from each diet group were run on a 2% agarose gel to verify amplicon singularity and size. Bands of the expected size were excised, gel purified (Qiaquick gel extraction kit, Qiagen #28706) and sequenced. Target specificity was confirmed by BLAST and comparing amplicon sequence with the NCBI macaque database.

TUNEL assay

We used an ApopTag® Peroxidase *In-Situ* TdT end-labeling apoptosis detection kit (Chemicon S7100) on fetal CTR, HFD, and REV paraffin embedded fetal liver sections (Right lobe, 5 microns thick) as per manufacturers instructions. Tissue was

deparaffinized in 3 washes of xylene, followed by graded alcohol (100%, 95% and 70%) rehydration. Proteinase K (20ug/ml) digestion for 15 min at room temperature was followed by 2 washes in ddH_2O. Endogenous peroxidases were quenched for 5 minutes in 3.0% hydrogen peroxide in 1X PBS. Following application of proprietary equilibration buffer, the TdT enzyme was incubated for one hour at 37^oC. The TdT reaction was stopped by immersion into wash buffer and the anti-digoxigenin conjugate was applied and incubated at room temperature for 30 minutes. Following 4 washes in 1X PBS, the peroxidase substrate was developed for 6 minutes at room temperature. Samples were then washed in ddH_2O and counterstained with methyl green.

TUNEL imaging and quantification

Imaging was performed on a Marianas Digital Imaging workstation equipped with a Zeiss Axiovert 200M inverted microscope (Zeiss Microimaging, Thornwood, NY) and a Coolsnap camera (Roper Scientific, Tucson, AZ) by a blinded observer. A montage of each liver section was created with the Marianas Digital Imaging workstation using a 2X objective. Stereological analysis of the 2X montage was performed by masking the hepatic area and placing 500 um X 500 um regions with spacings of 1000um X 1000 um and a random offset of 69.4 (x-coordinate) and 513.4 (y-coordinate), on the image (SlideBook, Intelligent Imaging Innovations, Denver, CO). Coordinates of each region were recorded and then each region was imaged using a 10X objective. Following acquisition of between 20 to 40 10X images per section, masking was used to threshold and calculate the total hepatic parenchyma area. An additional mask was used to threshold TUNEL positive cells. Within the stereology program we excluded TUNEL positive debris under 5 microns in width.

Following these adjustments, the number of TUNEL positive stained cells ("events") was automatically counted and the total hepatic area was recorded. The number of events was converted to a rate, defined as rate = (# events + 0.5)/ hepatic area, with the overall rate for each animal being summarized as the median rate among all animal-specific measurements. These rates were then log transformed (base 10) for analysis, with the log transformation aiding to stabilize variance and make the distribution of rates more symmetric. (The addition of 0.5 in the initial rate calculation was necessary to avoid taking the log of zero; among the 646 measurement only 7.6% were 0 counts). Analysis of variance was then used to determine whether the median rate differed according to diet.

Other analyses based on summarizing individual measurements in terms of mean rate, as well as non-parametric analysis (Kruskal-Wallis) applied to the current median-rate summary led to similar conclusions and are not reported. Similarly, a linear mixed effect model with count distributed according to a Poisson distribution (and area treated as an offset) found similar conclusions to our earlier and more simple approaches based on all summarizing forms (median/mean) of the log transformed rates; consequently, we present only results of the simpler analysis. All analyses performed using R version 2.6.1 (R Development Core Team (2007), R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL: http://www.R-project.org.) Graphs were produced with Prism software (GraphPad Software, Inc., La Jolla, CA).

Data analysis

Data for all analyses not explicitly described above, were first compiled and tested for normality by Shapiro-Wilk with STATA (College Station, Texas) statistical

software. Data were transformed to fit Gaussian distributions and tested for significance by ANOVA with a Bonferroni post-hoc analysis. Groups that did not attain a Gaussian distribution by transformation were first tested for overall significance by Kruskal-Wallis rank sum, followed by a Wilcoxon rank sum test with a Bonferroni adjusted alpha to determine significance between diet groups. Pair-wise analysis of the fatty acid association between each dam and their respective offspring was performed using pairwise correlation in STATA. We are reporting an overall Pearson correlation coefficient across our three diet groups for each analysis. In addition, maternal and fetal fatty acid samples were also tested by pairwise correlation in STATA for an overall association with maternal insulin resistance and glucose clearance. All graphs were made with Prism software (GraphPad Software, Inc., La Jolla, CA).

RESULTS

Maternal diet nutritional analysis

To initiate our analysis of the impact that maternal HFD has on the developing NHP fetus, we chose to first examine differences in fatty acid composition between the CTR and HFD diets. Using gas chromatography-mass spectroscopy, we found that compared to the CTR diet, the maternal HFD has higher levels of myristic (C14:0), myristoleic (C14:1), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1, N-9), linoleic (LA, C18:2, N-6), α-linolenic (ALA, C18:3, N-3), and arachidonic (AA, C20:4, N-6) fatty acids. In addition, the HFD had lower levels of eicosopentanoic acid (EPA, C20:5, N-3) and docosahexaenoic acid (DHA, C22:6, N-3) than the CTR diet. When fatty acid subtypes were combined into groups, the HFD contained much higher

levels of total fatty acids, saturated, monounsaturated, polyunsaturated, essential fatty acids (EFA, sum of C18:2 and C18:3) and total N-6 fatty acids (sum of C18:2 and C20:4) than in the CTR diet. Total N-3 fatty acids (sum of C18:3, C20:5 and C22:6) were similar between HFD and CTR maternal diets; however, a greater than 2-fold increase in HFD total n-6 fatty acids resulted in an N-6:N-3 ratio of 20:1 compared to the 9:1 N-6:N-3 ratio in the CTR diet (**Table 3-1)**. Thus, the HFD has an N-6:N-3 ratio that is reflective of current trends in the Western diet (Yacoubian et al., 2007).

Maternal plasma lipid profiles

Lipid analysis of maternal plasma total fatty acids revealed that fasting levels of total fatty acids, total N-6, saturated, monounsaturated, polyunsaturated, and EFA's were not significantly different between the CTR, HFD and REV maternal diet groups (**Table 3-2**). However, total N-3 fatty acids were significantly reduced in the HFD group when compared to CTR and REV diet groups. Comprising the reduction of total N-3 fatty acids were significant decreases in EPA (N-3) and DHA (N-3) in the HFD group when compared to both the CTR and REV dietary groups. There was no change in DHA between CTR and REV; however, EPA was higher in the REV group when compared to CTR. There were no significant differences observed in α-linolenic acid (N-3) and linoleic (N-6) across the three dietary groups. A trend was observed for lower levels of arachidonic acid (N-6) in HFD maternal plasma, but this did not reach statistical significance ($p = 0.06$).

No significant changes were observed in total circulating N-6 fatty acids between the three dietary groups. However, decreases in EPA and DHA in the HFD group were large enough to significantly lower total N-3 levels in maternal plasma. Consequently, the

HFD maternal plasma had a significantly higher N-6:N-3 ratio (20:1) when compared to CTR (5:1) or REV plasma (6:1).

Fetal plasma lipid profiles

Lipid analysis of fetal plasma also revealed that total fatty acids, saturated, EFA's and total N-6 fatty acid levels were not significantly different between the CTR, HFD and REV diet groups (**Table 3-3**). A significant increase in total polyunsaturated fatty acids was found in the REV group when compared to the HFD group but no statistical differences were observed when the CTR group was compared to either the HFD or REV diet groups. An increase in total monounsaturated fatty acids was observed in the HFD fetal plasma compared to the CTR and REV groups. The increase in monounsaturated fatty acids in the HFD group was due to higher levels of the major monounsaturated, oleic acid (C18:1, N-9), but the differences in oleic acid only reached statistical significance when the HFD was compared to the REV diet group.

As observed in the maternal circulation, total N-3 fatty acids were significantly lower in the HFD group when compared to CTR and REV. This decrease was due to significantly lower levels of DHA in the HFD fetal plasma when compared to CTR and REV. Again, due to the decreases in HFD circulating total N-3 fatty acids, a significant increase in the N-6:N-3 ratio was found between CTR (4:1) and the HFD group (9:1). In the REV diet group DHA and total N-3 fatty acids were normalized to CTR levels. However, due to an increase in REV plasma N-6 fatty acids, which was not itself significantly higher when compared to the CTR and HFD diet groups, the REV N-6:N-3 ratio (7:1) was only partially normalized to the CTR ratio. Thus, the reduced levels of N-

3 fatty acids and elevated N-6:N-3 fatty acid ratio found in the HFD maternal plasma were also observed in the HFD fetal plasma.

Plasma N-6:N-3 ratios are correlated between maternal and fetal circulation

The fasting levels of total fatty acids, saturated, monounsaturated and N-6 fatty acids are not different in the maternal plasma between dietary groups. Except for a small but significant increase in monounsaturated fatty acids, these findings are also observed in fetal plasma. However, we observed significant decreases in DHA and total N-3 fatty acids in both maternal and fetal HFD circulation that were normalized with maternal diet reversal. These findings suggest that circulating N-3 fatty acids, as essential fatty acids, are dependent on dietary supply and availability in both mother and fetus across our three dietary groups.

Our model provides us with the ability to perform pair-wise analysis of circulating lipids between individual dams and their offspring to investigate associations between maternal and fetal parameters. Thus, we performed a pairwise correlation analysis and found that there was a significant correlation (**Figure 3-1A**) between the maternal N-6:N-3 ratio and the N-6:N-3 ratio found in the fetus ($R_{overall}$=.63, p =.002). In addition, we found a weaker but statistically significant association (**Figure 3-1B**) between maternal EPA and fetal EPA (R=.43$_{overall}$, p =.045). However, we did not find a statistically significant association between maternal and fetal plasma DHA.

Maternal obesity versus maternal consumption of a high-fat diet

Previous work with this model has shown that following chronic maternal consumption of a HFD two maternal phenotypes emerge (McCurdy et al., 2009). Compared to CTR animals, diet-sensitive HFD dams are obese, hyperleptinemic, and

insulin resistant. In contrast, the diet-resistant HFD dams had normal insulin secretion during glucose tolerance tests, similar body weights, body fat and circulating leptin levels relative to the maternal CTR diet group even after four years on the high-fat diet. To date, the majority of findings regarding fetal development with this model have been independent of maternal obesity and diabetes.

We tested whether maternal sensitivity to the HFD is associated with either maternal or fetal fatty acid levels by pairwise correlation. We found that the maternal insulin secretion (insulin AUC) and maternal glucose clearance (glucose AUC), as determined by third trimester maternal i.v. glucose tolerance testing, were not correlated with fasting plasma levels of any maternal fatty acids acquired at time of cesarean section (G130).

Interestingly, we found that the fetal plasma N-6:N-3 ratio was positively correlated with maternal insulin AUC ($R_{overall}$=.61, p =.002, **Figure 3-2A**). No other fetal plasma fatty acids assayed were correlated with the maternal insulin AUC. Additionally, we found that the total fetal plasma levels of saturated fatty acids ($R_{overall}$=.54, p =.007, **Figure 3-2B**) were correlated with the maternal glucose AUC, as were the individual saturated fatty acids C14:0 (myristic acid; $R_{overall}$ = .43, p = .04), C16:0 (palmitic acid; $R_{overall}$= .45, p = .04). In addition, C18:1 (oleic acid; $R_{overall}$=.43, p =.04) and C18:3 (α-linolenic acid (n-3); $R_{overall}$ = .50, p = .01) were also positively correlated with maternal glucose AUC (**Figure 3-S1**). These data extend previous associations found between maternal diet sensitivity and fetal outcomes in our model (J. Cox et al., 2009). Our findings suggest that while maternal diet has been the primary predictor of fetal outcomes

thus far, other maternal factors (e.g. obesity, hyperinsulinemia, etc.) may also play important roles in determining the fatty acid milieu of the developing fetus.

Inflammation in the high-fat fetal liver

Recent work with this model suggested that non-alcoholic fatty liver disease (NAFLD) is present in the HFD fetal liver. McCurdy et al. demonstrated that oxidative damage, hepatic steatosis, and upregulation of phospho-JNK1 that was highly correlated with levels of fetal liver triglycerides were present in the HFD fetal livers (McCurdy et al., 2009). To extend these findings, we investigated whether inflammation and consequent evidence of non-alcoholic steato-hepatitis (NASH) was present in the HFD fetal liver. We used Real-time PCR to evaluate the expression of inflammatory markers between CTR, HFD and REV fetal livers (**Table 3-4**). We found that the expression of interleukin-10 (IL-10) was significantly different across the three dietary groups and lower in the REV group, but post hoc tests did not show statistical significance when compared to the CTR and HFD diet groups. Unexpectedly, the expression of Arginase-1 in the REV diet group, a marker of Th_2 macrophage activation (Gordon, 2003; Lumeng et al., 2007), was significantly decreased when compared to the CTR and HFD groups. No differences were observed in the expression of any of the other inflammatory markers we assayed between the CTR, HFD and REV maternal diet groups. These data suggest that the fetal liver is not the origin of the increased levels of pro-inflammatory cytokines found in fetal circulation in previous work with this model (McCurdy et al., 2009).

Apoptosis in high-fat fetal liver

During gestation the fetal liver directly receives about 50% of maternal blood flow via the branch of the umbilical vein that connects to the portal vein (Beckmann,

2006). Thus, the fetal liver, and particularly the right lobe of the fetal liver, is anatomically positioned to be directly affected by factors that are present in the umbilical circulation. Given the increases in pro-inflammatory cytokines found in the fetal umbilical circulation and the evidence of NAFLD reported in previous work (McCurdy et al., 2009), we performed a TUNEL assay to examine whether evidence of increased hepatic apoptosis was present in fetal livers exposed to a maternal HFD. We found that there is a significant increase in the number of apoptotic cells in the HFD fetal liver compared to CTR when normalized to hepatic area (2.14 fold increase, $p<.05$). Importantly, by switching a subgroup of HFD mothers to a control diet during a subsequent pregnancy (REV), apoptosis in the fetal liver was completely normalized to baseline (**Figure 3-3**).

Postnatal Studies

To begin to understand the long-term metabolic programming effects that a maternal high-fat diet has on the offspring, it is necessary to separate effects that occur *in-utero* from changes that occur after parturition. Lactation is a critical period of development for the offspring that may be sensitive to maternal obesity and consumption of a high-fat diet. To begin to address the effects that maternal obesity and overnutrition may have during lactation, we performed gas chromatograph mass spectroscopy on maternal breast milk to characterize postnatal exposure of the offspring to maternal fatty acids. In addition, we assayed levels of insulin, total protein, and leptin as well as interleukin-1β present in maternal breast milk.

Maternal breast milk lipid profiles

In the breast milk we found no changes in total fatty acids in the HFD group versus CTR. C14:0 (myristic), C16:0 (palmitic), C18:0 (stearic) and total saturated fatty acids were not higher in the HFD group when compared to control (**Table 3-5**). There were no differences in total levels of monounsaturated fatty acids as well as C16:1 (palmitoleic) and C18:1 (oleic). Total polyunsaturated fatty acids were unchanged in HFD breast milk when compared to CTR. Total N-6 fatty acids, as well as C18:2 (linoleic) and C20:4 (arachidonic), were unchanged in the HFD breast milk when compared to CTR. Total N-3 fatty acids were also unchanged between the CTR and HFD diet groups. However, C20:5 (EPA, N-3) and C22:6 (DHA, N-3) were significantly lower in the HFD breast milk when compared to CTR breast milk. C18:3 (linolenic, N-3) was the largest component of total N-3 fatty acids assayed and was unchanged between the CTR and HFD diet groups. Thus, the observed decreases in EPA and DHA were not large enough to significantly lower the total levels of breast milk N-3 fatty acids. The mean N-6:N-3 ratio was higher in the HFD (19:1) breast milk than CTR breast milk (9:1). However, a large variance in both groups prevented the increased HFD N-6:N-3 ratio from attaining significance. Overall, the significant decreases in EPA and DHA in HFD breast milk reflect what was also observed in maternal and fetal plasma.

To further characterize the effects that maternal HFD had on breast milk, we performed radio-immunoassays for insulin and leptin. We found that insulin levels in maternal breast milk are significantly higher (2-fold) in HFD mothers versus CTR (**Figure 3-4A**). We found no changes in the levels of leptin in maternal breast milk between CTR and HFD, although the levels were quite low in both groups (data not

shown). We also found no differences in the levels of the inflammatory cytokine IL-1β between CTR and HFD breast milk (data not shown). Total protein levels in HFD breast milk are significantly lower than in the CTR breast milk (**Figure 3-4B**).

Postnatal phenotype

Given the proinflammatory environment our cohort of HFD animals were exposed to *in-utero*, combined with increased apoptosis in the fetal liver and the significant changes in breast milk composition, we examined the offspring from CTR and HFD dams to determine if phenotype differences were apparent in the postnatal period. Previous work with this model demonstrated that the offspring of HFD dams had similar bodyweights at postnatal day 30 (P30) and post-natal day 90 (P90) as CTR offspring, and higher levels of body-fat at P90 (McCurdy et al., 2009). The current results again showed that body weights were similar between the CTR and HFD offspring at the P30 and P90 time points (**Figure 3-5A**) and that the HFD offspring had higher body fat at P90 than CTR offspring, as determined by DEXA scanning (**Figure 3-5B**). In addition, HFD offspring had significantly lower lean body mass than CTR offspring at P90 (**Figure 3-5C**). We measured bone mineral content as well and found no differences at either P30 or P90 offspring between the two diet groups (**Figure 3-5D**). Thus, while total body weights are similar at P90 between CTR and HFD offspring, the HFD offspring have higher body fat and lower lean body mass than CTR offspring.

DISCUSSION

While much work has been done in the NHP to highlight the effects that maternal nutrient deprivation has on development of metabolic systems in the offspring (L. A. Cox

et al., 2006; C. Li et al., 2007; Nijland et al., 2007; N. Schlabritz-Loutsevitch et al., 2007; N. E. Schlabritz-Loutsevitch et al., 2007), there is a fundamental gap in understanding the contributions that maternal obesity and maternal nutrient excess provide to metabolic programming. Previous work with this model has demonstrated that a maternal HFD altered fetal development and expression of key hypothalamic neuropeptides in the context of hypothalamic inflammation (B.E. Grayson et al., 2007). Furthermore, HFD fetal plasma contained elevated levels of inflammatory cytokines, and elevated triglycerides and glycerol that were highly correlated with maternal levels. In the liver, HFD fetuses had premature gluconeogenic gene expression, steatosis, elevated triglyceride content, and oxidative stress. The majority of these changes were observed irrespective of maternal obesity or maternal insulin resistance status and persisted into the early postnatal period. Importantly, switching HFD mothers to a control diet during pregnancy alone (diet-reversal; REV) normalized a number of the observed fetal hepatic pathologies towards control levels (McCurdy et al., 2009).

In the present study, we report in the nonhuman primate that chronic maternal HFD consumption, independent of maternal obesity or diabetes, leads to significantly reduced plasma levels of N-3 fatty acids in fasted HFD pregnant dams and third trimester fetuses. Our dietary model was designed to mimic the typical Western diet being consumed by a majority of pregnant women in the developed world. Whether the pathology is induced by elevated dietary fat content *per se* or is instead due to a change in dietary fatty acid composition (e.g. elevated N-6:N-3 ratio) was not addressed by this experimental design. Furthermore, we cannot draw conclusions regarding whether

specific fatty acid manipulations (e.g. N-3 supplementation) would have beneficial effects on the developing fetus.

In HFD dams we observed significantly reduced fasting plasma levels of DHA, EPA and total N-3 fatty acids. In the HFD fetal circulation, plasma levels of DHA and total N-3 fatty acids were also significantly reduced when compared to the CTR diet animals. HFD breast milk contained lower levels of EPA and DHA than CTR breast milk, however total N-3 fatty acids were not different between CTR and HFD breast milk. We also observed that apoptosis was significantly increased in the HFD fetal liver. Importantly, we found that returning HFD dams to a CTR diet during pregnancy normalized plasma N-3 fatty acids in pregnant dams and fetuses and returned fetal hepatic apoptosis to control levels.

The maternal HFD diet had by definition a much higher total fat content than the CTR diet including much higher levels of saturated, monounsaturated and polyunsaturated fats. Among the essential fatty acids, the HFD also contained slightly higher levels of linolenic acid (C18:3 N–3) but double the levels of the more abundant linoleic acid (C18:2 N–6) and total n-6 fatty acids. Consequently the HFD chow N-6:N-3 ratio was 2-fold higher than the CTR N-6:N-3 ratio. The high saturated and monounsaturated fatty acids in the HFD reflect that its fat largely came from animal sources (lard, animal fat, and butter), versus the CTR diet (grains and soybean oil). Also included in the maternal HFD were daily calorie-dense treats made from peanut butter.

In humans, a recent report showed that low-nutrient-density foods, consisting of refined carbohydrates and animal products high in saturated fat, were the major contributors to the total energy intake for a cohort of pregnant women (Siega-Riz et al.,

2002). While this study was small in scope, the consumption of a diet that is high in calories and saturated fats is similar to findings from nationally representative studies of children and non-pregnant women (Block et al., 1985; Subar et al., 1998). Thus, our NHP HFD has strikingly similar characteristics to what is known about human dietary choices during pregnancy.

The present data are particularly relevant in light of the fact that over the last 100 years dietary N-6:N-3 ratios have gone from being close to 1:1 to approximately 20:1 (Simopoulos, 1999). There is strong evidence suggesting that cellular membrane long chain polyunsaturated fatty acid composition is largely determined by dietary ratios (Simopoulos, 1991). Thus, the maternal and fetal plasma N-6:N-3 ratio mirrored the N-6:N-3 ratio of the diet in each diet group. The elevated N-6:N-3 ratio found in the HFD chow was a result of increases in N-6 fatty acids. Notably, the maternal and fetal HFD plasma N-6:N-3 ratio was driven by significant decreases in N-3 fatty acids when compared to CTR plasma. In particular, DHA was significantly decreased in both the maternal and fetal HFD circulation. Furthermore, significant decreases in EPA and DHA were observed in breast milk. Thus, the HFD offspring are provided with decreased levels of EPA, DHA and N-3 fatty acids during both fetal and early postnatal life, developmental periods that are dependent solely on maternal transfer of nutritional substrates for normal growth.

The N-3 and N-6 long chain polyunsaturated fatty acids, particularly DHA and AA, are critical for proper infant growth and neurodevelopment (reviewed in (Innis, 1991)). DHA and AA are both highly enriched in neural tissue while DHA is the major component of retinal photoreceptor membranes (Clandinin et al., 1980a, 1980b; M.

Martinez, 1992). While only non-esterified fatty acids (NEFA) can be transferred from mother to fetus directly, other mechanisms, including hydrolysis of triglycerides and receptor mediated transfer, allow fatty acids including docosahexaenoic acid (DHA, 22:6 N-3), eicosapentaenoic acid (EPA, 20:5 N-3) and arachidonic acid (AA, 20:4 N-6), to be transferred through the placenta to the fetus (Crabtree et al., 1998; Diamant et al., 1982; Herrera et al., 2006; Hudson et al., 1977; Ruyle et al., 1990).

In the human fetus, there is limited capacity for *de novo* lipogenesis and the precursors for fetal fat accretion are primarily supplied trans-placentally and consist of maternal substrates derived from lipids rather than from glucose (Haggarty, 2002; Herrera et al., 2000; Herrera et al., 2006). It has also been shown in baboons that while the fetus has the capacity to synthesize DHA from its EFA precursor, α-linolenic acid, preformed maternal DHA is preferentially used for DHA accretion in the fetal brain (Su, Bernardo et al., 1999; Su, Corso et al., 1999; Su et al., 2001). Thus, the composition of the fatty acid supply to the fetus is mainly determined by maternal lipid profile and suggests that modifications of maternal diet or metabolic homeostasis will affect delivery of lipid substrates to the fetus (Carlson et al., 1992; Olsen et al., 2000). Our results support these findings and demonstrate that decreased circulating levels of DHA, total N-3 fatty acids, and an elevated N-6:N-3 ratio was recapitulated in both maternal and fetal circulation.

Previous work in nonhuman primates demonstrated that dietary deprivation of N-3 fatty acids, and consequent decreases in plasma N-3 fatty acids during the prenatal and postnatal periods, had profound effects on brain and visual system fatty acid composition and retinal function of fetuses and infants (Neuringer et al., 1986; Neuringer et al., 1984).

Decreased levels of N-3 fatty acids during development have also been associated with altered acetylcholine and dopamine release in rodents (Aid et al., 2003; Kodas et al., 2002). Recently it was reported in our model, that fetuses exposed to a maternal HFD displayed significant changes in central serotonergic systems and nearly 78% of the HFD offspring displayed increased anxious or aggressive behavior during behavioral tests at postnatal day 130 (Sullivan et al., 2010). Our work demonstrates that circulating levels of DHA and total N-3 fatty acids are significantly reduced in the HFD maternal and fetal plasma. Thus while the changes in fetal brain development previously reported in our model are likely to be multifactorial, our data suggests that the lower levels of plasma DHA and N-3 fatty acids found in the HFD fetal circulation may be partly responsible.

While the maternal HFD chow has decreased levels of EPA and DHA when compared to the CTR chow, the levels of total N-3 fatty acids were not different between two diets. In addition, the maternal HFD has much higher levels of α-linolenic acid (C18:3, N-3), an essential fatty acid precursor necessary for DHA synthesis, than the CTR chow. Neuringer et al. (Neuringer et al., 1984) demonstrated in the NHP that plasma levels of DHA could be maintained in pregnant dams fed a diet containing 8% α-linolenic acid despite undetectable levels of pre-formed DHA. It has been well established that the desaturases responsible for synthesis of DHA from α-linolenic acid are subject to regulation from dietary and hormonal factors (Brenner, 1981; Castuma et al., 1972; Eck et al., 1979; Inkpen et al., 1969; Rosenthal, 1987; Sprecher, 1981); in particular, N–3 and N–6 fatty acids compete as substrates for these desaturases as well as for uptake into tissues. Thus it is reasonable to conclude that the decreased levels of

maternal plasma DHA and total N-3 fatty acids we observed are due to the high N–6:N–3 ratio of the HFD.

The present study expands upon previous findings that suggest that maternal diet can lead to severe inflammatory and oxidative stress in the fetal liver. McCurdy et al. explored the effects of maternal HFD in this model and demonstrated evidence of fetal hepatic steatosis, oxidative stress, upregulation of heat-shock proteins, and increased phosphorylation of *c*-Jun NH_2-terminal kinase (p-JNK), and increased inflammatory cytokines in the fetal circulation (McCurdy et al., 2009). While our data does not show that the HFD fetal liver is the primary site of cytokine synthesis (at least at a transcriptional level), the circulating inflammatory insult to the developing fetal liver in our model is nonetheless quite significant.

Hepatocyte apoptosis is a marker of disease severity in numerous hepatic disease states (Hatano, 2007; Ishii et al., 2003; Rudiger et al., 2002). In fact, the severity of hepatocyte apoptosis is significantly correlated with histopathological and biochemical markers of NASH and hepatic fibrosis (Feldstein et al., 2003). There is evidence that there are connections between fatty acids, hepatic steatosis and hepatic apoptosis. For example, incubation of HepG2 cells *in-vitro* with saturated and monounsaturated fatty acids produced steatosis and p-JNK-dependent apoptosis that was more pronounced with saturated fatty acids (Malhi et al., 2006). In primary rat hepatocytes, treatment with oleic and stearic acid induced steatosis and sensitized hepatocytes to cytotoxicity mediated by tumor necrosis factor related apoptosis inducing ligand (TRAIL) (Malhi et al., 2007). Our data show a significant increase in the numbers of apoptotic cells in fetal livers exposed to a maternal high-fat diet. Previous findings of severe hepatic steatosis,

increases in p-JNK, and high circulating levels of inflammatory cytokines (McCurdy et al., 2009), as well as our current findings of increased oleic acid in the fetal circulation are consistent with previously described mechanisms of hepatic apoptosis. To our knowledge, the increased apoptosis in the HFD fetal liver is a novel finding that reinforces the extent of damage occurring within the developing fetal liver. However, the regenerative capacity of the liver is formidable and studies already in progress will determine whether permanent hepatic damage is evident in these animals.

Our NHP model is a sophisticated and effective tool that makes it possible to quickly translate our findings into human clinical research studies. In addition to our findings, previous work with this model highlights the complex relationship between maternal diet and obesity. It has been shown that fetal serum metabolites are reduced under maternal high fat diet conditions. In agreement with our fatty acid findings, changes in specific fetal serum metabolites were associated with maternal diet, while others were associated with maternal obesity and insulin resistance (J. Cox et al., 2009). Future studies are needed to untangle the contribution of maternal phenotype from maternal diet and their combined effects on fetal development before comprehensive interventions are employed. Within the midst of the present childhood obesity epidemic, it is critical that we move our findings forward into human studies and potentially into the realm of public health policy and clinical practice.

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Table 3-1

Gas chromatography- mass spectrometry analysis of maternal chow.[1]

		DIET GROUP				
		CTR		HFD		
Fatty Acid	Common Name (Type)	Mean	SEM	Mean	SEM	HFD/CTR Ratio
C 14:0	Myristic	0.5	0.1	15.0	1.3	30
C 14:1	Myristoleic	0.004	0.001	0.4	0.1	100
C 16:0	Palmitic	6.1	2.0	24.7	5.7	4.1
C 16:1	Palmitoleic	0.2	0.0	3.4	0.5	17
C 18:0	Stearic	2.9	1.0	20.2	1.9	7
C 18:1	Oleic (N9)	9.5	2.1	52.9	15.4	5.6
C 18:2	Linoleic (N6)	18.9	5.0	40.6	14.6	2.1
C 18:3	Linolenic (N3)	0.5	0.1	1.2	0.4	2.4
C 20:4	Arachidonic (N6)	0.04	0.02	0.4	0.1	10
C 20:5	EPA (N3)	0.8	0.2	0.3	0.1	0.4
C 22:6	DHA (N3)	1.0	0.5	0.6	0.2	0.6
Total Fatty Acids		40.3	4.8	169.6	39.4	4.2
Total Saturated		9.4	3.0	59.9	8.8	6.4
Total Monounsaturated		9.7	2.0	56.6	15.9	5.8
Total Polyunsaturated		21.2	5.8	52.6	20.5	2.5
Total Essential Fatty Acids		19.4	5.1	41.8	15.0	2.2
Total N6		19.0	5.0	50.2	19.7	2.2
Total N3		2.2	0.8	2.4	0.8	0.9
N6:N3 Ratio		8.8	1.0	19.9	0.9	2.3

[1] All values are mean ± SEMs and expressed as mg/g of dry chow.

Table 3-2

Gas chromatography- mass spectrometry analysis of maternal plasma lipids[1]

		DIET GROUP						
		CTR		HFD		REV		
Fatty Acid	Common Name (Type)	Mean	SEM	Mean	SEM	Mean	SEM	*p*-value[2]
C 14:0	Myristic	290.7	61.8	347.5	84.8	226.09	35.6	0.68
C 14:1	Myristoleic	4.7	1.0	3.3	0.4	4.7029	0.9	0.52
C 16:0	Palmitic	2456.2	239.4	2187.7	209.8	2239.4	194.2	0.67
C 16:1	Palmitoleic	192.2	50.6	113.5	17.3	90.416	11.1	0.54
C 18:0	Stearic	796.7	60.1	1013.4	65.7	860.44	55.2	0.07
C 18:1	Oleic (N9)	828.7	129.0	1084.7	101.8	859.5	85.8	0.33
C 18:2	Linoleic (N6)	1167.9	150.6	1122.6	72.6	1395.6	231.8	0.73
C 18:3	Linolenic (N3)	27.8	4.0	28.6	2.1	31.344	4.5	0.81
C 20:4	Arachidonic (N6)	484.5	55.8	366.6	29.8	570.18	48.1	0.06
C 20:5	EPA (N3)	72.8[b,c]	9.4	**5.2**[a,c]	0.8	122.0[a,b]	13.1	**0.0001**
C 22:6	DHA (N3)	219.4[b]	33.8	**39.8**[a,c]	2.4	177.5[b]	21.2	**0.0001**
Total Fatty Acids		6541.5	632.3	6312.8	469.4	6377.7	568.6	0.9718
Total Saturated		3543.6	310.6	3548.6	310.8	3325.9	264.7	0.89
Total Monounsaturated		1025.5	170.5	1201.4	117.6	954.6	90.7	0.58
Total Polyunsaturated		1972.5	238.6	1562.8	81.8	2270.2	308.0	0.33
Total Essential Fatty Acids		1195.7	154.5	1151.2	74.7	1426.5	237.1	0.61
Total N6		1652.5	203.0	1489.2	80.3	1951.5	280.1	0.53
Total N3		320[b]	37.4	**73.7**[a,c]	2.7	330.8[b]	30.2	**0.0001**
N6:N3 Ratio		5.2[b]	0.2	**20.2**[a,c]	0.9	6.1[b]	0.5	**0.0009**

[1] All values are means ± SEMs and are expressed as µmol/L. n = 11 for CTR, n = 6 for HFD, n = 6 for REV.
[2] Overall significance as determined by ANOVA or Kruskal-Wallis rank sum test.
[a] Significantly different from CTR, p< .0167, Bonferroni adjusted α.
[b] Significantly different from HFD, p< .0167, Bonferroni adjusted α.
[c] Significantly different from REV, p< .0167, Bonferroni adjusted α.

Table 3-3

Gas chromatography- mass spectrometry analysis of fetal plasma lipids[1]

		DIET GROUP						
		CTR		HFD		REV		
Fatty Acid	Common Name (Type)	Mean	SEM	Mean	SEM	Mean	SEM	*p*-value[2]
C 14:0	Myristic	125.1	18.2	164.0	34.5	127.2	32.9	0.57
C 14:1	Myristoleic	4.4	1.0	6.3	1.9	5.5	1.9	0.8
C 16:0	Palmitic	708.3	108.3	647.5	98.4	737.4	146.3	0.88
C 16:1	Palmitoleic	146.1	19.7	118.8	10.7	115.2	15.6	0.49
C 18:0	Stearic	455.0	37.7	419.1	63.9	385.7	22.3	0.6
C 18:1	Oleic (N9)	473.2	56.5	**724.7**[c]	73.9	390.2[b]	103.1	**0.02**
C 18:2	Linoleic (N6)	498.6	73.0	450.0	31.9	976.9	293.5	0.17
C 18:3	Linolenic (N3)	13.9	1.8	13.7	1.5	14.2	1.9	0.98
C 20:4	Arachidonic (N6)	572.6	152.4	417.3	111.7	675.6	229.3	0.4
C 20:5	EPA (N3)	42.6	9.1	30.9	5.4	67.9	20.3	0.29
C 22:6	DHA (N3)	273.9[b]	78.7	**52.2**[a]	6.0	259.4	119.3	**0.01**
Total Fatty Acids		3313.7	404.9	3044.4	243.2	3755.1	566.3	0.65
Total Saturated		1288.4	134.8	1230.5	175.4	1250.2	164.9	0.96
Total Monounsaturated		623.7	70.5	**849.8**	82.1	510.8	111.7	**0.05**
Total Polyunsaturated		1401.7	279.8	964.1[c]	118.7	**1994.0**[b]	372.4	**0.047**
Total Essential Fatty Acids		512.5	74.8	463.7	32.4	991.1	294.3	0.19
Total N6		1071.2	201.1	867.3	114.1	1652.5	310.9	0.09
Total N3		330.5[b]	80.1	**96.8**[a,c]	6.8	341.5[b]	110.1	**0.001**
N6:N3 Ratio		3.6[b]	0.2	**8.9**[a]	0.9	6.7	1.7	**0.007**

[1] All values are means ± SEMs and are expressed as μmol/L. n = 11 for CTR, n = 7 for HFD, n = 6 for REV.
[2] Overall significance as determined by ANOVA or Kruskal-Wallis rank sum test.
[a] Significantly different from CTR, p< .0167, Bonferroni adjusted α.
[b] Significantly different from HFD, p< .0167, Bonferroni adjusted α.
[c] Significantly different from REV, p< .0167, Bonferroni adjusted α.

