INTERRELATIONSHIPS AMONG DIABETES, LONG CHAIN POLYUNSATURATED FATTY ACID NUTRITION AND BRAIN DEVELOPMENT IN RODENTS

Jinping Zhao

School of Dietetics and Human Nutrition

McGill University, Montréal

November 2010

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Doctor of Philosophy

ABSTRACT

Conversion of linoleic (LA) and α-linolenic acid (ALA) is vital in providing arachidonic (AA) and docosahexaenoic acid (DHA), and this capacity is up-regulated in pregnancy. However, the synthesizing enzymes are depressed in diabetes due to insulin deficiency or resistance, and the physiological adaptation in AA and DHA synthesis is absent in diabetic pregnant women. Newborn infants of diabetic mothers (IDM) have compromised AA and DHA status, while most diabetic rats have lower AA only. Follow-up studies reveal neurobehavioral deficits, bone abnormalities, and glucose intolerance in the IDM. Thus, the main objective of this thesis was to investigate if maternal AA supplementation improves neurodevelopment, bone health and glucose tolerance in weaning and adult offspring. Rat dams were randomized into 6 groups: Saline-Placebo, streptozotocin-induced diabetes (STZ) with glucose controlled at <13 mmol/L (STZ/GC), or poorly-controlled at 13-20 mmol/L (STZ/PC) using insulin; and fed either Control or AA (0.5% fat) diet throughout reproduction. The other objective was to test if brain DHA is maintained with a diet rich in ALA but absent in DHA in obese-insulin-resistant young rats. Male *fa/fa* and lean rats were fed diets enriched with flaxseed (35.5% ALA), menhaden (9.2% DHA) or safflower oil (54.1% LA) for 9 weeks. Results revealed that (i) for weaning-offspring: the liver AA was lower (17%) and the performances in negative geotaxis and rota rod were inferior in STZ/PC offspring, but this improved with maternal AA supplementation ($P \leq 0.003$), and AA-diet offspring had higher (16%) liver AA than Control-diet offspring; (ii) for adult-offspring: STZ/PC offspring showed

longer ($P \leq 0.018$) escape-latency on testing-day 1 water maze (WM), maternal glucose concentration positively correlated with ($P=0.006$) male offspring testing-day 1 WM latency, and AA diet improved the performances in WM and rota rod ($P \leq 0.032$); (iii) the STZ/GC offspring had greater ($P<0.030$) whole-body and regional bone area than STZ/PC offspring. Maternal glucose negatively correlated ($P<0.05$) with offspring whole-body bone area and mineral content at 4 weeks in all offspring and with tibia area in males at 12 weeks. Glucose tolerance was not affected by maternal treatment or diet; (iv) the forebrain DHA of *fa/fa* rats was lower ($P=0.011$) than lean rats when fed flaxseed but not different when fed menhaden or safflower oil diets, even with lower $\Delta 5$ ($P \leq 0.006$) desaturase indices. In conclusion, STZ/PC offspring have neurodevelopmental delays at weaning and adult age. Maternal AA supplementation improved learning outcomes. Maternal glucose control has long-term positive consequences to bone health of adult offspring. Dietary DHA may be needed to maintain forebrain DHA in insulin-resistant young rats.

RÉSUMÉ

La conversion de l'acide linoléique (LA) et l'acide α-linolénique (ALA) en acide arachidonique (AA) et acide docosahexaénoïque (DHA) est essentielle à la vie et cette capacité de conversion est augmentée pendant la grossesse. Cependant, les enzymes de synthèse sont diminuées dans le diabète en raison d'une déficience en insuline ou une résistance à son action et l'adaptation physiologique augmentant la synthèse de AA et l'acide DHA est absente chez les femmes enceintes diabétiques. L'état physiologique en AA et DHA est compromis chez les nouveau-nés de mères diabétiques, tandis que chez la plupart des rats diabétiques, seul celui de l'AA l'est. De plus, des déficits neurocomportementaux, des anomalies osseuses et l'intolérance au glucose ont été démontrés chez les nouveau-nés de mères diabétiques dans des études de suivi. L'objectif principal de cette thèse était de déterminer si une supplémentation maternelle en AA améliore le développement neurologique, la santé des os et la tolérance au glucose de la progéniture au moment du sevrage et à l'âge adulte. Pour ce, des rates fertiles ont été randomisées en 6 groupes: salin-placebo, diabète induit par la streptozotocine (STZ) avec glycémie bien contrôlée [<13 mmol/L (STZ/GC)], ou mal contrôlée [13-20 mmol/L (STZ/PC)] par insuline; ces 3 groupes ont été supplémentés ou non d'AA (0.5% des matières grasses) tout au long de la reproduction. Un deuxième objectif était de déterminer si le contenu en DHA du cerveau est maintenu avec un régime riche en ALA, mais sans DHA, chez de jeunes rats obèses insulino-résistants. Pour ce, des rats mâles *fa/fa* et des rats maigres ont reçu des rations enrichies d'huile de lin (35.5% ALA), de menhaden

(9.2% DHA) ou de carthame (54.1% LA) pendant 9 semaines. Nos résultats démontrent que: (i) au moment du sevrage, les taux hépatiques en AA qui sont plus faibles (17%) et les performances en géotaxie négative et tige de rotation inférieures chez les rats nés de mères STZ/PC, s'améliorent avec la supplémentation maternelle en AA ($P \leq 0.003$); les rats issus des mères aux rations enrichies en AA, ont des taux hépatiques en AA plus élevés de 16% que ceux de mères non-supplémentées; (ii) à l'âge adulte, les rats nés des mères STZ/PC requièrent plus de temps ($P \leq 0.018$) pour s'échapper du labyrinthe d'eau (WM) au jour 1, la concentration plasmatique en glucose des mères est en corrélation positive ($P=0.006$) avec celle de leur progéniture de sexe masculin au jour 1, la supplémentation en AA améliore les performances du WM et celles de la tige de rotation ($P \leq 0.032$); (iii) la progéniture des mères STZ/GC a une plus grande ($P<0.030$) surface osseuse corporelle et régionale que la progéniture des mères STZ/PC. La glycémie maternelle est en corrélation négative ($P<0.05$) avec la surface osseuse corporelle et le contenu minéral des descendants à 4 semaines et avec la surface du tibia chez les mâles à 12 semaines. La tolérance au glucose chez les rejetons, n'est pas affectée par le traitement de la mère diabétique ni sa supplémentation en AA; (iv) le contenu prosencéphal en DHA des rats *fa/fa* est plus bas ($P=0.011$) que celui des rats maigres quand nourris d'huile de lin, mais pas différent quand nourris d'huile de menhaden ou de carthame, même si l'indice de la $\Delta 5$ désaturase est bas ($P \leq 0.006$). En conclusion, chez les rats nés de mères STZ/PC, des retards du développement neurologique sont mesurables au moment du sevrage et à l'âge adulte. Une supplémentation maternelle en AA améliore l'apprentissage chez le rejeton. Le contrôle de la glycémie maternelle à long

terme a des effets positifs pour la santé osseuse des descendants adultes. Un apport alimentaire en DHA peut être nécessaire pour maintenir la concentration prosencéphale en DHA chez les jeunes rats insulino-résistants.

ADVANCE OF SCHOLARLY KNOWLEDGE

1. Original contribution to knowledge

The following are the contributions of this thesis to knowledge in the field of maternal and infant nutrition demonstrates for the first time:

- The potential efficacy of maternal dietary AA supplementation on the development of the offspring from rat dams with and without diabetes. Although there is strong evidence that the avoidance of hyperglycemia is essential in optimizing pregnancy outcome, poor control of all types of diabetes exists in women with diabetes during pregnancy and this occurs despite clear educational goals, recommended treatments and adherence by many women. Offspring of diabetic mothers show disadvantages in neurobehavioral, bone development and as reported by others glucose intolerance.
- Offspring of poorly-controlled diabetic dams have lower liver AA that is associated with poorer neurobehavioral performance at weaning age. Although diabetic rats have lower AA and newborn IDM have lower AA and DHA, this is the first study that systematically demonstrates the associations among maternal hyperglycemia (indicator of insulin deficiency), offspring long-chain polyunsaturated fatty acid (LC-PUFA) aberrations and neurobehavioral, bone and glucose tolerance outcome measurements.
- Maternal AA supplementation improved AA status and neurobehavioral performance in all offspring at weaning age. Provides

groundwork leading to future trials in humans, in whom the prevalence of diabetes is growing, particularly in Canada.

- A positive correlation between dams' gestational glucose concentration and the initial water maze escape latency of young adult offspring, and maternal AA supplementation positively influencing learning outcomes, indicating the importance of maternal glucose control and AA dietary intervention.
- A long term adverse relationship between maternal hyperglycaemia and skeletal size of adult offspring. Although there are some reports regarding the negative effects of maternal diabetes on offspring bone health, this is the first report of smaller bone size in the young adult, a time period (12 weeks of age in rats) at which peak bone mass is considered well established. The findings suggest that skeletal size is programmed by fetal exposure to maternal hyperglycemia.
- Maternal dietary AA supplementation is associated with higher bone mineral density in male rats. Liver AA at 4 weeks positively correlated with lumbar spine mineral density in males.
- Mammals with obesity and insulin resistance may have suboptimal DHA in forebrain even at young adult age. Insulin resistance and related metabolic disorders are associated with a unique fatty acid pattern with low in DHA in plasma and tissues in human adults and youth. No study, however, has examined the effect of insulin resistance on brain LC-PUFA metabolism especially in youth.

2. Research publications in refereed scientific journals

- Jinping Zhao, Marc R. Del Bigio, Hope A. Weiler. Maternal arachidonic acid supplementation improves neurodevelopment of offspring from healthy and diabetic rats. *Prostaglandins, Leukotrienes and Essential Fatty Acids* 2009;81:349-356. (Chapter 3, Manuscript 1)
- Jinping Zhao, Marc R. Del Bigio, Hope A. Weiler. Maternal arachidonic acid supplementation improves neurodevelopment in young adult offspring from rat dams with and without diabetes. *Prostaglandins, Leukotrienes and Essential Fatty Acids* 2011;84:63-70. (Chapter 4, Manuscript 2)
- Jinping Zhao and Hope A. Weiler. Long-term effects of gestational diabetes on offspring health are more pronounced in skeletal growth than body composition and glucose tolerance. *British Journal of Nutrition* 2010;104:1641-1649. (Chapter 5, Manuscript 3)
- Jinping Zhao, Melani E. Gillam, Carla G. Taylor, Hope A. Weiler. Deposition of docosahexaenoic acid (DHA) is limited in forebrain of young obese *fa/fa* Zucker rats fed a diet high in α-linolenic acid but devoid of DHA. *Journal of Nutritional Biochemistry* 2010 Dec 1 [Epub ahead of print]. (Chapter 6, Manuscript 4)

3. Abstracts and presentations

- Jinping Zhao, Marc R. Del Bigio, Hope A. Weiler. Maternal arachidonic acid and iron supplementation improves performance in Morris Water Maze in 28-day old offspring from diabetic rats – a pilot study. Experimental Biology 2011. Washington D.C., April 9 – 13, 2011. (Oral presentation)
- Jinping Zhao, Hope A. Weiler. Maternal glucose control and arachidonic acid status have long-term consequences to bone health in the adult offspring. *International Society for the Study of Fatty Acids and Lipids (ISSFAL) 2010* Abstract # P182, page 163, May 29 – June 2nd 2010, Maastricht, the Netherlands. (Poster)
- Jinping Zhao, Hope A. Weiler. Maternal dietary arachidonic acid supplementation and rapid catch up growth of offspring of diabetic dams are not associated with glucose tolerance or body composition at young adult age. *International Society for the Study of Fatty Acids and Lipids (ISSFAL) 2008* page 121, May 18 – 22nd 2008, Kansas City, MO, USA. (Oral presentation)
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brain tissue in rats fed diets high in n-3 fatty acids but devoid of DHA. Experimental Biology 2002. *The FASEB Journal*, 2002:16(4), Abstract # 188.7, page A222. (Oral Presentation)

CONTRIBUTIONS OF CO-AUTHORS TO MANUSCRIPTS

This thesis consists of four published manuscripts, all of which were co-authored with my supervisor, Dr. Hope Weiler. I was responsible for the experimental design, experimental manipulation of rats, sample collection, sample analysis, statistical analysis of the data, and interpretation of the results within the context of the available literature. I was responsible for writing all the manuscripts as well as the thesis. Dr. Weiler was involved in study design plus the animal experimentation, provided guidance on the interpretation of the data and was extremely helpful in revising the manuscripts and the thesis. Dr. Marc Del Bigio, former thesis committee member at University of Manitoba, was co-author of two of the manuscripts. Dr. Del Bigio was involved in the neurobehavioral testing method development and provided valuable comments in the revising the manuscripts. Dr. Carla Taylor, at University of Manitoba, was co-author of one of the manuscripts and designed the diets used to test for the benefits of preformed DHA. Dr. Taylor helped on revising the manuscript. Mrs. Melani Gillam, master student of University of Manitoba, was co-author of one of the manuscripts, and was involved in the Zucker rat study animal work.

ACKNOWLEDGEMENTS

I would like to acknowledge the scientific and financial support of my supervisor Dr. Hope Weiler. Your guidance, inspiration, encouragement and patient make my research project completed successfully, I am eternally grateful.

I would like to thank my thesis committee members Dr. Kubow and Dr. Gougeon for their suggestions, involvement in my graduate program and thesis correction. Thanks to Dr. Gougeon helped me tremendously on my thesis French abstract. I thank Dr. Thibault as my thesis committee member for a period of time.

I would like to acknowledge my co-authors Dr. Del Bigio and Dr. Taylor (former committee members at University of Manitoba) for their reviewing my papers and providing valuable comments. I also would like to acknowledge Dr. Wilson sitting in my former thesis committee at University of Manitoba.

I would like to thank Osaama Khan, Andrea MacIntosh, Sarah Finch and many other summer students in the Department of Human Nutritional Sciences, University of Manitoba for their help on animal work and testing.

I would like to acknowledge Shirley Fitzpatrick-Wong, Marilyn Latta, Lisa Maximiuk, Terry Enno, and Dennis Labossiere for technical support. Special thanks to Gerald Nolette and Denise Borowski for animal facility support. Thank

Pat Parish, Department Office Assistant in the Department of Human Nutritional Sciences, University of Manitoba.

I am thankful for Dr. Koski for help on my PhD study. I am thankful for everyone in the graduate office, but mostly to Lise Grant, Francine Tardif and Liz Ansell for their support. Thanks to Sherry Agellon for your support.

I am thankful Macdonald Campus Library especially Vincci Lui, James Ranjitsingh and David Kingsland.

I am thankful Roula Barake and Niloofar Hariri for kind help and friendship, my graduate student fellow in University of Manitoba and in McGill University.

I would like to acknowledge Marian and Ralph Sketch Fellowship of McGill University for the three years financial support.

I would like to thank Professor Hong Jin from Department of Nutrition, Institute of Hygiene and Environmental Medicine, Academy of Military Medical Sciences, Tianjin, P. R. China.

My deepest gratitude goes out for my family, my mother, my husband Chun Wang and my daughter Krystyna Zhaorong Wang. I thank my dear brothers and sister.

DEDICATION

I dedicate this thesis to my FATHER, Mr. SHUYU ZHAO, my father passed away on April 14, 2006.

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LIST OF ABBREVIATIONS

A1C: Glycated hemoglobin

AA: Arachidonic acid (20:4 n-6)

AI: Adequate intake

ALA: α-linolenic acid (18:3 n-3)

AMDR: Acceptable macronutrient distribution range

BBB: Blood brain barrier

CNS: Central nervous system

(COX)-2: Cyclooxygenase-2

CPG: Choline phosphoglycerides

d: Day

DHA: Docosahexaenoic acid (22:6 n-3)

DM: Diabetes mellitus

ELISA: Enzyme-linked-immunosorbent assay

EPA: Eicosapentaenoic acid (20:5 n-3)

EPG: Ethanolamine phosphoglycerides

FABP: Fatty acid binding protein

FXO: Flaxseed oil diet

GLA: γ-linoleic acid (18:3 n-6).

h: Hour

HOMA-IR: Homeostasis model assessment of insulin resistance

IDM: Infants of mothers with all types of diabetes

IGT: Impaired glucose tolerance

LA: Linoleic acid (18:2 n-6)

LC-PUFA: Long-chain polyunsaturated fatty acids

MBP: Myelin basic protein

MO: Menhaden oil

MRI: Magnetic resonance imaging

MUFA: Monounsaturated fatty acid

MWM: Morris water maze

NG test: Negative geotaxis test

PBS: Phosphate buffer solution

PGE_2: Prostaglandin E_2

PLA_2: Phospholipase A_2

PPAR: Peroxisome proliferator-activated receptor

RR: Rota rod

RR-A: Rota rod accelerating-speed

RR-C: Rota rod constant-speed

S+A: Saline-Placebo + AA diet

S+C: Saline-Placebo + Control diet

SFA: Saturated fatty acids

SO: Safflower oil

SR: Surface righting response

STZ: Streptozotocin

STZ/GC: STZ induced diabetes with mean gestational glucose <13 mmol/L as good controlled group

STZ/GC+A: STZ/GC + AA diet

STZ/GC+C: STZ/GC + Control diet

STZ/PC: STZ induced diabetes with mean gestational glucose 13-20 mmol/L as poor controlled group

STZ/PC+A: STZ/PC + AA diet

STZ/PC+C: STZ/PC + Control diet

TRT: Maternal treatments

WH: Wire hanging

WM: Water maze

WM: Weight-matched

CHAPTER 1. INTRODUCTION

1.1. BACKGROUND AND RATIONALE

In Canada, the prevalence of gestational diabetes varies from 3.7% in the non-aboriginal population to 8-18% in aboriginal populations (Dyck et al., 2002; Godwin et al., 1999; Harris et al., 1997). Overall, approximately 10% of pregnant women have some form of diabetes in pregnancy (Georgieff, 2006). Despite the importance of managing diabetes, the compliance to recommended management regimens is poor as evidenced by the high rate (4%) of newly diagnosed diabetic persons in an emergency department or hospital for acute complications of their condition (2008). Compared to the general population, women with diabetes during pregnancy continue to show higher rates of complications (Boulot et al., 2003; Evers et al., 2004; Jensen et al., 2004) including perinatal mortality, congenital malformations, preterm delivery, and neonatal morbidities.

The conversion of linoleic (18:2 n-6; LA) to arachidonic (20:4 n-6; AA) and α-linolenic (18:3 n-3; ALA) to docosahexaenoic acid (22:6 n-3; DHA) is vital in providing AA and DHA to all tissues (Bakewell et al., 2006). This is particularly important since in the Western diet LA intake is about 20-fold greater than that of AA, and ALA intake is about 20-30 fold greater than DHA intake (Yogev et al., 2007). Under normal physiological conditions, the adult liver has ample capacity to synthesize AA and DHA from circulating LA and ALA (Igarashi et al., 2007). In adult rats the estimated hepatic synthesis-secretion rate of AA is 27 times (Gao et al., 2010) while DHA is 43 times (Rapoport et al., 2010) the consumption rate by the brain. This high synthesis rate is supported by

the high expression and high activity of the Δ6- and Δ5- desaturase, enzymes required for the synthesis of AA and DHA in the liver (Igarashi et al., 2007; Rapoport et al., 2007). These activities, however, are depressed and the levels of AA and DHA are reduced in diabetes owing to insulin deficiency or resistance, which is the most potent activator of both enzymes (Brenner et al., 2000; Holman et al., 1983; Hu et al., 1994). Faas and Carter reported a 48% inhibition of Δ6 desaturase activity in liver microsomes of the diabetic rat (Faas et al., 1983). Insulin therapy ameliorates both the activity of the enzymes (Brenner, 2003; Mimouni et al., 1992) and the plasma and erythrocyte membrane fatty acid abnormalities in various experimental designs (Jones et al., 1983; Tilvis et al., 1985).

Normal pregnancy is associated with greater capacity for synthesizing AA and DHA, and the concentrations of AA and DHA are higher in plasma and liver than in non-pregnant women (2002; Boulot et al., 2003; Cordero et al., 1998; Innis, 2003; Melamed et al., 2008; Otto et al., 1997; Shao et al., 2006; Sibai et al., 2000). This physiological adaptation in hepatic lipid metabolism (Melamed et al., 2008) is required to meet the high demand of AA and DHA by the fetal brain especially during the growth spurt which begins at about 20 weeks gestation in humans (Dobbing et al., 1973). In the course of pregnancy, the mother deposits approximately the same weight in fat (3500 g) as the entire weight of the average newborn (Haggarty, 2010). An appreciable amount of DHA and AA are intended to be stored in the maternal adipose tissue, but it can vary over a wide range depending on the habitual dietary intake of LC-PUFA and maternal LC-PUFA metabolism. Healthy omnivores typically have DHA stores of around 19 g in the

adipose tissue (Leaf et al., 1995), and this amount of deposition is necessitated as it will be mobilized to make the entire 19 g fully available to the developing fetus. Young women have a higher concentration of DHA in adipose tissue compared with men (Bitsanis et al., 2006; Patel et al., 2006), while lower DHA and AA stores are expected in diabetic pregnant women as significantly lower adipose tissue AA and DHA content has been seen in STZ-diabetic rats (Makrides et al., 2000).

Despite the importance of the enhanced synthesizing capacity in meeting the high fetal requirements of AA and DHA (2002; Boulot et al., 2003; Cordero et al., 1998; Innis, 2003; Melamed et al., 2008; Otto et al., 1997; Shao et al., 2006; Sibai et al., 2000), this physiological adaptation may be absent in diabetic pregnant women (Lakin et al., 1998). The synthesis of the final products of the chain elongation and desaturation pathway is the most difficult to carry out, thus their concentrations have been used as indicators of rates of desaturation (Lakin et al., 1998; Sprecher et al., 1995). Compared to healthy omnivorous full-term pregnant women, the percentage of 22:4 n-6 and 22:5 n-6 in erythrocytes was significantly higher (28% and 40% respectively, $P<0.05$) in healthy vegetarian full-term pregnant women, but was significantly lower (42% and 46%) in diabetic omnivorous full-term pregnant women. Although they had not eaten meat and fish for more than a year, the vegetarians had similar percentages of DHA in the erythrocyte and umbilical cord as the diabetic women, both were about 41% lower than healthy omnivores. This observation is hypothesized to be due to impaired endogenous synthesis in women with diabetes (Lakin et al., 1998).

The human fetus depends on maternal supply of AA and DHA (Koletzko et al., 1996; Lakin et al., 1998) and accumulates about 70 mg/day of n-3 LC-PUFA, mainly DHA, during the 3rd trimester (Clandinin et al., 1980; Martinez, 1992). The accumulation of AA is thought to be substantially higher since it is omnipresent in most tissues in significant amounts. Much higher amounts (50 times more) of AA and DHA are deposited in fetal adipose tissue than deposited in brain during *in utero* life (Haggarty, 2004), which is crucial to support brain and retinal development during the critical first months of postnatal life. The extent of LC-PUFA incorporation into membranes modulates their functional properties such as fluidity, permeability for metabolite exchange, activity of membrane-bound enzymes and receptors, and electrical and humoral signal transduction (Clandinin et al., 1991). The fatty acid composition of the developing fetal brain is very sensitive to perturbation by the n-6 and n-3 fatty acid composition of the maternal diet. Numerous studies have shown that diet-induced reductions in DHA and AA in the central nervous system during development result in decreased visual function and alterations in behavior and learning (Bourre et al., 1989; Enslen et al., 1991; Frances et al., 1996; Neuringer et al., 1986; Yamamoto et al., 1988).

Newborn infants of diabetic mothers (IDM) have lower AA and DHA in umbilical cord blood (Ghebremeskel et al., 2004; Min et al., 2005; Wijendran et al., 2000). By contrast, compared with normal controls, most rat models of diabetes are characterized by lower tissue AA only, and not DHA (Holman et al., 1983; Hu et al., 1994). Even though both AA and DHA are important to neurodevelopment, brain accretion of AA exceeds that of DHA during gestation

(Hadders-Algra, 2008). AA metabolite, prostaglandin E_2 (PGE_2), plays an important role in neurogenesis (Uchida et al., 2002). Dietary AA supplementation in the dam enhances hippocampal neurogenesis in 31-day-old offspring by 32%, whereas no benefit is observed with combined AA plus DHA or DHA alone (Maekawa et al., 2009). Thus, low AA status in the offspring following a pregnancy complicated by diabetes might explain the learning and memory deficits in the offspring of diabetic dams (Kinney et al., 2003).

There are also other benefits of AA during development. AA is a precursor to PGE_2 that has a biphasic effect on bone by enhancing bone formation at lower concentrations in animals, while stimulating resorption at higher concentrations (Watkins et al., 2001). Alteration in the precursor pool affects PGE_2 metabolism, while modification of dietary fatty acids leads to parallel alterations in bone fatty acids (Weiler, 2000) and PGE_2 metabolism (Alam et al., 1993; Watkins et al., 1997). Altered calcium and magnesium homeostasis exists in newborn infants of mothers with diabetes (Mimouni et al., 1988; Tsang et al., 1972; Verhaeghe et al., 1995) that persists beyond infancy to at least 16 weeks in rats (Bond et al., 2005) and 10 years in children (Mughal et al., 2005). In a recent study, the serum AA was positively associated with whole body bone mass in 8-year-old children (Eriksson et al., 2009).

Other longer-term sequealae of being born to a mother with diabetes include a higher incidence of impaired glucose tolerance (IGT) and type 2 diabetes mellitus (DM) (Dabelea et al., 2008; Metzger, 2007; Silverman et al., 1995). In the Northwestern University Diabetes in Pregnancy follow-up study, 40% of offspring of diabetic mothers by age 16 years had IGT (Metzger, 2007).

Based on the SEARCH Case-Control study, 30.4% of youth with type 2 DM were exposed to maternal diabetes *in utero* compared with 6.3% of nondiabetic control youth (Dabelea et al., 2008). In cell culture, AA is fundamental to functional integrity of the pancreatic β-cell (Dixon et al., 2004), while glucose-stimulated insulin release is dependent on plasma membrane release of AA (Konrad et al., 1994). In rats, dietary AA supplementation lowers fasting blood glucose and insulin concentrations (Sasagawa et al., 2001), prevents high-fat diet-induced insulin resistance (Wu et al., 2007) and enhances glucose disposal (Song et al., 2003).

The prevalence of obesity and insulin resistance are high in youth and young adults (Lambert et al., 2004; Lee et al., 2006), in part due to intrauterine exposure to hyperglycemia as a component of the fetal origins of disease (Clark, 1998). Insulin resistance is associated with low Δ5 desaturase activity (Vessby, 2003; Vessby et al., 2002), which is required for the synthesis of DHA. Pima Indians, a population with the highest reported incidence of insulin resistance in the world, have low skeletal muscle DHA (Pan et al., 1995). Brain is rich in DHA and normal brain DHA can be maintained in adult rats by hepatic conversion plasma-derived ALA to DHA even without dietary DHA (Igarashi et al., 2007). It is unknown if brain DHA can be maintained in insulin resistant states but it is suspected to be inadequate since people with insulin resistance have greater cognitive decline (van Oijen et al., 2008; Young et al., 2006).

As summarized in this chapter, insulin is a strong stimulus in the synthesis of AA and DHA. There are a number of fundamental knowledge gaps regarding AA and DHA status in offspring of mothers with diabetes and the consequences

to brain function. Likewise, the commonly observed sequelae following a pregnancy complicated with diabetes include obesity and insulin resistance in the child; yet the impact on brain AA and DHA has not been investigated. Therefore, the following objectives were set to narrow these knowledge gaps.

1.2. THESIS OBJECTIVES

The objectives of the work contained in this thesis were:

1. To determine if the offspring of diabetic rat dams have lower AA status and the associations with neurobehavioral deficit (Chapter 3: Manuscript 1).
2. To determine if maternal AA supplementation throughout pregnancy and lactation improves neurodevelopment of offspring (up to day 29) from healthy and diabetic rat dams (Chapter 3: Manuscript 1).
3. To determine if the neurobehavioral deficit in the offspring of diabetic dams persist to young adult age and if maternal AA supplementation improves neurodevelopment in young adult offspring from healthy and diabetic rat dams (up to 12 week) (Chapter 4: Manuscript 2).
4. To test the effect of maternal diabetes and AA supplementation on offspring body composition, bone mass and glucose tolerance from 4 to 12 weeks (Chapter 5: Manuscript 3).
5. To investigate brain fatty acid composition and desaturase (Δ5, Δ6 and Δ9) activities in obese and insulin resistant young rats on diets with and without DHA (Chapter 6: Manuscript 4).

1.3. THESIS HYPOTHESES

Four main hypotheses are at the core of the stated objectives of this thesis:

1. The offspring of diabetic rat dams have lower AA status that is associated with neurobehavioral deficits. Maternal AA supplementation through pregnancy and lactation improves offspring neurodevelopment (up to day 29) (Chapter 3: Manuscript 1).
2. The neurobehavioral deficits in the offspring of diabetic dams persist to young adult age. Maternal AA supplementation has a long-lasting beneficial effect on offspring neurodevelopment (Chapter 4: Manuscript 2).
3. Maternal AA supplementation prevents subsequent development of IGT in the offspring at young adult age. This supplementation is beneficial to offspring body composition and bone health (Chapter 5: Manuscript 3).
4. Endogenous synthesis of DHA by the brain would be compromised in the obese insulin resistant model. Brain DHA cannot be maintained by liver when DHA is absent from the diet which is otherwise adequate in ALA in obese and insulin resistant young rats (Chapter 6: Manuscript 4).

CHAPTER 2. LITERATURE REVIEW

2.1. Higher prevalence of neurobehavioral deficits in infants of diabetic mothers

While glycemic control is a fundamental aspect in the treatment of a diabetic pregnancy (Kitzmiller et al., 2008), the complex metabolic changes in maternal nutrient metabolism ascribed to diabetes pose risks to brain development in the fetus (Rizzo et al., 1997). Outcome studies of IDM have yielded short- and long-term neurobehavioral deficits in both sensory-cognitive and psychomotor functions. These include altered auditory recognition memory processing at birth (Siddappa et al., 2004), reduced visual and memory performance at 8 and 12 months (DeBoer et al., 2005; Nelson et al., 2000; Nelson et al., 2003), poorer performance on tests of general development in infants and toddlers (Deregnier et al., 2000; Rizzo et al., 1991), and inferior performance in elementary school children (Rizzo et al., 1997). While the motor delay may be a sign of mild, nonspecific brain damage (Ornoy et al., 1998; Ornoy et al., 2001; Ornoy et al., 1999; Ratzon et al., 2000), the abnormalities in memory processing suggest alterations in hippocampal development and function (Nelson et al., 2000).

2.1.1. Infants of diabetic mothers

2.1.1.1. Definition, classification and diagnosis of diabetes

Diabetes mellitus is a metabolic disorder characterized by the presence of hyperglycemia due to defective insulin secretion, defective insulin action or both (2008). Diabetes mellitus mainly includes type 1, type 2 and gestational DM.

Type 1 DM is primarily a result of pancreatic β-cell destruction and a person with type 1 DM is prone to ketoacidosis. This form includes cases due to an autoimmune process and those for which the etiology of β-cell destruction is unknown. Type 2 DM may range from predominant insulin resistance with relative insulin deficiency to a predominant secretory defect with insulin resistance. Gestational DM refers to glucose intolerance with onset or first recognition during pregnancy.

Diabetes is diagnosed at a fasting plasma glucose level of 7.0 mmol/L, which correlates most closely with a 2-h plasma glucose value of 11.1 mmol/L in a 75-g oral glucose tolerance test and best predicts the development of microvascular disease (Sanmartin et al., 2008). The criteria for gestational DM are (i) 1-h plasma glucose value at ≥10.3 mmol/L in a 50-g glucose load; (ii) if 1-h plasma glucose value at 7.8 to 10.2 mmol/L is observed in a 50-g glucose load, then further testing with a 75-g glucose load is conducted. Criteria for diagnosis using 75-g glucose load are if fasting plasma glucose ≥5.3 mmol/L, 1-h plasma glucose ≥10.2 mmol/L or 2-h plasma glucose ≥8.9 mmol/L; if 2 values are met or exceeded then gestational DM is diagnosed (2008).

2.1.1.2. Incidence of diabetes in pregnancy

Diabetes in pregnancy includes both pregnancy in pre-existing DM (type 1 and type 2 DM) and gestational DM. The prevalence of gestational DM (GDM) is population-specific and reflects the underlying incidence of DM in that population (Dyck et al., 2002). Recent high powered epidemiological studies of women with pregestational DM continue to show higher rates of complications compared to the general population (1996; Boulot et al., 2003; Clausen et al.,

2005; Cundy et al., 2000; Dunne et al., 2003; Evers et al., 2004; Farrell et al., 2002; Gunton et al., 2002; Hadden et al., 2003; Jensen et al., 2004; Penney et al., 2003; Platt et al., 2002; Roland et al., 2005; Silva Idos et al., 2005; Wylie et al., 2002).

2.1.2. The challenge of diabetes in pregnancy

Diabetes is a serious condition with potentially devastating complications that affect all age groups worldwide. In 1985, an estimated 30 million people around the world were diagnosed with diabetes; in 2000, that figure rose to over 150 million, and it is projected to rise further to 380 million by 2025 (Sanmartin et al., 2008). The International Diabetes Federation states that "every ten seconds, two people are diagnosed with diabetes somewhere in this world," and given the current trend, more people will have diabetes in 2025 than the current population of the United States, Canada and Australia combined (Yogev et al., 2004). The impact of diabetes is felt in Canada, where 1.8 million adult Canadians, 5.5% of the population, had diagnosed diabetes in 2005 (Bhattacharyya et al., 2009). That is an increase from 1998, when the physician-diagnosed prevalence of diabetes in Canada was 4.8% (1 054 000 adult Canadians). Health services research indicates that 280 330 admissions into Canadian acute care hospitals in 2006 – or 10% of all such admissions – were related to diabetes or its complications (2008). Thus, in this regard, it is necessary to establish research to improve the lives of people living with diabetes.

2.2. Newborn infants of all types of diabetic mothers have lower AA and DHA status

The disturbances of fatty acid metabolism in various experimental models of diabetes are well established. The impairment is often manifested by an increase in LA and a concomitant decrease in AA in tissues and plasma in STZ induced diabetic rats (Brenner, 2003; Brenner et al., 2000; Clark et al., 1983; Dang et al., 1988; Faas et al., 1983; Gudbjarnason et al., 1987; Holman et al., 1983; Hu et al., 1994; Hurtado de Catalfo et al., 1998; Lin et al., 1985; Ramsammy et al., 1993). These alterations can be explained by the diminished activities of the Δ6- and/or Δ5-desaturase due to the relative lack of insulin, a hormone which is the most potent activator of both enzymes (Brenner, 1981; Brenner, 1990). Insulin therapy ameliorates both the activity of the enzymes (Brenner, 2003; Mimouni et al., 1992) and the plasma and erythrocyte membrane fatty acid abnormalities in various experimental designs (Jones et al., 1983; Tilvis et al., 1985).

In human observations, the effects of diabetes are much less consistent than those in animal studies, lower plasma and/or red cell AA and/or DHA have been reported in some (Decsi et al., 2002; Jones et al., 1983; Tilvis et al., 1985), but not in other studies (Baldini et al., 1989; Bassi et al., 1996; Ruiz-Gutierrez et al., 1993; Seigneur et al., 1994). The controversial results may be due to the differences in characteristics of the subjects (i.e., age, sex, diet, duration of diabetes, treatment options, presence of diabetes related complications, individual AA and DHA status) or to the analysis of different lipid fractions (whether testing plasma or red cell, measuring whole lipid, choline phosphoglycerides,

ethanolamine phosphoglycerides, triglycerides, or cholesterol ester), and so on (Decsi et al., 2007; Min et al., 2005).

Recently, there are studies investigating the effect of maternal diabetes during pregnancy on fetal and maternal LC-PUFA status (Ghebremeskel et al., 2004; Lakin et al., 1998; Min et al., 2005), which have shown consistently that nondiabetic neonates born to mothers with type 1, and perhaps type 2 DM, as well as their mothers, have highly compromised AA and DHA status at birth (Ghebremeskel et al., 2004; Lakin et al., 1998; Min et al., 2005; Min et al., 2006; Thomas et al., 2005; Wijendran et al., 2000). At the same time, there is also compelling evidence emerging that the fetal intrauterine accretion of AA and DHA is suboptimal in pregnancies complicated by gestational DM (Bitsanis et al., 2006; Kuhn et al., 1990; Min et al., 2004; Min et al., 2005; Thomas et al., 2005; Wijendran et al., 2000; Wijendran et al., 1999).

2.3. Brief outline of the brain n-6 and n-3 LC-PUFA metabolism

Fatty acids are a major constituent of the brain: about 50-60% of the dry weight in adult brain consists of lipids, and approximately 35% of the lipids are PUFA, most of which are LC-PUFA (Martinez et al., 1998). Brain DHA levels increase 6-fold in the fetus from 25 gestational weeks to the first week after birth and at four weeks after birth have only reached 50% of the DHA levels of the adult brain. AA levels are consistently higher than DHA levels in fetal brain. AA levels increase 3-fold in the fetus from 25 gestational weeks to one week after birth and reach adult levels four weeks after birth (Martinez et al., 1998). AA and DHA, which largely are esterified at the stereospecifically numbered (*sn*)- 2 position of membrane phospholipids, are the two most abundant LC-PUFA in the

brain (Martinez, 1992), accounting for about 20% of fatty acids in the mammalian brain (Contreras et al., 2000).

Dietary intakes of AA and DHA are substantially lower in the Western diet compared with their essential fatty acid precursors; ALA intake is about 20-fold greater than that of AA, and ALA intake is about 20-30 fold greater than DHA intake (Yogev et al., 2007). In pregnant women living in the Pacific coastal city of Vancouver, the mean intake, estimated by food-frequency questionnaire at 28 and 35 weeks pregnancy, of LA was 11.2 $\pm$0.4 g/d (3.9 $\pm$0.2% total energy) and of ALA was 1.6 $\pm$0.1 g/d (0.5 $\pm$0.1 total energy). The mean intakes of AA and DHA were 121 $\pm$8 and 160 $\pm$20 mg/d, respectively (Innis et al., 2003). Others have reported a mean intake of 1.3 $\pm$0.2 g/d of ALA and 82 $\pm$33 mg/d of DHA during the 2nd trimester and 3rd trimester of pregnant women living in the Guelph area (inland Canada) (Denomme et al., 2005). This suggests that the conversion of LA to AA, and ALA to DHA, may be important for providing AA and DHA for incorporation into the tissues (Bakewell et al., 2006).

Since AA and DHA cannot be synthesized *de novo* and are converted minimally (<0.2%) from LA (DeMar et al., 2006; Gao et al., 2010) and ALA (Demar et al., 2005; Rapoport et al., 2010) that enter from plasma into brain, brain AA and DHA are thus mainly derived from hepatic conversion of LA and ALA by sequential Δ6 desaturation, elongation and Δ5 desaturation reactions (Sprecher et al., 1995). LA and ALA are essential fatty acids for mammals because of the absence of the Δ12 and Δ15 enzymes required to add double bonds at the n-6 or n-3 position of a fatty acid carbon chain (Innis, 1991). However, all the classic symptoms of essential fatty acid deficiency (dermatitis, growth retardation,

infertility) can be completely cured by the n-6 fatty acids alone (Lauritzen et al., 2001). Synthesis of AA and DHA occurs on an analogous pathway, LA and ALA compete for the same enzymes in the metabolic cascade to their respective LC-PUFAs. Dietary studies in rats and other animals have shown that 18:3 n-3 is a strong suppressor of n-6 fatty acid metabolism, whereas 10 times as much 18:2 n-6 is required to give an equal suppression of n-3 metabolism (Holman, 1998). The preferred substrate for the Δ6-desaturase, in brain and in many other tissues, is 18:3 n-3 over 18:2 n-6 (Cunnane et al., 1994). The main dietary sources of LA and ALA are in plant products (Singh, 2005), while the preformed AA and DHA are present in animal food sources (for AA ≤2% of total fatty acids) because the Δ5 desaturase and the enzymes that follow the Δ5 desaturase enzyme are only found in animal but not in plant cells (DeMar et al., 2006; Innis, 2000). ALA is particularly abundant in nuts, seeds and vegetable oils, such as linseed and soybean oil, DHA in fatty fish, such as mackerel, herring, salmon, tuna and trout; LA is common in vegetable oils such as sunflower and corn oil and AA in meat, eggs and lean fish (Kris-Etherton et al., 2000).

The current daily Adequate Intake (AI) values for ALA are 1.6 and 1.1 g/day for men and women respectively. The Acceptable Macronutrient Distribution Range (AMDR) for ALA is 0.6 – 1.2% of daily energy intake (2000 – 2500 Kcal/day) (2005). The EPA and DHA can supply 10% of the AMDR for n-3 PUFA (2005). This recommendation represents current mean intake for EPA and DHA in the United States (~100 mg/day), which is much lower than what many groups worldwide are currently recommending (Kris-Etherton et al., 2009). The optimal ratio of LA to ALA is proposed to range from 5 to 10.

Recommendations also have been made for DHA intake for pregnant women and vegetarians/vegans. Pregnant women should consume 200 – 300 mg/day of DHA (Simopoulos et al., 1999). For vegans, 2 – 4 g of ALA per day, and 100 – 300 mg/day of DHA (Davis et al., 2003).

2.3.1. The importance of AA in the brain may be overlooked

As AA and its eicosanoid metabolites have multiple actions within different organs (Brash, 2001; Funk, 2001), the AA concentration must be maintained within homeostatic limits and in balance with the concentration of the n-3 LC-PUFA, DHA for optimal organ function (Simopoulos, 2003). However, early studies of the role of PUFA have been largely thought to be structural, increasing the fluidity of cellular membranes (Hansen et al., 1946), and mainly focused on DHA, because autopsy analyses have found lower DHA but not AA in the brain of infants who had been fed formula without LC-PUFA (Farquharson et al., 1992; Makrides et al., 1994). In addition, the higher concentration of DHA in brains of breast-fed infants may explain the superior neurodevelopment reported in breast-fed compared with formula-fed infants (Makrides et al., 1994). However, less is known about the effect of fetal exposure to maternal diabetes on neonatal fatty acid status and neurodevelopmental outcome. Even though both AA and DHA are important to neurodevelopment (Saste et al., 1998), brain accretion of AA exceeds that of DHA during gestation (Hadders-Algra, 2008), a period that is crucial to brain development. Recent infant studies (Bouwstra et al., 2006; van Goor et al., 2010) indicate that lower AA status at birth and in infancy is associated with higher incidence of mildly-abnormal general-movement; and the presence of mildly-abnormal-general-movements is associated with an

increased risk of development of minor neurologic dysfunction and attention problems at school age (Groen et al., 2005; Hadders-Algra et al., 1999; Hadders-Algra et al., 2004).

In addition, several major breakthroughs, including the discovery of prostaglandins and the role of AA as a secondary messenger in signal transduction, have advanced our understanding of the importance of AA in the brain. AA is a precursor to a host of signaling molecules known as eicosanoids, which include thromboxanes, leukotrienes, prostacyclins, and prostaglandins (Stroud et al., 2009). AA and its end-products are involved in physiological processes in the central nervous system such as neuronal survival (Bazan, 2005; Maekawa et al., 2009), and neurotransmission (Chalon et al., 2001), synaptic signalling (Kaufmann et al., 1996), neuronal firing (Chapman et al., 1992), neurotransmitter release (Ojeda et al., 1989), neuronal gene expression (Lerea et al., 1997; Lerea et al., 1993), cerebral blood flow (Li et al., 1997; Pickard, 1981; Stefanovic et al., 2006), sleep/wake cycle (Hayaishi et al., 1995), and appetite (Baile et al., 1973). Altered brain AA metabolism has been associated with a number of neurological, neurodegenerative, and psychiatric disorders, including epilepsy (McCown et al., 1997), HIV-associated dementia (Griffin et al., 1994), Alzheimer's disease (Bazan et al., 2002), Parkinson's disease (Teismann et al., 2003), schizophrenia (Skosnik et al., 2003), bipolar disorder (Rao et al., 2008), and depression (Rapoport et al., 2002; Sublette et al., 2004).

Decreasing concentrations of brain DHA by feeding animals an n-3 PUFA deficient diet impairs brain function in experimental animals (Bourre et al., 1989; Fedorova et al., 2006; Moriguchi et al., 2000; Salem et al., 2001). Although the

cerebral effects of n-3 PUFA dietary deficiency in rodents have been widely reported, those of dietary n-6 PUFA deprivation have not been as closely studied. In a recent study, an n-6 PUFA deficient diet (LA: 2.3% of total fatty acids), with adequate n-3 PUFA (ALA 4.8%), fed to post-weaning rats for 15 weeks significantly reduced AA contents in multiple organs including brain (-28%) and liver (-84%). Dietary AA supplementation has been shown to improve membrane fluidity, synaptic plasticity, and spatial cognition in aged rats (Fukaya et al., 2007; Kotani et al., 2003; Okaichi et al., 2005), the effects of dietary n-6 PUFA deprivation on brain function and behavior in rodents have not been thoroughly studied.

2.3.2. The unique fatty acid composition of the brain

The unique fatty acid composition of the brain and retina has been recognized for almost 50 years (Brenna et al., 2007; Svennerholm, 1964). This unusual feature is that the concentrations of the dietary essential fatty acids, LA and ALA, are low (generally less than 2% of total fatty acids), whereas concentrations of the products AA and DHA are high (Innis, 2000; Sastry, 1985). In other tissues, LA can exceed 20% of total fatty acids and those concentrations increase with increasing dietary intake. Multiple aspects of brain metabolism, function and structure are thought to depend on having adequate brain concentrations of AA and DHA as well as on interactions among these PUFA and their metabolites (Rapoport, 2008).

2.3.3. Brain is the most cholesterol-rich organ in the body: cholesterol for synthesis of myelin is made locally, not imported into brain

The neuron is the functional unit of the brain, and composed of a cell body and an axon, and their primary function is the generation and propagation of electrical impulses (Bjorkhem et al., 2004). Rapid transmission of impulses along the length of the axon is facilitated by the presence of the myelin sheath (Bjorkhem et al., 2004). Myelination takes place between the second trimester of gestation and continues through the second year of life (de Graaf-Peters et al., 2006). A noteworthy characteristic of myelin is that it contains ~70% lipid and 30% protein (when related to its dry weight), which is approximately opposite to the situation found in most other cell membranes. Moreover, more than 25% of the total lipid content is cholesterol, compared to less than 20% in other plasma membranes (Morell et al., 1996). Brain thus is the most cholesterol-rich organ in the body as ~25% of the cholesterol in the body is localized in the brain. In the brain, ~70% to 80% of its cholesterol is presented as myelin sheaths (i.e., oligodendroglia) (Jurevics et al., 1995), and the rest as plasma membranes (astrocytes and neurons) (Bjorkhem et al., 2004).

In the brain, the blood brain barrier (BBB) effectively prevents cholesterol uptake from the circulation, and *de novo* synthesis is responsible for practically all cholesterol present in the brain. Almost all brain cholesterol is a product of local synthesis (Bjorkhem et al., 2004; Dietschy et al., 2004; Jurevics et al., 1995; Morell et al., 1996). The independence of the isolated pool of cholesterol in the brain is likely to reflect a high need for constancy in the cholesterol content of membranes and myelin, a constancy that would be difficult to keep if brain

cholesterol had been exchangeable with lipoprotein cholesterol (Jurevics et al., 1995).

2.3.4. Sources of non-essential fatty acids of brain

Another feature of brain fatty acids is that it is completely autonomous with respect to synthesis of non-essential fatty acids (Edmond et al., 1998; Edmond et al., 1991). Studies with rat pups reared on defined diets *via* gastrostomy tube feeding have provided basic evidence that the brain is autonomous in providing the bulk of its lipids, entirely by *de novo* synthesis (Edmond et al., 1991; Marbois et al., 1992). Neither saturated- nor mono-saturated fatty acids nor cholesterol are supplied from exogenous (diet/plasma) sources. All are provided within the brain. Due to the differential affinity of carnitine palmitoyltransferase for acyl-CoA moieties, AA and DHA are largely (90%) reacylated into brain phospholipid, but LA and ALA are almost entirely (about 99%) β-oxidized after entering brain (DeMar et al., 2006; Demar et al., 2005). Among other sources, carbon atoms from essential fatty acids are especially utilized in the brain for the synthesis of non-essential lipids (Cunnane et al., 1999; Cunnane et al., 1994). Sheaff et al (Sheaff Greiner et al., 1996) found that maternal dietary labeled 18:3 n-3 gives rise to more label in the pool of saturated and monounsaturated fatty acids of fetal rhesus monkey brain and retina than in the pool of the n-3 fatty acids. Thus, recycling of carbon from 18:3 n-3 and other essential fatty acids appears to be a major pathway in the fetal and infant rhesus monkey (Sheaff Greiner et al., 1996) and rat brain (Cunnane et al., 1999; Cunnane et al., 1994). As a result of this efficient oxidation of 18 carbon

PUFAs and conversion to LC-PUFA, 18:2 n-6 and especially 18:3 n-3 are almost absent in the brain lipids.

2.3.5. PUFA enter the brain from the plasma: passive diffusion model

Although the brain has genetically been thought to contain a very stable pool of lipids, mainly phospholipids and cholesterol, it is known that the phospholipids turn over rapidly. Measurements in adult rat brain suggest that as much as 5% of AA and 8% of DHA, which are present as esters in phospholipids, turn over each day (Rapoport, 2001). Therefore, AA and DHA, as well as respective precursors, must be supplied from exogenous sources.