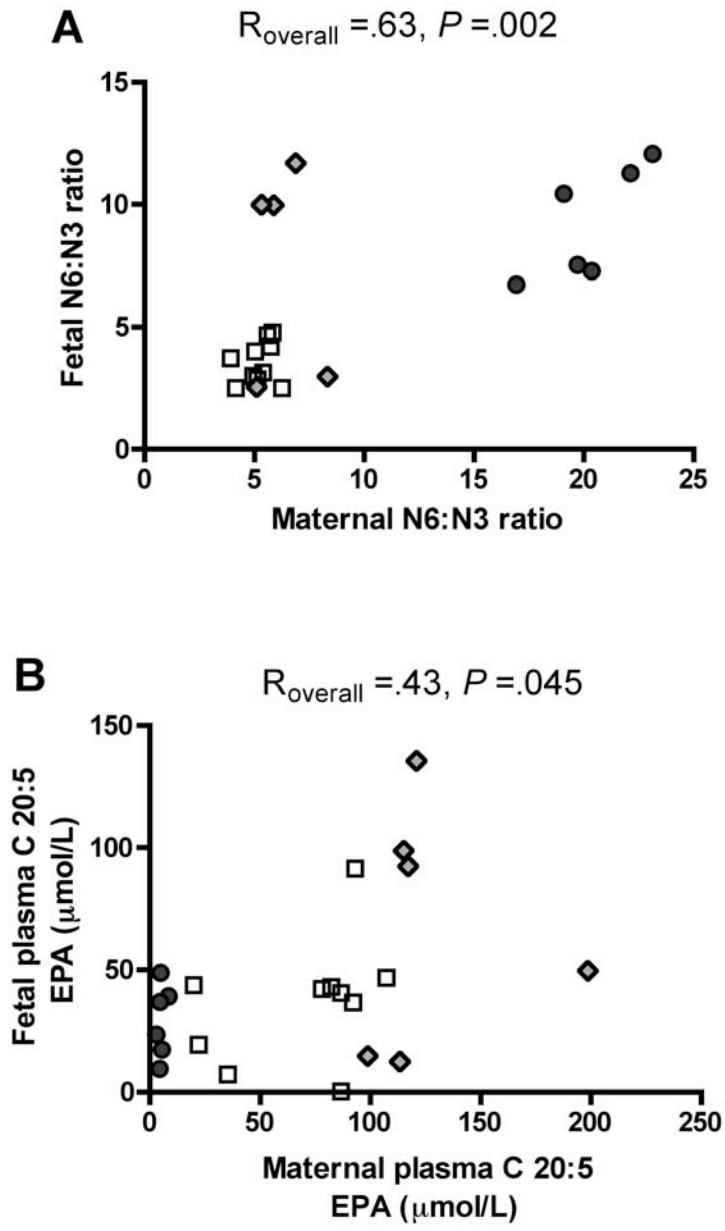


Figure 3-1. Pair-wise correlation analysis of the plasma N6:N3 fatty acid ratio (A), and plasma EPA levels (B), between CTR (□), HFD (●) and REV (◇) Japanese macaque dams and their respective third trimester fetuses (n = 22 maternal/fetal pairs). Both the plasma N6:N3 FA ratio and plasma EPA levels are correlated between maternal and fetal circulation.

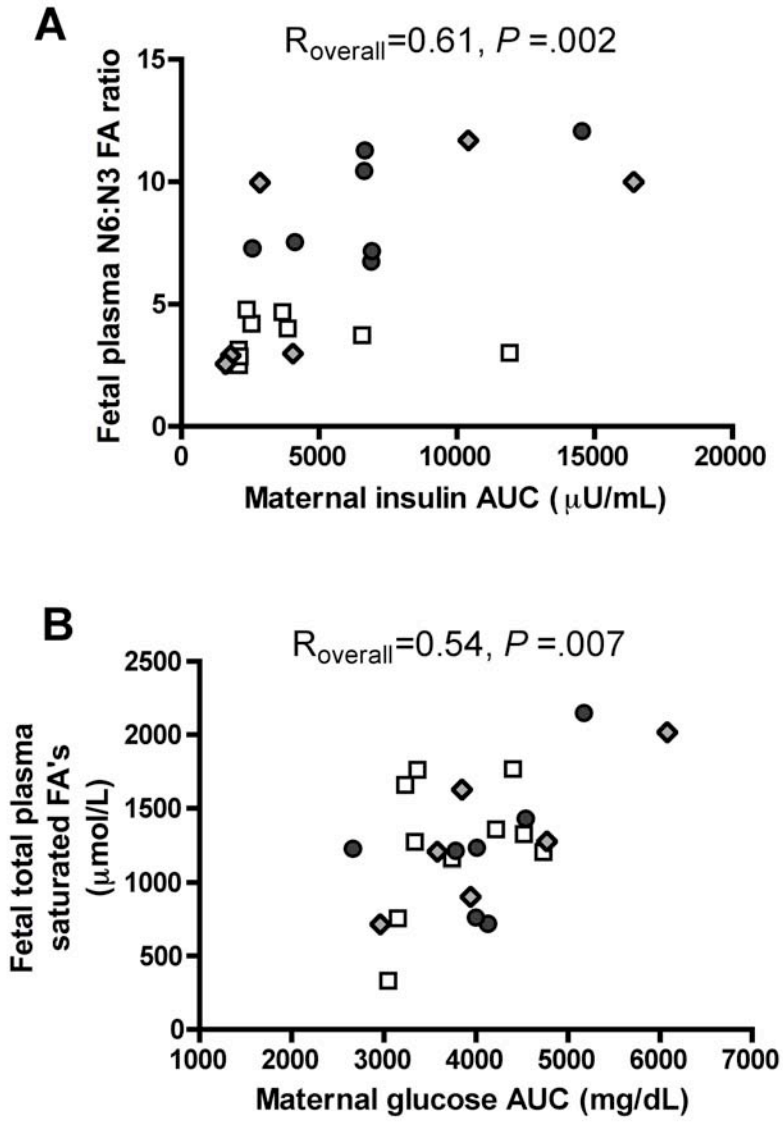


Figure 3-2. Pair-wise correlation analysis of fetal plasma N6:N3 fatty acid ratio with respective maternal insulin AUC (A), across CTR (□), HFD (●) and REV (◇) maternal diet groups. Pair-wise correlation analysis of total fetal plasma saturated FA's with respective maternal glucose AUC (B), across CTR (□), HFD (●) and REV (◇) maternal diet groups. (n = 23 maternal/fetal pairs). Fetal N6:N3 ratio is positively correlated with maternal insulin AUC. Total fetal plasma saturated FA's are correlated with maternal glucose AUC.

Table 3-4.

Inflammatory marker mRNA expression in fetal liver[1]

	DIET GROUP						
	CTR		HFD		REV		
Target	Mean	SEM	Mean	SEM	Mean	SEM	*p*-value[2]
Interferon-γ	0.42	0.11	0.38	0.11	0.32	0.06	0.87
Interleukin-1β	0.95	0.26	1.05	0.24	0.89	0.11	0.82
Interleukin-4	undetected		undetected		undetected		n/a
Interleukin-6	undetected		undetected		undetected		n/a
Interleukin-10	0.97	0.06	1.19	0.18	0.69	0.11	**0.045**
I-TAC (CXCL11)	0.93	0.12	1.06	0.08	0.82	0.15	0.1
Lymphotoxin-α	0.76	0.11	0.79	0.08	0.77	0.05	0.59
MCP-1 (CCL2)	0.78	0.1	0.81	0.14	0.75	0.06	0.91
Tumor Necrosis Factor-α	0.82	0.16	0.71	0.07	0.53	0.08	0.28
Arginase-1	1.01	0.09	1	0.07	**.65**[a,b]	0.04	**0.009**
C-Reactive protein	1.93	0.32	1.48	0.22	2.19	0.41	0.11

[1] All values are means ± SEMs and are expressed as relative fold to CTR calibrator sample.
[2] Overall significance as determined by Kruskal-Wallis rank sum test.
[a] Significantly different from CTR, p< .0167, Bonferroni adjusted α.
[b] Significantly different from HFD, p< .0167, Bonferroni adjusted α.
[c] Significantly different from REV, p< .0167, Bonferroni adjusted α.

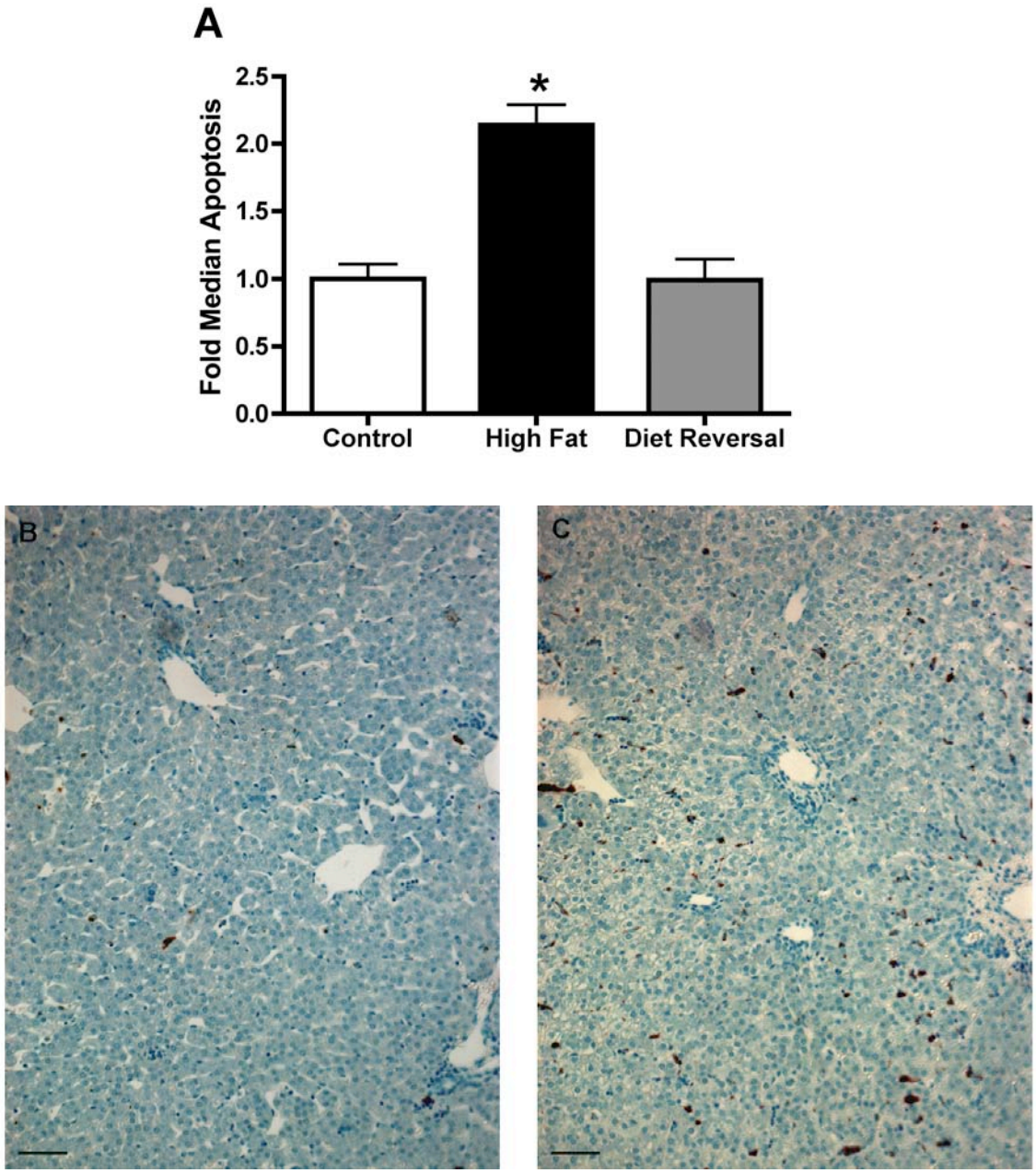


Figure 3-3. Quantification of TUNEL positive cells normalized to hepatic parenchyma area in G130 macaque fetal liver for CTR, HFD, and REV maternal diet groups (A). Data are expressed as fold increase over CTR of the median rate ± standard error (CTR; n=6, HFD; n=7, REV; n=6, $^{*}P < .05$ versus CTR, ANOVA). Representative brightfield images for TUNEL staining in CTR (B) and HFD (C) G130 fetal liver. TUNEL staining was returned to CTR levels following maternal diet reversal (REV), thus representative REV image is not pictured. Scale bar, 50μm.

Table 3-5.

Gas chromatography- mass spectrometry analysis of maternal breast milk lipids[1]

Fatty Acid	Common Name (Type)	DIET GROUP				*p*-value[2]
		CTR		HFD		
		Mean	SEM	Mean	SEM	
C 14:0	Myristic	25.8	16.1	49.8	12.4	0.18
C 14:1	Myristoleic	24.6	11.3	33.7	11.7	0.51
C 16:0	Palmitic	60.1	18.7	87.7	13.3	0.42
C 16:1	Palmitoleic	64.0	24.0	52.4	17.1	0.42
C 18:0	Stearic	27.9	8.3	46.4	6.8	0.10
C 18:1	Oleic (N9)	43.2	23.7	67.5	15.5	0.18
C 18:2	Linoleic (N6)	64.6	21.5	56.1	5.5	0.51
C 18:3	Linolenic (N3)	13.2	5.0	15.4	5.3	0.61
C 20:4	Arachidonic (N6)	1.7	0.5	1.9	0.3	0.48
C 20:5	EPA (N3)	0.6	0.2	**0.2**	0.1	**0.012**
C 22:6	DHA (N3)	2.3	0.6	**0.3**	0.0	**0.0007**
Total Fatty Acids		328.1	61.5	411.6	32.5	0.30
Total Saturated		113.9	41.2	183.9	29.9	0.21
Total Monounsaturated		131.8	24.1	153.7	18.6	0.61
Total Polyunsaturated		82.4	20.2	74.0	7.0	0.71
Total Essential Fatty Acids		77.8	19.0	71.5	6.7	0.71
Total N6		66.3	21.9	58.0	5.7	0.51
Total N3		16.1	4.9	16.0	5.4	0.51
N6:N3 Ratio		9.1	3.6	18.9	3.5	0.16

[1] All values are means ± SEMs and are expressed as µmolm/L of cream layer.
[2] Overall significance as determined by Student's T-test or Wilcoxon rank sum test.

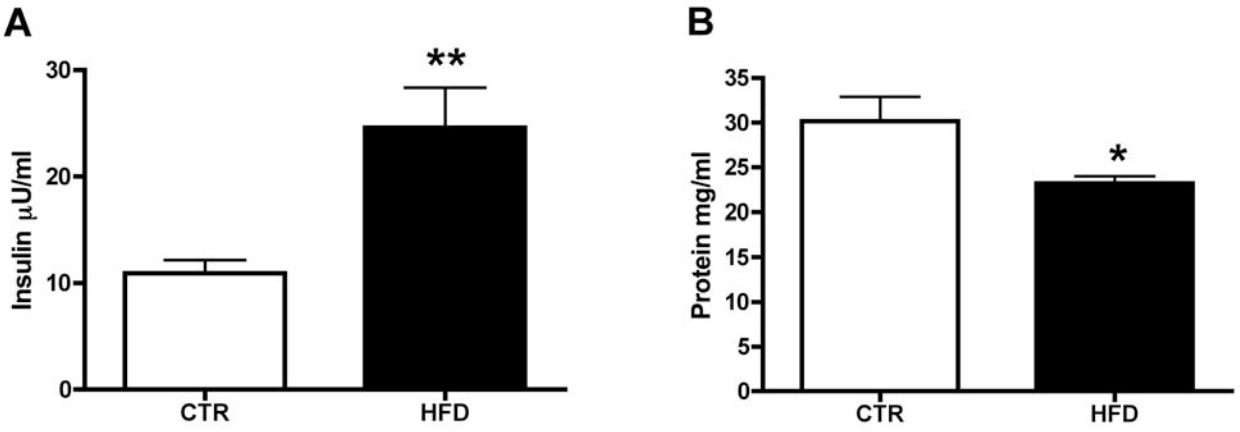


Figure 3-4. Analysis of insulin (A) and total protein levels (B) in breast milk from macaque dams in CTR (white bars) and HFD (black bars) maternal diet groups. **A.** Insulin was assayed by a commercially available primate RIA kit. HFD dams have significantly higher levels of insulin in their breast milk than CTR dams (CTR; $n = 11$, HFD; $n = 17$, $^{**}P < .01$ versus CTR, Wilcoxon rank sum test). **B.** Macaque breast milk total protein levels were measured from the aqueous layer using a BCA™ Protein Assay kit across CTR (white bars) and HFD (black bars) maternal diet groups. HFD breast milk contains significantly lower levels of total protein when compared to CTR (CTR; $n = 13$, HFD; $n = 17$, $^{*}P < .05$ versus CTR, Student's t-test).

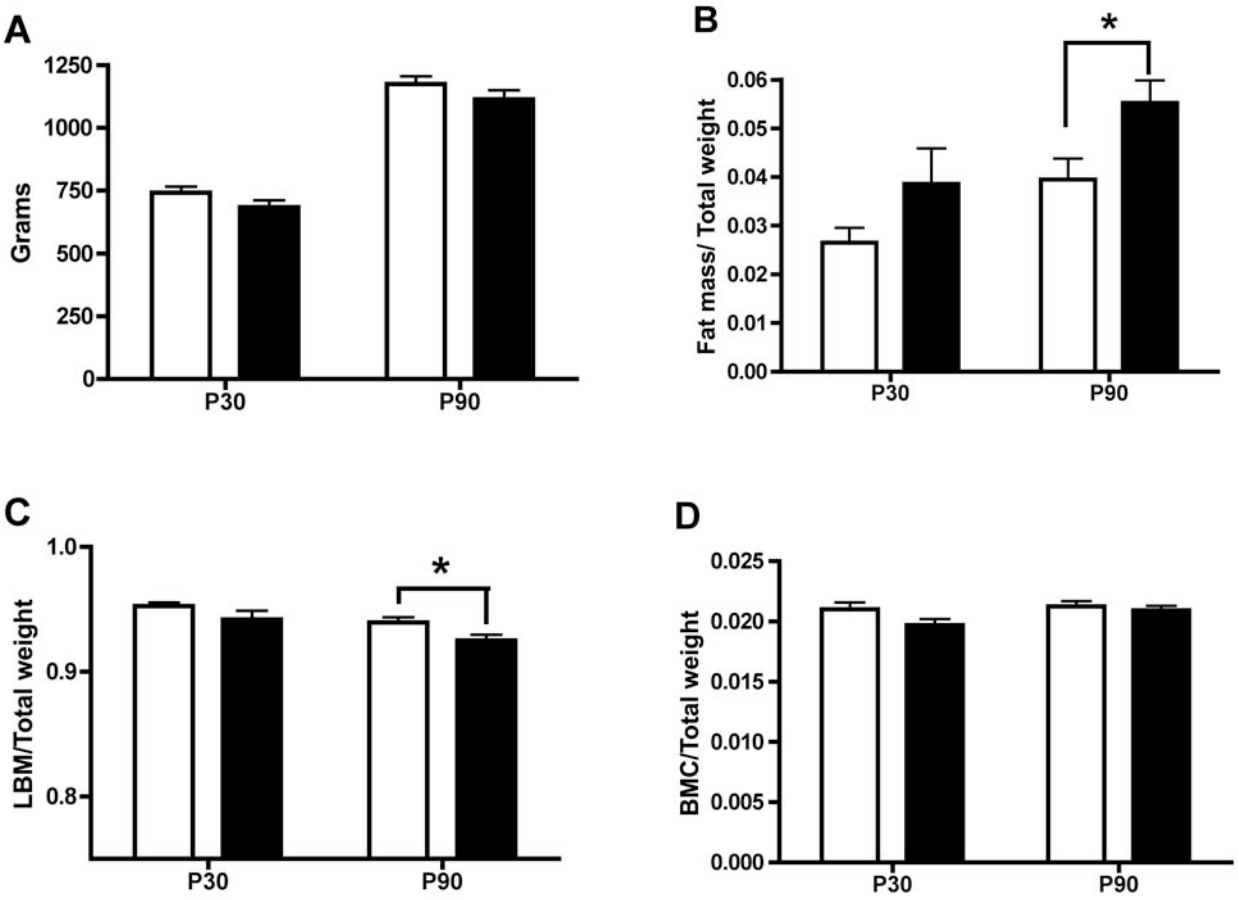
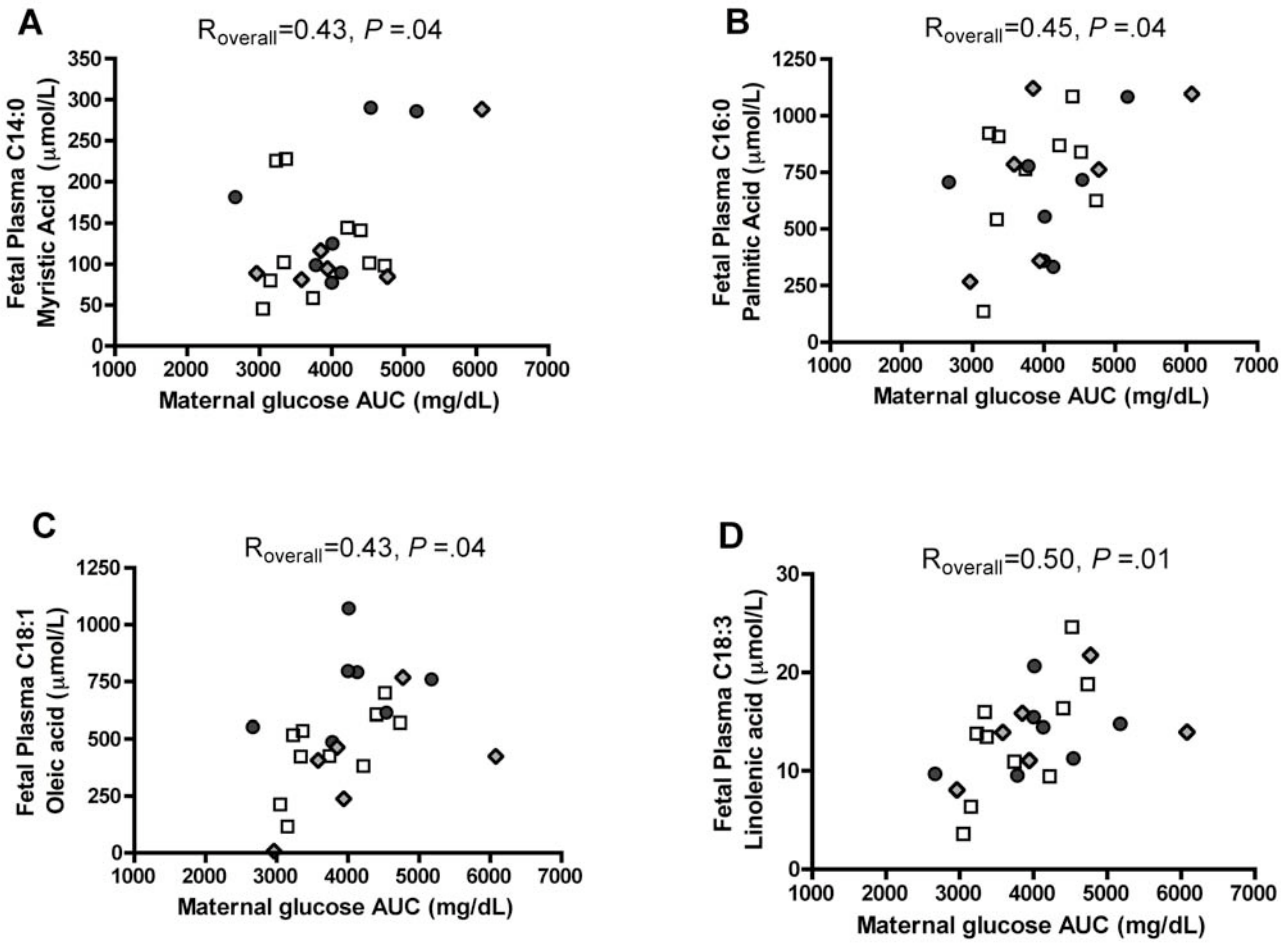


Figure 3-5. DEXA analysis of macaque offspring at post-natal day 30 (P30) and post-natal day 90 (P90) from CTR (white bars) and HFD (black bars) diet groups. **A.** Total weight of macaque offspring at P30 and P90. **B.** Normalized fat mass of macaque offspring between CTR and HFD diet groups at P30 and P90 ($^{*}P < .05$ versus CTR, Student's t-test). **C.** Normalized lean body mass (LBM) of macaque offspring between CTR and HFD diet groups at P30 and P90 ($^{*}P < .05$ versus CTR, Student's t-test). **D.** Normalized bone mineral content (BMC) of macaque offspring between CTR and HFD diet groups at P30 and P90. All data is expressed as mean ± standard error (P30 CTR; n = 15, P30 HFD; n = 17, P90 CTR; n = 13, P90 HFD; n = 19).



Supplemental Figure 3-S1. Correlation of fetal plasma fatty acids with maternal glucose clearance. Pair-wise correlation analysis of fetal Myristic; C 14:0 (A), Palmitic; C 16:0 (B), Oleic; C 18:1, and Linolenic; C 18:3 (D) FA's with respective maternal glucose AUC across CTR (□), HFD (●) and REV (◆) maternal diet groups (n = 22-23 maternal/fetal pairs).

SUPPLEMENTAL TABLE 3-S1.

Primer sequences for Real-time PCR amplification of macaque inflammatory markers.

mRNA	Sequence (5′-3′)	Genbank Accession No.
IFNG	F: TGTCCAACGCAAAGCAGTACA	NM_001032905
	R: AAAAGGAGTCAGATGTTTCGAGGT	
IL-1β	F: GACGTCGATGGCCCTAAACA	NM_001042756
	R: TGTAGTGCTCGTGGGAGATTTG	
IL-4	F: ACAACTGCCATATCGCCTTACG	NM_001032904
	R: CTTCTGCAGGGCTGCGAC	
IL-6	F: TGACAAACACATTCGGTACATCCT	NM_001042733
	R: AGCAAAGAGGCACTGGCAGA	
IL-10	F: CCGTGGAGCAGGTGAAGAAT	NM_001044727
	R: GACATCTTCATCAACTACATAGAAGCCTA	
CXCL11	F: AGAAAGCCTCCATAATTTACCCAAGT	NM_001032950
	R: GATTTGGGATTTAGGCATCGTT	
LTA	F: AGGATGGTTTCTCCTTGAGCAA	NM_001047148
	R: GGAGAGTAGGCTTTCCCAGAGAAG	
CCL2	F: AGTGTCCCAAAGAAGCTGTGATC	NM_001032821
	R: TCCAGGTGGTCCATGGAATC	
TNF-α	F: TGAGGCCAAGCCCTGGTA	NM_001047149
	R: CGAGATAGTCGGGCAGATTGA	
Arg-1	F: AACAGCTGGCTGGCAAGGT	NW_001116523
	R: TGGCCAGAGATGCTTCCAAT	
CRP	F: CTCATGCTTTTGGCCAGACA	NW_001108960
	R: GGCTTCGTTAACCGTGCTTT	

Definition of abbreviations: F, forward primer; R, reverse primer.

SUPPLEMENTAL METHODS:

Methods 3-S1. Maternal insulin sensitivity. Twice a year, i.v. glucose tolerance tests (IVGTT's) were performed on pregnant (early third trimester) and nonpregnant females after an overnight fast. Animals were sedated with ketamine (10 mg/kg) and administered a glucose bolus (50% dextrose solution) at a dose of 0.6 g/kg via the saphenous vein. Baseline blood samples were obtained prior to the infusion, and 1-ml blood samples were taken at 1, 3, 5, 10, 20, 40, and 60 min after infusion via the femoral artery. Glucose was measured immediately at each time-point using a OneTouch Ultra Blood Glucose Monitor (LifeScan), and the remainder of the blood was kept in heparinized tubes on ice for insulin measurement. After the IVGTT, samples were centrifuged, and plasma was stored at –80°C until assayed. Insulin was assayed in plasma by RIA (catalog no. RI-13K; Linco).

Methods 3-S2. Breast milk insulin, leptin, IL-1β and protein analysis. Insulin and leptin levels in the aqueous milk layer were assayed by commercially available primate radioimmmunoassay (RIA) kits (Insulin, Cat. # HI-14HK. leptin, Cat. # PL-84K; Linco, St. Charles, MO) according to the manufacturer's instructions. Briefly, samples (100 µl) were assayed in duplicate and added to tubes with 100 µl of hydrated ^{125}I-labeled human insulin or leptin and appropriate primate antiserum and incubated overnight at 4°C. Precipitating reagent (1 ml) was added, and tubes were centrifuged for 20 min at 2000-3000 *g*, then aspirated and total counts measured by gamma counter for 1 minute. The sensitivity of the assay was 2µU/ml for insulin and 0.5 ng/ml for leptin when using 100 µl of sample. IL-1β levels were determined using a monkey IL-1β ELISA (U-Cytech, Utrecht, The Netherlands) following the manufacturer's specifications and

assaying the samples in duplicate. Breast milk total protein levels were measured from the aqueous layer using BCA^{TM} Protein Assay Kit according to manufacturers specifications.

Methods 3-S3. RNA extraction protocol from liver tissue for Real-Time PCR. Total RNA was extracted from RNAlater (Ambion) stabilized primate fetal livers using an RNeasy Mini kit according to manufacturer's instructions (Qiagen, Appendix C, June 2001) with the following additions: Following disruption and homogenization of liver tissue, a 15 min Proteinase K incubation at 55 ^{o}C was performed. In addition, an optional on-column DNase treatment was also included. The RNA was eluted in RNase free water and the concentration and relative purity was determined by the 260/280 absorbance ratio. 1.3 ug of total RNA was reverse transcribed in a 75ul reaction using a Taqman Reverse Transcription kit (Applied Biosystems N808-0234) according to manufacturer's protocol.

Methods 3-S4. Real-Time PCR primer design. Target sequence was determined by using both the NCBI macaque database and the Ensembl macaque database. Sequences from each database were compared and only regions that had 100% homology between databases were used for primer design. Primers sets were designed by Primer Express (Applied Biosystems) to span exon-exon junctions where feasible and produce amplicons of ~100 bp in length.

Chapter 4

Perinatal Exposure to a High-Fat Diet is Associated with Reduced Hepatic Sympathetic Innervation in One-Year Old Male Japanese Macaques

ABSTRACT

Previous studies with our nonhuman primate (NHP) model demonstrated that exposure to a maternal high fat diet (HFD) *in-utero* induces nonalchoholic fatty liver disease (NAFLD), increased hepatic apoptosis and decreased plasma N3 fatty acids in the fetus. After partuition, evidence of insulin resistance and changes in body composition develop early in life, in the context of ongoing HFD exposure. The autonomic nervous system plays an important role in metabolic homeostasis through direct and indirect actions in the liver. In addition, the vagus nerve is also involved in regulating inflammation in the liver. The vagally mediated cholinergic anti-inflammatory pathway inhibits inflammation via the nicotinic acetylcholine receptor α7 subunit (CHRNA7). Our primary aim was to examine the effects that exposure to a HFD had on hepatic innervation. Thus, we evaluated fetal and juvenile tissue for changes in hepatic sympathetic innervation and expression of autonomic receptors. In addition, we examined the expression of inflammatory markers in the juvenile liver by Real-Time PCR.

In the fetal studies we established that robust sympathetic/peptigergic innervation is present in the portal triads, a novel finding in macaques. We evaluated two juvenile cohorts, CTR/CTR and HFD/HFD, for changes in hepatic sympathetic innervation and changes in the in NHP offspring chronically exposed to HFD from conception to one year of age. The nomenclature in our juvenile cohorts first lists the diet exposure prior to weaning, the second lists the post weaning diet (6 months). We observed a significant decrease in sympathetic innervation in the HFD/HFD males in portal and parenchymal regions. Significantly decreased expression of CHRNA7 was also observed in the

HFD/HFD juvenile liver. In addition, a significant increase in the expression of lymphotoxin-α, as well as trends for increased expression of TNF-α and Il-1β was observed in the HFD/HFD liver.

Our data suggest that sympathetic regulation of liver function is altered in the HFD/HFD males. In addition, increased hepatic inflammation indicates that a transition from benign fatty liver deposition to NAFLD is occurring in the HFD/HFD juvenile liver. Furthermore, the observed decrease in hepatic CHRNA7 expression could render the HFD/HFD juvenile offspring susceptible to local inflammation and consequent development of hepatic insulin resistance. These findings were observed independent of maternal obesity and underscore the importance of nutrition throughout gestation and postnatal life in hepatic development.

INTRODUCTION

Hepatic Innervation

The liver is innervated by both the sympathetic and parasympathetic nervous systems. These autonomic nerve fibers are primarily associated with the afferent vasculature and enter the liver at the hepatic hilus (Shimazu, 1996). The autonomic nerves form two communicating plexuses within the liver. An anterior plexus is associated with the hepatic artery and a posterior plexus is associated with the portal vein and the bile duct. Additional innervation may also be associated with the hepatic vein, but this has not been well described (M. I. Friedman, 1988).

Afferent as well as efferent nerves containing adrenergic, cholinergic, and peptidergic components are all found in the liver (Shimazu, 1996). Postganglionic sympathetic nerves are part of the splanchnic system originating in the celiac and superior mesenteric ganglia. The preganglionic parasympathetic nerves are part of the vagus, while postganglionic parasympathetic nerves are derived from ganglia presumed to be located at the hepatic hilus and within the portal regions (H. R. Berthoud, Neuhuber, W.L., 1998; M. I. Friedman, 1988).

Adrenergic and cholinergic nerves of the autonomic nervous system are found in distinct populations within and adjacent to the portal triads. Portal tract innervation by adrenergic nerves is well characterized and very consistent across mammalian species (Amenta et al., 1981; Burt et al., 1989; Feher et al., 1992; Feher et al., 1991; Forssmann et al., 1977; Goehler et al., 1991; Goehler et al., 1988; McCuskey, 2004; Metz et al., 1980; Nobin et al., 1978; Reilly et al., 1978; Skaaring et al., 1976). Interlobular innervation of autonomic nerve fibers however, is subject to much variation between

species. In higher-order primates, such as humans and macaques, parenchymal sympathetic nerve fibers are found within the space of Disse and are in close contact with hepatocyes HSC's, LSEC's and Kupffer cells (Bioulac-Sage et al., 1990; Burt et al., 1989; Forssmann et al., 1977; McCuskey, 2004; D. G. Tiniakos et al., 2008; Tsuneki et al., 1981). In the rhesus macaque, electron microscopy revealed nerve fibers, containing large dense-cored transmitter vesicles, embedded into grooves in parenchymal hepatocytes (Forssmann et al., 1977). No evidence of intralobular innervation by sympathetic fibers has been demonstrated to date in the rodent (Reilly et al., 1978; Skaaring et al., 1976; Ueno et al., 2004).

Neuropeptide-Y fibers have been shown to have a similar distribution and display co-localization with tyrosine hydroxylase (TH) immunoreactivity in the liver of humans and other mammals (Burt et al., 1989; Feher et al., 1991; Goehler et al., 1991). NPY positive fibers have also been found to be present in the hepatic lobule in close apposition with LSEC's and hepatocytes in the Japanese macaque (Ding et al., 1994). In addition, following specific denervation of sympathetic fibers with the neurotoxin, 6-hydroxydopamine, NPY-positive fibers were undetectable in the rat liver (Goehler et al., 1991). Taken together, these results suggest that NPY is involved with sympathetic function in the mammalian liver.

The neuroanatomy of the intrahepatic nerves as well as the role that these nerves play in regulating hepatic hemodynamics, hepatocyte function and metabolism, Kupffer cell activity, HSC function, and LSEC permeability are not well understood (Shimazu, 1996; Yi, la Fleur et al., 2010). Mechanisms involving direct neural stimulation of these cells as well as indirect mechanisms mediated by innervation of non-parenchymal cells

have been proposed (Anil et al., 1987; M. I. Friedman, 1988; Gardemann et al., 1992; Lautt, 1983; Lautt et al., 1987; McCuskey, 1966, 1967). In the rodent, it has been suggested that nervous control of hepatic metabolism is regulated by diffusion of released transmitters or indirectly through changes in blood flow or tissue oxygenation (Shimazu, 1996). Alternatively, neural signals might be propagated within the lobule through the large numbers of gap junctions present in the rat liver (Gilula, 1982; Seseke et al., 1992). In support of this idea, an inverse relationship between intralobular nerves and gap junctions has been reported (Forssmann et al., 1977; Reilly et al., 1978). The presence of intralobular nerves in species such as humans and macaques, and their close association with parenchymal and sinusoid cell types, suggest that nervous signals may be regulating hepatic function by directly acting on these cells (Anil et al., 1987; M. I. Friedman, 1988; Gardemann et al., 1992; Niijima, 1989a; Nobin et al., 1978).

Hepatic Nervous System Development

Few studies have specifically examined the ontogeny of hepatic innervation. Those that have are very limited in the species examined and the developmental timepoints examined. In the mouse, NPY immunoreactive fibers are present in low density in some portal regions in 19-day-old embryos. Following birth, NPY fibers are found in every portal region examined, reaching a peak density at one week after birth before decreasing to adult levels at two weeks after birth (Ding et al., 1997). To our knowledge, the ontogeny of hepatic innervation has never been explored in the macaque. However, in the adult macaque the organization of sympathetic and NPY innervation in portal and parenchymal regions are similar to that found in adult humans (Ding et al., 1997; Nobin et al., 1978).

In human liver, hepatic innervation in the portal triads generally began at about 20 weeks in gestation and increases in density reaching adult levels near term. Many fibers are found in close association with the hepatic artery, less surrounding the portal vein and only occasionally associated with the bile ducts. In the hepatic parenchyma, innervation is observed at very low density in isolated cases at 40 weeks, proceeding to moderate density in the adult. (D. G. Tiniakos et al., 2008). These findings support the idea that development of portal innervation occurs *in-utero*, while parenchymal innervation and maturity of liver innervation occurs after birth in humans (Delalande et al., 2004). The development of hepatic innervation in the macaque has not been thoroughly characterized.

Nervous Regulation of Hepatic Glucose Metabolism

Hepatic function is regulated by complex interactions between circulating factors, and the hepatic branches of the splanchnic and vagus nerves. Pioneering work by Shimazu et al., introduced the role that the hypothalamus and the peripheral nervous system play in the regulation of hepatic metabolism (Shimazu, 1962; Shimazu et al., 1965). Further work by this group delineated a reciprocal regulation of hepatic glycogen metabolism by the sympathetic and parasympathetic nervous systems in both rabbits and rats. Electrical stimulation of the sympathetic nervous system via the ventromedial hypothalamic nucleus (VMH) caused a rapid increase in blood glucose, activation of hepatic glycogen phosphorylase-*a*, a decrease in liver glycogen, increased hepatic gluconeogenesis and reduced glycolysis. Conversely, stimulation of the parasympathetic nervous system via the lateral hypothalamic nucleus (LHA) resulted in lower blood glucose, activation of hepatic glycogen synthetase, an increase in liver glycogen, and

decreased gluconeogenesis (Shimazu et al., 1966; Shimazu et al., 1978; Shimazu et al., 1975).

These studies were extended when selective activation of the VMH with physiological levels of norepinephrine produced a robust activation of hepatic glycogen phosphorylase-*a* within one minute after treatment. This effect was completely abolished by hypothalamic pretreatment with propranolol (a β-adrenergic antagonist) or systemic application of hexamethonium bromide (ganglionic blocker). Microinjection of acetylcholine, serotonin and γ-aminobutyric acid (GABA) into the VMH did not effect glycogen phosphorylase activity in the liver (Matsushita et al., 1980). Further, microinjection of the LHA with acetylcholine or carbachol (cholinergic agonist) significantly increased the activity of hepatic glycogen synthetase *I* within an hour after treament with no effects on hepatic phosphorylase-*a* activity. The increase in hepatic glycogen synthetase *I* was blocked following intrahypothalamic microinjection of atropine or scopolamine, or peripheral cholinergic blockade with hexamethonium or *N*-methylatropine (Matsushita et al., 1979).

Electrical stimulation of the exterior hepatic nerves around the hepatic artery and portal vein in isolated rat liver was reported to activate noradrenergic, cholinergic and peptidergic fibers. Within the liver itself, sympathetic output dominated the response which was marked by an overflow of noradrenaline into the hepatic vein, increased glucose output, a shift to lactate output and decreased oxygen uptake (Shimazu, 1996). Additional work in this model, demonstrated that vascular resistance was increased and a redistribution of blood flow was regulated by sinusoid contraction (Gardemann et al., 1992; Ji et al., 1984). Following hepatic sympathetic nerve stimulation, in addition to

noradrenaline release, NPY and galanin are also released with evidence that suggests hepatic production of both neuropeptides takes place in the rat liver (Kowalyk et al., 1992; Shimazu, 1996; Taborsky et al., 1994). NPY has been shown in other tissues to potentiate the vasoconstriction action of noradrenaline (Pernow et al., 1989). Galanin is localized to hepatic sympathetic nerve fibers and functions as a robust inhibitor of insulin release in the canine (McDonald et al., 1985; Mundinger et al., 1997).

While these data point strongly towards direct autonomic regulation of hepatic glucose metabolism, other studies provide a window into the complex neuroendocrine and autonomic processes that work as a single integrated system in the control of hepatic glucose homeostasis. For example, direct electrical stimulation of the splanchnic nerve increases adrenal catecholamine release (Damase-Michel et al., 1993; Takeuchi et al., 1993), and impacts glucagon and insulin release from the pancreas (Bloom et al., 1975, 1978; Kaneto et al., 1975). Injection of bombesin into the VMH or LHA causes hyperglycemia with increased glucagon secretion (Iguchi et al., 1984; Iguchi et al., 1983). In contrast, injection of insulin into the VMH or LHA causes hypoglycemia (Iguchi et al., 1981). Intravenous or intraventricular injection of pharmacological doses of leptin increases hepatic glucose production associated with reductions in hepatic glycogen content in mice (Kamohara et al., 1997). Further, reflex induced hyperglycemia, following surgical trauma or hemorrhage in rodents or felines, is not prevented by hepatic denervation or adrenalectomy alone, rather the combination of adrenalectomy and denervation is necessary to abolish the acute hyperglycemic response (Lautt et al., 1977; Lautt et al., 1982). However, species-specific differences in hepatic innervation also exist and may make the translation of findings from one species to another very complex

(Forssmann et al., 1977; Metz et al., 1980; Nonogaki, 2000). Thus, indirect mechanisms, neural regulation by glucoregulatory neuropeptides, redundant independent systems and species-specific considerations must be carefully addressed in functional studies of the liver.

The Cholinergic Anti-inflammatory Pathway

Early work in immune organs demonstrated that nerve fibers are localized in close apposition to the cellular mediators of innate and adaptive immunity (Felten et al., 1985). In the thymus and the spleen, robust sympathetic innervation in the absence of parasympathetic or sensory innervation, suggests that regulation of the immune function in these organs is provided by the efferent sympathetic nervous system (Bellinger et al., 1993; Cano et al., 2001; Nance et al., 1989; Nance et al., 1987; Schafer et al., 1998; Trotter et al., 2007; Wan et al., 1994). However, recent work has suggested that the role that the parasympathetic nervous system plays in the immune system is not so clearly defined (Huston et al., 2006; Nance et al., 2007).

During endotoxemia, the liver and the spleen are the primary sources of the inflammatory cytokine TNF-α found in the serum (Gregory et al., 1998; Huston et al., 2006; Kumins et al., 1996). Electrical stimulation of the peripheral vagus, in rats exposed to a lethal dose of lipopolysaccaride (LPS), significantly inhibited hepatic production of TNF-α and significantly attenuated the development of hypotension. These responses were observed in the absence of any measurable increases in corticosterone or IL-10 (Borovikova et al., 2000). These findings were extended and refined by demonstration that the cholinergic nicotinic α7 receptor (CHRNA7) and Kupffer cells are critically involved in vagally mediated protection from Fas-induced hepatic apoptosis in mice

(Hiramoto et al., 2008). Further, electrical stimulation of the vagus in CHRNA7 knockout mice failed to attenuate the cytokine release observed in wild-type littermates (Wang et al., 2003). Additional studies solidified the role that vagus nerve signaling plays in inhibiting cytokine effects and improving outcomes in models of sepsis, hemorrhagic shock, ischemia, ileus, arthritis and pancreatitis (Altavilla et al., 2006; Bernik et al., 2002; de Jonge et al., 2005; Guarini et al., 2003; Mioni et al., 2005; Saeed et al., 2005; van Westerloo et al., 2006).

The cholinergic anti-inflammatory pathway has been defined as the efferent arm of the inflammatory reflex. The inflammatory reflex is initiated by vagal afferent signals that are relayed to the nucleus of the solitary tract (NTS). The afferent signals have classically been assumed to be secondary to peripheral cytokine sensing by the vagus (Watkins et al., 1995). However, recent work demonstrated that dietary fat, acting via cholecystokinin (CCK) receptors in the gut, also activates the afferent vagal arm of the cholinergic anti-inflammatory pathway (Luyer et al., 2005). Afferent signals are relayed to the rostral ventrolateral medullary (RVLM), nucleus ambiguous (NA) and the dorsal vagal motor nucleus (DVM). Information is also sent to the hypothalamic-pituitary axis (HPA), activating humoral anti-inflammatory responses by the adrenal release of glucocorticoids via ACTH (Tracey, 2002, 2009).

Efferent signals are sent to the celiac ganglion via pre-ganglionic efferents originating in the sympathetic trunk of the spinal cord or through the vagus nerve (Tracey, 2009). Vagal efferents also travel through the subdiaphragmatic trunk, the celiac ganglion and the splenic nerve (Huston et al., 2006; Rosas-Ballina et al., 2008). At the level of the target tissue macrophage, acetylcholine release by the vagus binds

CHRNA7. Ligand binding enables sodium and calcium influx, however signal transduction in immune cells does not require ion channel activity or membrane depolarization (Tracey, 2009). Signal transduction in macrophages involves pleiotropic cascades that regulate the activation of NF-κB through the inhibition of phosphorylation of IκB (Yoshikawa et al., 2006). In addition, the JAK2/STAT3 pathway is activated, inhibiting NF-κB binding to DNA and increasing the expression of suppressor of cytokine signaling (SOCS3) (de Jonge et al., 2005). The net result is selective inhibition of inflammatory cytokines in macrophages while preserving production of anti-inflammatory cytokines (Borovikova et al., 2000; Parrish et al., 2008; Pavlov et al., 2007).

Initial work with this anti-inflammatory pathway demonstrated that incubation of human macrophages with acetylcholine, muscarine or α-bungarotoxin in the presence of LPS stimulation significantly reduced the release of pro-inflammatory cytokines *in-vitro* (Borovikova et al., 2000). Interestingly, it has been reported that following LPS exposure, norepinephrine, acting via β-adrenergic receptors, can also inhibit the production of TNF-α in macrophages (Ignatowski et al., 1996; Meltzer et al., 2004). Thus in addition to the vagally-mediated responses requiring CHRNA7 in target tissue, a synergistic relationship with sympathetic signaling may also exist in innate macrophage immune responses (Huston et al., 2006; Rosas-Ballina et al., 2008; Tracey, 2002, 2009).

Summary

Despite the clear evidence of sympathetic and parasympathetic innervation of the liver, the role that the autonomic nervous system plays in the control of hepatic energy metabolism remains unclear. There are still many details regarding the neuroanatomy of

the liver that remain to be addressed (Yi, la Fleur et al., 2010). To date, there is a paucity of data on the autonomic innervation of the nonhuman primate liver. While similarities exist in the hepatic innervation between man and the adult nonhuman primate (Forssmann et al., 1977; Nobin et al., 1978), the lack of more detailed knowledge in hepatic neuroanatomy or hepatic autonomic development in the nonhuman primate is a major obstacle for functional studies of liver metabolism in these animals.

In this study, we report that NPY nerve fibers are present and localized to the portal triad in fetal macaque liver. To our knowledge, this is a novel finding and underscores the importance of future inquiries into the hepatic development of autonomic innervation in the nonhuman primate. In addition, we report that robust sympathetic innervation is present in portal and parenchymal regions in the one-year old macaque. Further, we demonstrate that continuous exposure to a high-fat diet *in-utero* and through the post-natal period (HFD/HFD), is associated with decreased hepatic sympathetic innervation in parenchymal and portal regions in one-year old male macaques. We also report, that increased hepatic expression of inflammation is observed along with decreased expression of an integral component of the cholinergic anti-inflammatory pathway in the HFD/HFD juveniles. These data indicate that permanent changes in hepatic sympathetic innervation, and consequent functional effects, may be present in the HFD/HFD juvenile males. In addition, parasympathetic mechanisms shown to inhibit inflammation may be altered in the HFD/HFD animals.

MATERIALS AND METHODS

Macaque model of maternal overnutrition

All animal procedures have undergone an extensive review process and were in accordance with the guidelines of Institutional Animal Care and Use Committee of the Oregon National Primate Research Center (ONPRC) and Oregon Health & Science University. Our model has previously been described in detail (Grant et al., 2011; McCurdy et al., 2009).

Briefly, Japanese macaques matched for age (5-7 years at start) and weight (7-9 Kg) were randomly assigned to two dietary groups: 1: Control diet (CTR; 13% of calories from fat; Monkey Diet no. 5052, Lab Diet, Richmond, IN, USA) or 2: High-fat diet (HFD; 35.2% of calories from fat; Custom Diet 5A1F, Test Diet, Richmond, IN, USA). The HFD also included calorically dense treats made with peanut butter. The main source of fat in the CTR diet was soybean oil. The main sources of fat in the HFD were lard, animal fat, butter and safflower oil. For our fetal studies, we are reporting differences in HFD fetuses whose mothers were exposed to the maternal diet for at least four consecutive years. In the fifth year of our studies, a diet-reversal protocol (REV) was initiated to assess dietary impact independent of maternal obesity. This protocol entailed switching a subgroup of adult females that had been exposed to a high-fat diet for four consecutive years, to a control diet 1-3 months before becoming pregnant and throughout the pregnancy.

Manufacturers specifications provided for both diets show that the total metabolizable energy content of the CTR chow was 2.87 kcal/g and was apportioned at 26.8% energy from protein, 58.5% energy from carbohydrate, and 14.7% energy from

fat. The total energy content of the maternal HFD chow was 4.2 kcal/g and was apportioned at 16.7% energy from protein, 51.5% energy from carbohydrate, and 31.8% energy from fat. The animals were group housed and had *ad libitum* access to food and water.

Each maternal group was housed with two males so that pregnancies would occur during the yearly breeding season (November – February). The females were checked each successive year for pregnancies starting in November by ultrasound, which allows an estimate of gestational age ± 5 days. Twice a year the animals underwent IV glucose tolerance tests (IVGTT) (**Methods 3-S1**), once during the late summer (nonpregnant state) and once during the early 3rd trimester of pregnancy. All of the above procedures were done under ketamine sedation (5–10 mg/kg).

For our fetal studies, ONPRC veterinarians terminated singleton pregnancies from fasted dams by cesarean section at gestational day 130 (G130), as determined by ultrasound. All surgical procedures used in this study, were performed each scheduled day in an identical manner, following an *a priori* defined protocol in both technique and timing. After cesarean section, fetuses were deeply anesthetized with sodium pentobarbital (> 30 mg/kg i.v.) and exsanguinated. All peripheral tissues and brain were removed, weighed and stored for subsequent protein and RNA extractions or for histological analyses.

For our juvenile studies, the pregnancies were allowed to progress to natural birth. Infants and mothers were left undisturbed for the first 30 days after birth. On postnatal day 30, the offspring were weighed and dual energyX-ray absorptiometry (DEXA) scanned to examine body composition. Full-term offspring were maintained with their

birth mothers on the same diet as consumed during pregnancy until weaning. At postnatal day 180, the CTR and HFD offspring were weaned to create diet cohorts with the first dietary designation indicating the maternal diet *in-utero* and before weaning, and the second designation indicating the offspring's diet after weaning. The two juvenile diet groups examined in this study were CTR/CTR and HFD/HFD. The CTR/CTR cohort contained 7 males and 5 females and the HFD/HFD cohort contained 5 males and 4 females.

The mean age of the juvenile offspring at time of necropsy was 12.9 months with a 95% confidence interval of between 12.7-13.1 months. Necropsy was carried out by an *a-priori* protocol by the veterinary staff at the ONPRC. Briefly, the juvenile offspring were deeply anesthetized with sodium pentobarbital (> 30 mg/kg i.v.) and exsanguinated. All peripheral tissues and brain were removed, weighed and stored for subsequent protein and RNA extractions or for histological analyses. Head circumference and width as well as crown rump length were also included as a standard procedure in the necropsy protocol.

RNA extraction from tissue for Real-Time PCR

Total RNA was extracted from RNAlater (Ambion) stabilized primate fetal livers using an RNeasy Mini kit according to manufacturer's instructions (Qiagen, Appendix C, June 2001) with the following additions: Following disruption and homogenization of liver tissue, a 15 min Proteinase K incubation at 55 ^{o}C was performed. In addition, an optional on-column DNase treatment was also included. The RNA was eluted in RNase free water and the concentration and relative purity was determined by the 260/280 absorbance ratio. 1.3 ug of total RNA was reverse transcribed in a 75ul reaction using a

Taqman Reverse Transcription kit (Applied Biosystems N808-0234) according to manufacturer's protocol.

Primer design and sequences

Target sequence was determined by using both the NCBI macaque database and the Ensembl macaque database. Sequences from each database were compared and only regions that had 100% homology between databases were used for primer design. Primers sets were designed by Primer Express (Applied Biosystems) to span exon-exon junctions where feasible and produce amplicons of ~100 bp in length for Real-time PCR. An additional primer set for amplification of CHRNA7 was designed to produce an amplicon of 500 bp (**Supplemental Table 4-S1**).

Dynamic range and efficiency curves for Real-Time PCR

Macaque specific primer sets were evaluated to determine the efficiency of our primer sets within a working range of cDNA concentrations and identify an optimum concentration of cDNA to use in Real-time PCR assays. A cDNA dilution series was made from four random samples from each dietary group. The cDNA was diluted based on initial RNA concentration and the assumption of 100% reverse transcription efficiency. The dilution series was designed so that each primer set started at an upper limit of 50ng total cDNA/reaction and decreased in 10 ng increments to a minimum of 1ng/reaction (Grant et al., 2011; Pfaffl, 2001).

Primer validation Real-Time PCR reactions were run on an Applied Biosystems 7300 as relative quantification plates with SYBR master mix used at a 2x dilution. Following automatic thresholding and standard baseline adjustments after each run, Ct values were plotted as a function of the log(10) of the cDNA concentration and a linear

slope was calculated (ABI, 2006). Efficiency was calculated as 10^(-1/slope) and was used for our experimental quantification (Grant et al., 2011; Pfaffl, 2001). For experimental assays we chose a cDNA concentration that gave us Ct threshold values across all dietary groups of between 20 and 32 cycles. In cases where we could not detect the target of interest in any diet group; threshold Ct values >35, or SYBR fluorescence not rising above background at all, we confirmed the presence of the target and the specificity of our target primers on fetal spleen processed in an identical manner as our liver samples.

Real-Time PCR

Experimental Real-Time PCR reactions were run on an Applied Biosystems 7300 as relative quantification plates. Target and endogenous control primers were used at a final concentration of 471nM in a 21 μl reaction. SYBR master mix was used at the manufacturer's recommended 2x dilution. Dissociation curves were produced for every well to monitor primer amplification of a single target. Alg9 was used as an endogenous control for all our experiments. The Alg9 primer set was designed by core facilities at the Oregon National Regional Primate Center as an endogenous control for macaque gene expression analysis, and subjected to extensive gene stability validation in our model by use of the geNorm VBA applet (Kidd et al., 2007; Vandesompele et al., 2002).

Relative quantification of target gene expression was calculated for each animal using empirically derived efficiency values for each primer set and calculating an efficiency-corrected fold by the following formula:

$$\text{Fold} = (E_{target})^{\Delta Ct(\text{target control- target sample})} / (E_{endo})^{\Delta Ct(\text{endo control- endo sample})}$$

where E is the respective primer efficiency. The ΔCt_{target} was calculated by choosing one calibrator sample from the control diet group and subtracting subsequent target Ct values from that calibrator across all groups. In addition, ΔCt_{endo} was calculated by using endogenous control Ct values for the same calibrator sample as above, and subtracting subsequent endo Ct values from that calibrator (Grant et al., 2011; Pfaffl, 2001). Data was tested for significance between diet groups by a Kruskal-Wallis rank sum with STATA (College Station, Texas). Data is expressed as group mean fold ± SEM for each diet group. All graphs were made with Prism software (GraphPad Software, Inc., La Jolla, CA).

In instances where the optimum cDNA concentration of our endogenous control differed from our target, we produced cDNA dilutions for the endogenous control and target from the same reverse-transcriptase reaction. Following the Real-Time PCR reaction random target well reactions from each diet group were run on a 2% agarose gel to verify amplicon singularity and size. Bands of the expected size were excised, gel purified (Qiaquick gel extraction kit, Qiagen #28706) and sequenced. Target specificity was confirmed by BLAST and comparing amplicon sequence with the NCBI macaque database. Fetal studies were performed with 7 CTR, 8 HFD and 7 REV animals. Juvenile studies were performed with 7-10 CTR/CTR and 8-10 HFD/HFD animals.

Immunohistochemistry

For detection of NPY immunoreactivity in fetal macaque liver, a sheep polyclonal NPY antibody (Cat. #AB1583, Lot # JC1676120, Millipore, Temecula, CA), raised against synthetic NPY peptide conjugated to bovine thyroglobulin, was used (1:4000). For detection of tyrosine hydroxylase immunoreactivity in juvenile macaque liver, mouse

monoclonal ascites raised against tyrosine hydroxylase purified from PC12 cells was used (Cat. # MAB318 - clone LNC1, Millipore, Temecula, CA, 1:200). Both antibodies have been previously characterized for specificity in the macaque (Barraud et al., ; Glavas et al., 2008; Kanaan et al., 2007; Sanchez-Gonzalez et al., 2005).

Liver tissue postfixed in 10% zinc formalin was embedded in paraffin and sectioned at 16 μm. The sections were deparaffinized with xylene and rehydrated in a descending alcohol series (100%, 70%, 50%) and briefly rinsed rinsed in ddH_2O. Heat induced epitope retrieval (HIER) was performed on sections with a citrate based antigen unmasking solution (100X, Vector Laboratories, cat.# H-3300) in ddH_2O and heating sections to 250°F for approximately 15 minutes in a commercial autoclave. Following HIER, sections were washed in 1X PBST (1X PBS with 0.05% Tween 20) and blocked for 1 hour at room temperature in 5% normal serum (NPY; donkey, TH; goat) diluted in blocking buffer. The blocking buffer consisted of 1X PBS containing 1% BSA, 0.1% Triton X-100 and 0.05% Tween 20. Primary antibody was diluted in blocking buffer supplemented with the appropriate serum and was incubated for 48 hrs at 4°C in a humidified chamber. Sections were then washed in 1X PBST and blocked again for 30 min at room temperature in blocking buffer supplemented with 5% serum. The blocking reagent was quickly aspirated off each section and the appropriate secondary antibody (Molecular Probes, Invitrogen), diluted 1:500 in PBST, was applied for 1 hr at room temperature. For the NPY study an Alexa-fluor donkey anti-sheep 555 secondary antibody was used. An Alexa-fluor goat anti-mouse 555 secondary antibody was used for the TH study. For the doubled labeled study a cocktail of donkey anti-sheep 555 and donkey anti-mouse 488 was used for NPY and TH respectively. Following secondary

antibody incubation, sections were washed in 1X PBST, rinsed quickly with ddH_2O and coverslips were applied using elvanol mounting medium.

Microscopy, Imaging

For both the fetal and juvenile studies, liver sections were blinded and imaged with a C-APO 40X 1.2W Corr M27 objective on an LSM710 laser-scanning confocal microscope using Zen software for acquisition (Carl Zeiss, Thornwood, NY). A lambda scan was performed on unstained liver tissue, processed in an identical manner, to determine spectra contributing to autofluorescence in our samples. Based on the lambda scan, the excitation spectra for imaging the Alexa-Fluor 555 secondary was adjusted to 566- 614 nm. This excitation spectra adjustment had minimal effects on target immunofluorescence but greatly reduced non-specific tissue autofluorescence in our samples.

To minimize bias between samples, we imaged portal and parenchymal regions according to specific criteria. Utilizing epifluorescence from the GFP channel, which contained no visual emission signal from our alexa-fluor 555 secondary, we visually identified portal triads that: 1) contained a distinct portal vein, hepatic artery and bile canniculi and 2) would fit cleanly into our field of view. Thus, we were able to compare portal triads of similar size across our dietary groups. For the parenchymal investigation, we randomally chose regions, again using epifluorescence from the GFP channel, of hepatic parenchyma that had no discernable structural elements other than sinusoids.

Image acquisition of nerve fibers was performed with a 561nm laser and acquisition parameters were empirically determined to maximize the signal-to noise ratio of the 555 fluorophore without saturating individual pixel signal intensity. *Z*- stacks,

with a step-size of 1μm/section, were aquired for each target region using as many optical sections as necessary to image through the entire thickness of each liver section. For both fetal and juvenile studies, we attempted to acquire at least 10 images for regions (portal triad, parenchyma) conforming to our selection criteria for each animal. However, due tissue sample size, it was not always possible to acquire 10 images for each region for each animal. All sections for the fetal and juvenile studies were acquired in an identical manner.

Quantification of nerve fiber density

Fetal and juvenile sympathetic nerve fiber density was quantified in an identical manner. Sympathetic nerve fiber density was normalized to hepatic volume using Imaris 7.1 image analysis software (Bitplane, Zurich Switzerland). *Z*- stacks for each animal were imported into Imaris and volumes were calculated for hepatic tissue volume and sympathetic immunoreactivity. Background autofluorescence was utilized to calculate the volume of hepatic tissue in each image by constructing a volume object with the following parameters: Smoothing 5 μm, background 9 μm, threshold .250, number of voxels above 10. Our parameters were optimized to create a volume that included all hepatic tissue within a given image, yet for portal regions, did not include the empty space of the portal vein. An additional volume object was constructed to determine the volume of sympathetic immunoreactivity present in each image by the following parameters: Smoothing 0.2 μm, background 0.5 μm, threshold 5, maximum intensity 35, number of voxels above 10. Each image was visually inspected for the integrity of volume calculations before results were exported into Excel. The nerve fiber volume was then normalized to total hepatic volume for each image.

Our samples were unblinded and a median normalized nerve fiber density was calculated for each animal and then compiled into the appropriate dietary groups. These data were then tested for significance by Kruskal-Wallis rank sum with STATA (College Station, Texas). Data is expressed as the median normalized nerve density for each respective diet group. All graphs were made with Prism software (GraphPad Software, Inc., La Jolla, CA).

Reverse transcriptase-PCR

For reverse transcriptase-PCR (RT-PCR) of CHRNA7, 1.3 ug of total RNA prepared from two 1 year old Japanese macaque livers was reverse transcribed in a 75ul reaction using a Taqman Reverse Transcription kit (Applied Biosystems N808-0234) according to manufacturer's protocol. 50 ng of cDNA was amplified using Taq platinum polymerase in a 50 μl reaction. 200 pmol of the 5′ and 3′ primers were used in a Touchdown protocol to amplify a single 500 bp band (95°C for 2 min., [(95°C for 30 s, 64°C for 30 s, 72°C for 1 min) x 20 cycles], 95°C for 30 s, [(54°C for 30 s, 72°C for 1 min) x 20 cycles], 72°C for 2 min, 4°C hold). The primers used for this reaction are listed as $CHRNA7^{(500)}$ and are found in (**Supplemental Table 4-S1**). PCR reaction products were run on a 1.5% agarose gel to verify amplicon singularity and size. Bands of the expected size were excised, gel purified (Qiaquick gel extraction kit, Qiagen #28706) and sequenced. Target specificity was confirmed by BLAST and comparing amplicon sequence with the NCBI macaque database.

RESULTS

Hepatic Inflammation in HFD/HFD Juvenile Macaques

Previous work with this model suggested that non-alcoholic fatty liver disease (NAFLD) is present in the HFD fetal liver. McCurdy et al. demonstrated oxidative damage, hepatic steatosis, and upregulation of phospho-JNK1 that was highly correlated with levels of fetal liver triglycerides were present in the HFD fetal livers (McCurdy et al., 2009). In recent work, we investigated whether inflammation and consequent evidence of non-alcoholic steato-hepatitis (NASH) was present in the HFD fetal liver. We found no differences in the expression of any of the inflammatory markers we assayed in the fetal liver. However, we reported that significantly increased hepatic apoptosis was observed in the HFD fetal liver when compared to CTR. The apoptosis was returned to CTR levels following maternal diet-reversal during pregnancy (Grant et al., 2011). Taken together, these findings suggest the presence of severe NAFLD is present in the HFD fetal liver.

To extend these findings, we used Real-time PCR to evaluate the expression of inflammatory markers between the CTR/CTR and HFD/HFD diet groups in the juvenile liver (**Table 4-1**). No differences were observed in the expression of interleukin-1β, interleukin-10, tumor necrosis factor-α (TNF-α), interferon-γ, monocyte chemotactic protein-1(MCP-1, CCL2), or interferon-inducible T-cell alpha chemoattractant (I-TAC, CXCL11) between CTR/CTR and HFD/HFD diet groups. We found a significant upregulation in the expression of lymphotoxin-α (LTA, $\chi^2 = 5.1$, 1 d.f., $p = 0.02$) in the HFD/HFD group when compared to CTR/CTR. In contrast, we observed a significant reduction in the expression of Arginase-1 (Arg-1, $\chi^2 = 6.2$, 1 d.f., $p = 0.01$) in the

HFD/HFD juvenile liver when compared to CTR/CTR. Arginase-1 is a marker of Th_2 macrophage activation (Gordon, 2003; Lumeng et al., 2007), and may suggest a Th_1 response is prevailing in the HFD/HFD liver. C-reactive protein (CRP) was not differentially expressed in the juvenile liver when the data was analyzed without regard to the sex of the animal. When the analysis was performed separately for male and female offspring, a significant increase in CRP expression was observed in the female juvenile livers ($\chi^2 = 3.8$, 1 d.f., $p = 0.05$, **Supplementary Figure 4-S1**).

Phenotypic Changes in Juvenile Offspring

We previously reported in this model, that decreased levels of total N3 fatty acids and DHA as well as an elevated N6:N3 ratio were present in the maternal and fetal plasma and maternal breastmilk in the HFD diet group. In addition, high levels of apoptosis were observed in the fetal liver (Grant et al., 2011). These results suggest that gross brain and liver growth may be affected by exposure to a HFD either *in-utero* or after birth. Thus, we examined whether differences in head circumference or liver weight were different between the CTR/CTR and HFD/HFD juvenile offspring. We utilized occipito-frontal head circumference measurements, an established metric for brain development and neurological outcomes (Hack et al., 1991; Hansen et al., 2000; Stathis et al., 1999), and found that the HFD/HFD juvenile had a significantly reduced mean head circumference when compared to CTR/CTR animals (CTR/CTR = 24.61 ± .32 cm versus 23.86 ± .30 for HFD/HFD, (mean ± SEM) $\chi^2 = 3.8$, 1 d.f., $p = .05$). In addition, liver weight normalized to total body weight was significantly lower in the HFD/HFD animals when compared to CTR/CTR (CTR/CTR = 0.0262 ± 0.0004 versus 0.0249 ± 0.0005 for HFD/HFD,(mean ± SEM) $\chi^2 = 4.8$, 1 d.f., $p = .03$).

Gene Expression of Autonomic Targets in Fetal and Juvenile Liver

To investigate the effects that a maternal HFD has on the development of the autonomic nervous system in the nonhuman primate liver, we used Real-time PCR as a screening tool to identify differential expression of autonomic targets in both fetal and one-year old juvenile livers. In addition, we also included glucose transporter 2 (GLUT2) for its known role in vagally-mediated glucose sensing in the portal vein (Burcelin et al., 2000).

In the fetal liver, we found no changes in gene expression for adrenergic alpha 1A, 1B and 2A (ADRA1A, ADRA1B, ADRA2A) receptors. No differences were also observed across our three fetal dietary groups for cannabinoid 1 and 2 (CB1R, CB2R) receptors, the nicotinic cholinergic receptor alpha 7 subunit (CHRNA7) and GLUT2. Interestingly, we found that the expression of neuropeptide-Y Y1 receptor (NPYY1R) was significantly downregulated in the diet-reversed fetuses (REV) when compared to the HFD fetuses. There was no statistical difference in NPYY1R expression between CTR and REV fetal livers (**Table 4-2**).

In juvenile livers, again we found no changes in gene expression for adrenergic alpha 1A, 1B and 2A receptors. No differences were also observed for CB2R. Surprisingly, CB1R was not expressed in juvenile liver. This assay was performed with the identical primers used in our fetal assay and utilized cDNA and SYBR master mix that was successfully used for other autonomic targets. These data suggest that hepatic CB1R expression may be developmentally regulated in the nonhuman primate. We found no differences for NPYY1R and GLUT2 between the CTR/CTR and HFD/HFD animals. CHRNA7 expression was significantly lower in the HFD/HFD livers when

compared to CTR/CTR (χ^2 = 10.5, 1 d.f., p = 0.001), (**Table 4-3**).

While we routinely sequenced amplicons from our Real-Time PCR studies to confirm primer specificity, we also examined α7 subunit mRNA expression in the macaque liver by RT-PCR and sequence analysis. We developed primers complementary to the known macaque α7 sequence and overlapping the region we used for Real-Time PCR, performed RT-PCR with two randomly chosen juvenile samples and observed the expected 500 base pair band following electrophoresis (**Supplementary Figure 4-S2**). The resulting bands were gel-purified and sequenced. Sequence results compared with the NCBI macaque database confirmed the presence of authentic α7 subunit RNAs in the juvenile macaque liver.

Fetal Sympathetic Innervation

The distribution of Neuropeptide-Y (NPY) containing nerve fibers was evaluated in G-130 fetal liver by immunohistochemistry. NPY is a neuropeptide that has been shown to have a similar distribution and co-localization with tyrosine hydroxylase (TH) immunoreactive fibers in the mammalian liver (Burt et al., 1989; Goehler et al., 1991). Thus, it is a marker of sympathetic innervation. In addition, previous work in humans reported that NPY containing fibers were not detected in fetal liver and were only expressed in term and adult human livers (Shimazu, 1996). Our results show that NPY immunoreactive fibers are present in fetal macaque liver (**Figure 4-1**). The distribution of NPY immunoreactive fibers are localized to the portal triads at this gestational age as only in a few isolated instances were NPY-ergic fibers found in the hepatic parenchyma. A double-labeled immunofluorescence study was performed in a subset of fetal samples to qualitatively evaluate the colocalization of NPY with TH in the fetal macaque liver. A

representative image from this study shows that NPY is closely associated with TH immunoreactivity in the fetal macaque liver (**Figure 4-2**). TH and NPY immunofluoresence are localized to the portal triad with very little penetration into the surrounding lobule.

Maternal High-Fat Diet and Sympathetic Innervation in the Fetal Macaque

The density of NPY immunoreactive fibers was quantified between the CTR, HFD and REV diet groups to determine if differences were present in sympathetic innervation in the fetal liver. The volume of NPY immunofluorescence was normalized to the volume of hepatic tissue, less the lumen of the portal vein and other gaps in the tissue, to account for slight differences in the size of individual portal regions. Our results show that no differences exist in the density of NPY/sympathetic periportal innervation between the CTR, HFD and REV fetal diet groups (**Figure 4-3**).