Nutrients supplied to all organs must pass through microvascular endothelium, but the brain has a special endothelial barrier – blood brain barrier (BBB). The main architectural feature of the BBB is the tight junction between each endothelial cell. Thus glucose, fatty acids, amino acids, and ions cannot leak between the endothelial cells to reach the underlying brain cells. If the endothelial cell membranes were impermeable to fatty acids, the transport of fatty acids could be quite complex. Indeed ion membrane transporters are asymmetrically distributed between the luminal and transluminal membranes. The permeability of the BBB to LC-PUFAs is many orders of magnitude higher than glucose, amino acids, and ions. However, the mechanism by which PUFA enter the brain from the plasma is not agreed upon (Chen et al., 2009). One model proposes that fatty acids cross the BBB without specific transporters to reach brain cells. Most evidence shows that fatty acids derived from BBB transport are derived mainly from fatty acid/albumin complexes and, to a lesser extent, from circulating lipoproteins. The fatty acids cross the luminal and trans-

luminal leaflets of the endothelial cells and the plasma membrane of neural cells by a Mersible flip-flop (Hamilton et al., 2007). The other model describes LC-PUFA-influx into brain cell through a saturable, protein-mediated transport mechanism (Hamilton et al., 2007).

2.3.6. Brain AA and DHA cascades: contribution of brain vs. liver synthesis to AA and DHA homeostasis

Recent insights into brain PUFA metabolism have been accomplished with the integrated use of kinetic, enzymatic and molecular methods in animals as well as in humans. Intravenously injected radio-labeled AA (Rapoport, 2008) and DHA (Igarashi et al., 2007) have been used to quantify AA and DHA incorporation and turnover in brain phospholipids, and their loss from brain by metabolism. Pathways of plasma-brain exchange of AA and of its intravenously injected radiolabel (designated as AA*), as well as pathways and compartments of brain AA metabolism in what is termed the "brain AA cascade" (Rapoport, 2008). DHA participates in a comparable cascade (Igarashi et al., 2007).

Circulating radiolabelled AA* will rapidly exchange with unesterified AA in the brain endoplasmic reticulum. The brain AA cascade involves two major cycles. One cycle is a continuous process of AA de-acylation followed by re-acylation in individual phospholipids, with short half-lives (hours) and high turnover rates (e.g., 15 – 30%/h) in rodent brain (Rapoport, 2008). The other is a cycle involving metabolic AA loss within brain, compensated for by AA replenishment from plasma, with half-lives of weeks (Rapoport, 2008).

In the first cycle, AA is released from the *sn*-2 position of a synaptic membrane phospholipid by a phospholipase A_2 (PLA_2), whose activation is

coupled to activation of any of a number of postsynaptic neuroreceptors by a G-protein or by calcium. A small fraction of the released AA (~4%) is lost by metabolism to eicosanoids or other metabolites or by β-oxidation after being transferred to mitochondria from the acyl-CoA pool. This transfer requires carnitine palmitoyltransferase, whose affinity for arachidonoyl-CoA and other long-chain acyl-CoAs, compared with the affinity of acyltransferase, determines whether the fatty acid will be largely β-oxidized in mitochondria or esterified into lysophospholipid (Rapoport, 2008). AA and DHA are largely (90%) reacylated in brain, and palmitic acid (16:0) is 50% β-oxidized, but LA (18:2 n-6) and ALA (18:3 n-3) are almost entirely (about 99%) β-oxidized after entering brain (DeMar et al., 2006; Demar et al., 2005).

The larger fraction (~96%) of released AA in brain will be reincorporated into phospholipids after diffusing to the unesterified AA pool in the endoplasmic reticulum with the help of a fatty acid binding protein (FABP), then converted to arachidonoyl-CoA by an acyl-CoA synthetase with consumption of two ATP. For AA to be a substrate for cyclooxygenase (COX)-2, it first must be incorporated in phospholipids and then released by PLA_2. Circulating AA* rapidly reaches equilibrium with brain arachidonoyl-CoA during its intravenous infusion (Washizaki et al., 1994). Because AA cannot be synthesized *de novo* and is converted minimally in the brain from its shorter chain LA precursor (<0.2%) (DeMar et al., 2006; Gao et al., 2010), thus the rate of incorporation of plasma unesterified unlabeled AA into brain equals the quantity of AA that has been lost by metabolism, that is the rate of AA consumption within brain. A similar consideration applies to DHA.

Although the brain has the enzymes for complete synthesis of AA and DHA from their shorter-chain precursors (Hurtado de Catalfo et al., 1992; Matsuzaka et al., 2002; Wang et al., 2005), circulating unesterified LA and ALA precursors are almost completely β-oxidized after entering brain. The high concentrations of AA and DHA that are maintained within membrane phospholipids of these organs are controlled largely by their plasma concentrations (DeMar et al., 2006; Demar et al., 2005), thus hepatic regulation of plasma AA and DHA availability is critical for brain function.

Under normal physiological conditions, the adult liver has ample capacity to synthesize AA and DHA from circulating LA and ALA. In adult rats the estimated hepatic synthesis-secretion rate of AA is 27 times (Gao et al., 2010) while DHA is 43 times (Rapoport et al., 2010) the brain's consumption rate. However, in humans, below normal blood levels of DHA are common in liver disease, diabetes and aging (Brenner, 2003; Watanabe et al., 1999). Such reductions are postulated to be associated with reduced desaturase or elongase activity and are a risk factor for brain disease in the absence of dietary DHA supplementation (Rapoport et al., 2009). It is not clear if brain DHA can be sustained in obesity and insulin resistant states but it is suspected to be inadequate since people with insulin resistance have greater cognitive decline (van Oijen et al., 2008; Young et al., 2006).

2.4. Fetal and neonatal brain development and AA and DHA accretion

2.4.1. Brain undergoes remarkable structural and functional changes between 24 and 44 week after conception

The human brain undergoes remarkable structural and functional changes between 24 and 44 week after conception, progressing at the beginning of the third trimester from a smooth bi-lobed structure with few gyrations or sulcations to a complex one at term that morphologically resembles the adult brain (Georgieff, 2007). The increase in complexity largely reflects cortical neuronal growth, differentiation, and synaptic connections. In particular, the auditory and visual cortices begin to develop rapidly, as do areas underlying receptive language and higher cognitive function (Thompson et al., 2001). Major part of brain myelination occurs during the first year after birth. Most importantly, experience-dependent synapse formation occurs from 36 gestational weeks and provides a neuronal basis for the fetus to learn. The hippocampus, which is central to recognition memory processing, has established most of its connections from the entorhinal cortex and has begun to send projections through thalamic nuclear structures to the developing frontal cortex (Georgieff, 2007).

Nutrients and growth factors regulate brain development during fetal and early postnatal life. The developing brain between 24 and 42 weeks of gestation is particularly vulnerable to nutritional insults because of the rapid trajectory of several neurologic processes, including synapse formation and myelination. Conversely, the early postnatal young brain is remarkably plastic and therefore more amenable to repair after nutrient repletion. On balance, the brain's vulnerability to nutritional insults likely outweighs its plasticity, which explains

why early nutritional insults result in brain dysfunction not only while the nutrient is in deficit, but also after repletion (Georgieff, 2007).

2.4.2. Brain AA and DHA accretion

2.4.2.1. During early pregnancy

The LC-PUFA accretion in the brain starts at a relatively slow pace during early fetal life (Percy et al., 1996). The critical role of DHA (Coti Bertrand et al., 2006; Kawakita et al., 2006) and AA (Maekawa et al., 2009) in neurogenesis, however, suggests that adverse effects of inadequate AA and DHA in early gestation will be more severe and more difficult to overcome than deficiencies occurring later on (Innis, 2007). Available information indicates that accretion of AA in the fetal brain exceeds that of DHA, the accretion rates of n-6 fatty acids in fetal brain are approximately twice as high as those of n-3 fatty acids, in particular during the first two trimesters of gestation (Martinez, 1992; Svennerholm, 1968). As a result, the human brain contains relatively more AA than DHA at term (Martinez, 1992; Martinez et al., 1998). After birth, accretion of DHA gradually surpasses that of AA, so that in the adult brain, DHA is the major LC-PUFA (Clandinin et al., 1980; Farquharson et al., 1992; Martinez, 1992; Martinez et al., 1998; Svennerholm, 1968). The baboon study of Diau et al (Diau et al., 2005) confirmed that the brain LC-PUFA accretion is preferentially in grey matter, in particular in the synaptic membranes, and to a lesser extent in the white matter (Innis, 1991). Highest concentrations of LC-PUFA are found in the basal ganglia, the pre- and post-central cortices, hippocampus and thalamus, a finding which suggests that LC-PUFA, not only DHA but also AA, intake might affect particular circuitries involved in sensorimotor integration and memory.

2.4.2.2. Effects of LC-PUFA during the growth spurt of the brain

In humans the growth spurt of the brain occurs from approximately the beginning of the third trimester of pregnancy to 18 months after birth (Dobbing et al., 1979). The peak of the brain growth spurt occurs around the time of birth (Dobbing et al., 1979). In the human, neurogenesis takes place between gestational weeks 14 to 25. The adult neuronal number thus has already been achieved when the brain growth spurt begins (Dobbing et al., 1973; Dobbing et al., 1979). The role of LC-PUFA in brain development is thought to be at the level of nerve growth and synaptogenesis (Martinez, 1992; Martinez et al., 1978). The region of growth in an extending neuron is the growth cone region where the new membranes are actively being laid down to extend the axon into the dendritic processes in the direction towards glial cells. DHA is a major lipid constituent of synaptic end sites, while AA is present in both growth cones and synaptosomes. In the growing brain, thousands of new synapses are made every hour; there is thus a substantial amount of incorporation of AA and DHA containing lipids (Martinez, 1992). There are detectable changes both morphologically and biochemically (Martinez et al., 1978) that explain the sudden increase in LC-PUFA, particularly DHA and AA in the brain (Martinez, 1992). During the brain growth spurt there is a more than 10-fold increase in brain size, from about 100 g at the beginning of the third trimester to about 1100 g 18 months postnatally (Dobbing et al., 1973; Dobbing et al., 1979). By birth although the infant has only accumulated 5% of its adult body weight it has gained 70% of its adult brain size. The next 15% of brain growth occurs during the first year and another 10% is acquired before the age of 6 (Singh, 2005). Brain DHA levels increase 6-fold

from late gestation (25 gestational weeks) to the first week after birth and at four weeks after birth have only reached 50% of the DHA levels of the adult brain. AA levels are higher than DHA levels in fetal brain. AA increases 3-fold from the 25 genstational week to 1 week after birth and reaches adult levels four weeks after birth (Martinez et al., 1998). During the growth spurt of the human brain there is a 30-fold increase in the total amount of 22:6 n-3. In the human cerebrum and cerebellum, over a three-fold increase in AA and DHA occurs during the third trimester and another three-fold increase between birth and postnatal week 12 (Clandinin et al., 1980; Clandinin et al., 1980). During the last three months of pregnancy there is a rapid development of the neuronal system of the fetus, and thus lipid accretion in the developing brain increases significantly, with n-6 LC-PUFA deposition as great as n-3 LC-PUFA accretion (Koletzko, 1992; Koletzko et al., 1999).

The extent of LC-PUFA incorporation into membranes modulates their functional properties such as fluidity, permeability for metabolite exchange, activity of membrane-bound enzymes and receptors, and electrical and humoral signal transduction (Clandinin et al., 1991). The fatty acid composition of the developing fetal brain is very sensitive to perturbation by the n-6 and n-3 fatty acid composition of the maternal diet. Numerous studies have shown that diet-induced reductions in DHA and AA in the central nervous system during development result in decreased visual function and alterations in behavior and learning (Bourre et al., 1989; Enslen et al., 1991; Frances et al., 1996; Neuringer et al., 1986; Yamamoto et al., 1988).

2.5. Fetal adipose tissue AA and DHA accretion: 50 times more AA and DHA are stored in the adipose tissue than are deposited in the fetal brain during *in utero* life

Deposition of total lipid in the fetus increases exponentially with gestational age, reaching its maximal rate of accretion, around 7 g/day, just before term (Haggarty, 2010). Fat deposition in the fetus accounts for over half the energy accretion from the 27th week of gestation and as much as 90% of the energy accretion at term. The total *in utero* accretions of AA and DHA in the fetus are around 10 g respectively. Most is deposited in the last 10 weeks of gestation and most is found in the adipose tissue of the newborn.

Although the brain of the newborn has a relatively high concentration of DHA and AA, the absolute accretion during *in utero* life is small. The striking thing about *in utero* LC-PUFA accretion is the much higher concentrations of AA and DHA in the fetal adipose tissue than in the maternal adipose tissue and the fact that 50 times more DHA and AA are stored in the adipose tissue than are deposited in the fetal brain during *in utero* life (Haggarty, 2004). Within a few hours of birth there is a dramatic rise in plasma triglyceride and non-esterified fatty acid concentrations in the newborn, indicating mobilization of adipose tissue stores (Van Duyne et al., 1959). As a result, the concentration of DHA in the adipose tissue is undetectable, while only 12% of AA remains, after two months of postnatal life on a diet devoid of preformed DHA and AA (Farquharson et al., 1993). Therefore, the purpose of the adipose stores of AA and DHA would appear to support processes such as brain and retinal development during the critical first months of postnatal life. This suggests that the *in utero* requirement

for AA and DHA is likely to be complicated by its interaction with postnatal nutritional status. If the infant has a good supply of DHA and AA in the first months of life, then its adipose stores of these LC-PUFA may not be critical, but if it has a poor supply because it is given formula milk without added LC-PUFA, or the mother's breast milk is low in LC-PUFA such as diabetic mother's milk (Jackson et al., 1994; Thomas et al., 2000), or the infant does not feed well on any diet, then the availability *in utero* and the amount already laid down in the adipose tissue of the newborn may be critical (Haggarty, 2010). The DHA and AA deposited in the fetal fat may be considered as conditionally essential for pregnancy as it is not actually functional during *in utero* life, but in practical terms it may or may not be optional as it is an obligatory accompaniment to the normal deposition of adipose stores in the fetus (Haggarty, 2010).

2.6. The fetus depends on maternal supply of AA and DHA

The concentration of DHA and AA are 300- and 400-fold higher in fetal compared with maternal plasma phospholipids, whereas the LA and ALA precursors are lower (Innis, 2003). The fetus may contribute to its own LC-PUFA needs by synthesis. Although there is no direct evidence of fetal synthesis, the finding that preterm infants are capable of synthesizing both AA and DHA, at an age when they would still developmentally be dependent on the placenta, make this a viable possibility (Carnielli et al., 1996). However, there is a discrepancy between requirement and fetal ability to synthesize LC-PUFA. In order to estimate infantile capacity for endogenous synthesis of AA, Koletzko et al (Koletzko et al., 1996) fed four term neonates with newly diagnosed phenylketonuria (mean age 18 day) a formula with all fat contributed by corn oil,

which has a higher natural ^{13}C-enrichment than European human milk or formula. Analysis of ^{13}C-enrichment in plasma fatty acids over four days allowed researchers to estimate infantile AA synthesis. Koletzko et al found an increased ^{13}C-value in plasma AA of all infants, which indicates that term neonates can synthesize AA. With a simplified isotope balance equation, Koletzko et al estimated that endogenous synthesis contributed only about 23% of total plasma AA by day four. Similarly, Leaf et al (Leaf et al., 1992) have observed a one-third reduction in the relative proportion of AA despite a three-fold increase in the precursor LA in plasma of preterm neonates between birth and three weeks of age. The observation that the human placenta exhibits significant selectivity for the transfer of DHA from the maternal to fetal circulation, suggests that, even if the fetus has the capacity to synthesize LC-PUFA, the maternal supply is still important in fulfilling the requirement of the fetus for this fatty acid (Lakin et al., 1998). In addition, the strong, positive maternal-fetal correlations for all LC-PUFA are convincing evidence for this dependence (Al et al., 1996; Al et al., 1995).

2.7. Higher rate of preterm delivery in diabetic pregnancy

Preterm birth defined as birth less than 37 completed weeks of gestation and is responsible for three quarters of neonatal mortality and one half of long-term neurologic impairments in children (MacDorman et al., 2002). Preterm delivery is further stratified into spontaneous birth (as spontaneous delivery, cervical failure and premature rupture of membranes) and indicated birth, which follows maternal or fetal medical complications that necessitate iatrogenic prompt delivery (2002). Several studies have reported increased rates of preterm delivery

in association with pre-gestational diabetes, ranging from 24 to 62% (Boulot et al., 2003; Sibai et al., 2000; Yang et al., 2006). Yogev et al reported (Melamed et al., 2008) that rates of preterm delivery were 26.6% (119/448) and 6.0% (1,038/17,370) in the pre-gestational diabetes and control groups, respectively ($P<0.001$). The pre-gestational diabetes group had higher rates of both spontaneous (6.9% vs. 4.8%, $P<0.001$) and indicated (19.6% vs. 1.2%, $P<0.001$) preterm deliveries. Preconception care was not associated with lower rates of either spontaneous or indicated preterm delivery in the pre-gestational diabetes group. This lack of a protective effect may be explained by the inability of preconception care to alter some of the risk factors for preterm delivery, such as duration of diabetes, presence of nephropathy and nulliparity. Thus, while preconception care is successful in reducing the risk for fetal anomalies and spontaneous abortions (Temple et al., 2002), it may have little effect on preterm delivery (Melamed et al., 2008). Opinions differ whether the rate of spontaneous preterm delivery increases in pregnancies complicated with gestational diabetes (Yogev et al., 2007). The higher rate of preterm delivery is important to acknowledge in view of the higher rates of learning and cognitive deficits reported for infants of diabetic mothers. Whether such deficits are due more to preterm birth or to alterations in maternal-fetal LC-PUFA are not entirely clear.

Preterm infants were shown to have an essential PUFA and LC-PUFA status significantly lower than that of term neonates (Foreman-van Drongelen et al., 1995). Fetal plasma is characterized by low LA and high AA and DHA while human milk and infant formula are high in LA. Feeding with triglycerides high in LA as a major energy source make it unlikely that plasma AA and DHA levels

similar to the third trimester fetus can be achieved in parenterally or enterally fed infants (Georgieff et al., 2005). Autopsy tissue analyses have estimated an accretion of 552 mg/d n-6 fatty acids and 67 mg/d n-3 fatty acids during the last trimester of gestation (Clandinin et al., 1981). Most of the n-3 fatty acids accumulated in the brain is DHA, while fetal liver and adipose tissue contain about 2-fold more AA than LA (Clandinin et al., 1981). Fetal brain accretion has been estimated as 5.8 g n-6 and 3.1 g n-3 fatty acids/day, representing about 1.1% and 4.7% total body accretion (Clandinin et al., 1980). It is not known if the fetal brain is protected during limited DHA availability; however, prenatal n-3 fatty acid deprivation does result in a large deficit in fetal brain DHA in animals (Innis et al., 2001). Estimation of the essential fatty acid intakes of preterm infants fed at 120 mL/kg with human milk or formula shows the marked overabundance of LA and ALA compared with the estimated fetal accretion. Assuming 5% or 10% ALA is converted to DHA, a theoretical estimate of the total potential DHA available from 120 mL/kg/d milk or formula is 11-28 mg DHA, which is less than 50% of the estimated *in utero* accretion of 60 mg/d. Although these estimates are based on limited data, and growth and tissue DHA accretion are not linear, it is apparent that current approaches to lipid nutrition for preterm infants are likely to result in marked differences in tissue fatty acids from that achieved *in utero* (Georgieff et al., 2005).

2.8. Maternal LC-PUFA status during pregnancy

2.8.1. Dietary, synthesis and storage pools in adipose tissue

2.8.1.1. Dietary and synthesis of AA and DHA: vegetarian vs. diabetes

Conversion of LA to AA, and ALA to and DHA is important for providing AA and DHA for incorporation into the tissues. Because dietary intakes of AA and DHA are substantially lower in the Western diet compared with their essential fatty acid precursors; LA intake is about 20-fold greater than that of AA, and ALA intake is about 20-30 fold greater than DHA intake (Yogev et al., 2007).

Women of reproductive age have greater capacity for conversion of ALA to DHA than men (Burdge et al., 2002). AA and DHA proportions are also higher in liver and plasma phospholipids in female than male rats accompanied by greater expression of Δ6- and Δ5- desaturase activities (Burdge et al., 2008).

Pregnancy is associated with increased phospholipid DHA and AA concentrations in rat liver and plasma, guinea pig liver, and in plasma in humans (2002; Boulot et al., 2003; Cordero et al., 1998; Melamed et al., 2008; Otto et al., 1997; Sibai et al., 2000). These changes are the result of adaptations to hepatic phospholipid metabolism (Melamed et al., 2008). It is possible that the higher DHA and AA status in women, reflecting a greater capacity for DHA and AA synthesis, may provide DHA and AA for incorporation into phospholipids and in turn for supply to the fetus during pregnancy. This may occur by the establishment of reserves of DHA and AA in the adipose tissue, which can be mobilized during pregnancy (Al et al., 1997; Temple et al., 2002), and/or by synthesis of DHA and AA from its precursors during pregnancy. Because DHA and AA status and synthesis appear to be up-regulated by estrogen (Innis, 2003;

Shao et al., 2006), the increase in plasma estrogens that occurs during gestation may be an important determinant of maternal DHA and AA status and in turn DHA and AA supply to the fetus (Bakewell et al., 2006).

Montelongo et al (Montelongo et al., 1992) reported a longitudinal study of hormones during pregnancy in normal (n=12), gestational (n=9) and pre-gestational (n=12) diabetic women. Plasma levels of β-estradiol increase intensely with gestation, attaining the highest value at the 3rd trimester, and sharply fell at postpartum; this trend was repeated in all three groups. Values of β-estradiol levels during gestation were, however, always lower in diabetic women than in normal women - the difference being significant between pre-gestational diabetic women and normal control subjects at the 1st and 2nd trimester, but not at the 3rd trimester, nor between GDM.

Indeed the importance of this physiological adaptation in synthesizing LC-PUFA during late pregnancy has been demonstrated by Lakin et al (Lakin et al., 1998). In their study, dietary intake and tissue concentration of fatty acid status has been compared in omnivore, vegetarian and diabetic pregnancies. Although the dietary LC-PUFA intakes of the diabetic pregnant women are comparable to those of healthy omnivore pregnant mothers, the pattern of PUFA in their tissues resembles that of vegetarian mothers. This is because the rates of LA and ALA conversion to n-6 and n-3 LC-PUFA were increased in healthy vegetarians, but were impaired in diabetic mothers.

2.8.1.2. Storage pool in adipose tissues

In the course of pregnancy, appreciable amounts of DHA and AA are intended to be stored in the maternal adipose tissue (Haggarty, 2010). The pool of LC-PUFA serves as a dependable source of AA and DHA during the critical periods of fetal development and ensures a constant supply of substrates to the fetus, free of large diurnal fluctuations in supply corresponding to the timing of maternal meals (Haggarty, 2004). However, compromised DHA and AA stores are expected in pregnant women with diabetes as significantly lower adipose tissue AA and DHA stores have been reported in STZ-diabetic rats (Makrides et al., 2000).

2.8.2. Maternal AA and DHA status decline during normal pregnancy

It was estimated that the fetus accumulates about 70 mg of n-3 LC-PUFAs per day, mainly DHA, during the last trimester (Clandinin et al., 1980; Martinez, 1992). The accumulation of AA is thought to be substantially higher since it is omnipresent in most tissues in significant amounts. In order to cope with the fetal fatty acid demands, maternal circulating concentrations of triglyceride (Bakewell et al., 2006), phospholipids (Gu et al., 2006) and non-esterified fatty acids (Haggarty, 2010) increase throughout gestation, and this effect is particularly striking for maternal triglyceride, which increases 250% (Bakewell et al., 2006). Perhaps more important is the constancy of this level throughout the day. Even in the fasted state, the pregnant woman has a triglyceride concentration (Bakewell et al., 2006) that is almost twice the postprandial peak triglyceride in a non-pregnant individual following the consumption of a high-fat diet (Haggarty, 2010). Thus, the levels of all fatty acids increase, but the levels of AA and DHA increase

relatively less (Clandinin et al., 1981). Longitudinal data on fatty acid concentrations in pregnant women indicate that the total amounts (mg/L) of saturated, monounsaturated, and PUFA in the maternal plasma phospholipids start to increase in early (10 week) pregnancy until delivery (Al et al., 2000; Otto et al., 2001). This higher level of fatty acids is not brought about by altered dietary behavior, but by an accelerated breakdown of maternal fat stores during the last trimester (Al et al., 1995). In addition, nondiabetic women of childbearing age have a greater capacity for synthesizing DHA (Burdge et al., 2002; Pawlosky et al., 2003), and perhaps AA as well, since both fatty acids share the same synthetic pathway (Voss et al., 1991). However, in many longitudinal studies, a continuous decrease in the relative amounts (% of total fatty acids) of plasma AA and DHA was reported in plasma total phospholipids of British, Dutch, Hungarian, and Ecuadorian (Otto et al., 1997) and Dutch (Al et al., 1995) pregnant women.