Sympathetic Innervation in the Juvenile Macaque Liver

TH immunohistochemistry was utilized to evaluate the distribution of sympathetic innervation in one-year old juvenile macaque. In addition, we performed a quantitative evaluation of the effects that exposure to a HFD *in-utero* and in postnatal life had on hepatic sympathetic innervation these animals. In contrast to fetal liver, TH immunoreactivity was observed not only in the portal triad, but robust innervation of the hepatic parenchyma was also found in CTR/CTR liver. We observed that TH positive fibers were distributed throughout the stromal compartment of the triad, with concentrations of fibers localized around the portal vein, hepatic artery and bile duct. From the distribution of fibers in the portal triad, TH positive fibers were observed extending into the hepatic parenchyma (**Figure 4-4**). A qualitative assessment in the

CTR/CTR livers suggested that the density of parenchymal TH fibers were higher in the periportal region and less abundant in the distal lobule. No TH immunoreactivity was observed in association with the central vein (data not shown).

We evaluated the co-localization of TH and NPY immunoreactivity in the juvenile liver by double-labeled immunohistochemistry. As in fetal tissue, we observed clear co-localiation on NPY immunoreactive fibers with TH immunoreactive fibers in the portal triad (data not shown). Robust NPY and TH immunofluorescence was also present and clearly co-localized in the hepatic parenchyma of juvenile macaques (**Figure 4-5**).

High-Fat Diet and Sympathetic Innervation in the Juvenile Macaque

We quantified the density of TH immunoreactive fibers between the CTR/CTR and HFD/HFD juvenile diet groups to determine if differences were present in sympathetic innervation in the juvenile liver. We quantified and compared TH immunoreactivity from periportal regions as well as parenchymal regions, the latter randomly selected by the lack of hepatic structures other than sinusoids. As in the fetal studies, the volume of TH immunofluorescence was normalized to the volume of hepatic tissue, less the lumen of the portal vein and other gaps in the tissue, to account for slight differences in the size of individual portal regions.

Sexually dimorphic differences in sympathetic innervation between CTR/CTR and HFD/HFD juvenile liver were observed in both periportal and parenchymal hepatic regions. Without regard to the sex of the animal, no differences were seen in either periportal or parenchymal regions between CTR/CTR and HFD/HFD animals (**Figure 4-6**). When separated by sex, females displayed no differences in hepatic innervation in either periportal and parenchymal regions (**Figure 4-7**). Males on the

other hand, had significantly lower levels of sympathetic innervation in both periportal and parenchymal regions (**Figure 4-8**). We found no associations with these outcomes with maternal obesity or insulin resistance.

DISCUSSION

The present study focused on the impact that a maternal high-fat diet had on hepatic innervation in fetal and juvenile macaques. We evaluated changes in hepatic innervation by quantitative measurements of sympathetic nerve fiber density using immunohistochemical methods. Qualitative measures of hepatic sympathetic development were also examined. Real-Time PCR was also used in both fetal and juvenile macaque to determine the expression of autonomic nervous system receptors and their potential regulation by exposure to a HFD. In addition, expression of inflammatory markers were also explored in the juvenile animals to extend findings of NAFLD and hepatic apoptosis previously reported in HFD fetuses (Grant et al., 2011; McCurdy et al., 2009).

In the HFD/HFD juvenile liver we found increased expression of lymphotoxin-α (LTA). LTA, previously known as TNFβ, is a soluble factor produced by lymphocytes that is cytotoxic to tumor cells (G. A. Granger et al., 1968). In addition to being a soluble factor, LTA is also a component of a membrane heterocomplex with lymphotoxin-β (LTB) (Androlewicz et al., 1992; Browning et al., 1993). Lymphotoxin signaling has been shown to be involved with lymphoid organogenesis, splenic architecture, natural-killer (NK) and natural killer-T cell (NKT) differentiation, formation of tertiary lymphoid tissue and inflammation (S. W. Granger, Ware, C.F., 2003). In the present study, we

extracted mRNA from tissue homogenates. We believe that LTA is involved in a pro-inflammatory response to the HFD. However, distinguishing soluble signaling mechanisms from membrane type signaling mechanisms for this cytokine was not possible with our preparation.

In addition to increased expression of LTA in the HFD/HFD juvenile liver, we also observed decreased expression of Arginase-1. Increased arginase-1 is a marker of Th_2 macrophage activation (Gordon, 2003; Lumeng et al., 2007), thus a decrease suggests that a shift towards a Th_1 macrophage response is present in the HFD/HFD liver. While tissue resident macrophages are likely activated across a continuum somewhere between the polarized ends of the Th_1/ Th_2 spectrum, Th_1 activated macrophages are associated with increased pro-inflammatory cytokine production and increased production of reactive oxygen species (Lumeng et al., 2007). Evidence of oxidative damage was previously reported with this model in fetal liver (McCurdy et al., 2009). In addition, Th_1 type, pro-inflammatory activation of Kupffer cells is associated with fatty liver disease and obesity- induced insulin resistance in rodents (Marceau et al., 1999; Odegaard et al., 2008; Pelleymounter et al., 1995).

NK and NKT cells comprise a large proportion of the total lymphocyte populations in the liver and can participate in innate immune responses without prior antigenic stimulation (Z. Li et al., 2003). Following activation, NK and NKT cells can elicit either pro-inflammatory or anti-inflammatory responses that are modulated by Kupffer cells (Chen et al., 1997; Z. Li et al., 2003). In addition, consumption of a high-fat diet in mice leads to a depletion of hepatic NKT cells and an increase in Th_1 cytokine production that promotes sensitivity to LPS induced hepatotoxicity (Z. Li et al., 2005).

Other factors such as leptin and norepinephrine also regulate NKT populations in the rodent liver (Guebre-Xabier et al., 2000; Howard et al., 1999; Minagawa et al., 2000). Following chemical sympathectomy or application of an α-adrenergic receptor antagonist, hepatic NKT populations are significantly reduced in normal mouse livers. Alternatively, norepinephrine treatment significantly attenuated the increased NKT apoptosis normally observed in *ob/ob* mice (Z. Li et al., 2003). Thus, NKT mediated inflammation may also be present in juvenile HFD/HFD liver.

It is currently accepted that pathologies related to poor nutrition and obesity are associated with sustained low-grade inflammation in the circulation and in metabolic tissues (Barzilaym et al., 2003; Berg et al., 2005; Hotamisligil, 2006; Petersen et al., 2006; Pickup, 2004; Sutherland et al., 2004). Selective constitutive activation of NFκB signaling in hepatocytes of transgenic mice produced low-grade inflammation with elevations of TNF-α and IL-1β in the liver. In addition, impairments in hepatic insulin signaling and insulin-dependent glucose metabolism in the liver indicated that these mice had developed severe hepatic insulin resistance (Cai et al., 2005). In addition to the significant increase in LTA expression, our results also show trends for increased hepatic expression of TNF-α, IL-1β and C-reactive protein (CRP) in the HFD/HFD diet group. CRP is one of several NFκB dependent inflammatory mediators that are produced by hepatocytes in liver tissue (Cai, 2009; Patel et al., 2007).

Interestingly, hepatic CRP expression was significantly increased only in the female HFD/HFD juveniles in our model. However, a trend for higher expression was observed across both sexes in the HFD/HFD juvenile liver. We observed a large standard deviation for HFD/HFD CRP expression in the females, with higher than 15 fold

expression of CRP over the CTR/CTR reference sample, in two of the four total females in the HFD/HFD group. The reason for the large standard deviation within the HFD/HFD females as well as the apparent sexual dimorphism of CRP expression is unclear.

Taken together, the cumulative result of small increases in many pro-inflammatory mediators, along with increased hepatic expression of LTA, indicates a state of sustained low-grade inflammation is present in the HFD/HFD liver, although we did not define the cell type(s) responsible for the inflammation. These data also extend previous fetal studies with this model and suggest that non-alcoholic steato-hepatitis (NASH) was present in the HFD/HFD juvenile liver (Grant et al., 2011; McCurdy et al., 2009).

While the liver was the primary focus of this study, we previously reported that decreased levels of total N-3 fatty acids and DHA as well as an elevated N6:N3 ratio were present in the maternal and fetal plasma and maternal breastmilk in the HFD diet group (Grant et al., 2011). The N-3 fatty acids, particularly DHA are essential dietary nutrients that are critical for proper infant growth and neurodevelopment. DHA is highly enriched in neural tissue and is the major component of retinal photoreceptor membranes, cerebral cortex, testis and sperm (Clandinin et al., 1980a, 1980b; M. Martinez, 1992; Neuringer et al., 1984). Previous work with this model demonstrated that exposure to the maternal HFD *in-utero* led to changes in the melanocortin system of the hypothalamus and the serotonin system in the brainstem (B. E. Grayson, Levasseur et al., 2010; Sullivan et al., 2010). In addition, HFD offspring examined in the post-natal period before weaning, displayed significant behavioral changes (Sullivan et al., 2010). We reasoned

that decreased availability of DHA and N-3 fatty acids *in-utero* and during the post-natal period may effect brain development, which would be reflected in changes in occipito-frontal head circumference measurements, an established metric for brain development and neurological outcomes (Hack et al., 1991; Hansen et al., 2000; Stathis et al., 1999). We found that the HFD/HFD juveniles had significantly reduced mean head circumferences. These findings may indicate that structural changes as well as changes in important neuro-circuitry may be present in the brains of the HFD/HFD juvenile animals.

It has been reported that a linear increase in the percent of DHA and a decrease in the percent AA, is observed from week 30 to week 44 post-conception indicating selective accretion of N-3 fatty acids into the developing human liver (M Martinez, 1989). We previously reported that in addition to decreased levels of DHA and total N-3 fatty acids observed in the HFD fetal plasma and maternal breastmilk, significantly increased levels of hepatic apoptosis were observed in the developing fetal liver (Grant et al., 2011). Increased levels of fetal hepatic apoptosis and reduced availability of important structural fatty acids may impact hepatic development in a way that would be reflected in the wet weight of the liver itself. We observed a significant decrease in the HFD/HFD liver weight, normalized to total body weight, which indicates structural changes are present in the livers of these animals.

We investigated the expression levels of numerous neurotransmitter and neuropeptide receptors in both fetal and juvenile liver to determine if exposure to a maternal HFD was associated with differential expression. In the fetal liver we found that every receptor we assayed was expressed. We found no changes in expression of our

fetal targets between diet groups except for one. We found a significantly decreased expression of the NPY Y1 receptor in the REV fetal diet group. In the juvenile liver, all neurotransmitter and neuropeptide targets studied were expressed at detectable levels, with the exception of CB1R . Collectively, our results demonstrate that the fetal and juvenile liver is innervated and contains a robust complement of neurotransmitter and neuropeptide specializations.

Increasing evidence has implicated the cannabinoid system in the pathophysiology of liver diseases demonstrating involvement with hepatic injury and with complications of cirrhosis (Mallat et al., 2008). In rodents and humans upregulation of hepatic CB1R is associated with non-alcoholic fatty liver disease and cirrhosis, while CB2R promotes antifibrogenic effects associated with chronic liver injury (Mallat et al., 2006). We found no differences in expression of CB2R in juvenile liver between the CTR/CTR and HFD/HFD diet groups. In addition we observed that CB1R was no longer expressed in each diet group as it was in fetal liver. We believe this may reflect species-specific expression and developmental regulation of CB1R in the juvenile macaque. In addition, we found no changes in gene expression for adrenergic alpha 1A, 1B and 2A receptors between juvenile diet groups. These findings suggest that transcriptional expression of receptors of the sympathetic nervous system is unaltered in the HFD/HFD juvenile animals.

Importantly, we found that expression of CHRNA7, a critical component of the cholinergic anti-inflammatory pathway, was significantly reduced in the HFD/HFD juvenile liver. As part of the efferent arm of the inflammatory reflex, acetylcholine release in target tissue by the vagus binds CHRNA7. Signal transduction in macrophages

involves pleiotropic signaling cascades that regulate the activation of NF-κB through the inhibition of phosphorylation of IκB (Yoshikawa et al., 2006). In addition, the JAK2/STAT3 pathway is also activated, inhibiting NF-κB binding to DNA and increasing the expression of suppressor of cytokine signaling (SOCS3) (de Jonge et al., 2005). The net result in macrophages is selective inhibition of inflammatory cytokines with preservation of anti-inflammatory cytokine production (Borovikova et al., 2000; Parrish et al., 2008; Pavlov et al., 2007). While we were unable to conclusively determine the hepatic localization of CHRNA7 in the juvenile liver, we demonstrated through RT-PCR that authentic macaque CHRNA7 is present in the juvenile liver. Our results suggest that components of the hepatic parasympathetic nervous system are altered HFD/HFD juvenile liver. In addition, the presence of increased hepatic inflammation indicates that cholinergic anti-inflammatory pathway responses are altered in the HFD/HFD animals.

We compared the density of NPY fibers in fetal liver between our three diet groups; CTR, HFD and REV. In addition, we evaluated the distribution of NPY containing nerve fibers in the fetal liver. Previous work in humans reported that NPY containing fibers were not present in fetal liver and were only expressed in isolated cases at term in portal regions. However, in adult human livers, NPY innervation was observed in both parenchymal and portal regions (Dina G Tiniakos, 1996; D. G. Tiniakos et al., 2008). We found that robust NPY innervation is present in the fetal macaque liver and is localized to the portal triad. To our knowledge, this is the first demonstration of NPY innervation in the fetal macaque liver. In addition, we observed very little penetration of NPY fibers into the hepatic parenchyma and found no differences in the density of NPY

nerve fibers between the fetal diet groups.

We also observed very good co-localization with NPY and TH in the fetal liver that was localized to the portal triad with little penetration into the hepatic lobule. NPY is a neuropeptide that has previously been shown to be co-localized with tyrosine hydroxylase (TH) and to have a similar distribution as TH immunoreactive fibers in the mammalian liver (Burt et al., 1989; Feher et al., 1991; Goehler et al., 1991). In addition, hepatic sympathetic nerve stimulation leads to release of noradrenaline and NPY in the canine and rodent liver (Shimazu, 1996; Taborsky et al., 1994). Our findings suggest that NPY/sympathetic innervation is present in the early third trimester and localized to the portal region in fetal macaque liver.

In the juvenile liver, we evaluated the distribution of TH immunoreactive nerve fibers and observed robust TH immunoreactivity in the portal triads as well as the hepatic parenchyma. In addition, we extended our fetal findings and demonstrated that TH and NPY immunoreactivity are colocalized in portal regions and the hepatic parenchyma in the juvenile macaque. These data suggest that sympathetic and peptidergic innervation of the hepatic lobule occurs after the third trimester in the Japanese macaque. This finding is in good agreement with previous studies in humans that demonstrate that intralobular innervation occurs after birth (D. G. Tiniakos et al., 2008)

Importantly, we compared the density of TH nerve fibers, as a marker of sympathetic innervation, between the CTR/CTR and HFD/HFD juvenile diet groups. We observed significant decreases in sympathetic innervation in the portal and parenchymal regions of male HFD/HFD livers. In contrast, a trend for higher sympathetic innervation in both hepatic regions was observed in the female HFD/HFD liver. These findings

suggest a gender-specific effect is involved in hepatic sympathetic innervation in the macaque. The functional significance of this decrease in sympathetic innervation has yet to be evaluated in our model.

Limited data exists on effects that NAFLD or NASH has on heptatic innervation. Some work has been done with the effects that liver cirrhosis and hepatitis have on hepatic nerves with conflicting results. For example, proliferation of autonomic nerve fiber density and NPY nerve fibers were observed in the portal tracts of human patients with chronic active hepatitis and in the fibrous septa of patients with cirrhosis (Miyazawa et al., 1988). In contrast, others have reported the dissapearence of parenchymal nerve fibers in human patients with liver cirrhosis (Jaskiewicz et al., 1994; J. A. Lee et al., 1992). Given the evidence of NAFLD/NASH in the fetal liver and the increased inflammation observed in the juvenile liver, we cannot rule out that decreases in hepatic sympathetic innervation, secondary to liver disease, has occurred in the juvenile HFD/HFD animals in a sexually dependent manner.

We must also consider that the developmental program regulating the maturation of the hepatic nervous system and penetration of sympathetic nerves into the hepatic parenchyma may be altered by continuous exposure to a HFD in the perinatal period. There is very little data on the ontogeny of the hepatic nervous system in humans (Dina G Tiniakos, 1996; D. G. Tiniakos et al., 1996; D. G. Tiniakos et al., 2008), and virtually none reported in the nonhuman primate, with studies limited to adult tissue (Forssmann et al., 1977). Consequently, a paucity of data exists related to the mechanisms involved in the development of the hepatic nervous system in higher-order primates (Delalande et al., 2004).

Some work has been done on the expression of axon guidance molecules in the liver. *Sema4g* has been described as having strong expression in the developing liver of mouse embryos that persisted into adulthood. *Sema4g* is a membrane bound protein that most closely resembles class IV members of the semaphorin gene family (H. Li et al., 1999). While there are five distinct classes of semaphorins, their roles are best characterized as chemorepulsive factors involved in axon guidance in the CNS (Mark et al., 1997). Recently, it was reported that the expression of a number of semaphorins were significantly upregulated in the livers of morbidly obese adult humans with histologically severe steatosis and NASH (Bertola et al., 2010). While our study did not address the expression of axon guidance molecules in the liver, the above findings provide a logical starting point for future studies.

Gender-related differences in response to various types of stress have been demonstrated in the livers of rodent and humans (Colantoni et al., 2003; Harada et al., 2001; Poynard et al., 2003). In addition, the prevalence of NAFLD is higher in men (Suzuki et al., 2009), as is the prevalence of NASH in morbidly obese human patients (Bertola et al., 2010). A number of differences, including the levels of circulating sex hormones, growth hormone secretion, and hepatic expression of sex hormone receptors, have been proposed to mediate gender-specific pathophisiology in the liver (Eden, 1979; Francavilla et al., 1984; Francavilla et al., 1986).

In the rhesus macaque, morphological sexual differentiation occurs early in the prenatal period. In addition the fetal testes become steroidogenically active during this time (Resko, 1985; Wallen, 2005). Female fetal ovaries have been reported to be inactive in the prenatal period (Ellinwood et al., 1982). After birth, one-year old rhesus monkeys

display sexually differentiated patterns of behavior (Lovejoy, 1988). While the sexually differentiated patterns of behavior observed in the juvenile are initiated from a complex convergence of social and hormonal cues (Wallen, 2005), it provides support to the idea that differences in sex hormones may be involved in the sexually dimorphic changes we observed in hepatic sympathetic innervation in the juvenile macaque.

This study extends previous work with this model (Grant et al., 2011; B. E. Grayson, Levasseur et al., 2010; McCurdy et al., 2009; Sullivan et al., 2010), and suggests that chronic exposure to a HFD from conception through one year of age is associated with profound changes in the juvenile liver. Hepatic expression of inflammatory cytokines in the juvenile suggests that NAFLD is present in the HFD/HFD animals. In addition, significant changes in hepatic sympathetic innervation suggest that hepatic function is altered in male HFD/HFD offspring. In support of this idea, a metabolic phenotype has been observed in HFD/HFD juvenile males (K.L. Grove, personal communication). In good agreement with our previous work, our findings in this study were independent of maternal obesity and maternal insulin resistance (McCurdy et al., 2009). Taken together, these findings reinforce the impact that continuous exposure to a high-fat diet has in the development of hepatic pathologies and underscores the important public health implications of a modern diet in the emerging obesity epidemic.

Table 4-1.

Inflammatory marker mRNA expression in juvenile liver[1]

	DIET GROUP				
	CTR		HFD		
Target	Mean	SEM	Mean	SEM	*p*-value[2]
Arginase-1	**1.67**	0.15	**1.09**	0.15	**0.01**
C-Reactive protein	2.95	0.86	6.50	1.95	0.13
Interferon-γ	1.03	0.15	0.93	0.13	0.73
Interleukin-1β	0.97	0.14	1.23	0.17	0.35
Interleukin-10	1.38	0.22	1.09	0.15	0.33
I-TAC (CXCL11)	0.60	0.09	0.77	0.15	0.64
Lymphotoxin-α	**1.02**	0.11	**1.47**	0.16	**0.02**
MCP-1 (CCL2)	0.70	0.12	0.65	0.04	0.73
Tumor Necrosis Factor-α	0.93	0.13	1.40	0.21	0.15

[1] All values are means ± SEMs and are expressed as relative fold to CTR calibrator sample. n = 7-10 for CTR, n = 8-10 for HFD.
[2] Overall significance as determined by Kruskal-Wallis rank sum test.

Table 4-2.

Peripheral Nervous System mRNA expression in Fetal Macaque Liver[1]

		DIET GROUP						
		CTR		HFD		REV		
Target	Common Name (Type)	Mean	SEM	Mean	SEM	Mean	SEM	*p*-value[2]
ADRA1A	adrenergic, alpha-1A-, receptor	0.91	0.06	0.93	0.11	0.85	0.12	0.69
ADRA1B	adrenergic, alpha-1B-, receptor	0.89	0.05	1.00	0.09	1.08	0.10	0.28
ADRA2A	adrenergic, alpha-2A-, receptor	0.67	0.07	0.63	0.07	0.79	0.07	0.33
CB1R	cannabinoid 1 receptor	0.31	0.10	0.47	0.14	0.39	0.08	0.30
CB2R	cannabinoid 2 receptor	0.73	0.11	0.85	0.18	1.18	0.18	0.19
CHRNA7	cholinergic receptor, nicotinic, alpha 7	0.93	0.09	1.02	0.10	0.81	0.06	0.24
NPYY1R	neuropeptide-Y Y1 receptor	0.91	0.07	**0.95**[c]	0.06	**0.66**[b]	0.04	**0.01**
GLUT2	glucose transporter 2	1.33	0.10	1.36	0.11	1.38	0.03	0.82

[1] All values are means ± SEMs and are expressed as relative fold compared to CTR. n = 7 for CTR, n = 8 for HFD, n = 7 for REV.
[2] Overall significance as determined by Kruskal-Wallis rank sum test.
[a] Significantly different from CTR, p< .0167, Bonferroni adjusted α.
[b] Significantly different from HFD, p< .0167, Bonferroni adjusted α.
[c] Significantly different from REV, p< .0167, Bonferroni adjusted α.

Table 4-3.

Peripheral Nervous System mRNA expression in Juvenile Macaque Liver[1]

		DIET GROUP				
		CTR/CTR		HFD/HFD		
Target	Common Name (Type)	Mean	SEM	Mean	SEM	*p*-value[2]
ADRA1A	adrenergic, alpha-1A-, receptor	1.34	0.08	1.30	0.09	0.56
ADRA1B	adrenergic, alpha-1B-, receptor	0.86	0.08	0.83	0.08	0.73
ADRA2A	adrenergic, alpha-2A-, receptor	0.88	0.13	0.97	0.08	0.41
CB1R	cannabinoid 1 receptor	0		0		NA
CB2R	cannabinoid 2 receptor	1.26	0.13	1.71	0.24	0.11
CHRNA7	cholinergic receptor, nicotinic, alpha 7	**0.86**	0.05	**0.51**	0.03	**0.001**
NPYY1R	neuropeptide-Y Y1 receptor	0.71	0.06	0.85	0.08	0.20
GLUT2	glucose transporter 2	**0.94**	0.04	**0.80**	0.04	**0.03**

[1] All values are means ± SEMs and are expressed as relative fold compared to CTR. n = 7-10 for CTR, n = 8-10 for HFD.

[2] Overall significance as determined by Wilcoxon rank sum test.

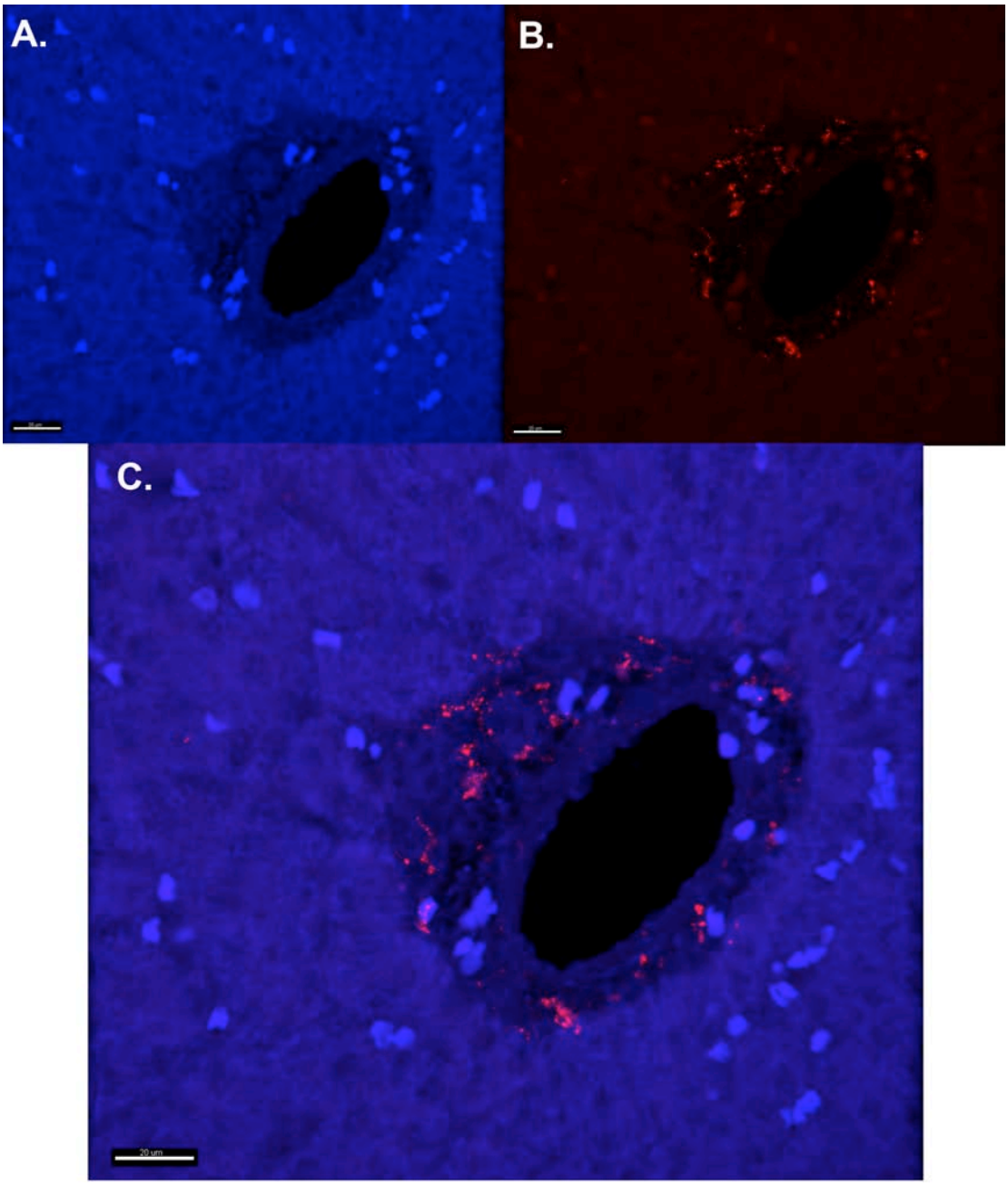


Figure 4-1. Immunohistochemical localization of NPY nerve fibers in fetal macaque liver by laser scanning confocal microscopy. (A). Emission of autofluorescence following excitation of fetal liver with 405 nm laser line allowed detailed acquisition of portal triad region. (B). NPY immunoreactive nerve fibers localized to the portal triad. (C). Overlay of A and B provides a representative image of the distribution of NPY immunoreactive nerve fibers in the fetal macaque liver. Scale bar = 20 μm.

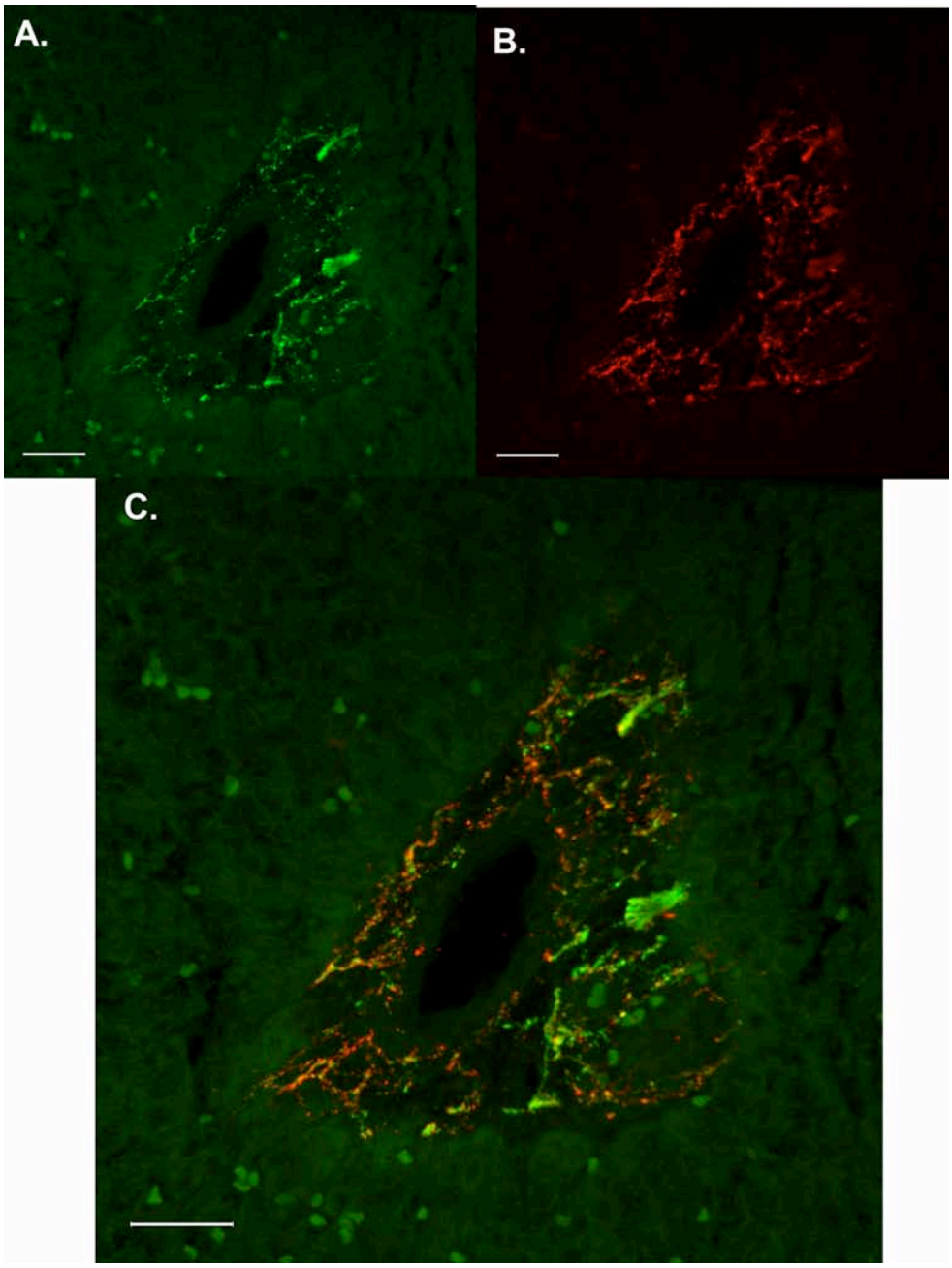


Figure 4-2. Co-localization of TH and NPY immunoreactivity in fetal macaque liver by double-labeled immunohistochemistry. (A). TH immunoreactivity in fetal liver acquired by excitation of tissue sample with 488 nm laser line. (B). NPY immunoreactivity in fetal liver acquired by excitation of tissue sample with 561 nm laser line. (C). Overlay of A and B provides a representative image of the robust colocalization of TH and NPY immunoreactive nerve fibers observed in the portal triads of fetal macaque liver. Scale bar = 40 μm.

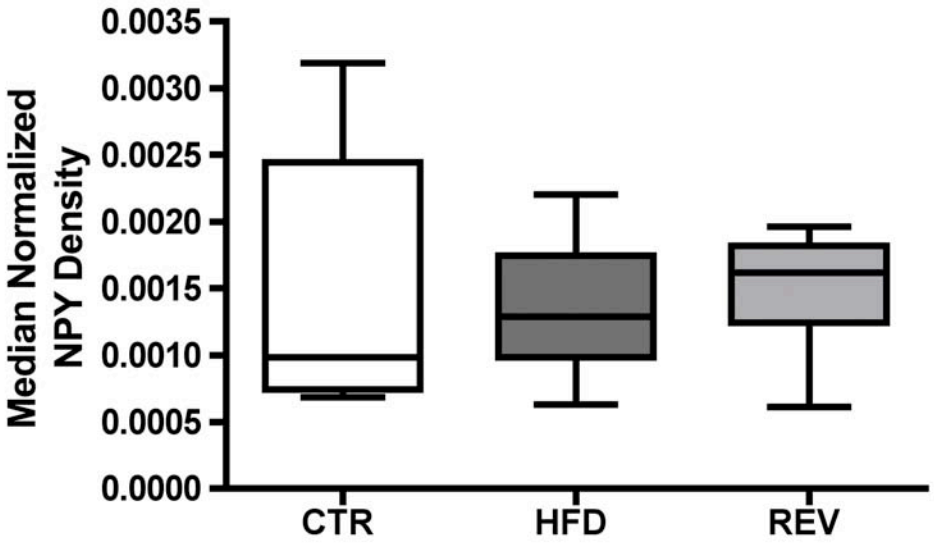


Figure 4-3. Quantification of the median density of portal NPY nerve fibers between maternal diet groups in the fetal macaque liver. NPY immunofluorescence was acquired in portal regions by laser scanning confocal microscopy. The volume of NPY immunoreactive fibers in each portal region was quantified and normalized to the volume of hepatic tissue in each image. No differences were observed in the density of NPY peptidergic innervation in fetal liver between maternal diet groups.
CTR; n = 6, HFD; n = 8, REV; n = 7.

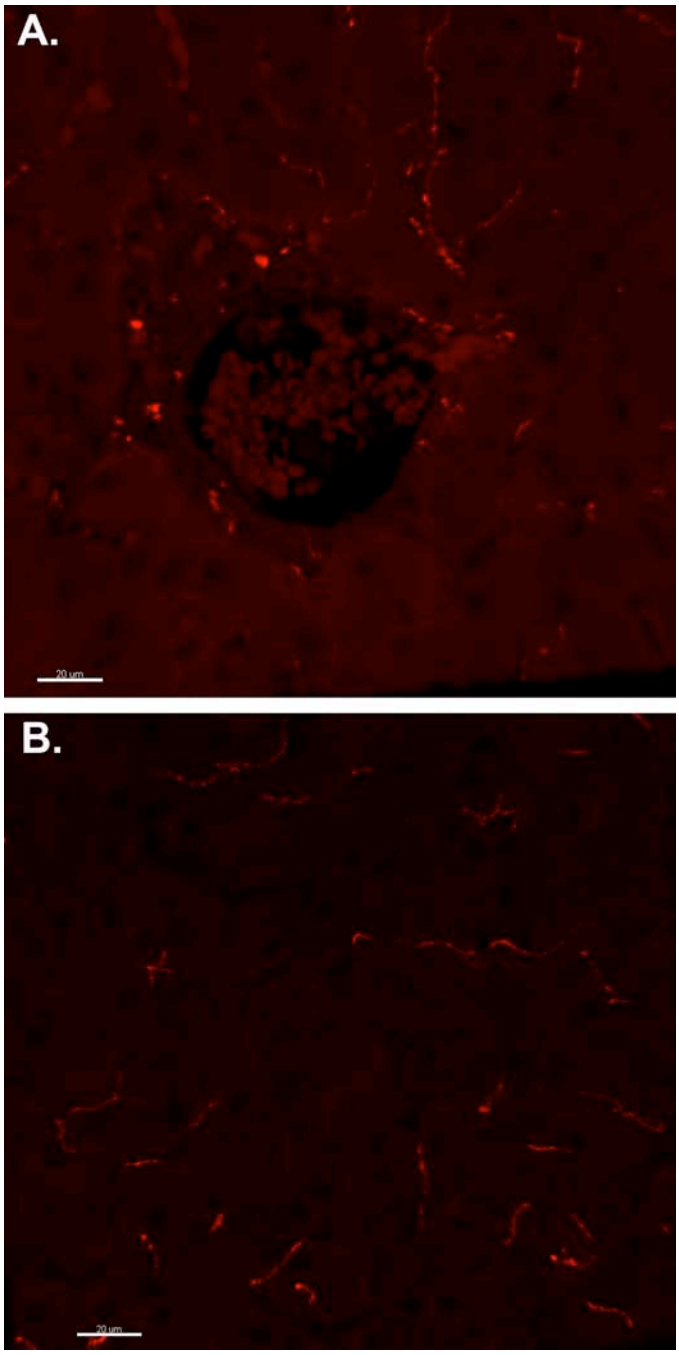


Figure 4-4. Hepatic sympathetic innervation in the one-year old juvenile macaque. Representative image of TH immunoreactive nerve fibers in the portal region (A) and parenchyma (B) in juvenile liver. Scale bar = 20 μm.

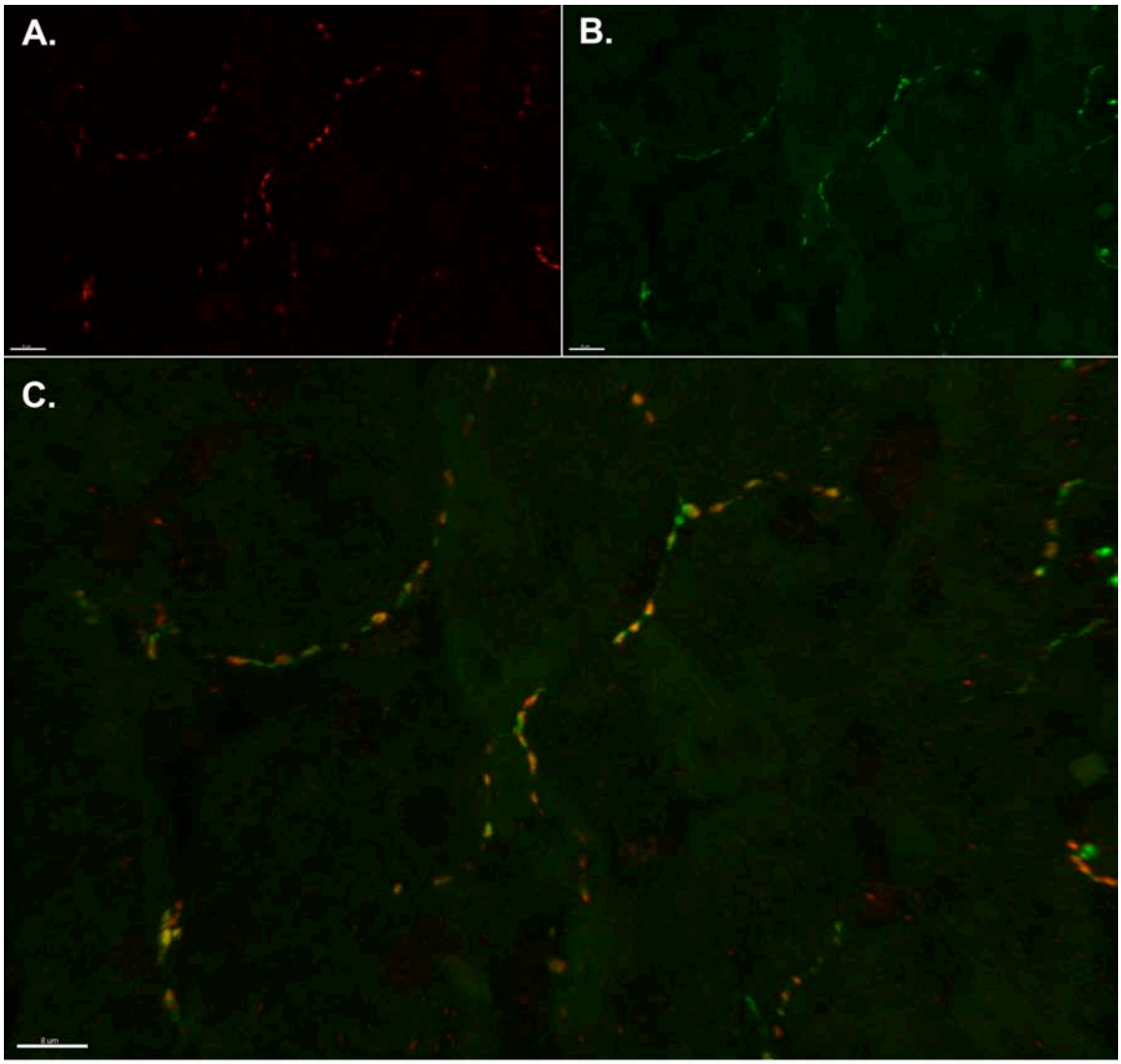


Figure 4-5. Colocalization of NPY and TH immunoreactivity in the hepatic parenchyma of a one-year old juvenile macaque. (A). NPY immunoreactive fibers in the hepatic parenchyma. (B). TH immunoreactive fibers in the hepatic parenchyma. (C). Overlay of A and B suggests that TH and NPY are sympathetic in origin and are tightly colocalized in the juvenile macaque liver. Scale bar = 8 μm.

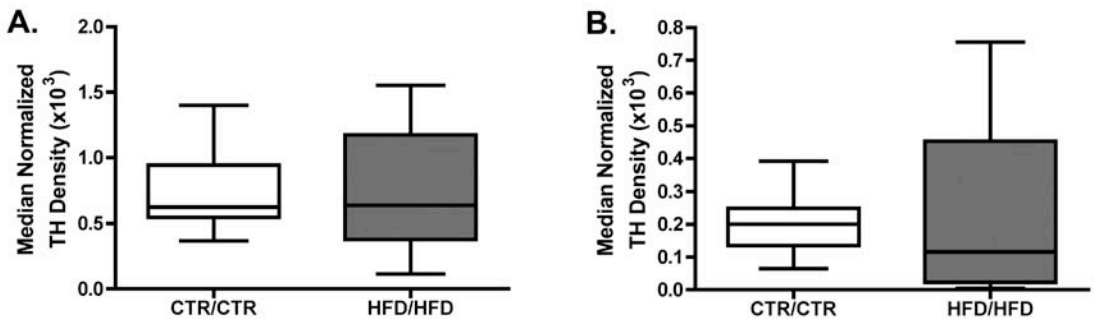


Figure 4-6. Quantification of the density of TH nerve fibers between CTR/CTR and HFD/HFD diet groups in the juvenile macaque liver. (A). Quantification of TH nerve fibers in the periportal region. (B). Quantification of TH nerve fibers in the hepatic parenchyma. TH immunofluorescence was acquired in each region by laser scanning confocal microscopy. The volume of TH immunoreactive fibers was normalized to the volume of hepatic tissue in each image. Data are expressed as the median normalized density for each juvenile diet group. No differences were observed in the density of sympathetic innervation in juvenile liver between diet groups.
CTR/CTR; n = 12, HFD/HFD; n = 9.

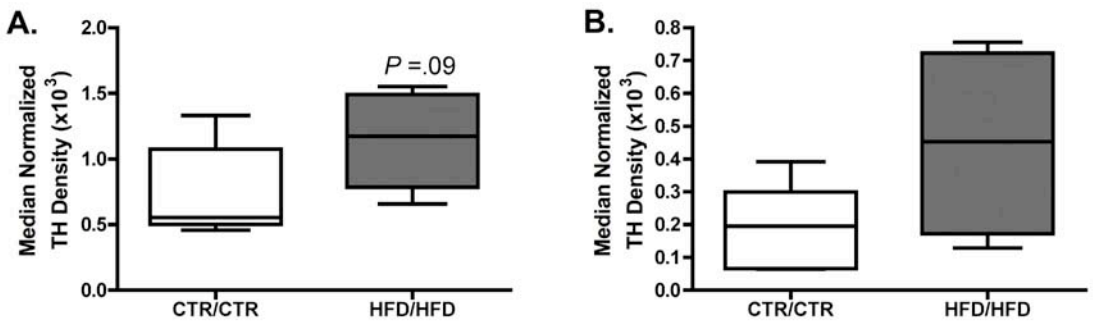


Figure 4-7. Quantification of the density of TH nerve fibers between CTR/CTR and HFD/HFD diet groups in the female juvenile macaque liver. (A). Quantification of TH nerve fibers in the periportal region. (B). Quantification of TH nerve fibers in the hepatic parenchyma. TH immunofluorescence was acquired in each region by laser scanning confocal microscopy. The volume of TH immunoreactive fibers was normalized to the volume of hepatic tissue in each image. Data are expressed as the median normalized density for each juvenile diet group. A nonsignificant trend for higher sympathetic innervation was observed in the female juvenile liver between diet groups. CTR/CTR; n = 5, HFD/HFD; n = 4.

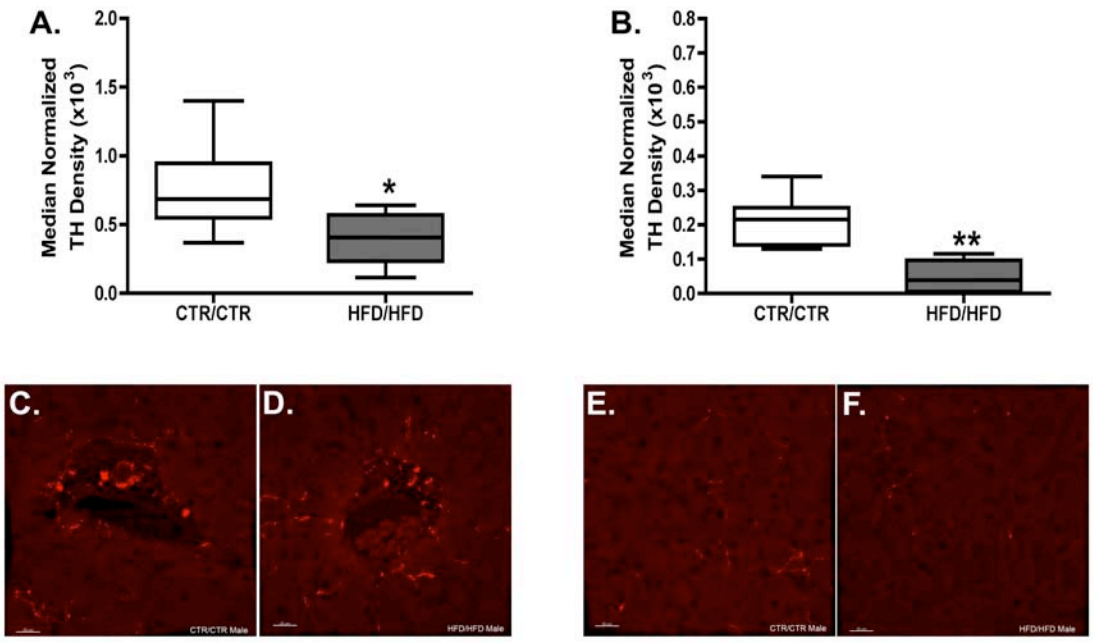


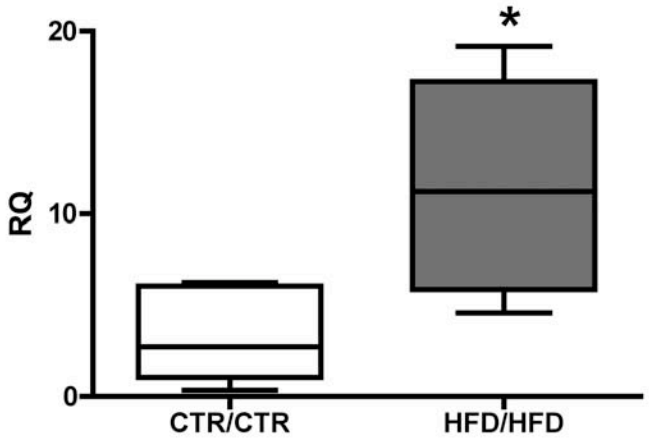
Figure 4-8. Quantification of the density of TH nerve fibers between CTR/CTR and HFD/HFD diet groups in the male juvenile macaque liver. (A). Quantification of TH nerve fibers in the periportal region. (B). Quantification of TH nerve fibers in the hepatic parenchyma. TH immunofluorescence was acquired in each region by laser scanning confocal microscopy. The volume of TH immunoreactive fibers was normalized to the volume of hepatic tissue in each image. Data are expressed as the median normalized density for each juvenile diet group. Representative images of TH immunoreactivity in the portal region for CTR/CTR (C) and HFD/HFD (D) males. Representative images of TH immunoreactivity in the hepatic parenchyma for CTR/CTR (E) and HFD/HFD (F) males. Significantly reduced sympathetic innervation was observed between diet groups in both portal and parenchymal regions in the male juvenile liver. CTR/CTR; n = 7, HFD/HFD; n = 5. (* = $p < 0.05$, ** = $p < 0.01$).

SUPPLEMENTAL TABLE 4-S1.

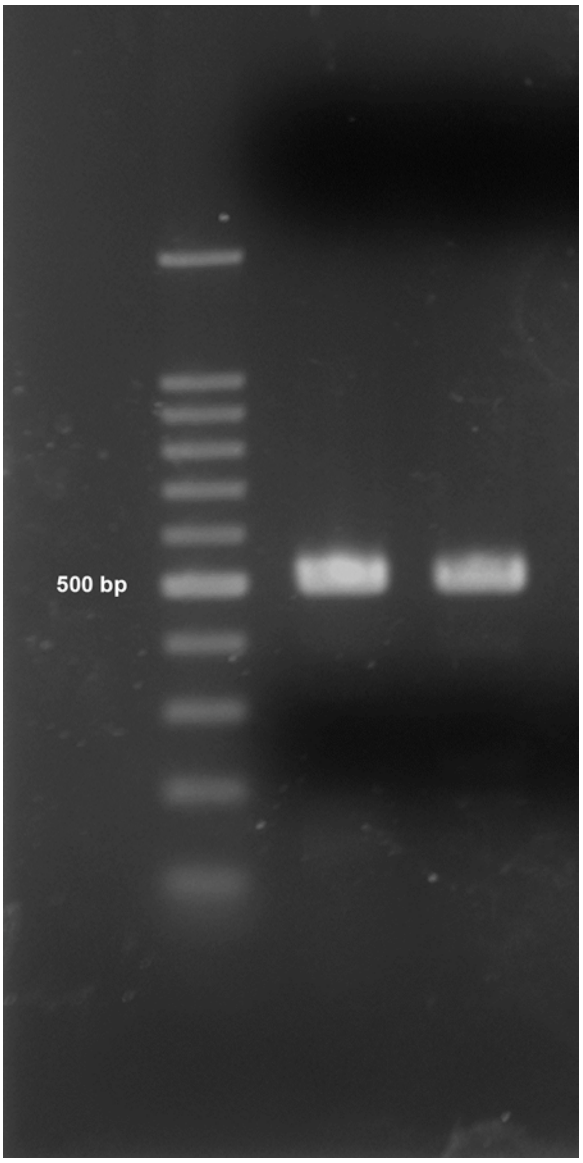
Primer sequences for Real-time PCR amplification of macaque inflammatory and neural markers.

mRNA	Sequence (5′-3′)	Genbank Accession No.
IFNG	F: TGTCCAACGCAAAGCAGTACA	NM_001032905
	R: AAAAGGAGTCAGATGTTTCGAGGT	
IL-1β	F: GACGTCGATGGCCCTAAACA	NM_001042756
	R: TGTAGTGCTCGTGGGAGATTTG	
IL-4	F: ACAACTGCCATATCGCCTTACG	NM_001032904
	R: CTTCTGCAGGGCTGCGAC	
IL-6	F: TGACAAACACATTCGGTACATCCT	NM_001042733
	R: AGCAAAGAGGCACTGGCAGA	
IL-10	F: CCGTGGAGCAGGTGAAGAAT	NM_001044727
	R: GACATCTTCATCAACTACATAGAAGCCTA	
CXCL11	F: AGAAAGCCTCCATAATTTACCCAAGT	NM_001032950
	R: GATTTGGGATTTAGGCATCGTT	
LTA	F: AGGATGGTTTCTCCTTGAGCAA	NM_001047148
	R: GGAGAGTAGGCTTTCCCAGAGAAG	
CCL2	F: AGTGTCCCAAAGAAGCTGTGATC	NM_001032821
	R: TCCAGGTGGTCCATGGAATC	
TNF-α	F: TGAGGCCAAGCCCTGGTA	NM_001047149
	R: CGAGATAGTCGGGCAGATTGA	
Arg-1	F: AACAGCTGGCTGGCAAGGT	NW_001116523
	R: TGGCCAGAGATGCTTCCAAT	
CRP	F: CTCATGCTTTTGGCCAGACA	NW_001108960
	R: GGCTTCGTTAACCGTGCTTT	
ADRA1A	F: CGACACCTGCACTCAGTCACA	NW_001122890
	R: CCTCGAAGATGGCGGAGAA	
ADRA1B	F: CAGCTAAGACGTTGGGCATTG	NW_001120992
	R: GGCTTCAGGGTGGAGAACAA	
ADRA2A	F: CTGGTGGCCACGCTTGTC	NW_001124223
	R: CGTCGAGCGCCAGGTAGAT	
CB1R	F: ACGCTTTCCGGAGCATGTT	NM_001032825
	R: GCGTTGTTTGCGTGTTTGTG	
CB2R	F: GGGCATGTTCTCTGGAAAGC	NW_001111036
	R: ACCTCACGTCCAGCCTCATT	
CHRNA7	F: TGGTGGTGACGGTGATCGT	NM_001032883
	R: CACGCGCACCAGTTCAGA	
CHRNA7$^{(500)}$	F: CGCTCACCGTCTACTTCTCC	NM_001032883
	R: GTAGGGCTCTTTGCAGCACT	
NPYY1R	F: ATTTCCGGTCTCGGGATGAT	NM_001032866
	R: AATGCGACTGGGCTTGCTT	
GLUT2	F: GACCACGTCCTGCTGCTTTAG	NW_001112558
	R: GGTCCACAGAAGTCCGCAAT	

Definition of abbreviations: F, forward primer; R, reverse primer.



Supplemental Figure 4-S1. Relative expression of C-Reactive protein in female juvenile liver. Real-Time PCR was used to assess the expression of CRP in one-year old juvenile liver between CTR/CTR and HFD/HFD diet groups. A significant increase in CRP expression was observed in female liver. CTR/CTR; n = 5, HFD/HFD; n = 4. (* = $p = 0.05$).



Supplemental Figure 4-S2. RT-PCR amplification of CHRNA7 from juvenile macaque liver. Following RT-PCR amplification of CHRNA7 from cDNA produced from two randomly chosen juvenile liver samples, the expected 500 bp bands were observed after gel electrophoresis. The presence of authentic macaque CHRNA7 in juvenile liver was confirmed by sequence analysis of the gel-purified bands.

OVERALL SUMMARY AND CONCLUSIONS

The research presented in this dissertation represents but a small part of a larger ongoing multi-institutional collaboration centered at the Oregon National Primate Research Center at Oregon Health & Science University. The long-term goals of this group are quite simple; to understand the causes of obesity in our modern industrialized world and to develop effective therapeutic interventions to halt the obesity epidemic.

The conception and development of the unique nonhuman primate model of maternal high-fat feeding utilized in this dissertation can be viewed in itself, as a quantum leap forward in obesity research. While rodent models have provided much useful information, important species-specific differences exist between humans and rodents that make translation of research findings into the clinical setting very difficult. Findings with our model can be directly translated and tested in human populations. An important human study, based on findings from the inaugural publication with this model and results described in Chapter 2 of this dissertation (Grant et al., 2011; McCurdy et al., 2009), is currently underway. This fact underscores the relevance and the importance of this nonhuman primate model as a research tool.

Within our model, an unintended and important initial finding was the segregation of HFD dams into diet-sensitive and diet-resistant cohorts. Diet-sensitive HFD dams became obese, hyperleptinemic and had higher insulin responses during glucose tolerance testing. Diet-resistant HFD dams did not become obese and were not different from CTR dams in any of the metabolic measures assayed (McCurdy et al., 2009). Previous reports from our group have demonstrated in fetuses that NAFLD, premature hepatic

gluconeogenic gene expression and alterations in the hypothalamic melanocortin system are associated with exposure to a maternal HFD independent of maternal phenotype.

The results presented in this dissertation support this previous work and suggest a primary role for the HFD in the development of fetal pathology. An example from this dissertation is the observation that increased hepatic apoptosis is present in the HFD fetal liver. Importantly, we found that hepatic apoptosis was returned to CTR levels in HFD fetuses with maternal diet reversal. The increase in HFD fetal hepatic apoptosis and its reversal occurred within a maternal background of both diet-sensitive and diet-resistant dams and was independent of maternal phenotype. Thus, these findings support the important role of the maternal diet in our model.

Taken together with previous work by McCurdy et al., the findings of increased hepatic apoptosis in the HFD fetal liver also suggest that severe NAFLD is present in HFD fetuses. Little is known about the effects that NAFLD *in-utero* may have on hepatic development and hepatic function. It is reasonable to conclude however, that NAFLD during gestation would lead to poor fetal outcomes. Functional tests in isolated fetal liver preparations could help define hepatic responses to such factors as glucose and insulin. In addition, an examination of cell proliferation would address whether the increased apoptosis we observed is the result of increased cell turnover within the fetal liver.

Of particular interest, is our finding that fetal plasma N6:N3 ratios are positively correlated to maternal plasma N6:N3 ratios as well as maternal insulin responses. Recently it was reported that higher N6:N3 ratios in the maternal diet and maternal and fetal plasma are associated with increased risk of obesity in 3 year-old human offspring (S. M. Donahue et al., 2011). While this important human study validates our approach

and findings, our nonhuman primate model also provides us with the opportunity to investigate the importance of the N6:N3 ratio in ways that would not be possible in humans. For example, dietary manipulation of the N6:N3 ratio alone could be examined in both fetal and juvenile nonhuman primates. We hypothesize that supplementation of the maternal and/or the postnatal HFD with N-3 fatty acid(s), effectively lowering dietary and plasma N6:N3 ratios to CTR levels, would have profound beneficial effects in fetal and juvenile HFD animals.

The hepatic innervation studies described in this dissertation provide basic knowledge on the ontogeny and structural morphology of sympathetic innervation in the macaque liver, an area of study that has not been well described. In the fetus, we observed that robust sympathetic/peptidergic innervation is present in portal regions and that adrenergic and peptidergic receptors are expressed at this point in gestation. While it is likely that the sympathetic nervous system is involved in liver function during this time, very little is known about the sympathetic regulation of hepatic function in the fetus.

Our analysis of hepatic sympathetic innervation in juvenile animals, suggests that parenchymal sympathetic innervation occurs sometime after gestational day 130. While the overall focus of this dissertation was to examine the effects that the HFD has on innervation in fetal and juvenile liver, valuable information would be provided by a thorough characterization of the ontogeny of sympathetic innervation in the macaque. A better understanding of hepatic sympathetic ontogeny in the macaque would have two very important results. First, a study of this kind would add greatly to the limited body of

knowledge that currently exists, and secondly this knowledge would provide an anatomical basis on which to design and interpret future studies with our model.

In the juvenile HFD/HFD male offspring we observed significant changes in hepatic sympathetic innervation. It is unclear to us whether the reduction in sympathetic innervation we observed is a consequence of faulty axonal outgrowth, or as a result of retraction of nerve fibers from a diseased liver. Reductions in parenchymal innervation have been reported in human cases of liver cirrhosis. From a functional perspective, we hypothesize that reduced hepatic sympathetic innervation will have little effect on basal glucose homeostasis. However, we expect to observe significant changes in these animals when a rapid need for glucose is required, such as in a fight or flight situation, or in response to severe hypoglycemia. Recently, functional studies with our juvenile macaques indicate that a metabolic phenotype is present in the HFD/HFD males. While this provides support for our findings, additional studies are needed to elucidate the functional role that reduced hepatic sympathetic innervation is playing in the HFD/HFD males.

We find it interesting that the changes in hepatic sympathetic innervation that we observed occurred in a sexually dimorphic manner. While the underlying mechanisms responsible for this finding are unclear at this time, the hormonal milieu must now be considered as a component in postnatal studies with this model. In addition to the impact that maternal phenotype has on some of our outcomes, sex differences now adds another level of complexity to the interpretation of our data.

A large body of evidence indicates that the brain directly regulates hepatic function. Transneuronal labeling studies using attenuated pseudorabies virus injected

directly into the liver, combined with either a hepatic sympathectomy or hepatic parasympathectomy, has revealed complex autonomic neurocircuitry extending from the liver to the brainstem and hypothalamus in the rodent. While assumptions can be made regarding the hepatic neurocircuitry found in other species based on these rodent studies, it is unknown whether the same brain regions are involved in hepatic function in the macaque. The technical challenges involved in a study of this nature may be very hard to replicate in the macaque. However, it is critical for the development of future studies with this model that a more complete understanding of the brain regions involved in liver function be elucidated in the macaque.

The publications thus far produced with our model overwhelmingly implicate the high-fat diet in the development of morphological, functional and behavioral abnormalities in the offspring. To that end, our analysis of the fatty acid profiles found in the maternal diet, maternal and fetal plasma and breastmilk provides fundamental information that will not only aid in the interpretation of data acquired with our model, but has also opened up new areas of investigation.

In addition to the overwhelming effects of the HFD itself, other data suggests that the effects of the diet may also be modulated by maternal phenotype and sex of the offspring. An early report from our group described that epigenetic changes in the fetal liver may be linked to maternal phenotype. Data presented in this dissertation has shown that the fetal plasma N6:N3 ratio is associated with maternal insulin resistance. In addition, we have shown clear effects of sex with decreased hepatic sympathetic innervation observed only in male juvenile HFD/HFD macaques.

Overall, our group is trying to understand the effects that exposure to a HFD has on the development and function of multiple metabolic organ systems across a wide range of developmental time-points. The studies outlined in this dissertation make an important contribution towards this goal, but they are a small part of a large and complex area of study. While our group has made great progress, it is becoming increasingly clear that additional studies, in both fetal and juvenile offspring, are needed to further define and understand the primary factors and temporal relationships involved in the effects that a HFD has on development, before a unified and satisfactory strategy be implemented to halt the epidemic of human obesity.

REFERENCES

Aagaard-Tillery, K. M., Grove, K., Bishop, J., Ke, X., Fu, Q., McKnight, R., et al. (2008). Developmental origins of disease and determinants of chromatin structure: maternal diet modifies the primate fetal epigenome. *J Mol Endocrinol, 41*(2), 91-102.

ABI. (2006). Amplification Efficiency of Taqman Expression Assays.

Ahima, R. S., & Flier, J. S. (2000). Adipose tissue as an endocrine organ. *Trends Endocrinol Metab, 11*(8), 327-332.

Aid, S., Vancassel, S., Poumes-Ballihaut, C., Chalon, S., Guesnet, P., & Lavialle, M. (2003). Effect of a diet-induced n-3 PUFA depletion on cholinergic parameters in the rat hippocampus. *J Lipid Res, 44*(8), 1545-1551.

Ailhaud, G. (2000). Adipose tissue as an endocrine organ. *Int J Obes Relat Metab Disord, 24 Suppl 2*, S1-3.

Akiyoshi, H., Gonda, T., & Terada, T. (1998). A comparative histochemical and immunohistochemical study of aminergic, cholinergic and peptidergic innervation in rat, hamster, guinea pig, dog and human livers. *Liver, 18*(5), 352-359.

Alling, C., Bruce, A., Karlsson, I., & Svennerholm, L. (1974). The effect of different dietary levels of essential fatty acids on lipids of rat cerebrum during maturation. *J Neurochem, 23*(6), 1263-1270.

Allison, D. B., Fontaine, K. R., Manson, J. E., Stevens, J., & VanItallie, T. B. (1999). Annual deaths attributable to obesity in the United States. *Jama, 282*(16), 1530-1538.

Allison, D. B., Heshka, S., Neale, M. C., & Heymsfield, S. B. (1994). Race effects in the genetics of adolescents' body mass index. *Int J Obes Relat Metab Disord, 18*(6), 363-368.

Allison, D. B., Heshka, S., Neale, M. C., Lykken, D. T., & Heymsfield, S. B. (1994). A genetic analysis of relative weight among 4,020 twin pairs, with an emphasis on sex effects. *Health Psychol, 13*(4), 362-365.

Allison, D. B., Kaprio, J., Korkeila, M., Koskenvuo, M., Neale, M. C., & Hayakawa, K. (1996). The heritability of body mass index among an international sample of monozygotic twins reared apart. *Int J Obes Relat Metab Disord, 20*(6), 501-506.

Altavilla, D., Guarini, S., Bitto, A., Mioni, C., Giuliani, D., Bigiani, A., et al. (2006). Activation of the cholinergic anti-inflammatory pathway reduces NF-kappab activation, blunts TNF-alpha production, and protects againts splanchic artery occlusion shock. *Shock, 25*(5), 500-506.

Amatruda, J. M., Harman, S. M., Pourmotabbed, G., & Lockwood, D. H. (1978). Depressed plasma testosterone and fractional binding of testosterone in obese males. *J Clin Endocrinol Metab, 47*(2), 268-271.

Amenta, F., Cavallotti, C., Ferrante, F., & Tonelli, F. (1981). Cholinergic nerves in the human liver. *Histochem J, 13*(3), 419-424.

Anderson, G. J., Neuringer, M., Lin, D. S., & Connor, W. E. (2005). Can prenatal N-3 fatty acid deficiency be completely reversed after birth? Effects on retinal and brain biochemistry and visual function in rhesus monkeys. *Pediatr Res, 58*(5), 865-872.

Anderson, R. E. (1970). Lipids of ocular tissues. IV. A comparison of the phospholipids from the retina of six mammalian species. *Exp Eye Res, 10*(2), 339-344.

Andreasen, C. H., Stender-Petersen, K. L., Mogensen, M. S., Torekov, S. S., Wegner, L., Andersen, G., et al. (2008). Low physical activity accentuates the effect of the FTO rs9939609 polymorphism on body fat accumulation. *Diabetes, 57*(1), 95-101.

Androlewicz, M. J., Browning, J. L., & Ware, C. F. (1992). Lymphotoxin is expressed as a heteromeric complex with a distinct 33-kDa glycoprotein on the surface of an activated human T cell hybridoma. *J Biol Chem, 267*(4), 2542-2547.

Anguita, R. M., Sigulem, D. M., & Sawaya, A. L. (1993). Intrauterine food restriction is associated with obesity in young rats. *J Nutr, 123*(8), 1421-1428.

Anil, M. H., & Forbes, J. M. (1987). Neural control and neurosensory functions of the liver. *Proc Nutr Soc, 46*(1), 125-133.

Arias, I. M. (Ed.). (2009). *The Liver: Biology and pathobiology* (Fifth ed.). Oxford: Wiley-Blackwell.

Athari, A., Hanecke, K., & Jungermann, K. (1994). Prostaglandin F2 alpha and D2 release from primary Ito cell cultures after stimulation with noradrenaline and ATP but not adenosine. *Hepatology, 20*(1 Pt 1), 142-148.

Baik, I., Ascherio, A., Rimm, E. B., Giovannucci, E., Spiegelman, D., Stampfer, M. J., et al. (2000). Adiposity and mortality in men. *Am J Epidemiol, 152*(3), 264-271.

Barja, F., & Mathison, R. (1982). Adrenergic and peptidergic (substance P and vasoactive intestinal polypeptide) innervation of the rat portal vein. *Blood Vessels, 19*(5), 263-272.

Barja, F., & Mathison, R. (1984). Sensory innervation of the rat portal vein and the hepatic artery. *J Auton Nerv Syst, 10*(2), 117-125.

Barker, D. J., Osmond, C., Golding, J., Kuh, D., & Wadsworth, M. E. (1989). Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *Bmj, 298*(6673), 564-567.

Barker, D. J., Osmond, C., & Law, C. M. (1989). The intrauterine and early postnatal origins of cardiovascular disease and chronic bronchitis. *J Epidemiol Community Health, 43*(3), 237-240.

Barraud, Q., Obeid, I., Aubert, I., Barriere, G., Contamin, H., McGuire, S., et al. Neuroanatomical study of the A11 diencephalospinal pathway in the non-human primate. *PLoS One, 5*(10), e13306.

Barzilaym, J. I., & Freedland, E. S. (2003). Inflammation and its relationship to insulin resistance, type 2 diabetes mellitus, and endothelial dysfunction. *Metab Syndr Relat Disord, 1*(1), 55-67.

Baskin, M. L., Ard, J., Franklin, F., & Allison, D. B. (2005). Prevalence of obesity in the United States. *Obes Rev, 6*(1), 5-7.

Bazan, N. G. (1982). Metabolism of phospholipids in the retina. *Vision Res, 22*(12), 1539-1548.

Beckmann, C. R. B. (2006). *Obstetrics and gynecology* (5th ed.). Philadelphia, PA: Lippincott Williams & Wilkins.

Bellinger, D. L., Lorton, D., Hamill, R. W., Felten, S. Y., & Felten, D. L. (1993). Acetylcholinesterase staining and choline acetyltransferase activity in the young adult rat spleen: lack of evidence for cholinergic innervation. *Brain Behav Immun, 7*(3), 191-204.

Berg, A. H., & Scherer, P. E. (2005). Adipose tissue, inflammation, and cardiovascular disease. *Circ Res, 96*(9), 939-949.

Bernik, T. R., Friedman, S. G., Ochani, M., DiRaimo, R., Ulloa, L., Yang, H., et al. (2002). Pharmacological stimulation of the cholinergic antiinflammatory pathway. *J Exp Med, 195*(6), 781-788.

Berra, B., Lindi, C., & Omodeo-Sale, F. (1980). Ganglioside distribution in foetal rat brains from dams fed on diets with different amount of essential fatty acids. *Prog Food Nutr Sci, 4*(5), 71-74.

Berson, A., De Beco, V., Letteron, P., Robin, M. A., Moreau, C., El Kahwaji, J., et al. (1998). Steatohepatitis-inducing drugs cause mitochondrial dysfunction and lipid peroxidation in rat hepatocytes. *Gastroenterology, 114*(4), 764-774.

Berthoud, H. R. (2004). Anatomy and function of sensory hepatic nerves. *Anat Rec A Discov Mol Cell Evol Biol, 280*(1), 827-835.

Berthoud, H. R., Kressel, M., & Neuhuber, W. L. (1992). An anterograde tracing study of the vagal innervation of rat liver, portal vein and biliary system. *Anat Embryol (Berl), 186*(5), 431-442.

Berthoud, H. R., Neuhuber, W.L. (1998). Peripheral and central functional neuroanatomy of sensory and motor innervation of the portal-hepatic axis and biliary system. D. J. K. Haussinger (Ed.), In *Liver and nervous system* (pp. 17-33). Lancaster. U.K.: Kluwer.

Bertola, A., Bonnafous, S., Anty, R., Patouraux, S., Saint-Paul, M. C., Iannelli, A., et al. (2010). Hepatic expression patterns of inflammatory and immune response genes associated with obesity and NASH in morbidly obese patients. *PLoS One, 5*(10), e13577.

Bioulac-Sage, P., Lafon, M. E., Saric, J., & Balabaud, C. (1990). Nerves and perisinusoidal cells in human liver. *J Hepatol, 10*(1), 105-112.