Maternal AA status may be further compromised by enhanced DHA consumption, as the DHA consumption of pregnant and lactating women can be expected to increase considerably in the near future as a result of the new recommendation for at least 200 mg/day (Koletzko et al., 2007; Makrides, 2009) and the claim that amounts of up to 3 g/day of DHA are safe (Makrides, 2009). However, an increase in the consumption of n-3 LC-PUFA often causes a concomitant decrease of circulating n-6 LC-PUFA concentrations and of AA in particular (Helland et al., 2006; Makrides et al., 1996). In pregnant and lactating women, this may not be desirable, because AA is considered essential for fetal and neonatal development (Innis et al., 2002; Muskiet et al., 2006). Concerns in diabetic pregnancy would be greater as they have compromised AA and DHA

status. Because both insulin and estrogen enhance fatty acid synthesizing enzymes, but are lower in diabetic pegnant women (Montelongo et al., 1992).

In addition, studies (Ghebremeskel et al., 2000; Min et al., 2000; Min et al., 2001; Sanjurjo et al., 1993) have reported the striking differences in the relative composition of plasma and red cell fatty acids among pregnant and nonpregnant women of the same ethnic origin and comparable age and parity. Pregnancy is associated with a reduction in AA and DHA and elevation in palmitic and oleic acid in plasma and red cell membranes. These pregnant women had not changed their dietary habits as a result of their pregnancy. Their response was consistent with the classical longitudinal study covering 20 years (Beal, 1971) which demonstrated that women change diets very little upon becoming pregnant. Hence, the observed difference in fatty acid status between the two groups of women appears to be primarily a reflection of a shift in metabolism in response to the demands of pregnancy. It is not clear whether the lower AA and DHA in the pregnant women is a physiological response to pregnancy or a reflection of the depletion of maternal AA and DHA stores due to the demand for these nutrients by the developing fetus (Ghebremeskel et al., 2000). If it is the latter, it should be possible to prevent the depletion by raising maternal status before or during pregnancy. Indeed, studies demonstrate that maternal and neonatal DHA status can be enhanced by administration of fish oil (van Houwelingen et al., 1995) and sardines and fish oil (Connor et al., 1996) sources of DHA, during pregnancy.

Healthy women may have limited ability to mobilize AA and DHA in order to meet the high demand of pregnancy for these fatty acids. However,

regardless of the capacity for mobilization, the requirement for AA and DHA is unlikely to be met if the mothers had low status prior to their pregnancy, and/or impairment of PUFA metabolism, such as diabetes (Ghebremeskel et al., 2000).

2.8.3. Diabetes in pregnancy

2.8.3.1. Compared with normal controls, STZ-induced diabetic rats are characterized by a lower AA only, but not DHA

Several studies have revealed substantial changes in the relative levels and metabolism of AA in a variety of tissues from human patients (Chase et al., 1979; Schrade et al., 1963; Watala et al., 1990) and in those from animals with experimentally induced diabetes (Dang et al., 1988; Faas et al., 1983; Lin et al., 1985). Such studies have indicated that there are appreciable changes in the relative levels of PUFA in phospholipids. A decrease in the level of AA and an increase in the level of ALA are common changes found in the tissues of diabetic rats. Faas and Carter found a 48% inhibition of Δ6 desaturase activity in microsomes of livers of the diabetic rats (Faas et al., 1983). Hu et al (Hu et al., 1994) reported a substantial decrease in the relative level of AA and an increase in that of ALA in the tissues of 8-week-diabetic rats. The relative levels of AA in phospholipids of liver and plasma showed significant changes as early as 7 days after the induction of diabetes and they decreased rapidly for up to 4 weeks. In contrast, the levels of AA in the heart, kidney, and testis did not change at the early stage of diabetes but significantly decreased after 3 weeks of diabetes. Lefkowith et al. (Lefkowith et al., 1985) suggested that the liver supplies AA to other tissues when the animal is deprived of essential fatty acids. Thus the delay in the reduction in levels of AA in the brain, kidney, heart, testis, and spleen

might be caused, in part, by the secondary effects of lipid modification in the liver in the diabetic state.

There is no similar study conducted in female rats. It is possible that the LC-PUFA deficiency in ovary and uterine tissue of female rat is in the similar order as testis of male rat. With progression of diabetes, ovaries and uterus will become LC-PUFA deficient, which is at the late stage of LC-PUFA deficiency, but before brain becomes AA deficient.

As for the comparison between males and females, it is dependent on reproductive status. If the ovaries or uterus belong to pregnant women, LC-PUFA deficiency in female's ovaries and uterus may have a greater implication than if LC-PUFA deficiency is observed in male testes. The female rat is at a lower level of LC-PUFA status since she has to transfer significant amount of LC-PUFA to her fetus. For non-pregnant women, the implications of LC-PUFA status may be comparable between female ovary, uterine and male testes when diabetes control is at the same levels.

2.8.3.2. Lower endogenous synthesis, compromised body stores and inability to increase the conversion rate during the 3rd trimester: newborn infants of all types of diabetic mothers have lower AA and DHA status

In human (types 1 and 2) and experimental diabetes, the activity of Δ6 and Δ5 desaturase, enzymes that are vital for the synthesis of AA and DHA, is impaired (Arisaka et al., 1991; Brenner et al., 2000; el Boustani et al., 1989), and the levels of AA and DHA are reduced in both circulation and in body stores in fat tissue (Igal et al., 1991; Mikhailidis et al., 1986; Tilvis et al., 1985). Pregnancy is diabetogenic, it has been found that late normal pregnancy in lean

and moderately obese women was associated with a two-third reduction in insulin sensitivity, as compared with the nongravid state (Buchanan et al., 1990). In normal pregnancy, the conversion rates of LA and ALA to AA and DHA are increased in the last trimester of pregnancy but this is not the case in diabetic pregnancy (Lakin et al., 1998). Thus, for those pre-gestational diabetic pregnancies, due to the combination of pre-diabetes and pregnancy-induced metabolic changes, the synthesis of AA and DHA would be diminished further compared with non-pregnant diabetic status. There is evidence that insulin treatment ameliorates both the activity of Δ6 and Δ5 desaturase and membrane AA and DHA abnormality in general population and in experimental animals (Ghebremeskel et al., 2004). However, although type 1 diabetic women were on insulin therapy throughout their pregnancy, and usually they had acceptable glycemic control [mean glycosylated hemoglobin (A1C) 5.72 $\pm$0.69%] and they had received individualized dietary counseling (Thomas et al., 2006), the levels of plasma AA and DHA of pregnant type 1 diabetic women were 14 and 38.9% lower than those of healthy nondiabetic women (Ghebremeskel et al., 2004; Min et al., 2005). It is plausible that the combination of diabetes- and pregnancy-induced metabolic changes (Montelongo et al., 1992) and the consumption of a high-saturated-fat "Western diet" may have depressed the AA and DHA levels in the pregnant type 1 diabetic women and consequently the fetus (Lakin et al., 1998).

Thus, in pregnancy complicated with diabetes, owing to the maternal impairment of AA and DHA synthesis, compromised body AA and DHA stores, and maybe impaired placental transfer, there may be an imbalance between the

fetal requirement and maternal supply. Indeed, there is evidence that AA and DHA levels are compromised in neonates of women who have type 1 (Ghebremeskel et al., 2004), type 2 (Min et al., 2005), and gestational (Min et al., 2005; Thomas et al., 2005) diabetes. Although these infants were born with no malformations, the levels of AA and DHA, relative to the cord value of infants of the nondiabetic women, were reduced by 15.1 and 26.6% and by 20 and 41.9% in plasma choline phosphoglycerides and cholesterol esters, respectively (Min et al., 2005; Thomas et al., 2005).

It has been claimed that a low level of AA in cord plasma and umbilical artery is associated with low birth weight (Crawford et al., 1990; Crawford et al., 1989; Leaf et al., 1992); and cord plasma levels of both AA and DHA are positively correlated with head circumference (Leaf et al., 1992; Leaf et al., 1992). Biochemical signs of essential fatty acid deficiency have been reported in the endothelium and umbilical arteries of low birth weight infants with reduced synthesis of prostacyclin by the umbilical endothelium (Ongari et al., 1984). These studies suggest that an imbalance between maternal supply and fetal demand for AA and DHA during the prenatal period may explain impaired development.

2.8.3.3. Augmented *de novo* fatty acid synthesis from the abundant glucose - dilute essential fatty acids

The AA and DHA status in the fetus of diabetic mothers is to a lesser extent determined by maternal LC-PUFA status than in normal pregnancies (Muskiet et al., 2006). Wijendran et al reported that erythrocyte AA and DHA in newborns of mothers with gestational diabetes were lower (Wijendran et al.,

2000), despite higher erythrocyte DHA and equal AA in the mothers (Wijendran et al., 1999), as compared with respective controls. This discrepancy, between the mother and fetus, has been explained by the existence of a relative, rather than an absolute, essential fatty acid deficiency. With higher maternal glucose, there would be increased glucose flux into the fetus. This would result in an increase of *de novo* synthesized fatty acids (notably 16:0, 18:1 n-9 and 16:1 n-7) from glucose and possibly lactate and in turn a substantial accumulation of fetal fat. The increase of fetal *de novo* synthesized fatty acids is likely to cause dilution of maternally derived LC-PUFA and essential fatty acids and, consequently, a relative essential fatty acid deficiency. Thus, it has been proposed that children born to mothers with poor glucose homeostasis have lower essential fatty acids and LC-PUFA status (Dijck-Brouwer et al., 2005). Indeed Rump et al (Rump et al., 2001) reported the negative correlation between maternal fat mass and fetal cord plasma AA and DHA. This resulting relative essential fatty acid and LC-PUFA deficiency might interfere with neurological development.

2.9. Placental transfer of PUFA

2.9.1. Preferential transfer DHA: placenta retain AA

Fetal blood is separated from maternal blood by the placental membrane (placental barrier). Placenta is a vascular organ rich in phospholipids (Bayon et al., 1993; Klingler et al., 2003; Nikolasev et al., 1973), which in turn are rich in PUFA, particularly AA (Bitsanis et al., 2005). The placenta might have a role in modulating the fatty acid supply to the fetus, although it lacks the Δ5- and Δ6-desaturases for the conversion of essential fatty acids to LC-PUFA (Chambaz et al., 1985).

Placenta plasma membrane fatty acid binding protein exhibits a hierarchy of binding affinities for different fatty acids and may be important in facilitating placental transfer LC-PUFA to the fetus (Campbell et al., 1998; Campbell et al., 1996; Campbell et al., 1998; Dutta-Roy, 2000). Many studies described significantly higher AA and DHA concentrations in fetal blood than in the maternal circulation (Al et al., 1995). More recently, it has been found that human placental membranes accumulate substantially higher proportions of AA than maternal and cord blood phospholipids (Bitsanis et al., 2005; Crawford et al., 2003; Min et al., 2004; Min et al., 2005). When perfusing the placenta with a mixture of fatty acids designed to mimic that of the circulating triglyceride in the last trimester of pregnancy, the order of selectivity for uptake is the AA>DHA>ALA>LA (Haggarty et al., 1997). However, the placenta appears to retain AA in preference to the other fatty acids, resulting in a quite different order of selective transfer to the fetal circulation as DHA>ALA>LA>AA (Haggarty et al., 1999). Similar results have been obtained from *in vivo* experiments (Ruyle et al., 1990). Placenta is an important site of production of the prostacyclins, prostaglandins, thromboxanes and leukotrienes and AA is the precursor of the eicosanoids. Therefore one possible explanation for AA retention may be that the placenta has a minimum requirement for AA to produce these metabolites and that it is only when this requirement is met that the remaining AA becomes available for transfer to the fetal circulation (Haggarty et al., 1997). Indeed that conversion of AA to prostaglandin has been measured directly within the perfused human placenta (Kuhn et al., 1986).

For comparing the placenta size between diabetic and healthy pregnancy, Nelson (Nelson et al., 2008) reported that placental weight wassignificantly greater [700 (600 - 800) g vs. 627 (551 - 700) g], and birth weight greater (3778 ± 701 g vs. 3553 ± 520 g) in the offspring of mothers with type 1 diabetes (n=122) than controls (n=46). Lakin reported (Lakin et al., 1998) that the placentas were significantly heavier (728 ± 180 g vs. 554 ± 43 g) in type 1 diabetic omnivore women (n=5) than normal vegetarian women (n=4), but no differences with omnivore controls (692 ± 162 g, n=10). There were no significant differences in the weight of newborn, the length of gestation among the three groups. Bitsanis reported (Bitsanis et al., 2006) that there was no difference in placental weight between the gestational diabetes (n=11) and the healthy control groups (640 ± 176.7 g vs. 573 ± 148.6 g, n=25).

This differential handling of AA relative to DHA is consistent with the observation that the fetus appears to be less dependent on a placental supply of AA than of DHA. Stable isotope studies carried out *in vivo* have demonstrated that the premature human infant can synthesize both AA and DHA at an age when it would still developmentally be dependent on the placenta (Carnielli et al., 1996; Salem et al., 1996), but that the rate of AA synthesis is significantly greater than that for DHA. Salem et al have shown that the rate of DHA synthesis in newborn human infants is extremely low and the ratio of synthesis of AA to DHA is around 60:1 (Salem et al., 1996). This differential synthesis is presumably due to the additional complexity of DHA biosynthesis; involving the participation of enzymes in both peroxisomes and the endoplasmic reticulum as well as the

controlled movement of fatty acids between these two cellular compartments (Sprecher et al., 1999; Sprecher et al., 1995).

2.9.2. Mobilization and release of AA and DHA from placenta is compromised in diabetes: exaggerated activities of placental lipoprotein lipase and fatty acid binding protein, diminish the transfer of the AA and DHA to the fetal circulation

In gestational diabetes a significant discrepancy between normal LC-PUFA contents in erythrocyte and plasma phospholipids of the mother compared to lower LC-PUFA contents in the neonate was found (Wijendran et al., 2000). Furthermore, the placenta of women with gestational diabetes had higher levels of AA and DHA compared to healthy control subjects (Bitsanis et al., 2006). These data suggest that placental LC-PUFA uptake is enhanced but transport from the placenta to the fetal circulation is impaired in women with gestational diabetes. Human placental membranes accumulate substantially higher proportions of AA than maternal and cord blood phospholipids, especially in pregnancy complicated with diabetes (Bitsanis et al., 2005; Crawford et al., 2003; Min et al., 2004; Min et al., 2005). It has been hypothesized these changes may be related to the alteration in the fatty acid transporters, in the activities of lipoprotein lipase and plasma membrane fatty acid binding protein that display high affinity for LC-PUFA (Bitsanis et al., 2006; Kaminsky et al., 1991; Magnusson et al., 2004). This mechanism has also been proposed to explain the lower fetal AA and DHA status in type1 and type 2 diabetic pregnancies (Bitsanis et al., 2006; Kaminsky et al., 1991; Magnusson et al., 2004).

Indeed, Chabowski (Chabowski et al., 2004) reported that insulin induced the expression of fatty acid translocase (FAT/CD36), one kind of fatty acid

transport protein. The impaired glucose homeostasis thus may interfere with placental FAT/CD36 expression then fatty acid transport. Anti-diabetic drugs are agonists of different peroxisome proliferator-activated receptor (PPAR) subtypes and can modify mRNA expression of lipid carriers, and potentially modify placental fatty acid transfer. In rat placental trophoblast cells, anti-diabetic drugs, fenobibrate, up-regulated acyl-CoA-synthetase activity (FATP) and fatty acid binding protein (FABP) with significant uptake of AA and DHA.

2.10. Characteristics of LC-PUFA in maternal milk

2.10.1. In normal maternal milk

The LC-PUFA in human milk is derived from diet, liberation from maternal body stores, or endogenous synthesis from precursor fatty acids (Sauerwald et al., 2001). Tracer studies indicate that vast majority of milk essential fatty acids do not come directly from the diet, but from maternal stores (Sauerwald et al., 2001). For instance, 30% of milk LA has been estimated to be derived directly from the diet and 70% from maternal stores. AA might be subjected to a large distribution volume, because of its high tendency to become incorporated into membrane phospholipids, and to a lesser extent in plasma and adipose tissue triglycerides (Calder, 2007; Nelson et al., 1997). Thus, only 1.2 (Koletzko et al., 1999) to 3% (Sauerwald et al., 2001) of AA in milk is derived from endogenous conversion of dietary LA and the rest is derived from maternal stores (Koletzko et al., 1999). It is conceivable that PUFA and particularly LC-PUFA content in human milk may be largely affected if the mother has chronic metabolic disorders such as diabetes.

With about 4% in mature milk, human milk fat contributes about 40-55% of the total energy intake to the breastfed infant. The most abundant PUFA in human milk is LA (~10 to 15% in most studies), and there is ~2-fold increase over the last decade in the content of LA in human milk (Jensen, 1999). Few studies have considered the effects of dietary ALA on human milk ALA; however, human milk levels of ALA vary widely, from 0.1 to about 4%, with an average of 1.4%, which increased to 2.6% among women taking a supplement of 2 g ALA /day (Innis, 1992; Innis, 2004; Innis et al., 1999). The LA/ALA ratio is on the order of 5:1 to 15:1 in most milks (Sauerwald et al., 2001). The two LC-PUFA found in the highest proportion are AA (~0.4 to 0.6%) and DHA (~0.2 to 0.4%). Regression analyses and calculation of Pearson correlation coefficients of the change in DHA and AA in mature human milk from predominantly white women in Vancouver shows that DHA decreases by 50% from 0.4% to 0.2% ($P<0.001$), whereas AA declines from 0.7% to 0.4% ($P<0.001$) over the period from 1988 to 1998 in this segment of the population (Innis, 2003). A similar decline in human milk DHA has been reported in Australia (Makrides et al., 1995), suggesting a global dietary trend.

The fatty acid composition of human milk varies as lactation progresses. The DHA content of colostrum is typically around twice as high as that of mature milk (Gibson et al., 1981; Jensen, 1996). From colostrum to mature milk, the contents of LA and ALA in human milk increase, whereas the percentage of AA and DHA decrease markedly (Koletzko et al., 1999). The percentage content of AA decreased by about 38% and that of DHA by about 50% during the first month of lactation (Genzel-Boroviczeny et al., 1997). Makrides et al (Makrides

et al., 1995) found the percentage of DHA decreases about 20% from the 6th to the 16th week of lactation, and remains constant until the 30th week. However, because total fat content increases with advancing lactation, the absolute amounts of LC-PUFA excretion remain relatively stable (Bitman et al., 1983). Hornstra (Weseler et al., 2008) reported that duration of lactation was associated with significant reductions of the AA (P=0.011 after 2 week, P=0.0004 after 8 week), but not DHA.

Maternal diet is an important factor influencing human milk fatty acid composition. Milk from women following vegan and vegetarian diets contains over 30% LA (Innis, 2004) and about 0.1% DHA, reflecting their lack of a dietary intake of DHA (Sanders et al., 1992); whereas much higher amounts are found among women with high habitual intakes of fish and other marine animals. For example, the average amount of DHA as a percentage of fat in human milk was 2.8% in Zhangzi, China, 1.0% in Japan (Innis, 2004).

2.10.2. In diabetic maternal milk

Although maternal diet is a key factor affecting human milk composition, other factors such as stage of lactation, nutritional status and maternal metabolic disease are known to influence the fat content and fatty acid composition in human milk (Koletzko et al., 1999). Indeed, Jackson et al (Jackson et al., 1994) reported that, in comparison to a healthy control group, milk LC-PUFA were lower in women with type 1 diabetes from 14 to 82 day postpartum. Reduced activity of liver desaturase enzymes involved in LC-PUFAs synthesis has been proposed as a possible cause of theses alterations. Similarly, Thomas et al

(Thomas et al., 2000) reported that AA and DHA (percentage) in maternal milk are depressed in diabetes.

2.10.3. AA dose-dependently increases the AA concentration in human milk

The biological variation of milk AA content is among the lowest of all milk fatty acids (CV 28%,; median 0.42 mol%; range 0.19-0.99), while those of EPA (100%; 0.05 mol%; 0.00-1.18) and DHA (86%; 0.21 mol%; 0.08-1.63) constitute the highest (Smit et al., 2002). The apparently low biological variation of AA may however derive from a sampling bias, which is caused by studying samples from populations with relatively low AA intakes (Muskiet et al., 2007). To illustrate, milk from women living in Doromoni (Tanzania), exhibit both high milk AA (median 0.70 mol%; range 0.50-0.93) and high DHA (0.75 mol%; 0.25-1.47). These women have high lifetime intakes of local AA and DHA rich fish as their only source of animal fat (Kuipers et al., 2005). Van Goor et al reported that breast milk AA increases after supplementation of a relatively low dose of AA (200 mg/day) during pregnancy and lactation. They concluded that breast milk AA content is sensitive to dietary AA intake. The influence of dietary AA intake becomes noticeable after prolonged supplementation, which is in line with the notion that milk PUFA derive notably from maternal body stores. Similarly Weseler et al reported (Weseler et al., 2008) that the consumption by lactating women of additional AA and DHA increased the AA and DHA concentrations of their milk total lipids. For AA, this effect appeared to be dose dependent.

The addition of some fish oils to formula to provide DHA reduces growth and alters neurodevelopment either positively or negatively in premature infants (Carlson et al., 1992; Carlson et al., 1996; Carlson et al., 1994; Carlson et al.,

1996) and to possibly reduce language development in term infants (Scott et al., 1998). These effects seem strikingly similar to the reduced brain growth seen in piglets fed formula with high ALA or DHA concentrations (Arbuckle et al., 1992; Arbuckle et al., 1994; Arbuckle et al., 1992; Innis, 2000). As with fish oils, high intakes of DHA also lowered brain weight and AA concentration in mice (Wainwright et al., 1997). Recent information suggests that this effect of dietary DHA may be offset by including AA in the diet (Wainwright et al., 1997). The metabolic explanation, however, is not known. In another animal study, high intakes of DHA resulted in increased concentrations of DHA and reduced concentrations of AA in some lipid classes in the brain and retina; these fatty acid compositional changes were accompanied by reduced visual function (Weisinger et al., 1996). A biochemical explanation for the adverse effect of high tissue DHA concentration on visual function is not available.

However, despite selectivity in placental n-6 and n-3 fatty acid transport, women with higher plasma AA and DHA during gestation give birth to infants with higher AA and DHA, respectively (Connor et al., 1996; Elias et al., 2001; Helland et al., 2001). This is important because in addition to potential positive effects on fetal growth and neural development (Elias et al., 2001; Helland et al., 2001), a higher n-6 and n-3 fatty acid status at birth does result in higher infant blood levels of AA and DHA for several weeks after birth (Foreman-van Drongelen et al., 1995; Guesnet et al., 1999).

2.11. AA, DHA status of newborns: deficiency (sub-optimal) vs. optimal

Optimal fetal growth and tissue function require an adequate supply of PUFA at defined periods in gestation. Failure to acquire sufficient and

appropriate PUFA at such critical periods in fetal development may have irreversible and harmful consequences for post-natal growth and function (Burdge et al., 1994).

As reviewed above, newborn infants of all types of diabetic mothers have lower AA and DHA status (Ghebremeskel et al., 2004; Min et al., 2005; Min et al., 2006; Thomas et al., 2005; Wijendran et al., 2000). Although newborns of diabetic mothers do not appear to exhibit the symptoms of AA and DHA deficiency, overt AA and DHA deficiency is a rare and extreme condition and much of the work on LC-PUFA in infant nutrition, for example, has focused on relatively subtle effects on clinical outcomes such as intelligence quotient and visual acuity. It may be the adverse effects arise from sub-optimal intakes of LC-PUFA, even in the absence of overt signs of deficiency. It is tenable to assume that prenatal and postnatal insufficiency of AA and DHA may contribute to cognitive impairment and/or behavior disorders in children of diabetic mothers.

2.12. Consequences to newborn infants of diabetic mothers with lower AA and DHA status: neurobehavioral performance (short-term and long-term)

Follow-up studies concerning the adverse effects of diabetic pregnancy on the developing brain have revealed neurobehavioral deficits in both sensory-cognitive and psychomotor functions. These include altered auditory recognition memory processing at birth (Siddappa et al., 2004), reduced visual and memory performance at 8 and 12 months (DeBoer et al., 2005; Nelson et al., 2003), poorer performance on tests of general development in infants and toddlers (Deregnier et al., 2000; Rizzo et al., 1991), and inferior performance in elementary school children (Ornoy, 2005; Rizzo et al., 1997). While motor delay may be a sign of

mild, nonspecific brain damage (Ornoy, 2005), the abnormalities in memory processing suggest alterations in hippocampal development and function (Nelson et al., 2000).