Bispham, J., Gardner, D. S., Gnanalingham, M. G., Stephenson, T., Symonds, M. E., & Budge, H. (2005). Maternal nutritional programming of fetal adipose tissue development: differential effects on messenger ribonucleic acid abundance for uncoupling proteins and peroxisome proliferator-activated and prolactin receptors. *Endocrinology, 146*(9), 3943-3949.

Bjorntorp, P. (1997). Body fat distribution, insulin resistance, and metabolic diseases. *Nutrition, 13*(9), 795-803.

Block, G., Dresser, C. M., Hartman, A. M., & Carroll, M. D. (1985). Nutrient sources in the American diet: quantitative data from the NHANES II survey. II. Macronutrients and fats. *Am J Epidemiol, 122*(1), 27-40.

Blomhoff, R., & Wake, K. (1991). Perisinusoidal stellate cells of the liver: important roles in retinol metabolism and fibrosis. *Faseb J, 5*(3), 271-277.

Bloom, S. R., & Edwards, A. V. (1975). The release of pancreatic glucagon and inhibition of insulin in response to stimulation of the sympathetic innervation. *J Physiol, 253*(1), 157-173.

Bloom, S. R., & Edwards, A. V. (1978). Certain pharmacological characteristics of the release of pancreatic glucagon in response to stimulation of the splanchnic nerves. *J Physiol, 280*, 25-35.

Boden, G., Chen, X., & Urbain, J. L. (1996). Evidence for a circadian rhythm of insulin sensitivity in patients with NIDDM caused by cyclic changes in hepatic glucose production. *Diabetes, 45*(8), 1044-1050.

Bodurtha, J. N., Mosteller, M., Hewitt, J. K., Nance, W. E., Eaves, L. J., Moskowitz, W. B., et al. (1990). Genetic analysis of anthropometric measures in 11-year-old twins: the Medical College of Virginia Twin Study. *Pediatr Res, 28*(1), 1-4.

Bolli, G. B., De Feo, P., De Cosmo, S., Perriello, G., Ventura, M. M., Calcinaro, F., et al. (1984). Demonstration of a dawn phenomenon in normal human volunteers. *Diabetes, 33*(12), 1150-1153.

Bonacchi, A., Petrai, I., Defranco, R. M., Lazzeri, E., Annunziato, F., Efsen, E., et al. (2003). The chemokine CCL21 modulates lymphocyte recruitment and fibrosis in chronic hepatitis C. *Gastroenterology, 125*(4), 1060-1076.

Boney, C. M., Verma, A., Tucker, R., & Vohr, B. R. (2005). Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. *Pediatrics, 115*(3), e290-296.

Boon, A. P., Hubscher, S. G., Lee, J. A., Hines, J. E., & Burt, A. D. (1992). Hepatic reinnervation following orthotopic liver transplantation in man. *J Pathol, 167*(2), 217-222.

Borovikova, L. V., Ivanova, S., Zhang, M., Yang, H., Botchkina, G. I., Watkins, L. R., et al. (2000). Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature, 405*(6785), 458-462.

Bouret, S. G., Draper, S. J., & Simerly, R. B. (2004). Formation of projection pathways from the arcuate nucleus of the hypothalamus to hypothalamic regions implicated in the neural control of feeding behavior in mice. *J Neurosci, 24*(11), 2797-2805.

Bourre, J. M., Durand, G., Pascal, G., & Youyou, A. (1989). Brain cell and tissue recovery in rats made deficient in n-3 fatty acids by alteration of dietary fat. *J Nutr, 119*(1), 15-22.

Bourre, J. M., Francois, M., Youyou, A., Dumont, O., Piciotti, M., Pascal, G., et al. (1989). The effects of dietary alpha-linolenic acid on the composition of nerve membranes, enzymatic activity, amplitude of electrophysiological parameters, resistance to poisons and performance of learning tasks in rats. *J Nutr, 119*(12), 1880-1892.

Bourre, J. M., Youyou, A., Durand, G., & Pascal, G. (1987). Slow recovery of the fatty acid composition of sciatic nerve in rats fed a diet initially low in n-3 fatty acids. *Lipids, 22*(7), 535-538.

Brenner, R. R. (1981). Nutritional and hormonal factors influencing desaturation of essential fatty acids. *Prog Lipid Res, 20*, 41-47.

Browning, J. L., Ngam-ek, A., Lawton, P., DeMarinis, J., Tizard, R., Chow, E. P., et al. (1993). Lymphotoxin beta, a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface. *Cell, 72*(6), 847-856.

Budge, H., Gnanalingham, M. G., Gardner, D. S., Mostyn, A., Stephenson, T., & Symonds, M. E. (2005). Maternal nutritional programming of fetal adipose tissue development: long-term consequences for later obesity. *Birth Defects Res C Embryo Today, 75*(3), 193-199.

Burcelin, R., Dolci, W., & Thorens, B. (2000). Glucose sensing by the hepatoportal sensor is GLUT2-dependent: in vivo analysis in GLUT2-null mice. *Diabetes, 49*(10), 1643-1648.

Burcelin, R., & Thorens, B. (2001). Evidence that extrapancreatic GLUT2-dependent glucose sensors control glucagon secretion. *Diabetes, 50*(6), 1282-1289.

Burdge, G. C., & Calder, P. C. (2005). Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. *Reprod Nutr Dev, 45*(5), 581-597.

Burdge, G. C., Slater-Jefferies, J., Torrens, C., Phillips, E. S., Hanson, M. A., & Lillycrop, K. A. (2007). Dietary protein restriction of pregnant rats in the F0 generation induces altered methylation of hepatic gene promoters in the adult male offspring in the F1 and F2 generations. *Br J Nutr, 97*(3), 435-439.

Burt, A. D., Tiniakos, D., MacSween, R. N., Griffiths, M. R., Wisse, E., & Polak, J. M. (1989). Localization of adrenergic and neuropeptide tyrosine-containing nerves in the mammalian liver. *Hepatology, 9*(6), 839-845.

Cai, D. (2009). NFkappaB-mediated metabolic inflammation in peripheral tissues versus central nervous system. *Cell Cycle, 8*(16), 2542-2548.

Cai, D., Yuan, M., Frantz, D. F., Melendez, P. A., Hansen, L., Lee, J., et al. (2005). Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med, 11*(2), 183-190.

Cailotto, C., La Fleur, S. E., Van Heijningen, C., Wortel, J., Kalsbeek, A., Feenstra, M., et al. (2005). The suprachiasmatic nucleus controls the daily variation of plasma glucose via the autonomic output to the liver: are the clock genes involved? *Eur J Neurosci, 22*(10), 2531-2540.

Cailotto, C., van Heijningen, C., van der Vliet, J., van der Plasse, G., Habold, C., Kalsbeek, A., et al. (2008). Daily rhythms in metabolic liver enzymes and plasma glucose require a balance in the autonomic output to the liver. *Endocrinology, 149*(4), 1914-1925.

Cano, G., Sved, A. F., Rinaman, L., Rabin, B. S., & Card, J. P. (2001). Characterization of the central nervous system innervation of the rat spleen using viral transneuronal tracing. *J Comp Neurol, 439*(1), 1-18.

Carey, V. J., Walters, E. E., Colditz, G. A., Solomon, C. G., Willett, W. C., Rosner, B. A., et al. (1997). Body fat distribution and risk of non-insulin-dependent diabetes mellitus in women. The Nurses' Health Study. *Am J Epidemiol, 145*(7), 614-619.

Carlson, S. E., Cooke, R. J., Rhodes, P. G., Peeples, J. M., & Werkman, S. H. (1992). Effect of vegetable and marine oils in preterm infant formulas on blood arachidonic and docosahexaenoic acids. *J Pediatr, 120*(4 Pt 2), S159-167.

Carter, A. M. (2007). Animal models of human placentation--a review. *Placenta, 28 Suppl A*, S41-47.

Cassiman, D., & Roskams, T. (2002). Beauty is in the eye of the beholder: emerging concepts and pitfalls in hepatic stellate cell research. *J Hepatol, 37*(4), 527-535.

Castuma, J. C., Catala, A., & Brenner, R. R. (1972). Oxidative desaturation of eicosa-8,11-dienoic acid to eicosa-5,8,11-trienoic acid: comparison of different diets on oxidative desaturation at the 5,6 and 6,7 positions. *J Lipid Res, 13*(6), 783-789.

Cawley, J. (2004). The impact of obesity on wages. *The Journal of Human Resources, 39*, 451-474.

Cetin, I., Alvino, G., & Cardellicchio, M. (2009). Long chain fatty acids and dietary fats in fetal nutrition. *J Physiol, 587*(Pt 14), 3441-3451.

Chan, J. M., Rimm, E. B., Colditz, G. A., Stampfer, M. J., & Willett, W. C. (1994). Obesity, fat distribution, and weight gain as risk factors for clinical diabetes in men. *Diabetes Care, 17*(9), 961-969.

Chang, G. Q., Gaysinskaya, V., Karatayev, O., & Leibowitz, S. F. (2008). Maternal high-fat diet and fetal programming: increased proliferation of hypothalamic peptide-producing neurons that increase risk for overeating and obesity. *J Neurosci, 28*(46), 12107-12119.

Chen, H., & Paul, W. E. (1997). Cultured NK1.1+ CD4+ T cells produce large amounts of IL-4 and IFN-gamma upon activation by anti-CD3 or CD1. *J Immunol, 159*(5), 2240-2249.

Cherrington, A. D. (1999). Banting Lecture 1997. Control of glucose uptake and release by the liver in vivo. *Diabetes, 48*(5), 1198-1214.

Clandinin, M. T., Chappell, J. E., Heim, T., Swyer, P. R., & Chance, G. W. (1981). Fatty acid accretion in fetal and neonatal liver: implications for fatty acid requirements. *Early Hum Dev, 5*(1), 7-14.

Clandinin, M. T., Chappell, J. E., Leong, S., Heim, T., Swyer, P. R., & Chance, G. W. (1980a). Extrauterine fatty acid accretion in infant brain: implications for fatty acid requirements. *Early Hum Dev, 4*(2), 131-138.

Clandinin, M. T., Chappell, J. E., Leong, S., Heim, T., Swyer, P. R., & Chance, G. W. (1980b). Intrauterine fatty acid accretion rates in human brain: implications for fatty acid requirements. *Early Hum Dev, 4*(2), 121-129.

Colantoni, A., Idilman, R., De Maria, N., La Paglia, N., Belmonte, J., Wezeman, F., et al. (2003). Hepatic apoptosis and proliferation in male and female rats fed alcohol: role of cytokines. *Alcohol Clin Exp Res, 27*(7), 1184-1189.
Cole, R. L., & Sawchenko, P. E. (2002). Neurotransmitter regulation of cellular activation and neuropeptide gene expression in the paraventricular nucleus of the hypothalamus. *J Neurosci, 22*(3), 959-969.
Corssmit, E. P., Romijn, J. A., & Sauerwein, H. P. (2001). Review article: Regulation of glucose production with special attention to nonclassical regulatory mechanisms: a review. *Metabolism, 50*(7), 742-755.
Coti Bertrand, P., O'Kusky, J. R., & Innis, S. M. (2006). Maternal dietary (n-3) fatty acid deficiency alters neurogenesis in the embryonic rat brain. *J Nutr, 136*(6), 1570-1575.
Cox, J., Williams, S., Grove, K., Lane, R. H., & Aagaard-Tillery, K. M. (2009). A maternal high-fat diet is accompanied by alterations in the fetal primate metabolome. *Am J Obstet Gynecol, 201*(3), 281 e281-289.
Cox, L. A., Nijland, M. J., Gilbert, J. S., Schlabritz-Loutsevitch, N. E., Hubbard, G. B., McDonald, T. J., et al. (2006). Effect of 30 per cent maternal nutrient restriction from 0.16 to 0.5 gestation on fetal baboon kidney gene expression. *J Physiol, 572*(Pt 1), 67-85.
Crabtree, J. T., Gordon, M. J., Campbell, F. M., & Dutta-Roy, A. K. (1998). Differential distribution and metabolism of arachidonic acid and docosahexaenoic acid by human placental choriocarcinoma (BeWo) cells. *Mol Cell Biochem, 185*(1-2), 191-198.
Crawford, M. A., Casperd, N. M., & Sinclair, A. J. (1976). The long chain metabolites of linoleic avid linolenic acids in liver and brain in herbivores and carnivores. *Comp Biochem Physiol B, 54*(3), 395-401.
Crawford, M. A., Costeloe, K., Ghebremeskel, K., Phylactos, A., Skirvin, L., & Stacey, F. (1997). Are deficits of arachidonic and docosahexaenoic acids responsible for the neural and vascular complications of preterm babies? *Am J Clin Nutr, 66*(4 Suppl), 1032S-1041S.
Crawford, M. A., Doyle, W., Williams, G., Drury, P.J. (1989). A. J. Vergroesen, Crawford, M. A (Ed.), In *The Role of Fats in Human Nutrition* (pp. 81-115). New York: Academic Press.
Crawford, M. A., Hassam, A. G., & Stevens, P. A. (1981). Essential fatty acid requirements in pregnancy and lactation with special reference to brain development. *Prog Lipid Res, 20*, 31-40.
Crawford, M. A., Sinclair, S.J. (1972). K. Elliot, Knight, J. (Ed.), In *Lipids, Malnutrition and the Developing Brain* (pp. 267-287). North Holland: Elsevier, Excerpta Medica.
Crispe, I. N. (2009). The liver as a lymphoid organ. *Annu Rev Immunol, 27*, 147-163.
Dabelea, D., Hanson, R. L., Lindsay, R. S., Pettitt, D. J., Imperatore, G., Gabir, M. M., et al. (2000). Intrauterine exposure to diabetes conveys risks for type 2 diabetes and obesity: a study of discordant sibships. *Diabetes, 49*(12), 2208-2211.
Dabelea, D., Knowler, W. C., & Pettitt, D. J. (2000). Effect of diabetes in pregnancy on offspring: follow-up research in the Pima Indians. *J Matern Fetal Med, 9*(1), 83-88.
Dabelea, D., Pettitt, D. J., Hanson, R. L., Imperatore, G., Bennett, P. H., & Knowler, W. C. (1999). Birth weight, type 2 diabetes, and insulin resistance in Pima Indian children and young adults. *Diabetes Care, 22*(6), 944-950.
Daemen, F. J. (1973). Vertebrate rod outer segment membranes. *Biochim Biophys Acta, 300*(3), 255-288.

Dam-Larsen, S., Franzmann, M., Andersen, I. B., Christoffersen, P., Jensen, L. B., Sorensen, T. I., et al. (2004). Long term prognosis of fatty liver: risk of chronic liver disease and death. *Gut, 53*(5), 750-755.

Damase-Michel, C., Tavernier, G., Giraud, P., Montastruc, J. L., Montastruc, P., & Tran, M. A. (1993). Effects of clonidine, dihydralazine and splanchnic nerve stimulation on the release of neuropeptide Y, MET-enkephalin and catecholamines from dog adrenal medulla. *Naunyn Schmiedebergs Arch Pharmacol, 348*(4), 379-384.

Daoust, R. (1958). The cell population of liver tissue and the cytological reference bases. *American Institute of Biological Sciences, 4*, 3-10.

Davidowa, H., Heidel, E., & Plagemann, A. (2002). Differential involvement of dopamine D1 and D2 receptors and inhibition by dopamine of hypothalamic VMN neurons in early postnatally overfed juvenile rats. *Nutr Neurosci, 5*(1), 27-36.

Davidowa, H., Li, Y., & Plagemann, A. (2002). Hypothalamic ventromedial and arcuate neurons of normal and postnatally overnourished rats differ in their responses to melanin-concentrating hormone. *Regul Pept, 108*(2-3), 103-111.

Davidowa, H., Li, Y., & Plagemann, A. (2003). Altered responses to orexigenic (AGRP, MCH) and anorexigenic (alpha-MSH, CART) neuropeptides of paraventricular hypothalamic neurons in early postnatally overfed rats. *Eur J Neurosci, 18*(3), 613-621.

Davidowa, H., & Plagemann, A. (2000a). Decreased inhibition by leptin of hypothalamic arcuate neurons in neonatally overfed young rats. *Neuroreport, 11*(12), 2795-2798.

Davidowa, H., & Plagemann, A. (2000b). Different responses of ventromedial hypothalamic neurons to leptin in normal and early postnatally overfed rats. *Neurosci Lett, 293*(1), 21-24.

Davidowa, H., & Plagemann, A. (2001). Inhibition by insulin of hypothalamic VMN neurons in rats overweight due to postnatal overfeeding. *Neuroreport, 12*(15), 3201-3204.

Davidowa, H., & Plagemann, A. (2004). Hypothalamic neurons of postnatally overfed, overweight rats respond differentially to corticotropin-releasing hormones. *Neurosci Lett, 371*(1), 64-68.

Davison, A. N., & Dobbing, J. (1966). Myelination as a vulnerable period in brain development. *Br Med Bull, 22*(1), 40-44.

Day, C. P. (2005). Natural history of NAFLD: remarkably benign in the absence of cirrhosis. *Gastroenterology, 129*(1), 375-378.

Day, C. P., & James, O. F. (1998). Steatohepatitis: a tale of two "hits"? *Gastroenterology, 114*(4), 842-845.

de Jonge, W. J., van der Zanden, E. P., The, F. O., Bijlsma, M. F., van Westerloo, D. J., Bennink, R. J., et al. (2005). Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway. *Nat Immunol, 6*(8), 844-851.

Delalande, J. M., Milla, P. J., & Burns, A. J. (2004). Hepatic nervous system development. *Anat Rec A Discov Mol Cell Evol Biol, 280*(1), 848-853.

Di Cianni, G., Miccoli, R., Volpe, L., Lencioni, C., Ghio, A., Giovannitti, M. G., et al. (2005). Maternal triglyceride levels and newborn weight in pregnant women with normal glucose tolerance. *Diabet Med, 22*(1), 21-25.

Diamant, Y. Z., Metzger, B. E., Freinkel, N., & Shafrir, E. (1982). Placental lipid and glycogen content in human and experimental diabetes mellitus. *Am J Obstet Gynecol, 144*(1), 5-11.

Ding, W. G., Kitasato, H., & Kimura, H. (1997). Development of neuropeptide Y innervation in the liver. *Microsc Res Tech, 39*(4), 365-371.

Ding, W. G., Tooyama, I., Kitasato, H., Fujimura, M., & Kimura, H. (1994). Phylogenetic and ontogenetic study of neuropeptide Y-containing nerves in the liver. *Histochem J, 26*(5), 453-459.
Dobbing, J. (1972). K. Elliot, Knight, J. (Ed.), In *Lipids, Malnutrition and the Developing Brain* (pp. 9-29). North Holland: Elsevier, Excerpta Medica.
Dobbing, J., & Sands, J. (1979). Comparative aspects of the brain growth spurt. *Early Hum Dev, 3*(1), 79-83.
Donahue, R. P., Abbott, R. D., Bloom, E., Reed, D. M., & Yano, K. (1987). Central obesity and coronary heart disease in men. *Lancet, 1*(8537), 821-824.
Donahue, S. M., Rifas-Shiman, S. L., Gold, D. R., Jouni, Z. E., Gillman, M. W., & Oken, E. (2011). Prenatal fatty acid status and child adiposity at age 3 y: results from a US pregnancy cohort. *Am J Clin Nutr, 93*(4), 780-788.
Drewnowski, A., Rehm, C. D., & Solet, D. (2007). Disparities in obesity rates: analysis by ZIP code area. *Soc Sci Med, 65*(12), 2458-2463.
Eck, M. G., Wynn, J. O., Carter, W. J., & Faas, F. H. (1979). Fatty acid desaturation in experimental diabetes mellitus. *Diabetes, 28*(5), 479-485.
Eden, S. (1979). Age- and sex-related differences in episodic growth hormone secretion in the rat. *Endocrinology, 105*(2), 555-560.
Eichenberg, J., Hauser, G., Karnovsky, M.L. (1969). G. H. Bourne (Ed.), In *The Structure and Function of Nervous Tissue* (Vol. 3, pp. 185-287). New York, NY: Academic Press.
Elias, S. L., & Innis, S. M. (2001). Infant plasma trans, n-6, and n-3 fatty acids and conjugated linoleic acids are related to maternal plasma fatty acids, length of gestation, and birth weight and length. *Am J Clin Nutr, 73*(4), 807-814.
Ellinwood, W. E., Baughman, W. L., & Resko, J. A. (1982). The effects of gonadectomy and testosterone treatment on luteinizing hormone secretion in fetal rhesus monkeys. *Endocrinology, 110*(1), 183-189.
Fabsitz, R. R., Carmelli, D., & Hewitt, J. K. (1992). Evidence for independent genetic influences on obesity in middle age. *Int J Obes Relat Metab Disord, 16*(9), 657-666.
Farooqi, I. S., Keogh, J. M., Yeo, G. S., Lank, E. J., Cheetham, T., & O'Rahilly, S. (2003). Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene. *N Engl J Med, 348*(12), 1085-1095.
Farquharson, J., Jamieson, E. C., Abbasi, K. A., Patrick, W. J., Logan, R. W., & Cockburn, F. (1995). Effect of diet on the fatty acid composition of the major phospholipids of infant cerebral cortex. *Arch Dis Child, 72*(3), 198-203.
Feher, E., Fodor, M., & Feher, J. (1992). Ultrastructural localization of somatostatin- and substance P-immunoreactive nerve fibers in the feline liver. *Gastroenterology, 102*(1), 287-294.
Feher, E., Fodor, M., Gorcs, T., Feher, J., & Vallent, K. (1991). Immunohistochemical distribution of neuropeptide Y and catecholamine-synthesizing enzymes in nerve fibers of the human liver. *Digestion, 50*(3-4), 194-201.
Fejes, I., Koloszar, S., Szollosi, J., Zavaczki, Z., & Pal, A. (2005). Is semen quality affected by male body fat distribution? *Andrologia, 37*(5), 155-159.
Feldstein, A. E., Canbay, A., Angulo, P., Taniai, M., Burgart, L. J., Lindor, K. D., et al. (2003). Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. *Gastroenterology, 125*(2), 437-443.
Felten, D. L., Felten, S. Y., Carlson, S. L., Olschowka, J. A., & Livnat, S. (1985). Noradrenergic and peptidergic innervation of lymphoid tissue. *J Immunol, 135*(2 Suppl), 755s-765s.

Ferrannini, E., Haffner, S. M., Mitchell, B. D., & Stern, M. P. (1991). Hyperinsulinaemia: the key feature of a cardiovascular and metabolic syndrome. *Diabetologia, 34*(6), 416-422.
Ferretti, A., & Flanagan, V. P. (1986). Mass spectrometric evidence for the conversion of exogenous adrenate to dihomo-prostaglandins by seminal vesicle cyclo-oxygenase. A comparative study of two animal species. *J Chromatogr, 383*(2), 241-250.
Finkelstein, E. A., Fiebelkorn, I. C., & Wang, G. (2003). National medical spending attributable to overweight and obesity: how much, and who's paying? *Health Aff (Millwood), Suppl Web Exclusives*, W3-219-226.
Fisher, R. A., Robertson, S. M., & Olson, M. S. (1987). Stimulation of glycogenolysis and vasoconstriction in the perfused rat liver by the thromboxane A2 analogue U-46619. *J Biol Chem, 262*(10), 4631-4638.
Fishman, P. H., & Brady, R. O. (1976). Biosynthesis and function of gangliosides. *Science, 194*(4268), 906-915.
Flegal, K. M., Carroll, M. D., Ogden, C. L., & Curtin, L. R. (2010). Prevalence and trends in obesity among US adults, 1999-2008. *Jama, 303*(3), 235-241.
Flegal, K. M., Carroll, M. D., Ogden, C. L., & Johnson, C. L. (2002). Prevalence and trends in obesity among US adults, 1999-2000. *Jama, 288*(14), 1723-1727.
Flegal, K. M., Shepherd, J. A., Looker, A. C., Graubard, B. I., Borrud, L. G., Ogden, C. L., et al. (2009). Comparisons of percentage body fat, body mass index, waist circumference, and waist-stature ratio in adults. *Am J Clin Nutr, 89*(2), 500-508.
Fliesler, S. J., & Anderson, R. E. (1983). Chemistry and metabolism of lipids in the vertebrate retina. *Prog Lipid Res, 22*(2), 79-131.
World Cancer Research Fund, American Institute of Cancer Research. (1997). *Food, Nutrition and the Prevention of Cancer: A Global Perspective*. Washington DC: American Institute of Cancer Research.
Ford, E. S., Li, C., Zhao, G., & Tsai, J. (2010). Trends in obesity and abdominal obesity among adults in the United States from 1999-2008. *Int J Obes (Lond)*.
Forssmann, W. G., & Ito, S. (1977). Hepatocyte innervation in primates. *J Cell Biol, 74*(1), 299-313.
Francavilla, A., di Leo, A., Eagon, P. K., Wu, S. Q., Ove, P., van Thiel, D. H., et al. (1984). Regenerating rat liver: correlations between estrogen receptor localization and deoxyribonucleic acid synthesis. *Gastroenterology, 86*(3), 552-557.
Francavilla, A., Eagon, P. K., DiLeo, A., Polimeno, L., Panella, C., Aquilino, A. M., et al. (1986). Sex hormone-related functions in regenerating male rat liver. *Gastroenterology, 91*(5), 1263-1270.
Freinkel, N. (1980). Banting Lecture 1980. Of pregnancy and progeny. *Diabetes, 29*(12), 1023-1035.
Frias, A. E., Morgan, T. K., Evans, A. E., Rasanen, J., Oh, K. Y., Thornburg, K. L., et al. (2011). Maternal High-Fat Diet Disturbs Uteroplacental Hemodynamics and Increases the Frequency of Stillbirth in a Nonhuman Primate Model of Excess Nutrition. *Endocrinology*.
Friedman, M. I. (1988). Hepatic nerve function. I. M. Arias, Popper, H., Jacoby, W.B., Schacter, D., Shafritz, D.A. (Ed.), In *The Liver: Biology and pathobiology* (pp. 949-959). New York: Raven Press.
Friedman, S. L. (2000). Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J Biol Chem, 275*(4), 2247-2250.
Friedman, S. L. (2008). Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev, 88*(1), 125-172.

Fu, Q., McKnight, R. A., Yu, X., Callaway, C. W., & Lane, R. H. (2006). Growth retardation alters the epigenetic characteristics of hepatic dual specificity phosphatase 5. *Faseb J, 20*(12), 2127-2129.

Fu, Q., McKnight, R. A., Yu, X., Wang, L., Callaway, C. W., & Lane, R. H. (2004). Uteroplacental insufficiency induces site-specific changes in histone H3 covalent modifications and affects DNA-histone H3 positioning in day 0 IUGR rat liver. *Physiol Genomics, 20*(1), 108-116.

Gaillard, D., Negrel, R., Lagarde, M., & Ailhaud, G. (1989). Requirement and role of arachidonic acid in the differentiation of pre-adipose cells. *Biochem J, 257*(2), 389-397.

Galli, C., Agradi, E., & Paoletti, R. (1975). Accumulation of trienoic fatty acids in rat brain after depletion of liver (n-6) polyunsaturated fatty acids. *J Neurochem, 24*(6), 1187-1190.

Garcia Carrapato, M. R. (2003). The offspring of gestational diabetes. *J Perinat Med, 31*(1), 5-11.

Gardemann, A., & Jungermann, K. (1986). Control of glucose balance in the perfused rat liver by the parasympathetic innervation. *Biol Chem Hoppe Seyler, 367*(7), 559-566.

Gardemann, A., Puschel, G. P., & Jungermann, K. (1992). Nervous control of liver metabolism and hemodynamics. *Eur J Biochem, 207*(2), 399-411.

Gilula, N. B., Hertzberg, E.I. (1982). Communication and gap junctions. I. M. Arias, Popper, H., Jacoby, W.B., Schacter, D., Shafritz, D.A. (Ed.), In *The Liver: Biology and pathobiology* (pp. 615). New York: Raven Press.

Glass, A. R., Swerdloff, R. S., Bray, G. A., Dahms, W. T., & Atkinson, R. L. (1977). Low serum testosterone and sex-hormone-binding-globulin in massively obese men. *J Clin Endocrinol Metab, 45*(6), 1211-1219.

Glavas, M. M., Grayson, B. E., Allen, S. E., Copp, D. R., Smith, M. S., Cowley, M. A., et al. (2008). Characterization of brainstem peptide YY (PYY) neurons. *J Comp Neurol, 506*(2), 194-210.

Gluckman, P. D., Lillycrop, K. A., Vickers, M. H., Pleasants, A. B., Phillips, E. S., Beedle, A. S., et al. (2007). Metabolic plasticity during mammalian development is directionally dependent on early nutritional status. *Proc Natl Acad Sci U S A, 104*(31), 12796-12800.

Goehler, L. E., & Sternini, C. (1991). Neuropeptide Y immunoreactivity in the mammalian liver: pattern of innervation and coexistence with tyrosine hydroxylase immunoreactivity. *Cell Tissue Res, 265*(2), 287-295.

Goehler, L. E., Sternini, C., & Brecha, N. C. (1988). Calcitonin gene-related peptide immunoreactivity in the biliary pathway and liver of the guinea-pig: distribution and colocalization with substance P. *Cell Tissue Res, 253*(1), 145-150.

Gordon, S. (2003). Alternative activation of macrophages. *Nat Rev Immunol, 3*(1), 23-35.

Granger, G. A., & Williams, T. W. (1968). Lymphocyte cytotoxicity in vitro: activation and release of a cytotoxic factor. *Nature, 218*(5148), 1253-1254.

Granger, S. W., Ware, C.F. (2003). Lymphotoxins. A. W. Thomson, Lotze, M.T. (Ed.), In *The Cytokine Handbook* (4th ed., Vol. 1, pp. 825-835). London: Elsevier Science Ltd.

Grant, W. F., Gillingham, M. B., Batra, A. K., Fewkes, N. M., Comstock, S. M., Takahashi, D., et al. (2011). Maternal High Fat Diet is Associated With Decreased Plasma n–3 Fatty Acids and Fetal Hepatic Apoptosis in Nonhuman Primates. *PLoS One, 6(2)*, e17261.

Grayson, B. E., Allen, S. E., Billes, S. K., Williams, S. M., Smith, M. S., & Grove, K. L. (2006). Prenatal development of hypothalamic neuropeptide systems in the nonhuman primate. *Neuroscience, 143*(4), 975-986.

Grayson, B. E., Kievit, P., Smith, M. S., & Grove, K. L. (2010). Critical determinants of hypothalamic appetitive neuropeptide development and expression: species considerations. *Front Neuroendocrinol, 31*(1), 16-31.

Grayson, B. E., LeVasseur, P., Williams, S. M., Smith, S. M., Marks, D. L., & Grove, K. L. (2007). Changes in Melanocortin Expression and Inflammatory Pathways in Fetal Offspring of Nonhuman Primates Fed a High Fat Diet. *in preparation*.

Grayson, B. E., Levasseur, P. R., Williams, S. M., Smith, M. S., Marks, D. L., & Grove, K. L. (2010). Changes in melanocortin expression and inflammatory pathways in fetal offspring of nonhuman primates fed a high-fat diet. *Endocrinology, 151*(4), 1622-1632.

Gregory, S. H., & Wing, E. J. (1998). Neutrophil-Kupffer-cell interaction in host defenses to systemic infections. *Immunol Today, 19*(11), 507-510.

Gressner, A. M., Lotfi, S., Gressner, G., & Lahme, B. (1992). Identification and partial characterization of a hepatocyte-derived factor promoting proliferation of cultured fat-storing cells (parasinusoidal lipocytes). *Hepatology, 16*(5), 1250-1266.

Grove, K. L., Allen, S., Grayson, B. E., & Smith, M. S. (2003). Postnatal development of the hypothalamic neuropeptide Y system. *Neuroscience, 116*(2), 393-406.

Grove, K. L., Brogan, R. S., & Smith, M. S. (2001). Novel expression of neuropeptide Y (NPY) mRNA in hypothalamic regions during development: region-specific effects of maternal deprivation on NPY and Agouti-related protein mRNA. *Endocrinology, 142*(11), 4771-4776.

Grove, K. L., Grayson, B. E., Glavas, M. M., Xiao, X. Q., & Smith, M. S. (2005). Development of metabolic systems. *Physiol Behav, 86*(5), 646-660.

Grove, K. L., & Smith, M. S. (2003). Ontogeny of the hypothalamic neuropeptide Y system. *Physiol Behav, 79*(1), 47-63.

Grunstein, R. R., Stenlof, K., Hedner, J., & Sjostrom, L. (1995). Impact of obstructive sleep apnea and sleepiness on metabolic and cardiovascular risk factors in the Swedish Obese Subjects (SOS) Study. *Int J Obes Relat Metab Disord, 19*(6), 410-418.

Guarini, S., Altavilla, D., Cainazzo, M. M., Giuliani, D., Bigiani, A., Marini, H., et al. (2003). Efferent vagal fibre stimulation blunts nuclear factor-kappaB activation and protects against hypovolemic hemorrhagic shock. *Circulation, 107*(8), 1189-1194.

Guebre-Xabier, M., Yang, S., Lin, H. Z., Schwenk, R., Krzych, U., & Diehl, A. M. (2000). Altered hepatic lymphocyte subpopulations in obesity-related murine fatty livers: potential mechanism for sensitization to liver damage. *Hepatology, 31*(3), 633-640.

Gupta, S. C., Gupta, C. D., & Gupta, S. B. (1981). Hepatovenous segments in the human liver. *J Anat, 133*(Pt 1), 1-6.

Hack, M., Breslau, N., Weissman, B., Aram, D., Klein, N., & Borawski, E. (1991). Effect of very low birth weight and subnormal head size on cognitive abilities at school age. *N Engl J Med, 325*(4), 231-237.

Haggarty, P. (2002). Placental regulation of fatty acid delivery and its effect on fetal growth--a review. *Placenta, 23 Suppl A*, S28-38.

Hales, C. N., & Barker, D. J. (2001). The thrifty phenotype hypothesis. *Br Med Bull, 60*, 5-20.