Normal peri-natal brain development is dependent on maternally derived LC-PUFA (Lauritzen et al., 2001). Newborn IDM have lower AA and DHA in plasma and tissues (Ghebremeskel et al., 2004; Min et al., 2005; Min et al., 2006; Thomas et al., 2005; Wijendran et al., 2000). The low availability of LC-PUFA might be implicated in impaired neurodevelopment because lower fetal status of AA and DHA and lower milk LC-PUFA is associated with a less favorable neonatal neurological condition (Dijck-Brouwer et al., 2005).

Most rat models of diabetes are characterized by a deficiency of AA only, and not of DHA (Brenner, 2003; Faas et al., 1983; Holman et al., 1983; Hu et al., 1994). Even though both AA and DHA are important to neurodevelopment (Saste et al., 1998), brain accretion of AA exceeds that of DHA during gestation (Hadders-Algra, 2008). AA metabolites, including prostaglandin E_2 and endocannabionids, play an important role in neurogenesis (Molina-Holgado et al., 2007; Uchida et al., 2002). Dietary AA supplementation in the dam enhances hippocampal neurogenesis in 31-day-old offspring by 32%, whereas no benefit is observed with combined AA plus DHA or DHA alone (Maekawa et al., 2009). Thus, low AA status in the offspring following a pregnancy complicated by diabetes might explain the learning and memory deficits in the offspring (Kinney et al., 2003).

Peri-natal periods represent critical intervals when inadequate accumulation of DHA may be particularly detrimental (Enslen et al., 1991).

Abnormal neurobehavior has been seen in adult rats raised on a DHA restricted diet (Enslen et al., 1991; Kodas et al., 2004; Kodas et al., 2002; Levant et al., 2004). This is conceivable as LC-PUFAs are not only important structural elements of membranes, but, together with their eicosanoid products, they are also implicated in gene expression (Muskiet et al., 2006). Similarly, in infants of diabetic mothers, the suboptimal hippocampus-related memory performances persist from birth to 3 years of age (Riggins et al., 2009), while the abnormalities of psychomotor function have been seen in school-age children born to diabetic mothers (Ornoy, 2005). To date no study has explored the possible role of AA in presence of diabetic pregnancy on the offspring neurodevelopment.

2.13. Consequences to newborn infants of diabetic mothers with lower AA and DHA status: skeletal growth, body composition and glucose tolerance

Evidence is accumulating that the fatty acid composition of membrane lipid is a critical cellular factor that influences both insulin secretion and its biological action. AA and DHA, in particular, have been associated with insulin sensitivity. The insulin resistance was correlated with the fatty acid concentration of the membrane choline phosphoglycerides (CPG) but not with those of ethanolamine phosphoglycerides (EPG), CPG and EPG are the predominant phospholipids of the outer and inner leaflet of the membrane lipid bilayer respectively (Borkman et al., 1993). The lipid abnormality was more pronounced in the outer leaflet of the membrane phospholipids, CPG is where most of receptor binding and enzyme activities take place. Insulin resistance involves a loss of receptor and/or transporter efficiency in membrane (Borkman et al., 1993).

In human studies, the concentration of DHA in cell membrane has been linked to insulin sensitivity (the lower the membrane DHA, the greater the insulin resistance). It has also been shown that supplementation of experimental animals with AA improves insulin sensitivity by enhancing glucose uptake (Nugent et al., 2001; Song et al., 2003).

The prevalence of diabetes in pregnancy is rising in part due to the sharp increase in obesity and type 2 DM (Bell et al., 2008). Fetal exposure to hyperglycemia during pregnancy can have profound and long-lasting consequences to the offspring (Freinkel, 1980). In the Northwestern University Diabetes in Pregnancy follow-up study, 40% of offspring of diabetic mothers by age 16 years had IGT defined as blood glucose 7.8 to 11.0 mmol/L 2 h after receiving a 75 g glucose load (Metzger, 2007). Furthermore, the occurrence of IGT was associated with higher insulin in amniotic fluid (Silverman et al., 1995). Based on the SEARCH Case-Control study, 30.4% of youth with type 2 DM were exposed to maternal diabetes *in utero* compared with 6.3% of nondiabetic control youth (Dabelea et al., 2008).

Whether altered maternal-fetal nutrient transfer or fetal metabolism explain the propensity for offspring of mothers with diabetes to develop obesity, IGT and diabetes is not clear. In cell culture, AA is fundamental to functional integrity of the pancreatic β-cell (Dixon et al., 2004), while glucose-stimulated insulin release is dependent on plasma membrane release of AA (Konrad et al., 1994). Furthermore, insulin sensitivity is positively associated with higher AA status in humans (Borkman et al., 1993). In rats, dietary AA supplementation lowers fasting blood glucose and insulin concentrations (Sasagawa et al., 2001),

prevents high-fat diet induced insulin resistance (Wu et al., 2007) and enhances glucose disposal (Song et al., 2003). However, women with diabetes have lower AA in erythrocytes, particularly if obese (Min et al., 2004) and newborn infants of women with diabetes also have reduced AA in cord blood (Ghebremeskel et al., 2004; Min et al., 2005; Min et al., 2006; Ortega-Senovilla et al., 2009; Thomas et al., 2005; Wijendran et al., 2000). Lower neonatal status is likely a reflection of lower maternal AA status, placental sequestration of AA, and increased fetal utilization (Ortega-Senovilla et al., 2009). Several studies have explored the possibility of maternal dietary intervention in modifying the susceptibility of offspring to adult diseases (Ibrahim et al., 2009; Korotkova et al., 2005; Siemelink et al., 2002). The differences in body weight and fasting insulin levels in adult rat offspring relate to the n-6 to n-3 fatty acid ratio in the maternal diet during the perinatal period (Korotkova et al., 2005). Similar effects have also been observed in diabetic pregnant rats and their adult offspring (Soulimane-Mokhtari et al., 2005). It is thus hypothesized that maternal supplementation with AA during pregnancy and lactation would prevent subsequent development of IGT in the offspring at young adult age.

New evidence suggests that there are linkages between glucose metabolism and bone metabolism (Confavreux et al., 2009). Since newborn IDM have compromised bone growth and mineralization (Mimouni et al., 1988; Tsang et al., 1972; Verhaeghe et al., 1995), as well as reduced AA status (Ghebremeskel et al., 2004; Min et al., 2005; Min et al., 2006; Ortega-Senovilla et al., 2009; Thomas et al., 2005; Wijendran et al., 2000), it is also hypothesized that maternal AA supplementation may also be beneficial to offspring body composition and

bone health. AA is a precursor to prostaglandin E_2 (PGE_2) that has a biphasic effect on bone by enhancing bone formation at lower concentrations in animals, while stimulating resorption at higher concentrations (Watkins et al., 2001). Alteration in the precursor pool affects PGE_2 metabolism, while modification of dietary fatty acids leads to parallel alterations in bone fatty acids (Weiler, 2000) and PGE_2 metabolism (Alam et al., 1993; Watkins et al., 1997). In a recent study, serum AA was positively associated with whole body bone mass in 8-year-old children (Eriksson et al., 2009). Dietary γ-linoleic acid (GLA, a precursor to AA) supplementation enhanced bone mass in young male rats (Claassen et al., 1995) and was effective against diabetes-induced fetal bone development defects (Braddock et al., 2002). Similarly (Korotkova et al., 2005), variations in fatty acid composition in the maternal diet during the perinatal period caused changes in bone growth in adult rat offspring (Korotkova et al., 2005; Korotkova et al., 2004).

2.14. Impact of childhood obesity and insulin resistance on brain fatty acid composition

The rising prevalence of childhood obesity is a major public health concern worldwide in view of the associated insulin resistance (Druet et al., 2006). A US population-based study revealed a prevalence of insulin resistance of 3.1% among normal weight, 15.0% among overweight and 52.1% among obese adolescents (Lee et al., 2006). The insulin resistance syndrome is highly prevalent in Canadian youth as well (Lambert et al., 2004). Academic achievement (Krukowski et al., 2009) and intelligence (Yu et al., 2010) are lower in obese children, the cause of which is multifactorial. Lipids such as DHA are

postulated to affect cognition in obesity (Morley et al., 2010). Brain is rich in DHA as a fundamental component of neural cell membranes (Salem et al., 2001) where it modulates functional properties such as fluidity, permeability for metabolite exchange, activity of membrane-bound enzymes and receptors, and electrical and humoral signal transduction (Clandinin et al., 1991). To date, whether DHA is implicated in the reduced academic achievement of children has not been addressed.

Numerous studies have reported on plasma and tissue fatty acids in adults (Sjogren et al., 2008; Warensjo et al., 2009) and youth (Decsi et al., 2000; Murakami et al., 2008; Steffen et al., 2008) with insulin resistance and related disorders. This pattern is characterized by a decrease in LA (18:2 n-6) and an increase in palmitoleic (16:1 n-7), γ-linolenic (18:3 n-6) and dihomo-γ-linolenic (20:3 n-6) acids. Since the content of these increased fatty acids is normally very small in the diet, this augmentation in plasma lipids is thus indicative of increasing endogenous desaturation of palmitic acid (16:0) by Δ9 desaturase (leading to increased 16:1 n-7) and of linoleic acid by Δ6 desaturase (increasing the proportions of 18:3 n-6 and 20:3 n-6) (Vessby, 2000). This may reflect a high intake of 16:0 and low intake of 18:2n-6 in the diet and a concomitant increase in Δ9 and Δ6 desaturases but low Δ5 desaturase activity (Vessby, 2003; Vessby et al., 2002), the latter which is required for the synthesis of DHA. Indeed, Pima Indians, a population with the highest reported incidence of insulin resistance in the world, have low skeletal muscle DHA (Pan et al., 1995). This striking difference cannot be explained by diet since among the Australian population,

even individuals with little or no discernible DHA intake had muscle DHA much higher than the Pima study group (Borkman et al., 1993; Pan et al., 1995).

Under normal physiological conditions, the adult liver has ample capacity to synthesize DHA from circulating ALA. In adult rats the estimated rate of DHA synthesis by liver is about 10 × the DHA consumption rate in brain tissue (Igarashi et al., 2007). This high synthesis rate is supported by the high expression and high activity of the elongase and desaturase enzymes in the liver (Igarashi et al., 2007; Rapoport et al., 2007). However, in humans below normal blood levels of DHA are common in liver disease, diabetes and aging (Brenner, 2003; Watanabe et al., 1999). Such reductions are postulated to be associated with reduced desaturase or elongase activity and are a risk factor for brain disease in the absence of dietary DHA supplementation (Rapoport et al., 2009). It is not clear if brain DHA can be sustained in obesity and insulin resistant states but it is suspected to be inadequate since people with insulin resistance have greater cognitive decline (van Oijen et al., 2008; Young et al., 2006). In fact, insulin activity is required for elongase and desaturase enzymes in liver (Brenner, 1990). Very few studies have examined the effect of insulin resistance on brain fatty acid metabolism (Guesnet et al., 1990). Although one study in the Zucker model of insulin resistance observed no difference in brain DHA compared with non-insulin resistant rats, the diet provided preformed EPA+DHA which bypasses Δ5 desaturase (Guesnet et al., 1990). It is well known that deposition of DHA is higher in brain when it is provided in the diet compared to ALA (Abedin et al., 1999). Thus, it is unknown if Δ5 desaturase activity is sufficient to compensate for a diet devoid of DHA.

Since diabetes in pregnancy is associated with development of diabetes in youth and since LC-PUFAs are implicated in learning deficits, it was hypothesized that endogenous synthesis of DHA by the brain would be compromised in an obese hyperinsulinemic state.

BRIDGE 1

From reviewing the literature, it is clear that the conversion of LA and ALA is vital in providing AA and DHA, and this capacity is up-regulated in pregnancy. However, the synthesizing enzymes are depressed in diabetes due to insulin deficiency, and the physiological adaptation in AA and DHA synthesis is absent in pregnant women with diabetes. Newborn infants of all types of diabetic mothers have compromised AA and DHA status, while most STZ-induced diabetic rats have lower AA only. Follow-up studies concerning the adverse effects of diabetic pregnancy on the developing brain reveal neurobehavioral deficits in both sensory-cognitive and psychomotor functions. Normal peri-natal brain development is dependent on maternally derived LC-PUFA (Lauritzen et al., 2001). Thus, low AA status in the offspring following a pregnancy complicated by diabetes might explain the learning and memory deficits in the STZ-offspring (Kinney et al., 2003). A rodent study thus has been carried out to investigate the effect of maternal AA supplementation on offspring neurodevelopment and the study was reported in Chapter 3.

CHAPTER 3. MANUSCRIPT 1.

MATERNAL ARACHIDONIC ACID SUPPLEMENTATION IMPROVES NEURODEVELOPMENT OF OFFSPRING FROM HEALTHY AND DIABETIC RATS

Jinping Zhao[a], Marc R. Del Bigio[b], Hope A. Weiler[a]*

Author affiliation:

[a]School of Dietetics and Human Nutrition, McGill University,

Ste-Anne-de-Bellevue, Québec, Canada

[b]Department of Pathology, University of Manitoba,

Winnipeg, Manitoba, Canada

*Corresponding author:

Hope A. Weiler, RD, PhD

School of Dietetics and Human Nutrition, McGill University,

Quebec, Canada H9X 3V9

Telephone: (514) 398-7905, Fax: (514) 398-7739

e-mail: hope.weiler@mcgill.ca

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3.1. ABSTRACT

Maternal diabetes may compromise infant arachidonic acid status and development. This study tested if maternal arachidonic acid supplementation improves neurodevelopment in rat offspring. Dams were randomized into 6 groups using a 3 × 2 design: saline-placebo, streptozotocin-induced diabetes with glucose controlled at <13 mmol/L, or poorly-controlled at 13-20 mmol/L using insulin; and fed either control or an arachidonic acid (0.5% of fat) diet throughout reproduction. Offspring were tested on postnatal days 3 and 5 for righting response, days 7 and 9 for negative geotaxis, day 14 for wire hanging endurance, days 18 and 24 for rota rod endurance, and day 28 for Morris water maze performance. Only the poorly-controlled group had impaired day 7 geotaxis and day 18 rota rod performance (P<0.02), but this improved with maternal arachidonic acid supplementation (P<0.0006). Arachidonic acid improved the wire hanging endurance (P=0.0003) and water maze latency (P=0.002), suggesting enhanced neurodevelopment in all offspring.

Keywords: Maternal arachidonic acid supplementation, Infants of diabetic mothers, Neurodevelopment, Rat

3.2. INTRODUCTION

While glycemic control is a fundamental aspect in the treatment of a diabetic pregnancy (Feig et al., 2006; Report., 1990), the complex metabolic changes in maternal nutrient metabolism ascribed to diabetes pose risks to brain development in the fetus (Rizzo et al., 1997). Compared with healthy infants, newborn IDM do not show evidence of discrimination, as detected by less sucking and heart rate changes in event-related potentials techniques (Deregnier et al., 2000; Georgieff, 2006). Similar observations exist in 8-month-old infants, and at 12 months developmental delay is observed using the Bayley Scales of Infant Development (Nelson et al., 2003). These studies suggest abnormal hippocampal processing of recognition memory that persists from birth to 1 year of age with implications for longer-term consequences to learning and neurobehavior.

Normal pre- and post-natal development of the brain is dependent on maternally derived long-chain polyunsaturated fatty acids (LC-PUFA) (Saste et al., 1998). Newborn infants of all types of diabetic mothers have lower concentrations of the key LC-PUFA, arachidonic acid (AA, 20:4 n-6) and docosahexaenoic acid (DHA, 22:6 n-3) in plasma and tissues (Ghebremeskel et al., 2004; Min et al., 2005; Min et al., 2006; Thomas et al., 2005; Wijendran et al., 2000). This is likely a reflection of lower maternal status combined with placental sequestration, and increased fetal utilization (Bitsanis et al., 2006; Ortega-Senovilla et al., 2009). Postnatal supply may also be a limiting factor in development since milk AA and DHA are lower in women with diabetes (Jackson

et al., 1994; Thomas et al., 2000). The low availability of LC-PUFA through gestation and postnatally might be implicated in impaired neurodevelopment since lower fetal status of AA and DHA is associated with a less favorable neonatal neurological condition (Dijck-Brouwer et al., 2005) and lower postnatal supply of AA and DHA limits developmental outcomes (Carlson, 2009; Ward et al., 1998).

Even though both AA and DHA are important to neurodevelopment (Saste et al., 1998), brain accretion of AA exceeds that of DHA during gestation (Hadders-Algra, 2008), suggesting that potential fatty acid interventions should be studied separately and possibly at different stages of development. Furthermore, dietary AA supplementation in the dam enhances hippocampal neurogenesis in 31 day old offspring by 32%, whereas no benefit is observed with combined AA plus DHA or DHA alone (Maekawa et al., 2009). Thus low AA status in infants following a pregnancy complicated with diabetes (Kuhn et al., 1990) might explain the adverse developmental outcomes related to hippocampal function (Deregnier et al., 2000; Georgieff, 2006; Nelson et al., 2003) and is postulated to have more pronounced effects on neurodevelopment early postnatally than the reduced DHA status. This theory is further supported by the observation that a deficiency of AA, but not DHA, is associated with hyperglycemia-induced teratogenesis in the offspring (Goldman et al., 1985). Feeding diets high in linoleic acid (LA, 18:2 n-6) to rats with diabetes increases serum levels of AA with a concomitant decline in the malformation rate in the offspring without altering glucose concentration (Reece et al., 1996). AA is greatly depressed in a

number of organs and serum of rats with diabetes (Brenner, 2003; Brenner et al., 2000; Clark et al., 1983; Dang et al., 1988; Faas et al., 1983; Gudbjarnason et al., 1987; Holman et al., 1983; Hu et al., 1994; Hurtado de Catalfo et al., 1998; Lin et al., 1985; Ramsammy et al., 1993). The decreased availability of AA is a possible teratogen in diabetic pregnancy (Eriksson et al., 2003), but has not been tested for its ability to normalize neurodevelopment when present in adequate amounts. Thus this study was conducted in pups of healthy and diabetic rat dams to determine if maternal AA supplement one week prior to mating through lactation would improve neurodevelopment in the offspring.

3.3. MATERIALS AND METHODS

3.3.1. Dam streptozotocin (STZ) treatment and dietary AA supplementation

This study was approved by the University of Manitoba Protocol Management and Review Committee in accordance with the guidelines of the Canadian Council for Animal Care. Nine-week-old female Sprague Dawley rats (University of Manitoba breeding colony) were randomized to 6 groups using a 3 × 2 factorial design (n=5/group) and permitted 1 week adaptation and all fed the Control diet. Diabetes was then induced on experimental day 0 using streptozotocin (STZ: 60 mg/kg, dissolved in saline at 15 mg/ml, Sigma-Aldrich, St Louis, MO, USA) i.p. once or twice in order to establish diabetes. Controls received an equivalent amount of saline i.p. denoted as Saline-Placebo. Diabetes was confirmed by blood glucose >13 mmol/L using a glucometer (One Touch® Ultra®) and blood sampling from saphenous vein 5 days later at 16:00 h. One week after STZ injection (experimental day 7), all diabetic rats received dosages

of insulin s.c. (glargine Lantus®, 0.5-10 unit/day) right after daily glucose checking to maintain glucose at designated ranges thereafter to day 28 postpartum. Half of the diabetic rats had glucose controlled to <13 mmol/L as Good Control (STZ/GC group,), and the other half as Poor Control (STZ/PC group) with glucose 13-20 mmol/L. These targets are higher compared to the human scenario as the Canadian Diabetes Association recommends glycemic targets for pregnant women at <5.3 mmol/L for fasting and <7.8 mmol/L 2-h postprandial (2006). This higher level was judged appropriate for 3 reasons: 1) rat gestation is only 21-22 days compared with 40 weeks in humans; 2) many diabetic women do experience high glucose prior to diagnosis so our model reflects undiagnosed diabetes, and 3) daily blood sampling was conducted in the non-fast state 16:00 h. Limiting the STZ/PC to <20 mmol/L was required because in our experience cataracts readily develop over 10 weeks when glucose exceeds 20 mmol/L.

The AA dietary intervention also started at experimental day 7 as half of the dams received a control diet which was modified from the AIN-93G (Reeves, 1997) with cornstarch as the sole source of carbohydrate; and the other half were fed the AA diet, which was the same diet but with 0.5 g/100g of fat as AA in place of soybean oil. The AA was derived from common soil fungi and provided in the form of RBD-ARASCO® (40.6 g/100 g of fatty acids as AA). The major diet ingredients are listed in **Table 3.1**. The level of dietary supplementation of AA in the dams diet (0.5% of fat) was based on Otto et al. (Otto et al., 2000). This amount of AA supplementation is also safe in rat dams and their offspring as

a 13-week toxicity study (Lina et al., 2006) demonstrated that supplementing AA (0.5% of fat) did not affect health, growth, fertility of the parental rats, nor pup characteristics (weight gain, viability, number per litter or gender ratio).

After 1 week adaptation to insulin treatment and diet (experimental day 14), dams were mated and continued on their respective diets and treatments to postpartum day 29. On postnatal day 3, the pups were weighed and randomized within litters to end-points (to be terminated at days 3, 14, 29 or 12 weeks of age), and the litters were culled to ≤8 pups, keeping equal numbers of each gender when possible. At day 29 half of the pups were euthanized with dams and are reported upon herein. The other half was transferred to a regular chow diet until 12 weeks of age for a long-term assessment to be reported upon separately.

Thus in this study, the 6 groups were: S+C (Saline-Placebo + Control diet); S+A (Saline-Placebo+ AA diet); STZ/GC+C (STZ/GC + Control diet); STZ/GC+A (STZ/GC + AA diet); STZ/PC+C (STZ/PC+ Control diet); STZ/PC+A (STZ/PC+ AA diet) (**Table 3.2**). A schematic of the study time-line is shown in **Figure 3.1**.

Throughout the acclimatization and experimental period, dams were housed individually and the pups were reared by their natural dams in plastic cages with wood shavings. Rats were housed with controlled temperature (21 °–23 ℃), humidity (55%), and 12-h light cycle and fed *ad libitum* with food intake monitored at each feeding. Pups were weighed using a digital scale (Mettler Toledo; SB32000, Columbus, OH, USA) at each test time-point (days 3, 5, 7, 9,

14, 18, 24 and 28) and dams were weighed daily while pregnant to enable insulin treatments and monitoring.

3.3.2. Offspring sensorimotor development testing

All sensorimotor tests used have been previously described and validated in rats (Altman et al., 1975), and are known to be reliable and indicative of the normal maturation of the central nervous system. All testing was conducted in a blinded manner and each of these tests was completed in duplicate and averaged. For the surface righting response (SR) test, pups were placed gently onto their backs on a cotton sheet; the time to return to prone position was recorded on days 3 and 5 whereby shorter time relates to normal development. The duration of the test was limited to 1 min, thus if a pup had not righted by 1 min, the time was recorded as 1 min. For the negative geotaxis (NG) test, each pup was placed head downward on an inclined plane with 25 °slope with the expectation that normal pups reorient face upward. The time to orient upward was measured for each pup on days 7 and 9, ages when the head starts raising from the ground in its typical sniffing response (Altman et al., 1975). The maximum allotted time was 1 min and recorded as such if the pup failed the test. At day 14, the wire hanging (WH) test was used to assess forelimb grip strength and climbing ability; rats were placed on a wire and allowed to grasp it with their forepaws. The wire was 1.5 mm in diameter, 70 cm long, strung between two poles at a height of 40 cm, with padding placed underneath. The time until falling was measured; the maximum allotted time was 2 min and recorded as such if the pup was able to sustain their grip. At days 18 and 24, the rota rod (RR) test was used to assess pup ambulatory

agility on a rotating cylinder (7 cm diameter; Economex, Columbus Instruments, Columbus, OH, USA). First, endurance at a constant rod rotating speed of 5 rpm was assessed for a maximum of 2 min. Second, the ability to stay on the rod at an accelerating speed was tested beginning at 2.5 rpm and increasing at a rate of 0.1 rpm every second for up to 2 min. The time was measured from the moment the rat was placed on the cylinder until it fell off. The Accelerated RR (RR-A) test requires a higher level of coordination than does the Standard RR (RR-S) test. Longer duration in this test reflects better agility and motor coordination. For both speeds, pups remaining on the rod were removed after 2 min and this time recorded.

3.3.3. Offspring learning and memory assessment - Morris water maze (MWM) test

Spatial recognition and memory were tested using a variation of the MWM test. Pups were placed in a pool of opaque water (90 × 90 cm, 25 cm deep, temperature 27 ℃) in which a 13 cm round platform was hidden in a standardized location 1 cm below the water surface. The room was dark except for one red light placed in one corner to provide direction cues. Rats were placed in the center of the pool and allowed to swim randomly until they found the platform. Those that failed the task after 1 min were placed on the platform for a 1 min rest period. Each pup was trained on day 26 and day 27, and had 4 trials per day. Each trial consisted of the pup being placed consecutively in 4 different orientations (facing N, S, E, W) in the water maze. Day 28 was the test date and the test results were expressed as mean of these 4 trials. Shorter time, or latency,

reflects better performance in the MWM. For the test day a maximum of 1 min was recorded if the pup had failed to locate the platform.

3.3.4. Tissue collection

On day 3 post-natally, pups were anaesthetized with isoflurane gas (^{Pr}AErrane, Baxter Corp. Mississauga, ON, Canada) and decapitated. Brain and liver were quickly removed without vascular perfusion and stored at -80^{o}C. Older pups at days 14 and 29 were anesthetized similarly and perfused transcardially with ice-cold 0.1 M PBS. Organs were collected and the brains were immediately sectioned into the anterior portion (frontal lobes) and posterior portion (cerebellum and brainstem) and flash frozen for storage at -80^{o}C. The middle portion (parietal cortex and hippocampus) was immersion fixed in 10% buffered formalin and the brain sections (6-μm thickness) were stained with solochrome cyanine for visualization of myelin and routine histological examination. Half of each forebrain and cerebellum was mixed for fatty acid analysis and the remaining forebrain was used for myelin basic protein (MBP). Dams were terminated at postpartum day 29 using similar methods.

3.3.5. Brain myelin basic protein analysis

Myelin is the lipid-enriched axon-ensheathing membrane and is essential for saltatory conduction of action potentials in the central nervous system (CNS) (Jakovcevski et al., 2009). Myelin is produced by oligodendrocytes in the CNS and forebrain production begins post-natally at about 10-12 days; the maximal rate of accumulation of myelin occurs at about 20 days of age (Cohen et al., 1976). Oligodendrocytes are also the cell population in the central nervous

system with the most significant turnover (Dawson et al., 2003). The deposition of MBP is regarded as an accurate index of myelin synthesis (Cohen et al., 1976) and measuring MBP has been used as a marker of oligodendrocyte maturity in the developing rat (Roussel et al., 1981). Since myelin basic protein is a sensitive biochemical indicator of brain development and maturation in rats (Norton et al., 1973) and was thus measured as a possible confounder in the developmental testing. As described previously in detail (Khan et al., 2006), ELISA was used to quantify brain MBP at days 14 and 29. Briefly, ELISA plates were coated with rabbit anti-human MBP (Dako A0623, Carpinteria, CA, USA). Brain homogenates (7.5 and 17.5 μg protein/ml) or MBP standards (M1891, Sigma) were measured in duplicate. MBP was quantified at 405 nm after sequential application of mouse anti-human monoclonal MBP antibody (US Biological, M9758-01), alkaline phosphatase goat anti-mouse antibody (Cedarlane/Biocan, 115-055-146), and phosphatase substrate (Pierce, 37620).

3.3.6. Brain and liver fatty acid measurement

Brain and liver tissue fatty acids were measured to reflect body stores of AA and DHA in response to maternal treatments (Innis, 2005). Total lipids from tissues were extracted according to a method (Weiler et al., 2002) adapted from Folch et al. (Folch et al., 1957). Briefly, after adding an internal standard, heptadecaenoic acid (C17:0), brain and liver tissue were extracted in chloroform:methanol 2:1 containing 0.01% BHT. The brain and liver tissues were homogenized. Crude lipid extracts were transmethylated in 1.2 m of methanolic HCl (3 mol/L, Supelco) at 80 ℃ for 1 h. Fatty acids from 12 to 22

carbons were quantified (mg/g tissue) using gas chromatography (Varian Star 3400, Mississauga, Canada).

3.3.7. Statistical analysis

Data are presented as means with their standard errors of the mean and analyzed by factorial ANOVA using the general linear model procedure of SAS (version 9.1). Significant differences ($P<0.05$) among means of groups by maternal treatments (TRT) or any interaction effects (TRT × Diet) were assessed using Bonferroni post hoc analysis. A repeated measures design was not selected due to the changing sample size at each test.

3.4. RESULTS

3.4.1. Experimental system

Induction and control of diabetes was successful (**Table 3.2**) as indicated by the mean gestational glucose concentrations at targeted ranges and thus by design there was a significant difference between the glucose levels in the STZ/PC and STZ/GC groups ($P<0.0001$) as well as a significant difference in the mean insulin glargine dose used to achieve target glucose concentrations ($P=0.0061$). There was no significant difference in the mean gestational weight gain among groups regardless of maternal treatment and diet ($P>0.05$). No significant difference was observed among the mean number of pups per litter/group, or between the number of males and females in each group (all $P>0.05$). There were no differences in feed intake among study groups of dams during gestation or lactation. Regardless of diet group or treatment group, the

range of intakes from mating to delivery was 21 to 36 g/d and during lactation 39 to 58 g/d.

3.4.1.1. Pup body weight

As shown in **Table 3.2**, the diet by treatment interaction at day 3 revealed that pups from maternal Saline-Placebo and STZ/PC groups fed the Control diet were all heavier than those receiving STZ/GC fed the Control diet; additionally, AA diet did not significantly alter body weight in pups of dams from STZ/GC and STZ/PC compared with Control diet group. At day 14, there was a significant diet by treatment interaction. The pups from STZ/PC dams were the lightest. AA diet supplementation elevated the body weight of pups from Saline-Placebo dams, but not those from STZ/GC and STZ/PC dams.

On day 29, there was again a significant diet by treatment interaction, with the pups from Saline-Placebo plus Control diet dams being significantly heavier than the pups from the STZ/GC consuming Control diet dams and STZ/PC dams receiving either diet. Pups from the Saline-Placebo and STZ-GC groups on the AA diet group were intermediate.

*3.4.2. Pup neurodevelopment tests (**Table 3.3**)*

3.4.2.1. Surface righting response test

On day 3, there were no significant main effects of treatment or diet. At day 5 there was a main effect of diet with the pups from dams fed the Control diet requiring less time to return to prone positioning compared with pups from dams fed the AA diet (4.3 ± 0.7 vs. 7.6 ± 1.8 s).

3.4.2.2. Negative geotaxis test

There was a significant diet by treatment interaction on day 7. Pups from STZ/PC dams fed the Control diet performed the poorest of all groups. The AA diet significantly improved the scores of pups from STZ/PC dams; however, the AA diet elevated negative geotaxis test scores of pups from STZ/GC dams. Only a main treatment effect was observed on day 9, pups from STZ/PC dams performed significantly worse than pups from Saline-Placebo dams (23.9 ± 2.9 vs. 15.9 ± 1.7 s).

3.4.2.3. Wire hanging test

A main effect of diet indicated that pups from dams fed AA diet performed better on the wire hanging test as grip-time was significantly longer than pups from dams fed Control diet (13.9 vs. 17.4 s). No effects of treatment or interactions were observed.

3.4.2.4. Rota rod tests

Diet by treatment interactions were observed in the day 18 standard rota rod tests with AA diet significantly improving the performance of pups from STZ/PC dams compared with pups from STZ/PC dams fed Control diet. For the same test, AA diet reduced performance of pups from STZ/GC dams, although this was not sustained to day 24. There were no main effects of diet or treatment on day 24 standard rota rod test.

Main treatment effects were observed for the day 18 accelerated rota rod test, with pups from Saline-Placebo dams performing significantly better than all of the STZ groups regardless of maternal glucose control (5.3 ± 0.9 vs. 3.1 ± 0.3

s). Day 24 accelerated rota rod test showed a diet by treatment interaction with the AA diet significantly improving the performance of pups from Saline-Placebo dams compared with Saline-Placebo fed the Control diet. The AA diet had no effect on the STZ treatment groups.

*3.4.3. Learning and memory assessment (**Table 3.3** and **Figure. 3.2**)*

A main effect of dams' treatment (Saline vs. STZ) on pups was not observed for the MWM test, but an effect of diet was observed. Pups from dams fed Control diet had a significantly longer (5.0 ± 0.5 vs. 7.0 ± 0.7 s, 40% longer) swim time to find the platform compared with pups from dams fed AA diet. No interaction effects were observed.

3.4.4. Brain myelin basic protein

There was no main effect or interaction at day 14 for MBP, perhaps in part because the protein is present at fairly low concentrations at this age. There was only a main effect of treatment on pup brain MBP at day 29; pups from STZ/PC dams had significantly lower brain MBP than those of pups from Saline-Placebo and STZ/GC dams (0.91 ± 0.01 vs. 1.00 ± 0.002 vs. 0.99 ± 0.03 mg/g protein, P=0.0045 and P=0.0179). There was no difference between pups from Saline-Placebo dams and pups from STZ/GC dams, and there was no diet effect (data not shown). On routine histological examination of mid-cerebrum sections (including hippocampus), there were no obvious abnormalities as a consequence of diabetes or diet (data not shown).

*3.4.5. Pup brain and liver AA and DHA (**Table 3.4 and Table 3.5**)*

There were no effects of diet, treatment or interactions for brain AA and DHA among the 6 groups of pups regardless of age (days 3, 14 and 29). For day 3 liver AA, no main or interaction effects of the maternal diet or treatments were observed. However**,** a diet by treatment interaction for liver DHA on day 3 revealed that pups from maternal groups receiving STZ/GC plus the AA diet had significantly higher DHA than those pups of the dams receiving the Control diet. The AA diet did not improve DHA status of pups from Saline-Placebo or STZ/PC dams.

By days 14 and 29, supplementation of the dams' diet with AA lead to significantly higher AA in the offspring livers (day 14: 7.4 $\pm$0.5 vs. 6.6 $\pm$0.2; and day 29: 4.4 $\pm$0.5 vs. 3.8 $\pm$0.5 mg/g liver). On day 14 liver AA, there was a main treatment effect with pups from STZ/PC dam being significantly lower than that of pups from STZ/GC dams (6.5 $\pm$0.4 vs. 7.8 $\pm$0.4 mg/g liver); the Saline-Placebo group was intermediate. There was a diet by treatment interaction on day 14 liver DHA with pups from dam STZ/PC + Control diet having significantly lower DHA than pups from Saline-Placebo + Control diet and STZ/GC on either diet, but there were no differences between pups from Saline-Placebo plus control diet dam and STZ/PC plus AA diet dam. The STZ-GC groups were not different from Saline-Placebo groups regardless of diet.

3.5 DISCUSSION AND CONCLUSIONS

The most significant finding of this study is that dietary AA supplemented (0.5% fat) 1 week before mating through lactation in both healthy and poorly-

controlled diabetic dams improved sensorimotor and developmental performances in the offspring, especially in the MWM test. Without the supplement, the offspring of the STZ/PC dams had neurodevelopmental delay as indicated by the 57% reduction in performance compared to Saline-Placebo in the day 18 rota rod standard test. Furthermore, they took over twice the time to complete the negative geotaxis on day 7. The maternal dietary AA intervention also elevated AA status in all offspring, implying enhanced maternal transfer from conception to weaning despite our low amount of AA. Higher dietary supplementation at 4% of dietary fat given shorter-term during lactation elevates brain AA and confers benefit to hippocampal neurogenesis by 31 days postnatally (Maekawa et al., 2009), suggesting an explanatory mechanism for our observations.

In this study, although the dams underwent insulin treatment, the pups from poorly-controlled diabetic dams had significantly lower liver AA than pups from good-controlled diabetic dams at day 14 of age. Thus poor glucose control did in fact yield lower AA in the offspring in our model. This finding expanded our knowledge of the effect of experimental diabetes on AA metabolism as not only do diabetic-rats have greatly depressed AA status in a number of organs and serum (Brenner, 2003; Brenner et al., 2000; Clark et al., 1983; Dang et al., 1988; Faas et al., 1983; Gudbjarnason et al., 1987; Holman et al., 1983; Hu et al., 1994; Hurtado de Catalfo et al., 1998; Lin et al., 1985; Ramsammy et al., 1993), but such reductions in AA status are also observed in the offspring. This observation aligns with those of with a growing number of human studies reporting that maternal diabetes during pregnancy reduces newborn AA status (Ghebremeskel et

al., 2004; Min et al., 2005; Thomas et al., 2006). In these studies, in women with type 1 diabetes with nearly optimal glycemic control (A1C: 4-6%), the cord plasma AA was decreased. Thus both mother and infant under the diabetic scenario are at risk for lower AA status even with a high degree of blood glucose control.

Biosynthesis of AA from LA in mammals is mainly modulated by the Δ6 and Δ5 desaturases (Brenner, 2003). STZ-induced diabetes depresses Δ6 and Δ5 desaturases due to the relative lack of insulin which is the most potent activator of both enzymes (Ramsammy et al., 1993). The putative effects in the fatty acid composition of tissue lipids thus would be an increase of LA and a decrease of AA that is indeed observed in tissues and plasma in STZ-induced diabetic rats (Brenner, 2003; Brenner et al., 2000; Clark et al., 1983; Dang et al., 1988; Faas et al., 1983; Gudbjarnason et al., 1987; Holman et al., 1983; Hu et al., 1994; Hurtado de Catalfo et al., 1998; Lin et al., 1985; Ramsammy et al., 1993). Most rat models of diabetes are characterized by a deficiency of AA only (Brenner, 2003; Brenner et al., 2000; Clark et al., 1983; Faas et al., 1983; Gudbjarnason et al., 1987; Holman et al., 1983; Hu et al., 1994; Hurtado de Catalfo et al., 1998; Lin et al., 1985; Ramsammy et al., 1993) and not DHA possibly since a low dietary n-6:n-3 fatty acid ratio (~8:1) used in rodent diets supports DHA status well. In fact, significantly higher DHA has been found in tissues and plasma of diabetic rats (Brenner et al., 2000; Faas et al., 1983; Ghebremeskel et al., 2002; Hurtado de Catalfo et al., 1998; Kuwahara et al., 1997), despite reduced Δ6 and Δ5 desaturase activity (Brenner et al., 2000) suggesting another factor preserves DHA, at least in

the adult rat. In contrast to the experimental diabetes, a deficiency in both AA and DHA is common in newborn IDM (Ghebremeskel et al., 2004; Min et al., 2005; Thomas et al., 2005; Wijendran et al., 2000). This may be ascribed to an additional combined effect of a higher dietary n-6:n-3 fatty acid ratio of ~16:1 that would not favor endogenous synthesis of DHA (Reeves, 1997) and thereby limit supply to the placenta. Furthermore, placental transfer regardless of DHA supply is limited in diabetes (Bitsanis et al., 2006). The net maternal-offspring transfer of DHA appears limited in our study based on reductions in liver DHA, suggesting our model is suitable to study offspring development following diabetes in pregnancy. Further animal studies with combined AA (early gestation) and DHA (postnatal) intervention at separate times might thus be warranted.

A concern regarding supplementing AA alone is that it might affect the maternal and/or offspring DHA status (Weiler et al., 2002). Such concern is not supported however by this study since the dams receiving dietary AA supplementation during pregnancy improved liver DHA in day 3 pups of the STZ/GC group and day 14 pups of the STZ/PC group. Otherwise there was no significant difference in DHA status of liver and brain between AA supplemented and non-supplemented groups in pups and dams (data not shown). Thus, this study provides evidence that maternal AA supplementation alone at low dose is feasible and effective in promoting the offspring developmental outcomes dependent on brain function, at least in an experimental model. Since there were no main effects of the intervention on DHA status, but there were for AA, we

surmise the mechanism behind the response is mainly ascribed to a novel function of AA in neurodevelopment. Further work is required to elucidate mechanisms prior to translating such intervention to humans.

Arachidonic acid is a major component of cell membranes and is of specific importance to the brain and blood vessels and the fetus predominantly relies on mother for accretion of AA (Hadders-Algra et al., 2007). In the current study, alterations in AA status were observed in conjunction with compromised performance in neurodevelopmental tests such as day 7 negative geotaxis and day 18 rota rod. This is similar to humans whereby infants of mothers with diabetes prior to pregnancy have significantly lower scores on the Bayley Scales of Mental Development Index and Psychomotor Development Index than did infants of mothers in the nondiabetic group at 1 year of age (Levy-Shiff et al., 2002). Suboptimal performances on neurodevelopmental tests, and lower psychomotor performance score and IQ have been reported in other studies of infants (Rizzo et al., 1994) and children (Rizzo et al., 1997; Yamashita et al., 1996) of diabetic mothers. It is thus tenable to postulate that the imbalance between maternal supply and fetal demand for AA during the perinatal period may contribute to the cognitive impairment and/or behavioral disorders in children of diabetic mothers (Ghebremeskel et al., 2004).

This study also demonstrates the importance of maternal glucose control on the offspring's fatty acids, myelin status and their normal development. On day 14, pups from STZ/PC dams had significantly lower liver AA and DHA than pups from STZ/GC dams. Additionally, day 29 pups from STZ/PC dams had

significantly lower brain MBP content than those of pups from Saline-Placebo and STZ/GC dams, while there was no difference in brain MBP between pups from Saline-Placebo dams and from STZ/GC dams. Since oleic acid requires Δ9 desaturase activity that is reduced in diabetes (Brenner et al., 2000) and is used in synthesis of MBP we explored if oleic acid was different in the brain, but it was not altered by maternal diabetes (data not shown, mean of 0.006 mg oleic acid/g brain tissue per group). Similarly, other studies demonstrate that brain oleic acid remains optimal and constant whatever the amount of oleic acid in the diet (Bourre et al., 2003). Thus brain oleic acid does not explain the low MBP. Further investigation into maternal-fetal iron transport and tissue iron content of brain may elucidate the cause of low MBP since iron is a cofactor in the synthesis of MBP (Connor, 1994).

Myelin basic protein is a sensitive biochemical indicator of brain development and maturation in rats (Norton et al., 1973). In fact, Khan et al. (Khan et al., 2006) demonstrated impaired recognition memory on the MWM test at 20 days of age in pups with a 50% reduction in MBP due to hydrocephalus. However, the magnitude of reduction in MBP in the present study was only in the order of 10% and did not limit improvements in MWM performance in the STZ/PC offspring from the AA supplemented dams; thus it was not likely the limiting factor. Notably, our MBP measurements were at day 29 as opposed to day 20 when the maximum rate of myelination occurs (Norton et al., 1973). Whether limitations in recognition memory are observed earlier in the suckling period in offspring of diabetic dam remain to be established.

In conclusion, offspring of diabetic dams in good glycemic control experience some growth delay, but exhibit neurodevelopmental behavior similar to Saline-Placebo pups. Pups from dams with poor glycemic control exhibited alterations in neurodevelopmental behavior and benefited from the dietary AA the most. These findings are especially meaningful in light of the challenges in achieving optimal control of glucose. Further research needs to clarify if combined interventions of AA and DHA, as is more suitable for human intervention, will mitigate the adverse effects of diabetes in pregnancy on neurodevelopmental outcomes. Such inquiry is important in view of new evidence suggesting that combined supplementation of AA and DHA in the maternal diet may prohibit the beneficial effects of AA in hippocampal neurogenesis (Maekawa et al., 2009).

3.6. ACKNOWLEDGEMENTS

The authors thank Osaama H. Khan, Andrea MacIntosh and Sarah L. Finch for their help on animal work and testing; thank Denise Borowski for animal facility support, Lisa Maximiuk, Shirley C. Fitzpatrick-Wong, Terry Enno, and Dennis Labossiere for technical support.

Each author was involved in the study design, whether in relation to conception of the study or to method development, and was fully involved in study conduct (JZ), data analysis (JZ), and the final manuscript (all authors) and has no conflict of interest.

Table 3.1. Major diet composition

	Control diet	AA diet (0.5 g/100g fat as AA)
Cornstarch (g/kg diet)	629.486	629.486
Casein (≥85%) (g/kg diet)	200.0	200.0
Soybean oil (g/kg diet)	70.000	69.138
RBD-ARASCO® (40.6% AA) (g/kg diet)	-	0.862
Fatty acid composition (% of total FA)		
Σ SFA	10.30	10.21
Σ MUFA	16.36	16.22
18:2 n-6, LA	37.95	37.75
18:3 n-3, ALA	5.39	5.43
20:4 n-6, AA	0	0.39
Σ PUFA	43.34	43.57

SFA = saturated fatty acid, MUFA = monounsaturated fatty acid, LA = linoleic acid, ALA, α-linolenic acid.

All ingredients were purchased from Harlan Teklad (WI, USA) and conform to AIN-93G specifications for total energy, protein and micronutrients except for the AA which was provided in the form of RBD-ARASCO® (Martek Biosciences Corp., Columbia, MD, USA).

Table 3.2. Characteristics of dam pregnancy and pup body weight measured at d 3, d 14 and d 29 of life

Measurements	S+C	S+A	STZ/GC+C	STZ/GC+A	STZ/PC+C	STZ/PC+A	*P*- value		
							TRT	Diet	TRT × Diet
Glucose d5 post STZ injection (mmol/L)	6.0 ±0.3^{X}	5.8 ±0.3^{X}	22.8 ±2.7^{Y}	21.0 ±4.8^{Y}	30.7 ±1.6^{Z}	30.4 ±2.6^{Z}	<0.0001	0.7027	0.9283
Gestational glucose (mmol/L)	5.5 ±0.2^{X}	5.4 ±0.4^{X}	11.0 ±1.2^{Y}	8.9 ±2.6^{Y}	19.0 ±1.3^{Z}	20.1 ±1.3^{Z}	<0.0001	0.7470	0.4525
Gestational insulin glargine (unit/day)	Saline	Saline	2.6 ±0.8^{X}	2.2 ±1.9^{X}	5.2 ±1.3^{Y}	6.9 ±0.8^{Y}	0.0061	0.7187	0.3667
Gestational wt gain (g)	150.1 ±8.6	133.8 ±10.9	115.7 ±8.4	129.3 ±19.6	127.3 ±17.3	113.3 ±8.6	0.2188	0.6078	0.4699
No. of pups born/litter	14 ±1	13 ±2	13 ±2	13 ±2	12 ±2	13 ±1	0.7277	0.9164	0.9510
(range pups/litter)	(10-17)	(9-17)	(8-16)	(9-16)	(8-16)	(11-15)			
No. of pups studied at each time-point									
Day 3	63	45	61	38	59	47			
Day 5 to 14	47	33	49	29	45	39			
Day 18 to 28	32	25	36	21	32	29			
Pup body wt (g)									
Day 3	7.1 ±0.1^{a}	7.2 ±0.1^{a}	6.2 ±0.2^{b}	6.7 ±0.1^{ab}	7.2 ±0.1^{a}	6.7 ±0.3^{ab}	0.0001	0.4217	0.0051
Day 14	29.3 ±0.4^{a}	32.2 ±0.6^{b}	27.2 ±0.5^{ac}	27.2 ±0.7^{ac}	26.7 ±0.5^{c}	26.8 ±1.0^{ac}	<0.0001	0.0235	0.0499
Day 29	89.6 ±1.7^{a}	77.9 ±2.8^{ab}	77.4 ±1.9^{b}	79.7 ±3.2^{ab}	70.2 ±2.7^{b}	68.9 ±3.0^{b}	<0.0001	0.1428	0.0193

Differences among groups (P<0.05) are identified by different superscripts: capital superscripts ^{X}, ^{Y} and ^{Z} for main effect of treatment, and the low-case superscripts $^{a\text{-}c}$ for diet and treatment interaction. Sample size is varied over the course of study as a result of tissue sampling at d 3 and d 14.

Table 3.3. Neurodevelopmental testing in offspring of control and diabetic dams between d 3 and d 28 of life

Age at testing	S+C	S+A	STZ/GC+C	STZ/GC+A	STZ/PC+C	STZ/PC+A	*P*- value		
							TRT	Diet	TRT × Diet
				Surface righting response (Seconds)					
d 3	18.1 ±2.5	17.0 ±3.9	17.7 ±2.0	13.8 ±1.7	15.5 ±1.7	13.0 ±1.2	0.6825	0.1007	0.9526
d 5	4.1 ±0.7^{A}	5.7 ±1.8^{B}	3.2 ±0.6^{A}	7.4 ±1.8^{B}	5.9 ±0.9^{A}	9.6 ±1.7^{B}	0.0591	0.0086	0.5519
				Negative geotaxis (Seconds)					
d 7	27.1 ±4.1^{a}	17.0 ±2.3^{a}	25.4 ±4.1^{a}	40.1 ±4.6^{b}	61.2 ±4.6^{c}	42.2 ±3.7^{b}	<0.0001	0.2019	0.0004
d 9	18.4 ±2.1^{X}	13.3 ±1.3^{X}	16.6 ±2.3^{XY}	23.7 ±3.6^{XY}	24.8 ±3.4^{Y}	23.0 ±2.3^{Y}	0.0119	0.9430	0.0637
				Wire hanging (Seconds)					
d 14	14.4 ±1.0^{A}	18.5 ±1.6^{B}	13.3 ±1.0^{A}	15.7 ±1.2^{B}	14.1 ±1.1^{A}	17.9 ±1.7^{B}	0.1891	0.0003	0.7691
				Rota rod (Seconds)					
d 18 Standard	5.9 ±0.7^{a}	5.9 ±0.8^{a}	5.6 ±0.7^{a}	3.3 ±0.3^{bc}	2.5 ±0.1^{c}	4.2 ±0.6^{ab}	0.0006	0.6412	0.0016
d 18 Accelerated	4.7 ±0.6^{X}	5.8 ±1.1^{X}	3.5 ±0.2^{Y}	3.1 ±0.3^{Y}	3.0 ±0.2^{Y}	2.7 ±0.3^{Y}	<0.0001	0.8544	0.4330
d 24 Standard	40.0 ±7.3	48.1 ±8.8	37.3 ±4.6	29.5 ±7.0	36.0 ±6.2	35.4 ±7.0	0.3068	0.8969	0.6306
d 24 Accelerated	27.7 ±4.3^{ad}	43.2 ±7.3^{b}	29.8 ±3.3^{a}	21.4 ±2.4^{ad}	17.8 ±1.7^{cd}	19.9 ±2.3^{d}	<0.0001	0.1684	0.0042
				Morris water maze (Seconds)					
d 28	6.1 ±0.5^{A}	5.4 ±0.6^{B}	6.1 ±0.7^{A}	4.7 ±0.4^{B}	8.8 ±0.9^{A}	4.9 ±0.5^{B}	0.1210	0.0021	0.0744

Differences among groups (P<0.05) are identified by different superscripts: capital superscripts ^{A}, ^{B} and ^{C} for main effect of diet, the ^{X}, ^{Y} and ^{Z} for treatment effect, and the low-case superscripts $^{a\text{-}d}$ for diet and treatment interaction. See Table 2 for sample sizes.