Hales, C. N., Barker, D. J., Clark, P. M., Cox, L. J., Fall, C., Osmond, C., et al. (1991). Fetal and infant growth and impaired glucose tolerance at age 64. *Bmj, 303*(6809), 1019-1022.
Hansen, D., & Lou, H. C. (2000). Brain development, head circumference and medication. *Acta Paediatr, 89*(5), 505-507.
Harada, H., Pavlick, K. P., Hines, I. N., Hoffman, J. M., Bharwani, S., Gray, L., et al. (2001). Selected contribution: Effects of gender on reduced-size liver ischemia and reperfusion injury. *J Appl Physiol, 91*(6), 2816-2822.
Hartman, C. G., & Straus, W. L. (1933). *The anatomy of the rhesus monkey (Macaca mulatta)*: The Williams & Wilkins Company.
Hatano, E. (2007). Tumor necrosis factor signaling in hepatocyte apoptosis. *J Gastroenterol Hepatol, 22 Suppl 1*, S43-44.
Hedley, A. A., Ogden, C. L., Johnson, C. L., Carroll, M. D., Curtin, L. R., & Flegal, K. M. (2004). Prevalence of overweight and obesity among US children, adolescents, and adults, 1999-2002. *Jama, 291*(23), 2847-2850.
Heird, W. C., & Lapillonne, A. (2005). The role of essential fatty acids in development. *Annu Rev Nutr, 25*, 549-571.
Helland, I. B., Saugstad, O. D., Saarem, K., Van Houwelingen, A. C., Nylander, G., & Drevon, C. A. (2006). Supplementation of n-3 fatty acids during pregnancy and lactation reduces maternal plasma lipid levels and provides DHA to the infants. *J Matern Fetal Neonatal Med, 19*(7), 397-406.
Helland, I. B., Smith, L., Saarem, K., Saugstad, O. D., & Drevon, C. A. (2003). Maternal supplementation with very-long-chain n-3 fatty acids during pregnancy and lactation augments children's IQ at 4 years of age. *Pediatrics, 111*(1), e39-44.
Herrera, E., & Amusquivar, E. (2000). Lipid metabolism in the fetus and the newborn. *Diabetes Metab Res Rev, 16*(3), 202-210.
Herrera, E., Amusquivar, E., Lopez-Soldado, I., & Ortega, H. (2006). Maternal lipid metabolism and placental lipid transfer. *Horm Res, 65 Suppl 3*, 59-64.
Herschkowitz, N. (1988). Brain development in the fetus, neonate and infant. *Biol Neonate, 54*(1), 1-19.
Hewitt, J. K. (1997). The genetics of obesity: what have genetic studies told us about the environment. *Behav Genet, 27*(4), 353-358.
Hiramoto, T., Chida, Y., Sonoda, J., Yoshihara, K., Sudo, N., & Kubo, C. (2008). The hepatic vagus nerve attenuates Fas-induced apoptosis in the mouse liver via alpha7 nicotinic acetylcholine receptor. *Gastroenterology, 134*(7), 2122-2131.
Hitzemann, R. (1981). Developmental changes in the fatty acids of synaptic membrane phospholipids: effect of protein malnutrition. *Neurochem Res, 6*(9), 935-947.
Homayoun, P., Durand, G., Pascal, G., & Bourre, J. M. (1988). Alteration in fatty acid composition of adult rat brain capillaries and choroid plexus induced by a diet deficient in n-3 fatty acids: slow recovery after substitution with a nondeficient diet. *J Neurochem, 51*(1), 45-48.
Horrocks, L. A. (1989). Sources for brain arachidonic acid uptake and turnover in glycerophospholipids. *Ann N Y Acad Sci, 559*, 17-24.
Hotamisligil, G. S. (2006). Inflammation and metabolic disorders. *Nature, 444*(7121), 860-867.
Howard, J. K., Lord, G. M., Matarese, G., Vendetti, S., Ghatei, M. A., Ritter, M. A., et al. (1999). Leptin protects mice from starvation-induced lymphoid atrophy and increases thymic cellularity in ob/ob mice. *J Clin Invest, 104*(8), 1051-1059.
Hsu, C. T. (1992). The role of the sympathetic nervous system in promoting liver cirrhosis induced by carbon tetrachloride, using the essential hypertensive animal (SHR). *J Auton Nerv Syst, 37*(3), 163-173.

Hudson, D. G., Elphick, M. C., & Hull, D. (1977). Distribution of label in maternal plasma, placenta, fetal plasma and tissues after injection of 14C-palmitate into the circulation of 21- and 28-day-pregnant rabbits. *Biol Neonate, 32*(5-6), 290-295.
Huston, J. M., Ochani, M., Rosas-Ballina, M., Liao, H., Ochani, K., Pavlov, V. A., et al. (2006). Splenectomy inactivates the cholinergic antiinflammatory pathway during lethal endotoxemia and polymicrobial sepsis. *J Exp Med, 203*(7), 1623-1628.
Ignatowski, T. A., Gallant, S., & Spengler, R. N. (1996). Temporal regulation by adrenergic receptor stimulation of macrophage (M phi)-derived tumor necrosis factor (TNF) production post-LPS challenge. *J Neuroimmunol, 65*(2), 107-117.
Iguchi, A., Burleson, P. D., & Szabo, A. J. (1981). Decrease in plasma glucose concentration after microinjection of insulin into VMN. *Am J Physiol, 240*(2), E95-100.
Iguchi, A., Matsunaga, H., Nomura, T., Gotoh, M., & Sakamoto, N. (1984). Glucoregulatory effects of intrahypothalamic injections of bombesin and other peptides. *Endocrinology, 114*(6), 2242-2246.
Iguchi, A., Sakamoto, N., & Burleson, P. D. (1983). The effects of neuropeptides on glucoregulation. *Adv Metab Disord, 10*, 421-434.
Inkpen, C. A., Harris, R. A., & Quackenbush, F. W. (1969). Differential responses to fasting and subsequent feeding by microsomal systems of rat liver: 6- and 9-desaturation of fatty acids. *J Lipid Res, 10*(3), 277-282.
Innis, S. M. (1991). Essential fatty acids in growth and development. *Prog Lipid Res, 30*(1), 39-103.
Innis, S. M. (2005). Essential fatty acid transfer and fetal development. *Placenta, 26 Suppl A*, S70-75.
Innis, S. M., & de La Presa Owens, S. (2001). Dietary fatty acid composition in pregnancy alters neurite membrane fatty acids and dopamine in newborn rat brain. *J Nutr, 131*(1), 118-122.
Ishii, H., Adachi, M., Fernandez-Checa, J. C., Cederbaum, A. I., Deaciuc, I. V., & Nanji, A. A. (2003). Role of apoptosis in alcoholic liver injury. *Alcohol Clin Exp Res, 27*(7), 1207-1212.
Ito, Y., Magari, S., & Sakanaka, M. (1990). Immunoelectron-microscopic localization of peptidergic nerve fibers around lymphatic capillaries in the rat liver. *Arch Histol Cytol, 53 Suppl*, 199-208.
Iwai, M., Miyashita, T., & Shimazu, T. (1991). Inhibition of glucose production during hepatic nerve stimulation in regenerating rat liver perfused in situ. Possible involvement of gap junctions in the action of sympathetic nerves. *Eur J Biochem, 200*(1), 69-74.
Iwai, M., & Shimazu, T. (1996). Exaggeration of acute liver damage by hepatic sympathetic nerves and circulating catecholamines in perfused liver of rats treated with D-galactosamine. *Hepatology, 23*(3), 524-529.
Jaffee, S. R., & Price, T. S. (2007). Gene-environment correlations: a review of the evidence and implications for prevention of mental illness. *Mol Psychiatry, 12*(5), 432-442.
Jaskiewicz, K., Voigt, M. D., & Robson, S. C. (1994). Distribution of hepatic nerve fibers in liver diseases. *Digestion, 55*(4), 247-252.
Jensen, C. L., Voigt, R. G., Prager, T. C., Zou, Y. L., Fraley, J. K., Rozelle, J. C., et al. (2005). Effects of maternal docosahexaenoic acid intake on visual function and neurodevelopment in breastfed term infants. *Am J Clin Nutr, 82*(1), 125-132.
Ji, S., Beckh, K., & Jungermann, K. (1984). Regulation of oxygen consumption and microcirculation by alpha-sympathetic nerves in isolated perfused rat liver. *FEBS Lett, 167*(1), 117-122.

Kalsbeek, A., Foppen, E., Schalij, I., Van Heijningen, C., van der Vliet, J., Fliers, E., et al. (2008). Circadian control of the daily plasma glucose rhythm: an interplay of GABA and glutamate. *PLoS One, 3*(9), e3194.

Kalsbeek, A., La Fleur, S., Van Heijningen, C., & Buijs, R. M. (2004). Suprachiasmatic GABAergic inputs to the paraventricular nucleus control plasma glucose concentrations in the rat via sympathetic innervation of the liver. *J Neurosci, 24*(35), 7604-7613.

Kalsbeek, A., Ruiter, M., La Fleur, S. E., Cailotto, C., Kreier, F., & Buijs, R. M. (2006). The hypothalamic clock and its control of glucose homeostasis. *Prog Brain Res, 153*, 283-307.

Kamohara, S., Burcelin, R., Halaas, J. L., Friedman, J. M., & Charron, M. J. (1997). Acute stimulation of glucose metabolism in mice by leptin treatment. *Nature, 389*(6649), 374-377.

Kanaan, N. M., Kordower, J. H., & Collier, T. J. (2007). Age-related accumulation of Marinesco bodies and lipofuscin in rhesus monkey midbrain dopamine neurons: relevance to selective neuronal vulnerability. *J Comp Neurol, 502*(5), 683-700.

Kaneto, A., Kajinuma, H., & Kosaka, K. (1975). Effect of splanchnic nerve stimulation on glucagon and insulin output in the dog. *Endocrinology, 96*(1), 143-150.

Karnovsky, M. J., & Roots, L. (1964). A "Direct-Coloring" Thiocholine Method for Cholinesterases. *J Histochem Cytochem, 12*, 219-221.

Katagiri, H., Ito, Y., Ishii, K., Hayashi, I., Suematsu, M., Yamashina, S., et al. (2004). Role of thromboxane derived from COX-1 and -2 in hepatic microcirculatory dysfunction during endotoxemia in mice. *Hepatology, 39*(1), 139-150.

Ke, X., McKnight, R. A., Wang, Z. M., Yu, X., Wang, L., Callaway, C. W., et al. (2005). Nonresponsiveness of cerebral p53-MDM2 functional circuit in newborn rat pups rendered IUGR via uteroplacental insufficiency. *Am J Physiol Regul Integr Comp Physiol, 288*(4), R1038-1045.

Keil, U., & Kuulasmaa, K. (1989). WHO MONICA Project: risk factors. *Int J Epidemiol, 18*(3 Suppl 1), S46-55.

Kidd, M., Nadler, B., Mane, S., Eick, G., Malfertheiner, M., Champaneria, M., et al. (2007). GeneChip, geNorm, and gastrointestinal tumors: novel reference genes for real-time PCR. *Physiol Genomics, 30*(3), 363-370.

Kim, H. K., Della-Fera, M., Lin, J., & Baile, C. A. (2006). Docosahexaenoic acid inhibits adipocyte differentiation and induces apoptosis in 3T3-L1 preadipocytes. *J Nutr, 136*(12), 2965-2969.

Kishimoto, Y., Davies, W. E., & Radin, N. S. (1965). Developing rat brain: changes in cholesterol, galactolipids, and the individual fatty acids of gangliosides and glycerophosphatides. *J Lipid Res, 6*(4), 532-536.

Kjaer, M., Jurlander, J., Keiding, S., Galbo, H., Kirkegaard, P., & Hage, E. (1994). No reinnervation of hepatic sympathetic nerves after liver transplantation in human subjects. *J Hepatol, 20*(1), 97-100.

Kodama, H., Yamaguchi, R., Fukuda, J., Kasai, H., & Tanaka, T. (1997). Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil Steril, 68*(3), 519-524.

Kodas, E., Vancassel, S., Lejeune, B., Guilloteau, D., & Chalon, S. (2002). Reversibility of n-3 fatty acid deficiency-induced changes in dopaminergic neurotransmission in rats: critical role of developmental stage. *J Lipid Res, 43*(8), 1209-1219.

Kort, H. I., Massey, J. B., Elsner, C. W., Mitchell-Leef, D., Shapiro, D. B., Witt, M. A., et al. (2006). Impact of body mass index values on sperm quantity and quality. *J Androl, 27*(3), 450-452.

Koutcherov, Y., Mai, J. K., Ashwell, K. W., & Paxinos, G. (2002). Organization of human hypothalamus in fetal development. *J Comp Neurol, 446*(4), 301-324.
Kowalyk, S., Veith, R., Boyle, M., & Taborsky, G. J., Jr. (1992). Liver releases galanin during sympathetic nerve stimulation. *Am J Physiol, 262*(5 Pt 1), E671-678.
Kumins, N. H., Hunt, J., Gamelli, R. L., & Filkins, J. P. (1996). Partial hepatectomy reduces the endotoxin-induced peak circulating level of tumor necrosis factor in rats. *Shock, 5*(5), 385-388.
Kuperstein, F., Yakubov, E., Dinerman, P., Gil, S., Eylam, R., Salem, N., Jr., et al. (2005). Overexpression of dopamine receptor genes and their products in the postnatal rat brain following maternal n-3 fatty acid dietary deficiency. *J Neurochem, 95*(6), 1550-1562.
La Fleur, S. E. (2003). Daily rhythms in glucose metabolism: suprachiasmatic nucleus output to peripheral tissue. *J Neuroendocrinol, 15*(3), 315-322.
La Fleur, S. E., Kalsbeek, A., Wortel, J., & Buijs, R. M. (1999). A suprachiasmatic nucleus generated rhythm in basal glucose concentrations. *J Neuroendocrinol, 11*(8), 643-652.
Lagerstedt, S. A., Hinrichs, D. R., Batt, S. M., Magera, M. J., Rinaldo, P., & McConnell, J. P. (2001). Quantitative determination of plasma c8-c26 total fatty acids for the biochemical diagnosis of nutritional and metabolic disorders. *Mol Genet Metab, 73*(1), 38-45.
Lajunen, H. R., Kaprio, J., Keski-Rahkonen, A., Rose, R. J., Pulkkinen, L., Rissanen, A., et al. (2009). Genetic and environmental effects on body mass index during adolescence: a prospective study among Finnish twins. *Int J Obes (Lond), 33*(5), 559-567.
Lalor, P. F., & Adams, D. H. (1999). Adhesion of lymphocytes to hepatic endothelium. *Mol Pathol, 52*(4), 214-219.
Lamptey, M. S., & Walker, B. L. (1978a). Learning behavior and brain lipid composition in rats subjected to essential fatty acid deficiency during gestation, lactation and growth. *J Nutr, 108*(3), 358-367.
Lamptey, M. S., & Walker, B. L. (1978b). Physical and neurological development of the progeny of female rats fed on essential fatty acid-deficient diet during pregnancy and/or lactation. *J Nutr, 108*(3), 351-357.
Larsson, B., Svardsudd, K., Welin, L., Wilhelmsen, L., Bjorntorp, P., & Tibblin, G. (1984). Abdominal adipose tissue distribution, obesity, and risk of cardiovascular disease and death: 13 year follow up of participants in the study of men born in 1913. *Br Med J (Clin Res Ed), 288*(6428), 1401-1404.
Lautt, W. W. (1983). Afferent and efferent neural roles in liver function. *Prog Neurobiol, 21*(4), 323-348.
Lautt, W. W., & Cote, M. G. (1977). The effect of 6-hydroxydopamine-induced hepatic sympathectomy on the early hyperglycemic response to surgical trauma under anesthesia. *J Trauma, 17*(4), 270-274.
Lautt, W. W., Dwan, P. D., & Singh, R. R. (1982). Control of the hyperglycemic response to hemorrhage in cats. *Can J Physiol Pharmacol, 60*(12), 1618-1623.
Lautt, W. W., & Greenway, C. V. (1987). Conceptual review of the hepatic vascular bed. *Hepatology, 7*(5), 952-963.
Lean, M. E., Han, T. S., & Seidell, J. C. (1999). Impairment of health and quality of life using new US federal guidelines for the identification of obesity. *Arch Intern Med, 159*(8), 837-843.
Lee, J. A., Ahmed, Q., Hines, J. E., & Burt, A. D. (1992). Disappearance of hepatic parenchymal nerves in human liver cirrhosis. *Gut, 33*(1), 87-91.

Lee, Y., Hirose, H., Zhou, Y. T., Esser, V., McGarry, J. D., & Unger, R. H. (1997). Increased lipogenic capacity of the islets of obese rats: a role in the pathogenesis of NIDDM. *Diabetes, 46*(3), 408-413.
Lee, Y., Wang, M. Y., Kakuma, T., Wang, Z. W., Babcock, E., McCorkle, K., et al. (2001). Liporegulation in diet-induced obesity. The antisteatotic role of hyperleptinemia. *J Biol Chem, 276*(8), 5629-5635.
Lepercq, J. (2003). [The diabetic pregnant woman]. *Ann Endocrinol (Paris), 64*(3 Suppl), S7-11.
Levin, B. E., & Govek, E. (1998). Gestational obesity accentuates obesity in obesity-prone progeny. *Am J Physiol, 275*(4 Pt 2), R1374-1379.
Li, C., Levitz, M., Hubbard, G. B., Jenkins, S. L., Han, V., Ferry, R. J., Jr., et al. (2007). The IGF axis in baboon pregnancy: placental and systemic responses to feeding 70% global ad libitum diet. *Placenta, 28*(11-12), 1200-1210.
Li, H., Wu, D. K., & Sullivan, S. L. (1999). Characterization and expression of sema4g, a novel member of the semaphorin gene family. *Mech Dev, 87*(1-2), 169-173.
Li, Z., & Diehl, A. M. (2003). Innate immunity in the liver. *Curr Opin Gastroenterol, 19*(6), 565-571.
Li, Z., Soloski, M. J., & Diehl, A. M. (2005). Dietary factors alter hepatic innate immune system in mice with nonalcoholic fatty liver disease. *Hepatology, 42*(4), 880-885.
Lillycrop, K. A., Slater-Jefferies, J. L., Hanson, M. A., Godfrey, K. M., Jackson, A. A., & Burdge, G. C. (2007). Induction of altered epigenetic regulation of the hepatic glucocorticoid receptor in the offspring of rats fed a protein-restricted diet during pregnancy suggests that reduced DNA methyltransferase-1 expression is involved in impaired DNA methylation and changes in histone modifications. *Br J Nutr, 97*(6), 1064-1073.
Lim, S. Y., Hoshiba, J., & Salem, N., Jr. (2005). An extraordinary degree of structural specificity is required in neural phospholipids for optimal brain function: n-6 docosapentaenoic acid substitution for docosahexaenoic acid leads to a loss in spatial task performance. *J Neurochem, 95*(3), 848-857.
Limmer, A., Ohl, J., Kurts, C., Ljunggren, H. G., Reiss, Y., Groettrup, M., et al. (2000). Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance. *Nat Med, 6*(12), 1348-1354.
Lohse, A. W., Knolle, P. A., Bilo, K., Uhrig, A., Waldmann, C., Ibe, M., et al. (1996). Antigen-presenting function and B7 expression of murine sinusoidal endothelial cells and Kupffer cells. *Gastroenterology, 110*(4), 1175-1181.
Loureiro, M. (2004). Economic dimensions of a "super size" problem. *Choices, (3rd quarter)*, 1-5.
Lovejoy, J., Wallen, K. (1988). Sexually dimorphic behavior in group housed rhesus monkeys (*Macaca mulatta*) at one year of age. *Psychobiology, 16*, 348-356.
Lumeng, C. N., Bodzin, J. L., & Saltiel, A. R. (2007). Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest, 117*(1), 175-184.
Luyer, M. D., Greve, J. W., Hadfoune, M., Jacobs, J. A., Dejong, C. H., & Buurman, W. A. (2005). Nutritional stimulation of cholecystokinin receptors inhibits inflammation via the vagus nerve. *J Exp Med, 202*(8), 1023-1029.
Luzi, L., Perseghin, G., Regalia, E., Sereni, L. P., Battezzati, A., Baratti, D., et al. (1997). Metabolic effects of liver transplantation in cirrhotic patients. *J Clin Invest, 99*(4), 692-700.
MacLennan, N. K., James, S. J., Melnyk, S., Piroozi, A., Jernigan, S., Hsu, J. L., et al. (2004). Uteroplacental insufficiency alters DNA methylation, one-carbon metabolism, and histone acetylation in IUGR rats. *Physiol Genomics, 18*(1), 43-50.

Maes, H. H., Neale, M. C., & Eaves, L. J. (1997). Genetic and environmental factors in relative body weight and human adiposity. *Behav Genet, 27*(4), 325-351.
Magnusdottir, E. V., Thorsteinsson, T., Thorsteinsdottir, S., Heimisdottir, M., & Olafsdottir, K. (2005). Persistent organochlorines, sedentary occupation, obesity and human male subfertility. *Hum Reprod, 20*(1), 208-215.
Maher, J. J. (2001). Interactions between hepatic stellate cells and the immune system. *Semin Liver Dis, 21*(3), 417-426.
Makrides, M., Neumann, M. A., Byard, R. W., Simmer, K., & Gibson, R. A. (1994). Fatty acid composition of brain, retina, and erythrocytes in breast- and formula-fed infants. *Am J Clin Nutr, 60*(2), 189-194.
Malhi, H., Barreyro, F. J., Isomoto, H., Bronk, S. F., & Gores, G. J. (2007). Free fatty acids sensitise hepatocytes to TRAIL mediated cytotoxicity. *Gut, 56*(8), 1124-1131.
Malhi, H., Bronk, S. F., Werneburg, N. W., & Gores, G. J. (2006). Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis. *J Biol Chem, 281*(17), 12093-12101.
Mallat, A., & Lotersztajn, S. (2006). Endocannabinoids as novel mediators of liver diseases. *J Endocrinol Invest, 29*(3 Suppl), 58-65.
Mallat, A., & Lotersztajn, S. (2008). Endocannabinoids and liver disease. I. Endocannabinoids and their receptors in the liver. *Am J Physiol Gastrointest Liver Physiol, 294*(1), G9-G12.
Maltepe, E., Bakardjiev, A. I., & Fisher, S. J. (2010). The placenta: transcriptional, epigenetic, and physiological integration during development. *J Clin Invest, 120*(4), 1016-1025.
Manson, J. E., Stampfer, M. J., Hennekens, C. H., & Willett, W. C. (1987). Body weight and longevity. A reassessment. *Jama, 257*(3), 353-358.
Manson, J. E., Willett, W. C., Stampfer, M. J., Colditz, G. A., Hunter, D. J., Hankinson, S. E., et al. (1995). Body weight and mortality among women. *N Engl J Med, 333*(11), 677-685.
Marceau, P., Biron, S., Hould, F. S., Marceau, S., Simard, S., Thung, S. N., et al. (1999). Liver pathology and the metabolic syndrome X in severe obesity. *J Clin Endocrinol Metab, 84*(5), 1513-1517.
Marion, A. W., Baker, A. J., & Dhawan, A. (2004). Fatty liver disease in children. *Arch Dis Child, 89*(7), 648-652.
Mark, M. D., Lohrum, M., & Puschel, A. W. (1997). Patterning neuronal connections by chemorepulsion: the semaphorins. *Cell Tissue Res, 290*(2), 299-306.
Marks, J. L., & Waite, K. (1997). Intracerebroventricular neuropeptide Y acutely influences glucose metabolism and insulin sensitivity in the rat. *J Neuroendocrinol, 9*(2), 99-103.
Marra, F., & Pinzani, M. (2002). Role of hepatic stellate cells in the pathogenesis of portal hypertension. *Nefrologia, 22 Suppl 5*, 34-40.
Marszalek, J. R., & Lodish, H. F. (2005). Docosahexaenoic acid, fatty acid-interacting proteins, and neuronal function: breastmilk and fish are good for you. *Annu Rev Cell Dev Biol, 21*, 633-657.
Martin, D. S., Segura, T., & Haywood, J. R. (1991). Cardiovascular responses to bicuculline in the paraventricular nucleus of the rat. *Hypertension, 18*(1), 48-55.
Martinez, M. (1989). C. Galli, Simopoulos, A (Ed.), In *Dietary ω3 and ω6 Fatty Acids. Biological Effects and Nutritional Essentiality* (Vol. 171, pp. 123-133). New York, NY: Plenum Press.
Martinez, M. (1992). Tissue levels of polyunsaturated fatty acids during early human development. *J Pediatr, 120*(4 Pt 2), S129-138.

Matsushita, H., Ishikawa, K., & Shimazu, T. (1979). Chemical coding of the hypothalamic neurones in metabolic control. I. Acetylcholine-sensitive neurones and glycogen synthesis in liver. *Brain Res, 163*(2), 253-261.
Matsushita, H., & Shimazu, T. (1980). Chemical coding of the hypothalamic neurones in metabolic control. II. Norepinephrine-sensitive neurones and glycogen breakdown in liver. *Brain Res, 183*(1), 79-87.
Matteoni, C. A., Younossi, Z. M., Gramlich, T., Boparai, N., Liu, Y. C., & McCullough, A. J. (1999). Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology, 116*(6), 1413-1419.
Mayer-Davis, E. J., Rifas-Shiman, S. L., Zhou, L., Hu, F. B., Colditz, G. A., & Gillman, M. W. (2006). Breast-Feeding and Risk for Childhood Obesity: Does maternal diabetes or obesity status matter? *Diabetes Care, 29*(10), 2231-2237.
McCullough, A. J. (2006). Pathophysiology of nonalcoholic steatohepatitis. *J Clin Gastroenterol, 40*(3 Suppl 1), S17-29.
McCurdy, C. E., Bishop, J. M., Williams, S. M., Grayson, B. E., Smith, S. M., Friedman, J. E., et al. (2009). Maternal high-fat diet triggers lipotoxicity in the fetal livers of nonhuman primates. *The Journal of Clinical Investigation, 119*(2), 323-335.
McCuskey, R. S. (1966). A dynamic and static study of hepatic arterioles and hepatic sphincters. *Am J Anat, 119*(3), 455-477.
McCuskey, R. S. (1967). Dynamic microscopic anatomy of the fetal liver. II. Effect of pharmacodynamic substances on the microcirculation. *Bibl Anat, 9*, 71-75.
McCuskey, R. S. (2004). Anatomy of efferent hepatic nerves. *Anat Rec A Discov Mol Cell Evol Biol, 280*(1), 821-826.
McCuskey, R. S., & McCuskey, P. A. (1990). Fine structure and function of Kupffer cells. *J Electron Microsc Tech, 14*(3), 237-246.
McCuskey, R. S., & Reilly, F. D. (1993). Hepatic microvasculature: dynamic structure and its regulation. *Semin Liver Dis, 13*(1), 1-12.
McDonald, T. J., Dupre, J., Tatemoto, K., Greenberg, G. R., Radziuk, J., & Mutt, V. (1985). Galanin inhibits insulin secretion and induces hyperglycemia in dogs. *Diabetes, 34*(2), 192-196.
Mehran, R., Schneider, R., & Franchebois, P. (2000). The minor hepatic veins: anatomy and classification. *Clin Anat, 13*(6), 416-421.
Meisami, E., Timiras, P.S. (1988a). *Handbook of Human Growth and Developmental Biology* (Vol. I, Part B). Boca Raton, FL: CRC Press.
Meisami, E., Timiras, P.S. (1988b). *Handbook of Human Growth and Developmental Biology* (Vol. I, Part A). Boca Raton, FL: CRC Press.
Melton, A. C., & Yee, H. F. (2007). Hepatic stellate cell protrusions couple platelet-derived growth factor-BB to chemotaxis. *Hepatology, 45*(6), 1446-1453.
Meltzer, J. C., MacNeil, B. J., Sanders, V., Pylypas, S., Jansen, A. H., Greenberg, A. H., et al. (2004). Stress-induced suppression of in vivo splenic cytokine production in the rat by neural and hormonal mechanisms. *Brain Behav Immun, 18*(3), 262-273.
Metz, W., & Forssmann, W. G. (1980). Innervation of the liver in guinea pig and rat. *Anat Embryol (Berl), 160*(3), 239-252.
Minagawa, M., Oya, H., Yamamoto, S., Shimizu, T., Bannai, M., Kawamura, H., et al. (2000). Intensive expansion of natural killer T cells in the early phase of hepatocyte regeneration after partial hepatectomy in mice and its association with sympathetic nerve activation. *Hepatology, 31*(4), 907-915.
Mioni, C., Bazzani, C., Giuliani, D., Altavilla, D., Leone, S., Ferrari, A., et al. (2005). Activation of an efferent cholinergic pathway produces strong protection against myocardial ischemia/reperfusion injury in rats. *Crit Care Med, 33*(11), 2621-2628.

Miyazawa, Y., Fukuda, Y., Imoto, M., Koyama, Y., & Nagura, H. (1988). Immunohistochemical studies on the distribution of nerve fibers in chronic liver diseases. *Am J Gastroenterol, 83*(10), 1108-1114.
Moitra, J., Mason, M. M., Olive, M., Krylov, D., Gavrilova, O., Marcus-Samuels, B., et al. (1998). Life without white fat: a transgenic mouse. *Genes Dev, 12*(20), 3168-3181.
Mokdad, A. H., Ford, E. S., Bowman, B. A., Dietz, W. H., Vinicor, F., Bales, V. S., et al. (2003). Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. *Jama, 289*(1), 76-79.
Mokdad, A. H., Serdula, M. K., Dietz, W. H., Bowman, B. A., Marks, J. S., & Koplan, J. P. (1999). The spread of the obesity epidemic in the United States, 1991-1998. *Jama, 282*(16), 1519-1522.
Morgan, B. L., Oppenheimer, J., & Winick, M. (1981). Effects of essential fatty acid deficiency during late gestation on brain N-acetylneuraminic acid metabolism and behaviour in the progeny. *Br J Nutr, 46*(2), 223-230.
Motta, P. M. (1977). The three-dimensional fine structure of the liver as revealed by scanning electron microscopy. *Int Rev Cytol Suppl*(6), 347-399.
Mundinger, T. O., Verchere, C. B., Baskin, D. G., Boyle, M. R., Kowalyk, S., & Taborsky, G. J., Jr. (1997). Galanin is localized in sympathetic neurons of the dog liver. *Am J Physiol, 273*(6 Pt 1), E1194-1202.
Mustelin, L., Silventoinen, K., Pietilainen, K., Rissanen, A., & Kaprio, J. (2009). Physical activity reduces the influence of genetic effects on BMI and waist circumference: a study in young adult twins. *Int J Obes (Lond), 33*(1), 29-36.
Nance, D. M., & Burns, J. (1989). Innervation of the spleen in the rat: evidence for absence of afferent innervation. *Brain Behav Immun, 3*(4), 281-290.
Nance, D. M., Hopkins, D. A., & Bieger, D. (1987). Re-investigation of the innervation of the thymus gland in mice and rats. *Brain Behav Immun, 1*(2), 134-147.
Nance, D. M., & Sanders, V. M. (2007). Autonomic innervation and regulation of the immune system (1987-2007). *Brain Behav Immun, 21*(6), 736-745.
Nanji, A. A., Khettry, U., Sadrzadeh, S. M., & Yamanaka, T. (1993). Severity of liver injury in experimental alcoholic liver disease. Correlation with plasma endotoxin, prostaglandin E2, leukotriene B4, and thromboxane B2. *Am J Pathol, 142*(2), 367-373.
Needleman, P., Raz, A., Minkes, M. S., Ferrendelli, J. A., & Sprecher, H. (1979). Triene prostaglandins: prostacyclin and thromboxane biosynthesis and unique biological properties. *Proc Natl Acad Sci U S A, 76*(2), 944-948.
Neuringer, M., Anderson, G. J., & Connor, W. E. (1988). The essentiality of n-3 fatty acids for the development and function of the retina and brain. *Annu Rev Nutr, 8*, 517-541.
Neuringer, M., Connor, W. E., Lin, D. S., Barstad, L., & Luck, S. (1986). Biochemical and functional effects of prenatal and postnatal omega 3 fatty acid deficiency on retina and brain in rhesus monkeys. *Proc Natl Acad Sci U S A, 83*(11), 4021-4025.
Neuringer, M., Connor, W. E., Van Petten, C., & Barstad, L. (1984). Dietary omega-3 fatty acid deficiency and visual loss in infant rhesus monkeys. *J Clin Invest, 73*(1), 272-276.
Ng, S. F., Lin, R. C., Laybutt, D. R., Barres, R., Owens, J. A., & Morris, M. J. (2010). Chronic high-fat diet in fathers programs beta-cell dysfunction in female rat offspring. *Nature, 467*(7318), 963-966.
Niijima, A. (1983). Glucose-sensitive afferent nerve fibers in the liver and their role in food intake and blood glucose regulation. *J Auton Nerv Syst, 9*(1), 207-220.

Niijima, A. (1989a). Nervous regulation of metabolism. *Prog Neurobiol, 33*(2), 135-147.
Niijima, A. (1989b). Neural mechanisms in the control of blood glucose concentration. *J Nutr, 119*(6), 833-840.
Nijland, M. J., Schlabritz-Loutsevitch, N. E., Hubbard, G. B., Nathanielsz, P. W., & Cox, L. A. (2007). Non-human primate fetal kidney transcriptome analysis indicates mammalian target of rapamycin (mTOR) is a central nutrient-responsive pathway. *J Physiol, 579*(Pt 3), 643-656.
Nishizawa, M., Nakabayashi, H., Kawai, K., Ito, T., Kawakami, S., Nakagawa, A., et al. (2000). The hepatic vagal reception of intraportal GLP-1 is via receptor different from the pancreatic GLP-1 receptor. *J Auton Nerv Syst, 80*(1-2), 14-21.
Nobin, A., Baumgarten, H. G., Falck, B., Ingemansson, S., Moghimzadeh, E., & Rosengren, E. (1978). Organization of the sympathetic innervation in liver tissue from monkey and man. *Cell Tissue Res, 195*(3), 371-380.
Nonogaki, K. (2000). New insights into sympathetic regulation of glucose and fat metabolism. *Diabetologia, 43*(5), 533-549.
Oben, J. A., & Diehl, A. M. (2004). Sympathetic nervous system regulation of liver repair. *Anat Rec A Discov Mol Cell Evol Biol, 280*(1), 874-883.
Oben, J. A., Roskams, T., Yang, S., Lin, H., Sinelli, N., Torbenson, M., et al. (2004). Hepatic fibrogenesis requires sympathetic neurotransmitters. *Gut, 53*(3), 438-445.
Oben, J. A., Yang, S., Lin, H., Ono, M., & Diehl, A. M. (2003). Norepinephrine and neuropeptide Y promote proliferation and collagen gene expression of hepatic myofibroblastic stellate cells. *Biochem Biophys Res Commun, 302*(4), 685-690.
World Health Organization Expert Committee. (1998). *Obesity: preventing and managing the global epidemic* (Report of a World Health Organization consultation on obesity). Geneva.
Odegaard, J. I., Ricardo-Gonzalez, R. R., Red Eagle, A., Vats, D., Morel, C. R., Goforth, M. H., et al. (2008). Alternative M2 activation of Kupffer cells by PPARdelta ameliorates obesity-induced insulin resistance. *Cell Metab, 7*(6), 496-507.
Ogden, C. L., Carroll, M. D., Curtin, L. R., McDowell, M. A., Tabak, C. J., & Flegal, K. M. (2006). Prevalence of overweight and obesity in the United States, 1999-2004. *Jama, 295*(13), 1549-1555.
Ogden, C. L., Flegal, K. M., Carroll, M. D., & Johnson, C. L. (2002). Prevalence and trends in overweight among US children and adolescents, 1999-2000. *Jama, 288*(14), 1728-1732.
Ogden, C. L., Yanovski, S. Z., Carroll, M. D., & Flegal, K. M. (2007). The epidemiology of obesity. *Gastroenterology, 132*(6), 2087-2102.
Oken, E., Huh, S. Y., Taveras, E. M., Rich-Edwards, J. W., & Gillman, M. W. (2005). Associations of maternal prenatal smoking with child adiposity and blood pressure. *Obes Res, 13*(11), 2021-2028.
Oken, E., Taveras, E. M., Kleinman, K. P., Rich-Edwards, J. W., & Gillman, M. W. (2007). Gestational weight gain and child adiposity at age 3 years. *Am J Obstet Gynecol, 196*(4), 322 e321-328.
Oliveria, S. A., Felson, D. T., Cirillo, P. A., Reed, J. I., & Walker, A. M. (1999). Body weight, body mass index, and incident symptomatic osteoarthritis of the hand, hip, and knee. *Epidemiology, 10*(2), 161-166.
Olsen, S. F., Secher, N. J., Tabor, A., Weber, T., Walker, J. J., & Gluud, C. (2000). Randomised clinical trials of fish oil supplementation in high risk pregnancies. Fish Oil Trials In Pregnancy (FOTIP) Team. *Bjog, 107*(3), 382-395.
Ozanne, S. E., & Hales, C. N. (1999). The long-term consequences of intra-uterine protein malnutrition for glucose metabolism. *Proc Nutr Soc, 58*(3), 615-619.