Table 3.4. Pup brain and liver AA and DHA at d 3 and d 14 of life

Fatty acids (mg/g tissue)	S+C	S+A	STZ/GC+C	STZ/GC+A	STZ/PC+C	STZ/PC+A	*P*- value		
							TRT	Diet	TRT × Diet
				Day 3					
n	16	12	12	9	14	8			
Brain AA	0.9 ±0.1	0.9 ±0.2	0.8 ±0.1	0.8 ±0.2	0.8 ±0.2	0.8 ±0.1	0.7860	0.3954	0.2740
Brain DHA	0.8 ±0.0	0.8 ±0.0	0.7 ±0.0	0.7 ±0.0	0.7 ±0.0	0.7 ±0.0	0.5734	0.2589	0.1396
Liver AA	10.0 ±0.7	12.2 ±0.9	7.6 ±1.0	10.2 ±1.1	11.6 ±0.6	9.9 ±0.5	0.0851	0.1680	0.0529
Liver DHA	6.7 ±0.4^{a}	7.2 ±0.3^{a}	4.8 ±0.5^{b}	7.0 ±0.3^{a}	6.3 ±0.3^{a}	6.0 ±0.3^{ab}	0.0974	0.0370	0.0223
				Day 14					
n	15	8	13	8	13	10			
Brain AA	1.3 ±0.0	1.4 ±0.1	1.3 ±0.1	1.4 ±0.0	1.3 ±0.1	1.2 ±0.1	0.1820	0.7625	0.2586
Brain DHA	1.2 ±0.0	1.3 ±0.0	1.2 ±0.1	1.2 ±0.0	1.1 ±0.1	1.1 ±0.1	0.1504	0.9464	0.7335
Liver AA	6.7 ±0.2^{A,XY}	6.8 ±0.5^{B,XY}	7.0 ±0.3^{A,Y}	8.5 ±0.4^{B,Y}	6.1 ±0.2^{A,X}	6.8 ±0.5^{B,X}	0.0121	0.0130	0.3064
Liver DHA	4.0 ±0.1^{a}	3.6 ±0.3^{ab}	3.8 ±0.1^{a}	4.1 ±0.2^{a}	3.4 ±0.1^{b}	3.6 ±0.1^{ab}	0.0436	0.6770	0.0257

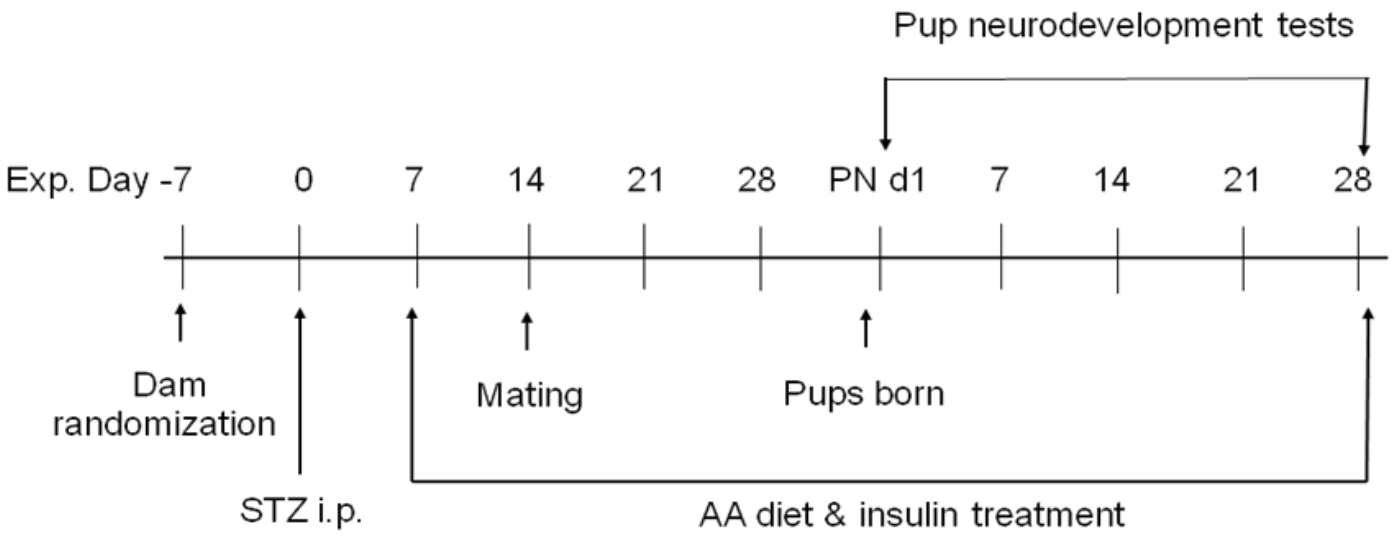
Differences among groups (P<0.05) are identified by different superscripts: capital superscripts ^{A}, ^{B} and ^{C} for main effect of diet, the ^{X}, ^{Y} and ^{Z} for treatment effect, and the low-case superscript $^{a\text{-}d}$ for diet and treatment interaction.

Table 3.5. Pup brain and liver AA and DHA at d 29 of life

Fatty acids (mg/g tissue)	S+C	S+A	STZ/GC+C	STZ/GC+A	STZ/PC+C	STZ/PC+A	*P*- value		
							TRT	Diet	TRT × Diet
				Day 29					
n	15	12	18	10	15	13			
Brain AA	1.7 ±0.0	1.7 ±0.0	1.5 ±0.1	1.6 ±0.1	1.7 ±0.0	1.6 ±0.0	0.1060	0.6510	0.2060
Brain DHA	2.2 ±0.0	2.1 ±0.0	1.9 ±0.1	2.1 ±0.0	2.1 ±0.0	2.1 ±0.0	0.5054	0.7994	0.0889
Liver AA	3.5 ±0.6^{A}	4.1 ±0.6^{B}	3.8 ±0.5^{A}	4.8 ±0.5^{B}	4.0 ±0.5^{A}	4.2 ±0.5^{B}	0.1735	0.0035	0.2374
Liver DHA	1.4 ±0.0	1.8 ±0.0	1.5 ±0.0	1.8 ±0.0	1.5 ±0.0	1.4 ±0.0	0.4253	0.0529	0.0954

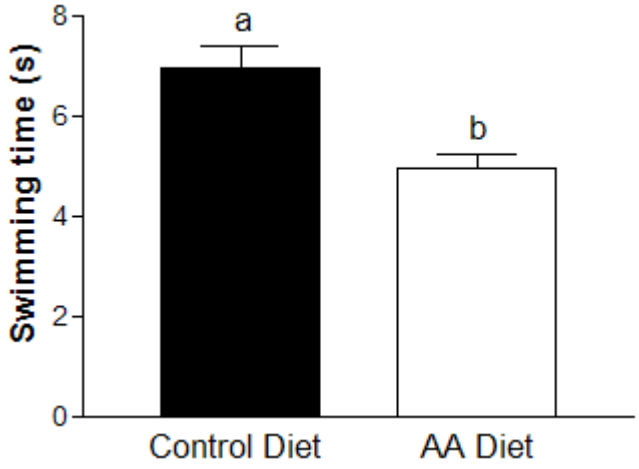
Differences among groups (P<0.05) are identified by different superscripts: capital superscripts ^{A}, ^{B} and ^{C} for main effect of diet.

Figure 3.1. Experimental time-course for each dam and litter



Exp., Experimental; STZ, streptozotocin; AA, arachidonic acid; PN d1, postnatal day1

Figure 3.2. Effect AA supplementation on Morris water maze test performance in the offspring at 28 days of age. Pooling of all offspring of Control-diet and all AA diet-fed dams was performed regardless maternal treatment. Pups from dams fed Control diet (n=100) needed longer (p=0.0021) time to find the platform than pups from dams fed AA diet (n=75). Data are mean ± SEM. Bars with different subscripts indicate significant differences (P<0.05).



BRIDGE 2

In Chapter 3, it was demonstrated for the first time that liver AA was lower in poorly-controlled offspring of diabetic dams than well-controlled offspring at day 14 of age. The performances in geotaxis and rota rod tests were inferior in the offspring of poorly-controlled dams, but this improved with maternal AA supplementation. Offspring of dams fed the AA supplement had higher liver AA than those of a Control diet group at day 14 and day 29 of age. The AA supplement also resulted in offspring with better performance in the water maze test than Control offspring. This finding expanded knowledge of the effect of experimental diabetes on AA metabolism as not only do diabetic rats have greatly depressed AA status (Holman et al., 1983; Hu et al., 1994), but such reductions in AA status are also observed in the offspring. These findings are significant in light of the challenges in achieving optimal control of glucose. Recently, the suboptimal neurodevelopment performances in the newborn IDM persist to 3 years age (Riggins et al., 2009) and are observed in school-age children (Ornoy, 2005). Infants with mildly abnormal movements at 12 weeks of age had a lower AA content at birth (Bouwstra et al., 2006) as well as at 3 months (van Goor et al., 2010). The presence of the mildly abnormal movements in infants is associated with an increased prevalence of minor neurologic dysfunction and attention deficit at school age (Groen et al., 2005). Taken together, these findings led to investigate if maternal AA supplementation improves neurodevelopment in adult offspring in the follow up study presented in Chapter 4.

CHAPTER 4. MANUSCRIPT 2.

MATERNAL ARACHIDONIC ACID SUPPLEMENTATION IMPROVES NEURODEVELOPMENT IN YOUNG ADULT OFFSPRING FROM RAT DAMS WITH AND WITHOUT DIABETES

Jinping Zhao[a], Marc R. Del Bigio[b], Hope A. Weiler[a]*

Author affiliation:

[a]School of Dietetics and Human Nutrition, McGill University,

Ste-Anne-de-Bellevue, Québec, Canada

[b]Department of Pathology, University of Manitoba,

Winnipeg, Manitoba, Canada

*Corresponding author:

Hope A. Weiler, RD, PhD

School of Dietetics and Human Nutrition, McGill University,

Quebec, Canada H9X 3V9

Telephone: (514) 398-7905, Fax: (514) 398-7739

e-mail: hope.weiler@mcgill.ca

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4.1. ABSTRACT

Maternal diabetes may compromise infant arachidonic acid (AA) status and development. This study tested if maternal AA supplementation improves neurodevelopment in adult offspring. Rat dams were randomized into 6 groups: Saline-Placebo, streptozotocin-induced diabetes with glucose controlled at <13 mmol/L, or poorly-controlled at 13-20 mmol/L using insulin; and fed either a Control or AA (0.5% fat) diet throughout reproduction. Weaned-offspring were fed regular chow to 12 weeks of age. Testing included exploratory behavior, rota rod and water maze (WM). Poorly-controlled offspring showed longer ($P \leq 0.018$) escape-latency on testing-day 1 WM but not thereafter ($P > 0.05$). Maternal glucose concentration positively correlated with ($P = 0.006$) male offspring testing-day 1 WM latency. The AA-diet offspring performed better in WM and rota rod ($P \leq 0.032$) and showed higher exploratory behavior ($P = 0.008$) than Control-diet offspring. These data suggest maternal hyperglycemia has longstanding consequences to initial stages of learning in the offspring. Maternal AA supplementation and training positively influence learning outcomes.

Keywords: Maternal arachidonic acid supplementation; Infants of diabetic mothers; Long-term neurodevelopment; Rat

4.2. INTRODUCTION

Follow-up studies concerning the adverse effects of diabetic pregnancy on the developing brain have revealed neurobehavioral deficits in both sensory-cognitive and psychomotor functions. These include altered auditory recognition memory processing at birth (Siddappa et al., 2004), reduced visual and memory performance at 8 and 12 months (DeBoer et al., 2005), poorer performance on tests of general development in infants and toddlers (Rizzo et al., 1991), and inferior performance in elementary school children (Ornoy, 2005). While motor delay may be a sign of mild, nonspecific brain damage, the abnormalities in memory processing suggest alterations in hippocampal development and function (Nelson et al., 2000).

Normal peri-natal brain development is dependent on maternally derived long-chain polyunsaturated fatty acids (LC-PUFA) (Lauritzen et al., 2001). Newborn IDM have lower arachidonic acid (AA: 20: 4 n-6) and docosahexaenoic acid (DHA: 22: 6 n-3) in cord blood (Ghebremeskel et al., 2004; Min et al., 2005; Wijendran et al., 2000). The low availability of LC-PUFA might be implicated in impaired neurodevelopment because lower fetal status of AA and DHA and lower milk LC-PUFA is associated with a less favorable neonatal neurological condition (Dijck-Brouwer et al., 2005).

Compared with normal controls, most rat models of diabetes are characterized by a lower AA only, and not DHA (Holman et al., 1983; Hu et al., 1994). Even though both AA and DHA are important to neurodevelopment, brain accretion of AA exceeds that of DHA during gestation (Hadders-Algra, 2008). AA metabolite, prostaglandin E_2 (PGE_2), plays an important role in neurogenesis

(Uchida et al., 2002). Dietary AA supplementation in the dam enhances hippocampal neurogenesis in 31-day-old offspring by 32%, whereas no benefit is observed with combined AA plus DHA or DHA alone (Maekawa et al., 2009). Thus, low AA status in the offspring following a pregnancy complicated by diabetes might explain the learning and memory deficits in the offspring (Kinney et al., 2003). A rodent study thus has been carried out in our laboratory to investigate the effect of maternal AA supplementation on offspring neurodevelopment. In our short-term report (up to post-natal day 29), the liver AA was lower in poorly-controlled (dam glucose 13-20 mmol/L) offspring than well-controlled (dam glucose <13 mmol/L) offspring at day 14 of age (Zhao et al., 2009). The performances in geotaxis and rota rod tests were inferior in the offspring of poorly-controlled dams, but this improved with maternal AA supplementation. Offspring of dams fed the AA supplement had higher liver AA than those of a Control diet group at day 14 and day 29 of age. The AA supplement also resulted in offspring with better performance in the water maze (WM) test than Control offspring (Zhao et al., 2009).

Peri-natal periods represent critical intervals when inadequate accumulation of DHA may be particularly detrimental. Abnormal neurobehavior has been seen in adult rats raised on a DHA restricted diet (Enslen et al., 1991). This is conceivable as LC-PUFAs are not only important structural elements of membranes, but, together with their eicosanoid products, they are also implicated in gene expression (Muskiet et al., 2006). Similarly, in infants of diabetic mothers, the suboptimal hippocampus-related memory performances persist from birth to 3 years of age (Riggins et al., 2009), while the abnormalities of

psychomotor function has been seen in school-age children born to diabetic mothers (Ornoy, 2005). To date no study has explored the possible role of AA in the long-term effect of diabetic pregnancy on the offspring neurodevelopment. We hypothesize that the neurobehavioral deficit in the offspring of diabetic dams will persist to young adult age. We also hypothesize that maternal AA supplementation will have a long-lasting beneficial effect on offspring neurodevelopment.

4.3. MATERIALS AND METHODS

4.3.1. Dam streptozotocin (STZ) treatment and dietary AA supplementation

The study design and maternal diet composition have been published in detail (Zhao et al., 2009). A schematic of the study time-line is shown in **Figure 4.1**. Briefly, nine-week-old female Sprague Dawley rats were randomized into 6 groups using a 3 × 2 design (n=5/group) and permitted 1 week adaptation (week -6) while fed the Control diet. Diabetes was induced on week -5 using STZ (Sigma-Aldrich, St Louis, MO; 60 mg/kg i.p.); controls received an equivalent amount of saline denoted as Saline-Placebo. Diabetes was confirmed by blood glucose >13 mmol/L using a glucometer (One Touch® Ultra®) and blood sampling from saphenous vein 5 days later at 16:00 h. One week after STZ injection (week -4), all diabetic rats received dosages of insulin (glargine Lantus®, 0.5-10 unit/d s.c.) until postpartum week 4. Half of the diabetic rats had glucose controlled at <13 mmol/L as Good Control (STZ/GC group), and the other half as Poor Control (STZ/PC group) with glucose 13-20 mmol/L.

The dietary AA intervention started on the same day as insulin treatment (week -4). Half of the dams continued on Control diet which was modified from

AIN-93G (Reeves, 1997) with cornstarch as the sole source of carbohydrate. The other half was fed the AA diet, which was the same diet but with 0.5 g/100 g of fat as AA (RBD-ARASCO: 40.6 %; Martek Biosciences Corp., Columbia, MD, USA). The energy provided by AIN-93G is 3.89 kcal per g diet and includes 0.637 kcal from fat. Thus, AA (0.5% of fat) contributes 0.082 kcal per 100 kcal of diet.

After 1 week adaptation to insulin treatment and diet (week -3), dams were mated and continued on their respective diets and treatments, and the pups were reared by their natural dams. On post-natal day 3, the litters were culled to ≤8 pups, keeping equal numbers of each sex when possible. At post-natal week 4, half of the pups were euthanized with dams for measurement of fatty acid status at weaning; the other half were transferred to a regular pelleted chow (LabDiet® 5012: 23% crude protein, 6% crude fiber, 4.5% fat as soybean oil) and housed in same-sex groups of up to 4 per cage until post-natal week 12 (12 weeks of age) for a long-term assessment. Thus based on dams' treatment and diet, the 6 groups of offspring were: S+C (Saline-Placebo + Control diet); S+A (Saline-Placebo + AA diet); STZ/GC+C (STZ/GC + Control diet); STZ/GC+A (STZ/GC + AA diet); STZ/PC+C (STZ/PC + Control diet); STZ/PC+A (STZ/PC + AA diet).

Throughout the entire study period, dams were housed individually or with pups. All rats were housed in standard box cages with controlled temperature (21-23 ℃), humidity (55%), reversed 12-h light cycle, and fed *ad libidum*. The maternal diet was prepared weekly and stored at -20 ℃ until fed. Rats were given fresh feed daily. Feed intake of dams (corrected for spillage) and weekly body weights of offspring were recorded. The institutional and national

guidelines for the care and use of animals were followed and that all experimental procedures involving animals were approved by the University of Manitoba Protocol Management and Review Committee.

4.3.2. Young adult offspring psychomotor activity assessment

The neurobehavioral tests used have been previously described and validated in rats (Altman et al., 1975), and all tests were conducted in a blinded manner and performed in the same order. On the test day rats were transported to the testing room in their home cages 1 hour prior to testing and weighed.

4.3.2.1. Exploratory behavior and locomotor activity

Exploratory behavior and basal locomotor activity in a novel environment was assessed as previously described (Enslen et al., 1991; Mills et al., 1988; Pierce et al., 2007). Rats were placed one at a time into a pre-cleaned test enclosure (43×43 cm^2 observation area; Opto-Varimex Minor, Columbus Instruments, Columbus, OH, USA) in which 15 photobeams positioned 2.5 cm apart from each other in each of 2 dimensions detect movements. The apparatus was located in a soundproof chamber illuminated with a dim red light. The rats were allowed to habituate for 10 min and then exploratory behavior and locomotor activity was measured by counting interruptions of infrared photobeams for 30 min and recorded as total, ambulatory and vertical counts. This exploratory behavior and locomotor activity was monitored at the same time of day for two consecutive days which started 1 day prior to 8 and 12 weeks of age; and the results of the two days were averaged and expressed as exploratory behavior and spontaneous activity of week 8 and 12.

4.3.2.2. Rota rod (RR) test

At 11 and 12 weeks of age, the rota rod test was conducted to assess rat ambulatory agility on a rotating cylinder (7-cm diameter; Economex, Columbus Instruments, Columbus, OH, USA). First, endurance on a rod rotating constantly at a speed of 5 rpm was assessed for a maximum of 3 min. Second, the ability to stay on the rod at an accelerating speed was tested beginning at 2.5 rpm and increasing at a rate of 0.1 rpm every second for up to 3 min. The time was measured from the moment the rat was placed on the cylinder until it fell off. If the rat did not fall from the rotating rod for more than 3 min, the test was stopped and time of 3 min recorded. The accelerating RR tests a higher level of coordination than does the constant RR test. Each of these tests was completed in duplicate and averaged. The rats in the present study also had the same testing conducted at 18 and 24 days of age (Zhao et al., 2009).

4.3.3. Young adult offspring learning and memory assessment – water maze test

A pool was filled with opacified water ($150 \times 150 cm^2$, 35 cm deep, temperature 25 ℃) in which a 13 cm in diameter round platform was hidden in a standardized location 1 cm below the water surface. The room was dark except for one red light placed in one corner to provide direction cues. Because there was nothing to directly show the location of the escape platform, rats had to memorize the platform location in relation to the light source. Therefore, this task is considered to assess spatial memory (Del Bigio et al., 2002; Morris et al., 1982). To acclimatize rats to the swimming task, they were allowed to swim in the water for 1 min on the day before starting the water maze test. Each rat was then tested on 3 consecutive days which started 2 days prior to 12 weeks of age.

On the 1st test day (testing-day 1), the rats were placed in the center of the pool and allowed to swim randomly until they found the platform. Those that failed the task after 1 min were placed on the platform for a 1 min rest period. There were 4 trials on each test day with a 20 min interval between each trial. Each trial consisted of 4 attempts to find the platform, each attempt beginning with the rat being placed in the center of the pool facing a different quadrant (N, S, E, and W, separated by a 30-second rest). The time of the 4 attempts was averaged for each trial. The result of each test day was expressed as the mean of these 4 trials. Shorter time, or latency, reflects better performance in the water maze. For the test day a maximum of 1 min was recorded if the rat had failed to locate the platform. The subject rats of the present report had water maze testing previously conducted at post-natal days 26, 27 and 28 in the short-term study (Zhao et al., 2009).

4.3.4. Tissue collection

At 12 weeks of age, rats were anaesthetized with isoflurane gas (PrAErrane, Baxter Corp. Mississauga, ON, Canada) and then exsanguinated via cardiac puncture. Blood was collected, stored on ice until centrifuged at 1500 rpm for 15 min at 4 ℃. Brain was removed and frozen in liquid nitrogen. Serum and brain were stored at -80 ℃ for further analysis.

4.3.5. Brain fatty acids analysis

To reflect fatty acids status in response to maternal treatments, brain AA and DHA were analyzed in the 12 week offspring using the method previously reported for 3, 14 and 29 day old offspring (Zhao et al., 2009). Briefly according to a method adapted from Folch et al (Folch et al., 1957), after adding the internal

standard heptadecaenoic acid (C17:0), lipid from homogenized brain was extracted in chloroform: methanol 2:1 containing 0.01% BHT. Crude lipid extracts were transmethylated in 1.2 ml of methanolic HCl (3N, Supelco) at 80 ℃ for 1 h. Fatty acids from 12 to 22 carbons were quantified (g/100g of fatty acids) using gas chromatography (Varian Star 3400, Mississauga, Canada).

4.3.6. Statistical Analysis

Data are presented as groups by maternal diets and treatments, offspring sex, and training effect where applicable (outcome measurements at 1st time point testing vs. 2nd time point testing). All data are expressed as mean ± SEM. All data were assessed for normality using the Kolmogorov-Smirnov goodness-of-fit test. The only data that failed normality was the rota rod dataset that was normalized using squaring prior to statistical analyses. Outliers, greater than 3 standard deviations from the mean, were removed. Main and interaction effects were identified using factorial (end-point, fatty acid results) and mixed (repeated measurements) model procedures of the Statistical Analysis Systems program (version 9.1 SAS Institute, Cary, NC, USA) as is suitable for the randomized 3 × 2 factorial design and recommended by the University of Manitoba Statistical Advisory Service. Significant differences ($P<0.05$) among means of groups by maternal treatments or any interaction effects were assessed using Bonferroni post-hoc testing.