Paik, Y. H., Schwabe, R. F., Bataller, R., Russo, M. P., Jobin, C., & Brenner, D. A. (2003). Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells. *Hepatology, 37*(5), 1043-1055.
Painter, R. C., de Rooij, S. R., Bossuyt, P. M., de Groot, E., Stok, W. J., Osmond, C., et al. (2007). Maternal nutrition during gestation and carotid arterial compliance in the adult offspring: the Dutch famine birth cohort. *J Hypertens, 25*(3), 533-540.
Palta, M., Prineas, R. J., Berman, R., & Hannan, P. (1982). Comparison of self-reported and measured height and weight. *Am J Epidemiol, 115*(2), 223-230.
Parrish, W. R., Rosas-Ballina, M., Gallowitsch-Puerta, M., Ochani, M., Ochani, K., Yang, L. H., et al. (2008). Modulation of TNF release by choline requires alpha7 subunit nicotinic acetylcholine receptor-mediated signaling. *Mol Med, 14*(9-10), 567-574.
Patel, D. N., King, C. A., Bailey, S. R., Holt, J. W., Venkatachalam, K., Agrawal, A., et al. (2007). Interleukin-17 stimulates C-reactive protein expression in hepatocytes and smooth muscle cells via p38 MAPK and ERK1/2-dependent NF-kappaB and C/EBPbeta activation. *J Biol Chem, 282*(37), 27229-27238.
Pavlov, V. A., Ochani, M., Yang, L. H., Gallowitsch-Puerta, M., Ochani, K., Lin, X., et al. (2007). Selective alpha7-nicotinic acetylcholine receptor agonist GTS-21 improves survival in murine endotoxemia and severe sepsis. *Crit Care Med, 35*(4), 1139-1144.
Pelleymounter, M. A., Cullen, M. J., Baker, M. B., Hecht, R., Winters, D., Boone, T., et al. (1995). Effects of the obese gene product on body weight regulation in ob/ob mice. *Science, 269*(5223), 540-543.
Perez-Martinez, P., Perez-Jimenez, F., & Lopez-Miranda, J. (2010). n-3 PUFA and lipotoxicity. *Biochim Biophys Acta, 1801*(3), 362-366.
Pernow, J., Schwieler, J., Kahan, T., Hjemdahl, P., Oberle, J., Wallin, B. G., et al. (1989). Influence of sympathetic discharge pattern on norepinephrine and neuropeptide Y release. *Am J Physiol, 257*(3 Pt 2), H866-872.
Perseghin, G., Regalia, E., Battezzati, A., Vergani, S., Pulvirenti, A., Terruzzi, I., et al. (1997). Regulation of glucose homeostasis in humans with denervated livers. *J Clin Invest, 100*(4), 931-941.
Peters, J. C. (2003). Dietary fat and body weight control. *Lipids, 38*(2), 123-127.
Petersen, K. F., & Shulman, G. I. (2006). Etiology of insulin resistance. *Am J Med, 119*(5 Suppl 1), S10-16.
Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res, 29*(9), e45.
Phillips, M. J., Poucell, S., Patterson, J. (1987). *The Liver: an Atlas and Text of Ultrastructural Pathology*. New York: Raven Press.
World Health Organization Expert Committee. (1995). *Physical status: the use and interpretation of anthropometry* (Report of a World Health Organization consultation on obesity). Geneva.
Pickup, J. C. (2004). Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. *Diabetes Care, 27*(3), 813-823.
Pinhas-Hamiel, O., & Zeitler, P. (2005). The global spread of type 2 diabetes mellitus in children and adolescents. *J Pediatr, 146*(5), 693-700.
Pinhas-Hamiel, O., & Zeitler, P. (2007). Acute and chronic complications of type 2 diabetes mellitus in children and adolescents. *Lancet, 369*(9575), 1823-1831.
Plagemann, A., Harder, T., Franke, K., & Kohlhoff, R. (2002). Long-term impact of neonatal breast-feeding on body weight and glucose tolerance in children of diabetic mothers. *Diabetes Care, 25*(1), 16-22.

Plagemann, A., Harder, T., Kohlhoff, R., Rohde, W., & Dorner, G. (1997a). Glucose tolerance and insulin secretion in children of mothers with pregestational IDDM or gestational diabetes. *Diabetologia, 40*(9), 1094-1100.
Plagemann, A., Harder, T., Kohlhoff, R., Rohde, W., & Dorner, G. (1997b). Overweight and obesity in infants of mothers with long-term insulin-dependent diabetes or gestational diabetes. *Int J Obes Relat Metab Disord, 21*(6), 451-456.
Popkin, B. M., Keyou, G., Zhai, F., Guo, X., Ma, H., & Zohoori, N. (1993). The nutrition transition in China: a cross-sectional analysis. *Eur J Clin Nutr, 47*(5), 333-346.
Poynard, T., Mathurin, P., Lai, C. L., Guyader, D., Poupon, R., Tainturier, M. H., et al. (2003). A comparison of fibrosis progression in chronic liver diseases. *J Hepatol, 38*(3), 257-265.
Price, R. A., Cadoret, R. J., Stunkard, A. J., & Troughton, E. (1987). Genetic contributions to human fatness: an adoption study. *Am J Psychiatry, 144*(8), 1003-1008.
Randich, A., Spraggins, D. S., Cox, J. E., Meller, S. T., & Kelm, G. R. (2001). Jejunal or portal vein infusions of lipids increase hepatic vagal afferent activity. *Neuroreport, 12*(14), 3101-3105.
Ravelli, A. C., van der Meulen, J. H., Michels, R. P., Osmond, C., Barker, D. J., Hales, C. N., et al. (1998). Glucose tolerance in adults after prenatal exposure to famine. *Lancet, 351*(9097), 173-177.
Ravelli, A. C., van Der Meulen, J. H., Osmond, C., Barker, D. J., & Bleker, O. P. (1999). Obesity at the age of 50 y in men and women exposed to famine prenatally. *Am J Clin Nutr, 70*(5), 811-816.
Reddy, J. K., & Rao, M. S. (2006). Lipid metabolism and liver inflammation. II. Fatty liver disease and fatty acid oxidation. *Am J Physiol Gastrointest Liver Physiol, 290*(5), G852-858.
Reilly, F. D., McCuskey, P. A., & McCuskey, R. S. (1978). Intrahepatic distribution of nerves in the rat. *Anat Rec, 191*(1), 55-67.
Resko, J., A. (1985). Gonadal hormones during sexual differentiation in vertabrates. R. Goy, W. (Ed.), In *Handbook of Behavioral Neurobiology* (pp. 21-42). New York: Plenum Press.
Rexrode, K. M., Carey, V. J., Hennekens, C. H., Walters, E. E., Colditz, G. A., Stampfer, M. J., et al. (1998). Abdominal adiposity and coronary heart disease in women. *Jama, 280*(21), 1843-1848.
Rissiter, R. J., Stickland, K.P. (1970). *Handbook of Neurochemistry* (Vol. 3). New York, NY: Plenum Press.
Rodes, J. (2007). *Textbook of hepatology: from basic science to clinical practice*: Blackwell.
Rosas-Ballina, M., Ochani, M., Parrish, W. R., Ochani, K., Harris, Y. T., Huston, J. M., et al. (2008). Splenic nerve is required for cholinergic antiinflammatory pathway control of TNF in endotoxemia. *Proc Natl Acad Sci U S A, 105*(31), 11008-11013.
Roseboom, T. J., van der Meulen, J. H., Osmond, C., Barker, D. J., Ravelli, A. C., Schroeder-Tanka, J. M., et al. (2000). Coronary heart disease after prenatal exposure to the Dutch famine, 1944-45. *Heart, 84*(6), 595-598.
Rosenthal, M. D. (1987). Apparent specificity of the thrombin-stimulated deacylation of endothelial glycerolipids for polyunsaturated fatty acids with a delta-5 desaturation. *Biochim Biophys Acta, 917*(2), 279-289.
Rowland, M. L. (1990). Self-reported weight and height. *Am J Clin Nutr, 52*(6), 1125-1133.

Rudiger, H. A., & Clavien, P. A. (2002). Tumor necrosis factor alpha, but not Fas, mediates hepatocellular apoptosis in the murine ischemic liver. *Gastroenterology, 122*(1), 202-210.
Runge, C. F. (2007). Economic consequences of the obese. *Diabetes, 56*(11), 2668-2672.
Ruyle, M., Connor, W. E., Anderson, G. J., & Lowensohn, R. I. (1990). Placental transfer of essential fatty acids in humans: venous-arterial difference for docosahexaenoic acid in fetal umbilical erythrocytes. *Proc Natl Acad Sci U S A, 87*(20), 7902-7906.
Saeed, R. W., Varma, S., Peng-Nemeroff, T., Sherry, B., Balakhaneh, D., Huston, J., et al. (2005). Cholinergic stimulation blocks endothelial cell activation and leukocyte recruitment during inflammation. *J Exp Med, 201*(7), 1113-1123.
Salvati, S., Attorri, L., Avellino, C., Di Biase, A., & Sanchez, M. (2002). Dietary prenatal lipids affect myelin gene expression in postnatal undernourished rats. *Nutr Neurosci, 5*(4), 243-250.
Samulski, M. A., & Walker, B. L. (1982). Maternal Dietary fat and polyunsaturated fatty acids in the developing foetal rat brain. *J Neurochem, 39*(4), 1163-1168.
Sanchez-Gonzalez, M. A., Garcia-Cabezas, M. A., Rico, B., & Cavada, C. (2005). The primate thalamus is a key target for brain dopamine. *J Neurosci, 25*(26), 6076-6083.
Sancho-Bru, P., Bataller, R., Colmenero, J., Gasull, X., Moreno, M., Arroyo, V., et al. (2006). Norepinephrine induces calcium spikes and proinflammatory actions in human hepatic stellate cells. *Am J Physiol Gastrointest Liver Physiol, 291*(5), G877-884.
Sasaki, Y., Hayashi, N., Kasahara, A., Matsuda, H., Fusamoto, H., Sato, N., et al. (1986). Calcitonin gene-related peptide in the hepatic and splanchnic vascular systems of the rat. *Hepatology, 6*(4), 676-681.
Sasaki, Y., Kamada, T., Hayashi, N., Sato, N., Kasahara, A., Fusamoto, H., et al. (1984). Immunohistochemical distribution of glucagon, substance P and vasoactive intestinal polypeptide in hepatic vasculature of the rat. *Hepatology, 4*(6), 1184-1189.
Sasse, D., Spornitz, U. M., & Maly, I. P. (1992). Liver architecture. *Enzyme, 46*(1-3), 8-32.
Sastry, P. S. (1985). Lipids of nervous tissue: composition and metabolism. *Prog Lipid Res, 24*(2), 69-176.
Satler, J. J., Predan, S., & Brzin, M. (1974). The activity and cytochemical localization of acetylcholinesterase and butyrylcholinesterase in the rat liver. *Histochemistry, 39*(1), 65-70.
Sawchenko, P. E., & Friedman, M. I. (1979). Sensory functions of the liver--a review. *Am J Physiol, 236*(1), R5-20.
Saxena, R., Theise, N. D., & Crawford, J. M. (1999). Microanatomy of the human liver-exploring the hidden interfaces. *Hepatology, 30*(6), 1339-1346.
Schafer, M. K., Eiden, L. E., & Weihe, E. (1998). Cholinergic neurons and terminal fields revealed by immunohistochemistry for the vesicular acetylcholine transporter. II. The peripheral nervous system. *Neuroscience, 84*(2), 361-376.
Schaffner, F. (1985). Structure of the Liver. J. Berk (Ed.), In *Bockus Gastroenterology* (pp. 2625-2658). Philidelphia: W.B. Saunders.
Schlabritz-Loutsevitch, N., Ballesteros, B., Dudley, C., Jenkins, S., Hubbard, G., Burton, G. J., et al. (2007). Moderate maternal nutrient restriction, but not glucocorticoid administration, leads to placental morphological changes in the baboon (Papio sp.). *Placenta, 28*(8-9), 783-793.

Schlabritz-Loutsevitch, N. E., Dudley, C. J., Gomez, J. J., Nevill, C. H., Smith, B. K., Jenkins, S. L., et al. (2007). Metabolic adjustments to moderate maternal nutrient restriction. *Br J Nutr, 98*(2), 276-284.
Schlesinger, M. H., Wilets, I. F., & Nagler, H. M. (1994). Treatment outcome after varicocelectomy. A critical analysis. *Urol Clin North Am, 21*(3), 517-529.
Schwabe, R. F., Bataller, R., & Brenner, D. A. (2003). Human hepatic stellate cells express CCR5 and RANTES to induce proliferation and migration. *Am J Physiol Gastrointest Liver Physiol, 285*(5), G949-958.
Scialli, A. R. (2006). Teratology public affairs committee position paper: maternal obesity and pregnancy. *Birth Defects Res A Clin Mol Teratol, 76*(2), 73-77.
Seidell, J. C., & Flegal, K. M. (1997). Assessing obesity: classification and epidemiology. *Br Med Bull, 53*(2), 238-252.
Seidell, J. C., Rissanen, A. (1997). *Time Trends in the Worldwide Prevalence of Obesity*. New York: Dekker.
Seki, E., De Minicis, S., Osterreicher, C. H., Kluwe, J., Osawa, Y., Brenner, D. A., et al. (2007). TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat Med, 13*(11), 1324-1332.
Seseke, F. G., Gardemann, A., & Jungermann, K. (1992). Signal propagation via gap junctions, a key step in the regulation of liver metabolism by the sympathetic hepatic nerves. *FEBS Lett, 301*(3), 265-270.
Shafik, A., & Olfat, S. (1981). Lipectomy in the treatment of scrotal lipomatosis. *Br J Urol, 53*(1), 55-61.
Shangraw, R. E. (2006). Metabolic issues in liver transplantation. *Int Anesthesiol Clin, 44*(3), 1-20.
Shimabukuro, M., Koyama, K., Chen, G., Wang, M. Y., Trieu, F., Lee, Y., et al. (1997). Direct antidiabetic effect of leptin through triglyceride depletion of tissues. *Proc Natl Acad Sci U S A, 94*(9), 4637-4641.
Shimazu, T. (1962). The effect of electric stimulation of hypothalamus on rabbit liver tryptophan pyrrolase. *Biochim Biophys Acta, 65*, 373-375.
Shimazu, T. (Ed.). (1996). *Liver Innervation and the control of hepatic function*. London: John Libbey & Company Ltd.
Shimazu, T., & Fukuda, A. (1965). Increased activities of glycogenolytic enzymes in liver after splanchnic-nerve stimulation. *Science, 150*(703), 1607-1608.
Shimazu, T., Fukuda, A., & Ban, T. (1966). Reciprocal influences of the ventromedial and lateral hypothalamic nuclei on blood glucose level and liver glycogen content. *Nature, 210*(5041), 1178-1179.
Shimazu, T., Matsushita, H., & Ishikawa, K. (1978). Hypothalamic control of liver glycogen metabolism in adult and aged rats. *Brain Res, 144*(2), 343-352.
Shimazu, T., & Ogasawara, S. (1975). Effects of hypothalamic stimulation on gluconeogenesis and glycolysis in rat liver. *Am J Physiol, 228*(6), 1787-1793.
Siega-Riz, A. M., Bodnar, L. M., & Savitz, D. A. (2002). What are pregnant women eating? Nutrient and food group differences by race. *Am J Obstet Gynecol, 186*(3), 480-486.
Silventoinen, K., Rokholm, B., Kaprio, J., & Sorensen, T. I. (2010). The genetic and environmental influences on childhood obesity: a systematic review of twin and adoption studies. *Int J Obes (Lond), 34*(1), 29-40.
Silverman, B. L., Landsberg, L., & Metzger, B. E. (1993). Fetal hyperinsulinism in offspring of diabetic mothers. Association with the subsequent development of childhood obesity. *Ann N Y Acad Sci, 699*, 36-45.
Simopoulos, A. P. (1991). Omega-3 fatty acids in health and disease and in growth and development. *Am J Clin Nutr, 54*(3), 438-463.

Simopoulos, A. P. (1999). Essential fatty acids in health and chronic disease. *Am J Clin Nutr, 70*(3 Suppl), 560S-569S.
Sinclair, A. J., & Crawford, M. A. (1972). The accumulation of arachidonate and docosahexaenoate in the developing rat brain. *J Neurochem, 19*(7), 1753-1758.
Singer, L. K., Kuper, J., Brogan, R. S., Smith, M. S., & Grove, K. L. (2000). Novel expression of hypothalamic neuropeptide Y during postnatal development in the rat. *Neuroreport, 11*(5), 1075-1080.
Sjostrom, L. V. (1992). Mortality of severely obese subjects. *Am J Clin Nutr, 55*(2 Suppl), 516S-523S.
Skaaring, P., & Bierring, F. (1976). On the intrinsic innervation of normal rat liver. Histochemical and scanning electron microscopical studies. *Cell Tissue Res, 171*(2), 141-155.
Skandalakis, J. E., Skandalakis, L. J., Skandalakis, P. N., & Mirilas, P. (2004). Hepatic surgical anatomy. *Surg Clin North Am, 84*(2), 413-435, viii.
Smedsrod, B., De Bleser, P. J., Braet, F., Lovisetti, P., Vanderkerken, K., Wisse, E., et al. (1994). Cell biology of liver endothelial and Kupffer cells. *Gut, 35*(11), 1509-1516.
Sorensen, T. I., Holst, C., Stunkard, A. J., & Skovgaard, L. T. (1992). Correlations of body mass index of adult adoptees and their biological and adoptive relatives. *Int J Obes Relat Metab Disord, 16*(3), 227-236.
Sorensen, T. I., Price, R. A., Stunkard, A. J., & Schulsinger, F. (1989). Genetics of obesity in adult adoptees and their biological siblings. *Bmj, 298*(6666), 87-90.
Sprecher, H. (1981). Biochemistry of essential fatty acids. *Prog Lipid Res, 20*, 13-22.
Srinivasan, M., Aalinkeel, R., Song, F., Mitrani, P., Pandya, J. D., Strutt, B., et al. (2006). Maternal hyperinsulinemia predisposes rat fetuses for hyperinsulinemia, and adult-onset obesity and maternal mild food restriction reverses this phenotype. *Am J Physiol Endocrinol Metab, 290*(1), E129-E134.
Srinivasan, M., Katewa, S. D., Palaniyappan, A., Pandya, J. D., & Patel, M. S. (2006). Maternal high-fat diet consumption results in fetal malprogramming predisposing to the onset of metabolic syndrome-like phenotype in adulthood. *Am J Physiol Endocrinol Metab, 291*(4), E792-799.
Stathis, S. L., O'Callaghan, M., Harvey, J., & Rogers, Y. (1999). Head circumference in ELBW babies is associated with learning difficulties and cognition but not ADHD in the school-aged child. *Dev Med Child Neurol, 41*(6), 375-380.
Stein, A. D., Ravelli, A. C., & Lumey, L. H. (1995). Famine, third-trimester pregnancy weight gain, and intrauterine growth: the Dutch Famine Birth Cohort Study. *Hum Biol, 67*(1), 135-150.
Stein, A. D., Zybert, P. A., van de Bor, M., & Lumey, L. H. (2004). Intrauterine famine exposure and body proportions at birth: the Dutch Hunger Winter. *Int J Epidemiol, 33*(4), 831-836.
Stein, Z., & Susser, M. (1975). The Dutch famine, 1944-1945, and the reproductive process. II. Interrelations of caloric rations and six indices at birth. *Pediatr Res, 9*(2), 76-83.
Stocker, C. J., Arch, J. R., & Cawthorne, M. A. (2005). Fetal origins of insulin resistance and obesity. *Proc Nutr Soc, 64*(2), 143-151.
Strain, G. W., Zumoff, B., Kream, J., Strain, J. J., Deucher, R., Rosenfeld, R. S., et al. (1982). Mild Hypogonadotropic hypogonadism in obese men. *Metabolism, 31*(9), 871-875.
Strauss, R. S. (1997). Effects of the intrauterine environment on childhood growth. *Br Med Bull, 53*(1), 81-95.

Stunkard, A. J., Foch, T. T., & Hrubec, Z. (1986). A twin study of human obesity. *Jama, 256*(1), 51-54.
Stunkard, A. J., Harris, J. R., Pedersen, N. L., & McClearn, G. E. (1990). The body-mass index of twins who have been reared apart. *N Engl J Med, 322*(21), 1483-1487.
Stunkard, A. J., Sorensen, T. I., Hanis, C., Teasdale, T. W., Chakraborty, R., Schull, W. J., et al. (1986). An adoption study of human obesity. *N Engl J Med, 314*(4), 193-198.
Sturm, R. (2002). The effects of obesity, smoking, and drinking on medical problems and costs. *Health Aff (Millwood), 21*(2), 245-253.
Su, H. M., Bernardo, L., Mirmiran, M., Ma, X. H., Corso, T. N., Nathanielsz, P. W., et al. (1999). Bioequivalence of dietary alpha-linolenic and docosahexaenoic acids as sources of docosahexaenoate accretion in brain and associated organs of neonatal baboons. *Pediatr Res, 45*(1), 87-93.
Su, H. M., Corso, T. N., Nathanielsz, P. W., & Brenna, J. T. (1999). Linoleic acid kinetics and conversion to arachidonic acid in the pregnant and fetal baboon. *J Lipid Res, 40*(7), 1304-1312.
Su, H. M., Huang, M. C., Saad, N. M., Nathanielsz, P. W., & Brenna, J. T. (2001). Fetal baboons convert 18:3n-3 to 22:6n-3 in vivo. A stable isotope tracer study. *J Lipid Res, 42*(4), 581-586.
Subar, A. F., Krebs-Smith, S. M., Cook, A., & Kahle, L. L. (1998). Dietary sources of nutrients among US children, 1989-1991. *Pediatrics, 102*(4 Pt 1), 913-923.
Sullivan, E. L., Grayson, B., Takahashi, D., Robertson, N., Maier, A., Bethea, C. L., et al. (2010). Chronic consumption of a high-fat diet during pregnancy causes perturbations in the serotonergic system and increased anxiety-like behavior in nonhuman primate offspring. *J Neurosci, 30*(10), 3826-3830.
Suter, M., Bocock, P., Showalter, L., Hu, M., Shope, C., McKnight, R., et al. (2011). Epigenomics: maternal high-fat diet exposure in utero disrupts peripheral circadian gene expression in nonhuman primates. *Faseb J*.
Sutherland, J. P., McKinley, B., & Eckel, R. H. (2004). The metabolic syndrome and inflammation. *Metab Syndr Relat Disord, 2*(2), 82-104.
Suzuki, A., & Abdelmalek, M. F. (2009). Nonalcoholic fatty liver disease in women. *Womens Health (Lond Engl), 5*(2), 191-203.
Svennerholm, L. (1968). Distribution and fatty acid composition of phosphoglycerides in normal human brain. *J Lipid Res, 9*(5), 570-579.
Taborsky, G. J., Jr., Beltramini, L. M., Brown, M., Veith, R. C., & Kowalyk, S. (1994). Canine liver releases neuropeptide Y during sympathetic nerve stimulation. *Am J Physiol, 266*(5 Pt 1), E804-812.
Takeuchi, A., Kimura, T., & Satoh, S. (1993). Effects of nifedipine and Bay-K-8644 on the release of catecholamines from the dog adrenal gland in response to splanchnic nerve stimulation. *Clin Exp Pharmacol Physiol, 20*(1), 35-39.
Teli, M. R., James, O. F., Burt, A. D., Bennett, M. K., & Day, C. P. (1995). The natural history of nonalcoholic fatty liver: a follow-up study. *Hepatology, 22*(6), 1714-1719.
Thone-Reineke, C., Kalk, P., Dorn, M., Klaus, S., Simon, K., Pfab, T., et al. (2006). High-protein nutrition during pregnancy and lactation programs blood pressure, food efficiency, and body weight of the offspring in a sex-dependent manner. *Am J Physiol Regul Integr Comp Physiol, 291*(4), R1025-1030.
Tiniakos, D. G. (1996). Development of intrahepatic nerves in human liver. T. Shimazu (Ed.), In *Liver innervation and the control of hepatic function* (pp. 23-29). London: John Libbey & Company Ltd.

Tiniakos, D. G., Lee, J. A., & Burt, A. D. (1996). Innervation of the liver: morphology and function. *Liver, 16*(3), 151-160.
Tiniakos, D. G., Mathew, J., Kittas, C., & Burt, A. D. (2008). Ontogeny of human intrahepatic innervation. *Virchows Arch, 452*(4), 435-442.
Torii, K., & Niijima, A. (2001). Effect of lysine on afferent activity of the hepatic branch of the vagus nerve in normal and L-lysine-deficient rats. *Physiol Behav, 72*(5), 685-690.
Tracey, K. J. (2002). The inflammatory reflex. *Nature, 420*(6917), 853-859.
Tracey, K. J. (2009). Reflex control of immunity. *Nat Rev Immunol, 9*(6), 418-428.
Trauner, M., Arrese, M., & Wagner, M. (2010). Fatty liver and lipotoxicity. *Biochim Biophys Acta, 1801*(3), 299-310.
Trotter, R. N., Stornetta, R. L., Guyenet, P. G., & Roberts, M. R. (2007). Transneuronal mapping of the CNS network controlling sympathetic outflow to the rat thymus. *Auton Neurosci, 131*(1-2), 9-20.
Tsai, T. L. (1958). [A histological study of sensory nerves in the liver]. *Acta Neuroveg (Wien), 17*(3-4), 354-385.
Tsuneki, K., & Ichihara, K. (1981). Electron microscope study of vertebrate liver innervation. *Arch Histol Jpn, 44*(1), 1-13.
Twigg, J., Fulton, N., Gomez, E., Irvine, D. S., & Aitken, R. J. (1998). Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. *Hum Reprod, 13*(6), 1429-1436.
Ueno, T., Bioulac-Sage, P., Balabaud, C., & Rosenbaum, J. (2004). Innervation of the sinusoidal wall: regulation of the sinusoidal diameter. *Anat Rec A Discov Mol Cell Evol Biol, 280*(1), 868-873.
Uneyama, H., Niijima, A., Tanaka, T., & Torii, K. (2002). Receptor subtype specific activation of the rat gastric vagal afferent fibers to serotonin. *Life Sci, 72*(4-5), 415-423.
Unger, R. H. (2002). Lipotoxic diseases. *Annu Rev Med, 53*, 319-336.
Unger, R. H. (2003). The physiology of cellular liporegulation. *Annu Rev Physiol, 65*, 333-347.
Unger, R. H., & Orci, L. (2001). Diseases of liporegulation: new perspective on obesity and related disorders. *Faseb J, 15*(2), 312-321.
Unger, R. H., & Orci, L. (2002). Lipoapoptosis: its mechanism and its diseases. *Biochim Biophys Acta, 1585*(2-3), 202-212.
Unger, R. H., Zhou, Y. T., & Orci, L. (1999). Regulation of fatty acid homeostasis in cells: novel role of leptin. *Proc Natl Acad Sci U S A, 96*(5), 2327-2332.
Vahratian, A. (2008). Prevalence of Overweight and Obesity Among Women of Childbearing Age: Results from the 2002 National Survey of Family Growth. *Matern Child Health J*.
Van Assche, F. A., Holemans, K., & Aerts, L. (2001). Long-term consequences for offspring of diabetes during pregnancy. *Br Med Bull, 60*, 173-182.
van den Hoek, A. M., van Heijningen, C., Schroder-van der Elst, J. P., Ouwens, D. M., Havekes, L. M., Romijn, J. A., et al. (2008). Intracerebroventricular administration of neuropeptide Y induces hepatic insulin resistance via sympathetic innervation. *Diabetes, 57*(9), 2304-2310.
van den Hoek, A. M., Voshol, P. J., Karnekamp, B. N., Buijs, R. M., Romijn, J. A., Havekes, L. M., et al. (2004). Intracerebroventricular neuropeptide Y infusion precludes inhibition of glucose and VLDL production by insulin. *Diabetes, 53*(10), 2529-2534.

van Houwelingen, A. C., Sorensen, J. D., Hornstra, G., Simonis, M. M., Boris, J., Olsen, S. F., et al. (1995). Essential fatty acid status in neonates after fish-oil supplementation during late pregnancy. *Br J Nutr, 74*(5), 723-731.

Van Rossen, E., Vander Borght, S., van Grunsven, L. A., Reynaert, H., Bruggeman, V., Blomhoff, R., et al. (2009). Vinculin and cellular retinol-binding protein-1 are markers for quiescent and activated hepatic stellate cells in formalin-fixed paraffin embedded human liver. *Histochem Cell Biol, 131*(3), 313-325.

van Westerloo, D. J., Giebelen, I. A., Florquin, S., Bruno, M. J., Larosa, G. J., Ulloa, L., et al. (2006). The vagus nerve and nicotinic receptors modulate experimental pancreatitis severity in mice. *Gastroenterology, 130*(6), 1822-1830.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol, 3*(7), RESEARCH0034.

Vermeulen, A., Kaufman, J. M., & Giagulli, V. A. (1996). Influence of some biological indexes on sex hormone-binding globulin and androgen levels in aging or obese males. *J Clin Endocrinol Metab, 81*(5), 1821-1826.

Visscher, T. L., & Seidell, J. C. (2001). The public health impact of obesity. *Annu Rev Public Health, 22*, 355-375.

Wake, K. (1980). Perisinusoidal stellate cells (fat-storing cells, interstitial cells, lipocytes), their related structure in and around the liver sinusoids, and vitamin A-storing cells in extrahepatic organs. *Int Rev Cytol, 66*, 303-353.

Wake, K. (1988). Liver perivascular cells revealed by gold and silver impregnation methods and electron microscopy. P. Motta (Ed.), In *Biopathology of the Liver, an Ultrastructural Approach* (pp. 23-26). Dordrecht: Kluwer.

Wake, K. (1995). Structure of the sinusoidal wall in the liver. E. Wisse, Knook, DL., Wake, K (Ed.), In *Cells of the Hepatic Sinusoid* (pp. 241-246). Leiden: Kupffer Cell Foundation.

Wake, K., Motomatsu, K., Ekataksin, W. (1991). Postnatal development of the perisinusoidal stellate cells in the rat liver. E. Wisse, Knook, DL., McCuskey, RS (Ed.), In *Cells of the Hepatic Sinusoid* (pp. 269-275). Leiden: Kupffer Cell Foundation.

Walker, B. L. (1967). Maternal diet and brain fatty acids in young rats. *Lipids, 2*(6), 497-500.

Wallen, K. (2005). Hormonal influences on sexually differentiated behavior in nonhuman primates. *Front Neuroendocrinol, 26*(1), 7-26.

Wan, W., Wetmore, L., Sorensen, C. M., Greenberg, A. H., & Nance, D. M. (1994). Neural and biochemical mediators of endotoxin and stress-induced c-fos expression in the rat brain. *Brain Res Bull, 34*(1), 7-14.

Wang, H., Yu, M., Ochani, M., Amella, C. A., Tanovic, M., Susarla, S., et al. (2003). Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. *Nature, 421*(6921), 384-388.

Watanabe, Y., Takahashi, A., & Shimazu, T. (1990). Neural control of biosynthesis and secretion of serum transferrin in perfused rat liver. *Biochem J, 267*(2), 545-548.

Watkins, L. R., Goehler, L. E., Relton, J. K., Tartaglia, N., Silbert, L., Martin, D., et al. (1995). Blockade of interleukin-1 induced hyperthermia by subdiaphragmatic vagotomy: evidence for vagal mediation of immune-brain communication. *Neurosci Lett, 183*(1-2), 27-31.

Weber, P. C., & Leaf, A. (1991). Cardiovascular effects of omega 3 fatty acids. Atherosclerosis risk factor modification by omega 3 fatty acids. *World Rev Nutr Diet, 66*, 218-232.

Whitaker, R. C. (2004). Predicting preschooler obesity at birth: the role of maternal obesity in early pregnancy. *Pediatrics, 114*(1), e29-36.
Whitaker, R. C., Wright, J. A., Pepe, M. S., Seidel, K. D., & Dietz, W. H. (1997). Predicting obesity in young adulthood from childhood and parental obesity. *N Engl J Med, 337*(13), 869-873.
White, H. B., Jr., Galli, C., & Paoletti, R. (1971). Ethanolamine phosphoglyceride fatty acids in aging human brain. *J Neurochem, 18*(7), 1337-1339.
WHO factsheet no. 311. (February, 2011). *Obesity and overweight*, from http://www.who.int/mediacentre/factsheets/fs311/en/index.html
Wiggins, R. C. (1982). Myelin development and nutritional insufficiency. *Brain Res, 257*(2), 151-175.
Wiggins, R. C. (1986). Myelination: a critical stage in development. *Neurotoxicology, 7*(2), 103-120.
Wilkin, T. J. (2002). Diabetes mellitus: Type 1 or type 2? The accelerator hypothesis. *J Pediatr, 141*(3), 449-450.
Winau, F., Hegasy, G., Weiskirchen, R., Weber, S., Cassan, C., Sieling, P. A., et al. (2007). Ito cells are liver-resident antigen-presenting cells for activating T cell responses. *Immunity, 26*(1), 117-129.
Wolf, A. M., & Colditz, G. A. (1998). Current estimates of the economic cost of obesity in the United States. *Obes Res, 6*(2), 97-106.
Xiao, X. Q., Williams, S. M., Grayson, B. E., Glavas, M. M., Cowley, M. A., Smith, M. S., et al. (2007). Excess weight gain during the early postnatal period is associated with permanent reprogramming of brown adipose tissue adaptive thermogenesis. *Endocrinology, 148*(9), 4150-4159.
Yacoubian, S., & Serhan, C. N. (2007). New endogenous anti-inflammatory and proresolving lipid mediators: implications for rheumatic diseases. *Nat Clin Pract Rheumatol, 3*(10), 570-579; quiz 571 p following 589.
Yamauchi, T., Kamon, J., Waki, H., Terauchi, Y., Kubota, N., Hara, K., et al. (2001). The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med, 7*(8), 941-946.
Yeh, Y. Y. (1988). Maternal dietary restriction causes myelin and lipid deficits in the brain of offspring. *J Neurosci Res, 19*(3), 357-363.
Yi, C. X., la Fleur, S. E., Fliers, E., & Kalsbeek, A. (2010). The role of the autonomic nervous liver innervation in the control of energy metabolism. *Biochim Biophys Acta, 1802*(4), 416-431.
Yi, C. X., Serlie, M. J., Ackermans, M. T., Foppen, E., Buijs, R. M., Sauerwein, H. P., et al. (2009). A major role for perifornical orexin neurons in the control of glucose metabolism in rats. *Diabetes, 58*(9), 1998-2005.
Yi, C. X., Sun, N., Ackermans, M. T., Alkemade, A., Foppen, E., Shi, J., et al. (2010). Pituitary adenylate cyclase-activating polypeptide stimulates glucose production via the hepatic sympathetic innervation in rats. *Diabetes, 59*(7), 1591-1600.
Yogev, Y., & Langer, O. (2008). Pregnancy outcome in obese and morbidly obese gestational diabetic women. *Eur J Obstet Gynecol Reprod Biol, 137*(1), 21-26.
Yoshikawa, H., Kurokawa, M., Ozaki, N., Nara, K., Atou, K., Takada, E., et al. (2006). Nicotine inhibits the production of proinflammatory mediators in human monocytes by suppression of I-kappaB phosphorylation and nuclear factor-kappaB transcriptional activity through nicotinic acetylcholine receptor alpha7. *Clin Exp Immunol, 146*(1), 116-123.
Young, T., Palta, M., Dempsey, J., Skatrud, J., Weber, S., & Badr, S. (1993). The occurrence of sleep-disordered breathing among middle-aged adults. *N Engl J Med, 328*(17), 1230-1235.

Youyou, A., Durand, G., Pascal, G., Piciotti, M., Dumont, O., & Bourre, J. M. (1986). Recovery of altered fatty acid composition induced by a diet devoid of n-3 fatty acids in myelin, synaptosomes, mitochondria, and microsomes of developing rat brain. *J Neurochem, 46*(1), 224-228.

Zhou, Y. T., Shimabukuro, M., Lee, Y., Koyama, K., Higa, M., Ferguson, T., et al. (1998). Enhanced de novo lipogenesis in the leptin-unresponsive pancreatic islets of prediabetic Zucker diabetic fatty rats: role in the pathogenesis of lipotoxic diabetes. *Diabetes, 47*(12), 1904-1908.

Zhou, Y. T., Shimabukuro, M., Wang, M. Y., Lee, Y., Higa, M., Milburn, J. L., et al. (1998). Role of peroxisome proliferator-activated receptor alpha in disease of pancreatic beta cells. *Proc Natl Acad Sci U S A, 95*(15), 8898-8903.