Correlations between dam mean gestational glucose (mmol/L) and all long-term offspring behavior test outcome measurements were tested using Pearson's correlation coefficient *r* to quantify the strength of the relationship. Any correlations involving rota rod data were conducted using Spearman

correlation. Correlations between offspring tissue fatty acids (brain and liver AA and DHA), tested at newborn, weaning (Zhao et al., 2009) and young adult (present study), and all long-term behavior test outcome measurements were examined. Correlations in the test performances, water maze and rota rod, between weaning (Zhao et al., 2009; Zhao et al., 2010) and young adult were also examined. Values at 4 weeks of age were averaged per litter per sex to examine longitudinal relationships with same-sex litter mates. Values at 12 weeks were available for all offspring and thus individual values were used in correlation analyses.

4.4. RESULTS

4.4.1. Experimental system

There were no effects of maternal treatment or diet on offspring body weight at 4, 8 and 12 weeks of age. At weaning (4 weeks), the body weight of male rats ranged from 76.1 to 88.5 g; and female rats ranged from 69.0 to 81.7 g. At 12 weeks of age, body weight of male rats ranged from 457.0 to 500.0 g; and female rats ranged from 255.1 to 281.5 g.

4.4.2. Psychomotor activity assessment

*4.4.2.1. Exploratory behavior and locomotor activity (**Table 4.1**)*

There were similar trends in the results between 8 and 12 weeks of age in the effects of maternal treatment, diet and offspring sex on the exploratory behavior and locomotor activity. There were also similar trends in the changes among total, ambulatory and vertical movements (data not shown for ambulatory and vertical movements). Maternal treatment effect indicated that STZ/GC offspring had significantly less movements in comparison to STZ/PC (P=0.007)

offspring and Saline-Placebo (P=0.001) offspring in both 8 and 12 weeks of age. There was no difference between STZ/PC offspring and Saline-Placebo offspring in all measured movements (all P>0.05). The maternal diet effect indicated that AA-diet offspring had significantly more (P=0.008) movements in comparison to Control-diet offspring in both 8 and 12 weeks of age. No maternal treatment and diet effect interaction was observed. The offspring sex effect indicated that female rats had more (P=0.0009) movements in comparison to males. There was a training effect as significantly more (P=0.011) movements were observed at the 2nd test (12 weeks of age) than at the 1st test (8 weeks of age).

*4.4.2.2. Rota rod (RR) test (**Table 4.2**)*

There were similar trends in the results between 11 and 12 weeks of age in the effects of maternal treatment, diet and offspring sex on accelerating- and constant-speed rota rod testing. Maternal AA dietary supplementation significantly improved young adult offspring performance in rota rod tests of accelerating speed at 11 (175%, P=0.017) and 12 weeks (177%, P=0.003), and also constant speed at 12 weeks of age (167%, P=0.032) but not at 11 weeks (P=0.246) regardless of maternal treatment and offspring sex.

Training had significant effect on rota rod performance as rat performance at the 2nd test (12 weeks of age) was significantly better than the performance at the 1st test (11weeks of age) in both constant (P<0.0001) and accelerating speed (P=0.0002). Females performed significantly better than male offspring in 11 week constant (P<0.0001) and accelerating (P=0.0003) speeds, as well as 12 week constant (P<0.0001) and accelerating (P=0.003) speeds. No maternal treatment effect was observed.

There were correlations in the performance on rota rod tests between weaning (days 18 and 24) (Zhao et al., 2009) and young adult age (11 and 12 weeks). The performance of constant-speed testing on post-natal day 18 was positively correlated with performance at 11 (r=0.29, P=0.008) and 12 (r=0.31, P=0.004) weeks; those rats who stayed longer on the constant-speed rod at day 18 also stayed on longer at 11 and 12 weeks of age. The day 24 accelerating-speed rota rod performance was only correlated with 12 week performance (r=0.28, P=0.010).

*4.4.3. Water maze test (**Table 4.3**)*

There was a significant training effect as the latency to find the hidden-platform decreased across testing days and trials (all P<0.05). At testing-day 3, rats were much quicker in finding the platform in comparison to both testing-days 1 (P<0.0001) and 2 (P=0.006), indicating learning in all of the rats. The maternal treatment effect indicated that STZ/PC offspring (both males and females combined) required significantly more time in finding the platform at testing-day 1 than did Saline-Placebo offspring (P=0.007), while the STZ/GC offspring were intermediate; this treatment effect disappeared at testing-days 2 (P=0.211) and 3 (P=0.190). There were no differences between STZ/GC offspring and Saline-Placebo offspring (P=0.501) at testing-days 1, 2 and 3. For the maternal diet effect, the AA-diet offspring had a shorter (P=0.010) latency in finding the platform than the Control-diet offspring at all 3 testing-days. No interaction effects between maternal treatment and diet were observed. No effect of offspring sex was observed (P=0.885).

Maternal gestational glucose concentration was positively correlated with testing-day 1 latency of 12 week old male offspring (**Figure 4.2**). The young adult male offspring from dams with higher gestational glucose required longer time to find the hidden-platform in the testing-day1 water maze test. However, this relationship disappeared at testing-days 2 and 3, and did not exist in female offspring. There were no other correlations between maternal glucose and offspring long-term neurobehavioral outcome measurements (all $P>0.05$).

There were positive correlations in the water maze performance between weaning and young adult age. Rats that had longer latency at post-natal day 27 also needed longer time in finding the platform in the testing-day 1 of 12 week WM (**Figure 4.3**). In addition, performance at post-natal day 26 was positively correlated with the performance at the testing-day 3 at 12 weeks of age (**Figure 4.4**).

*4.4.4. Brain AA and DHA (**Table 4.4**)*

At 12 week of age, no maternal treatment and diet effect was observed on offspring brain AA and DHA; while male rats had significantly lower ($P<0.0001$) brain DHA in comparison to female rats.

Newborn (post-natal day 3) brain AA (g/100g fatty acids) was positively correlated with the vertical ambulatory movement in female offspring at 8 weeks ($r=0.49$, $P=0.021$), which indicated that there were more grooming, scratching activities in the female offspring at 8 weeks when they had higher brain AA at birth. No other correlation was observed between brain and liver AA and DHA at 12 weeks with outcomes of long-term behavior.

4.5. DISCUSSION AND CONCLUSIONS

A novel finding of this study is the positive correlation between dams' gestational glucose concentration and the initial water maze escape latency of young adult offspring. Adult (12 week) male and female offspring prenatally exposed to hyperglycemia at 13 to 20 mmol/L (STZ/PC) required more time to find the hidden-platform than did control offspring on the first day of testing, while the offspring exposed to hyperglycemia <13 mmol/L STZ/GC were intermediate. Escape latency in the water maze is generally considered to be a parameter reflecting the spatial learning and cognitive capacity (Morris et al., 1982). Poor performance during initial exposure to the problem suggests a deficient search or problem solving capacity. No differences were observed among the three treatment groups during subsequent trials on days 2 and 3. These data are consistent with previous reports that rats prenatally exposed to cocaine are capable of exhibiting a variety of learned behaviours (Heyser et al., 1995). This is also consistent with infant studies (Riggins et al., 2009) as impairments occurred at the initial stage of memory processing when cognitive demands were high; when cognitive demands were moderate, there were no observable differences in performance of controls and infants of diabetic mothers. The dysfunction appears to be modulated by the nature and extent of the demands placed on the circuitry of memory (de Haan et al., 2006). The hippocampus, which is central to recognition and recall memory function, undergoes a growth spurt with a significant amount of LC-PUFA deposition during late fetal life and is particularly vulnerable to metabolic abnormalities (Georgieff, 2008).

Another possible explanation for the difference in performance of water maze between maternal treatment groups may be through an influence on the motivational state (i.e., fear of drowning). Although this hypothesis cannot be ruled out, there is some evidence that would mitigate against this interpretation. As in the various measures of the exploratory behavior and locomotor tests of the present study, the STZ/PC offspring were more active than STZ/GC offspring and as active as Saline-Placebo offspring. In addition, litter mates the STZ/PC had lower brain DHA than STZ/GC and Saline-Placebo at 4 weeks (Zhao et al., 2009). This increased exploratory behavior and locomotor activity has been observed in rats with lower brain DHA (Levant et al., 2004; Moriguchi et al., 2000) and in offspring of diabetic rats (Johansson et al., 1991). One may also argue that the water maze latency may be due to the swimming speed due to impaired motor activity. However, in the present study there was no treatment effect on either constant- or accelerating-speed rota rod tests among offspring of the maternal treatment groups. Thus, the combination of results suggests that adult offspring of poorly-controlled diabetic dams may have mild learning and memory delay when presented with a difficult test, although this can be overcome through training.

The other key observations of this study were the long-lasting benefits of maternal AA supplementation on offspring neurodevelopmental test performance regardless of diabetes. In rota rod and water maze tests, the offspring of dams fed AA diet during reproduction performed consistently better. This is similar to previous studies where AA supplementation prevented the age-related AA decline (~20% decrease compared to the young control) in the hippocampus (McGahon et

al., 1997) and improved the performance in a water maze test in aged rats (Kotani et al., 2003). Mice raised on a very high DHA diet without AA during both prenatal and postnatal periods showed a significantly longer latency to escape on water maze cue trial at 11 weeks of age; this adverse effect is overcome by adding AA in the diet (Wainwright et al., 1997).

Diabetes in pregnancy creates a suboptimal AA supply for fetal development, along with other diabetes-related disturbances (Siddappa et al., 2004). Compared with normal controls, STZ-induced diabetic rats are characterized by a lower AA (Brenner, 2003; Faas et al., 1983; Holman et al., 1983; Hu et al., 1994) due to lack of insulin, which is the most potent activator of both Δ6 and Δ5 desaturases, the enzymes necessary for AA and DHA synthesis (Ramsammy et al., 1993). Like DHA, AA is vital to brain development, as the accretion of AA in the fetal brain exceeds that of DHA, especially during the first two trimesters of gestation (Clandinin et al., 1980; Percy et al., 1996), the period of rapid proliferation of neuronal and glial elements (Dobbing et al., 1973). The consequence of the disturbances in the supply of AA during the development is significant as major developmental changes of neurobiological processes occur during prenatal life (Hadders-Algra et al., 2007). Our short-term study has shown that maternal AA supplementation improves neurodevelopment of offspring (up to day 29) of diabetic dams in poor glycemic control (glucose > 13 mmol/L) (Zhao et al., 2009).

The present study extends our previous observation of the beneficial effect of maternal AA supplementation on offspring neurodevelopment from weaning (Zhao et al., 2009) to young adulthood, in the absence of dietary effects in brain

fatty acid composition. The early maternal treatment and diet effects on liver AA at day 14 (i.e. lower liver AA in STZ-PC offspring and higher liver AA with supplementation) were likely not enough to affect brain AA. Lefkowith et al (Lefkowith et al., 1985) suggest that the liver supplies AA to other tissues when the animal is deprived of essential fatty acids. This hypothesis would help explain the lower liver, but not brain AA at day 14 in STZ-PC offspring. The ability to synthesize AA is significantly increased with age (Makrides et al., 1994), thus there was no difference in AA status of 12 week rats among treatment and dietary groups. It is also possible that STZ-PC offspring may have had lower brain AA than STZ-GC offspring at day 14 of age in specific phospholipids or brain region, but we only measured the total fatty acids in a sub-group analysis.

Recent evidence indicates that AA status, peri-natal in particular, is related to short and possibly long-term neurologic outcome (Groen et al., 2005; Hadders-Algra, 2008; Hadders-Algra et al., 2007). Infants with mildly abnormal movements at 12 weeks of age had a lower AA content at birth in cord blood (Bouwstra et al., 2006) as well as at 3 months in erythrocyte membranes (van Goor et al., 2010). The occurrence of this abnormal neurologic outcome was related to maternal supplementation of DHA but was not seen when DHA was combined with AA during pregnancy and lactation (van Goor et al., 2010). The presence of the mildly abnormal movements in infants is associated with an increased prevalence of minor neurologic dysfunction and attention deficit at school age (Groen et al., 2005). This could thus imply that during neonatal life, a period of rapid proliferation of the glial populations and formation of neuronal synapses (Svennerholm et al., 1989), the supply of AA to the fetus is more critical

than the supply of DHA. Furthermore, the accretion rate of AA in the fetal brain is approximately twice as high as DHA (Clandinin et al., 1980; Clandinin et al., 1980; Percy et al., 1996). The finding that manipulation of fatty acids during early development alters behavior and learning in adulthood following cessation of supplementation in our study, in the absence of demonstrable membrane lipid changes, suggests that early brain growth and/or differentiation may be affected (Mills et al., 1988). Our longitudinal observations are consistent with the observation that administration of lipid-free diets to rats during pregnancy results in offspring possessing small ganglion cells with abnormal morphology in the cortex of the frontal lobe (Mills et al., 1988).

In this follow-up study, the positive correlations in the performances on the water maze and rota rod tests between weaning and young adult age are similar to the early origins of disease hypothesis (Godfrey et al., 2000). In the present study, those who had performance delays in the water maze performance at weaning (post-natal day 27) also were slower at the 1st day of testing at 12 weeks of age. But these disadvantages in the performances can be overcome by both training and maternal AA supplementation. This implies that children of diabetic mothers might require supplemental teaching.

More active and better performance was observed in female rats in the locomoter activity and rota rod test in the present study. Sex specific effects on central nervous system development have previously been observed (Jonasson, 2005; Kinney et al., 2003). There is controversy regarding the cause of these differences (Jonasson, 2005). It seems that the presence of female sex hormones during testing does not play a role in the behavior testing based on studies in

ovariectomized rats (Kinney et al., 2003). In the present study, the female rats had 5% higher brain DHA than male rats. Typically behavior deficits are associated with over a 50% reduction in brain DHA (Fedorova et al., 2009; Moriguchi et al., 2000). Therefore, this study suggests that smaller reductions in brain DHA may affect behavior. Further investigations are warranted to clarify the cause of these sex differences and to establish if they disappear with further maturation.

In conclusion, young adult offspring of poorly-controlled diabetic rat dams may have mild cognitive delay. A small amount of AA supplementation in the maternal diet during gestation and lactation improved the offspring behavior and learning. These results suggest that maternal glucose control and AA status have long-term consequences on neurodevelopment in the adult offspring. A study of AA supplementation in pregnant diabetic mothers may also be needed to address this matter.

4.6. ACKNOWLEDGEMENTS

The authors would like to acknowledge the assistance of Andrea Maclntosh, Osaama H. Khan, and Sarah L. Finch on psychomotor testing; Lisa Maximiuk, Dennis Labossiere and Shirley C. Fitzpatrick-Wong for technical support; Gerald Nolette and Denise Borowski for providing diligent animal care in University Manitoba Health Sciences Centre animal facility.

Each author was involved in the study design, whether in relation to conception of the study or to method development, and was fully involved in study conduct (JZ), data analysis (JZ), and the final manuscript (all authors).

4.7. FIGURE LEGENDS

Figure 4.1. Experimental time-course for each dam and litter

Figure 4.2. Correlation between dam gestational glucose concentration (mmol/L) and 12 week male offspring testing-day 1 water maze latency (seconds)

Figure 4.3. Correlation of water maze performance (seconds) between 12 weeks of age and days 26, 27 and 28

Figure 4.4. Correlation of water maze performance (seconds) between day 26 and 12 week day 3 testing

Table 4.1. Total exploratory behavior and locomotor activity testing (counts/ 30 min) at 8 and 12 weeks of age

Age (week)	Sex	Diet	Saline-Placebo	STZ/GC	STZ/PC	***Diet mean***	*P*-value			
							TRT	Diet	Sex	Training
8	M	Control	2507 ±167 (*n* 9)	2215 ±198 (*n* 12)	2849 ±374 (*n* 10)	***2514 ±155^{B}***	0.0007	0.008	0.0009	0.011
		AA	3039 ±346 (*n* 4)	3011 ±527 (*n* 5)	3125 ±522 (*n* 8)	***3073 ±300^{A}***				
		Treatment mean	***2640 ±160^{X}***	***2464 ±224^{Y}***	***2972 ±303^{X}***					
	F	Control	3734 ±473 (*n* 8)	2050 ±139 (*n* 7)	3410 ±466 (*n* 7)	***3095 ±272^{B}***				
		AA	3887 ±496 (*n* 6)	3065 ±436 (*n* 6)	3956 ±288 (*n* 8)	***3656 ±232^{A}***				
		Treatment mean	***3793 ±335^{X}***	***2518 ±251^{Y}***	***3701 ±266^{X}***					
12	M	Control	3025 ±271	2744 ±307	2904 ±399	***2891 ±326^{B}***				
		AA	3881 ±506	3014 ±346	3504 ±312	***3466 ±388^{A}***				
		Treatment mean	***3453 ±389^{X}***	***2879 ±327^{Y}***	***3204 ±356^{X}***					
	F	Control	3852 ±340	3090 ±571	4023 ±553	***3655 ±488^{B}***				
		AA	5298 ±728	2978 ±397	3943 ±613	***4073 ±579^{A}***				
		Treatment mean	***4575 ±534^{X}***	***3034 ±484^{Y}***	***3983 ±583^{X}***					

Values are means ± SEM (n). The sample sizes are the same among week 8, 11 and 12. Differences among groups (P<0.05) are identified by different superscripts: capital superscript $^{X>Y}$ for maternal treatment effect; $^{A>B}$ for maternal diet effect.

Table 4.2. Rota rod testing (seconds) at 11 and 12weeks of age

	Sex	Age (week)	Diet	Saline-Placebo	STZ/GC	STZ/PC	***Diet mean***	*P*-value TRT	Diet	Sex	Training
Constant-speed	M	11	Control	10.8±2.1	21.1±4.2	15.3±4.9	***16.1±2.4^{B}***	0.171	0.031	<0.0001	<0.0001
			AA	30.1±20.2	16.2±4.8	20.3±5.7	***20.9±4.6^{A}***				
		12	Control	20.6±9.0	37.7±12.8	27.9±5.6	***29.3±5.7^{B}***				
			AA	35.0±19.4	78.2±41.8	35.6±8.5	***48.8±14.1^{A}***				
	F	11	Control	97.7±31.2	60.8±18.6	46.4±17.8	***46.4±17.8^{B}***				
			AA	125.4±30.9	50.8±21.3	99.3±27.1	***90.8±16.0^{A}***				
		12	Control	115.2±28.6	98.6±29.8	50.4±22.2	***50.4±22.2^{B}***				
			AA	180.0±0.0	99.4±39.8	116.0±31.2	***127.6±18.2^{A}***				
Accelerating-speed	M	11	Control	7.2±2.4	10.2±3.7	15.0±5.7	***10.9±2.4^{B}***	0.309	0.001	0.0001	0.0002
			AA	14.8±10.4	17.7±4.3	27.3±5.4	***21.9±3.6^{A}***				
		12	Control	14.7±3.3	20.2±5.6	15.9±4.4	***17.1±2.7^{B}***				
			AA	32.9±0.5	32.8±11.6	28.9±4.6	***30.9±4.0^{A}***				
	F	11	Control	36.2±10.1	30.8±9.5	18.8±2.3	***18.8±2.3^{B}***				
			AA	60.7±12.1	30.8±13.8	39.4±9.0	***42.3±6.6^{A}***				
		12	Control	45.4±16.0	32.6±9.2	20.4±4.9	***20.4±4.9^{B}***				
			AA	69.9±10.6	42.8±17.7	48.8±10.8	***52.4±7.4^{A}***				

Values are means ± SEM, sample size as per table 1. Analysis on square-transformed data to achieve normal distribution. Differences among groups (P<0.05) are identified by different superscripts: $^{A>B}$ for maternal diet effect.

Table 4.3. Water maze (seconds) testing at 12 weeks of age

Testing-day	Diet	Saline-Placebo	STZ/GC	STZ/PC	***Diet mean***	*P*-value			
						TRT	Diet	Sex	Training
1	Control	8.2 ±0.5	8.0 ±1.1	12.4 ±1.3	$\boldsymbol{9.5 \pm 0.7^{A}}$	0.013	0.010	0.885	<0.0001
	AA	6.2 ±1.1	8.6 ±1.3	9.1 ±1.5	$\boldsymbol{8.3 \pm 0.8^{B}}$				
	Treatment mean	$\boldsymbol{7.6 \pm 0.5^{Y}}$	$\boldsymbol{8.2 \pm 0.8^{XY}}$	$\boldsymbol{10.8 \pm 1.0^{X}}$					
2	Control	8.2 ±0.8	8.3 ±0.8	9.4 ±1.2	$\boldsymbol{8.6 \pm 0.5^{A}}$				
	AA	5.7 ±1.5	6.2 ±0.7	8.0 ±0.8	$\boldsymbol{6.9 \pm 0.5^{B}}$				
	Treatment mean	$\boldsymbol{7.4 \pm 0.8^{Y}}$	$\boldsymbol{7.5 \pm 0.6^{Y}}$	$\boldsymbol{8.7 \pm 0.7^{XY}}$					
3	Control	6.8 ±0.6	6.5 ±0.6	7.4 ±0.7	$\boldsymbol{6.9 \pm 0.4^{A}}$				
	AA	4.4 ±0.2	6.4 ±0.6	6.2 ±0.6	$\boldsymbol{5.8 \pm 0.4^{B}}$				
	Treatment mean	$\boldsymbol{6.0 \pm 0.5^{Y\,\S}}$	$\boldsymbol{6.4 \pm 0.4^{Y\,\S}}$	$\boldsymbol{6.8 \pm 0.5^{Y\,\S}}$					

Values are means ± SEM, sample size as per table 1.

Differences among groups ($P<0.05$) are identified by different superscripts: capital superscript $^{X>Y}$ for maternal treatment effect; $^{A>B}$ for maternal diet effect. § indicated the training effect that the latency in testing-day 3 was significantly shorter than that in both testing-days 1 and 2.

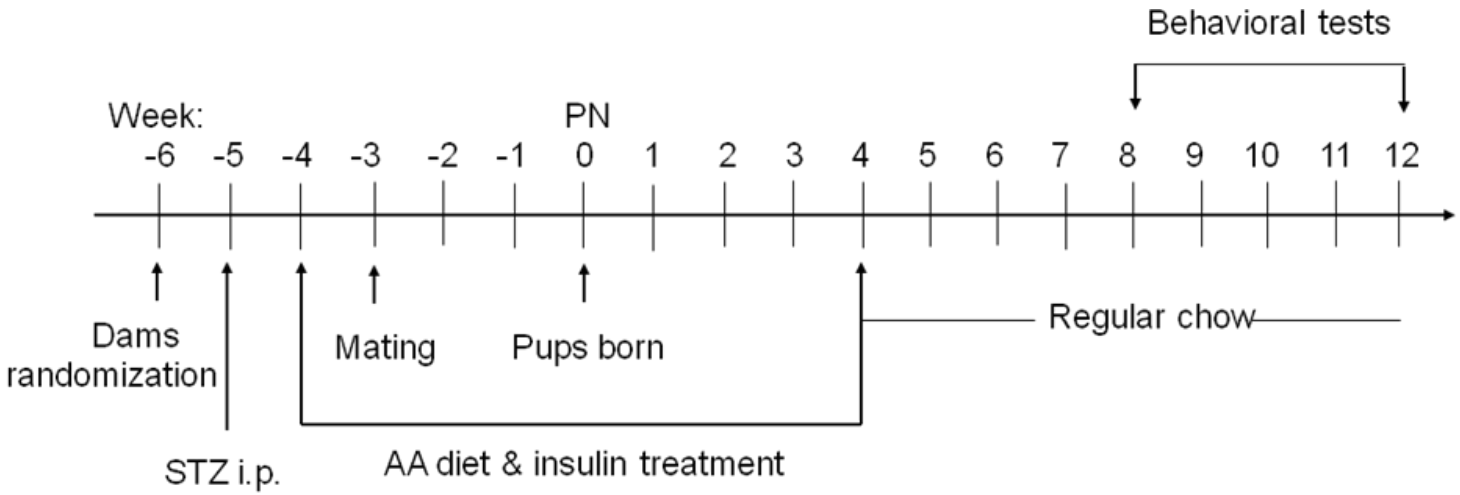
Table 4.4. Offspring brain AA and DHA (g/100g of fatty acids) measured at 12 weeks of age

	Sex	S+C	S+A	STZ/GC+C	STZ/GC+A	STZ/PC+C	STZ/PC+A	*P*-value TRT	Diet	Sex
AA	M	6.4 ± 0.1	6.5 ± 0.1	6.5 ± 0.1	6.5 ± 0.1	6.5 ± 0.04	6.6 ± 0.1	0.545	0.687	0.111
	F	6.5 ± 0.1	6.5 ± 0.1	6.3 ± 0.1	6.5 ± 0.1	6.3 ± 0.1	6.5 ± 0.1	0.575	0.127	
DHA	M	10.0 ± 0.2	9.7 ± 0.1	10.0 ± 0.1	10.0 ± 0.3	9.8 ± 0.1	10.1 ± 0.1	0.775	0.994	<0.0001
	F	10.6 ± 0.1	10.1 ± 0.1	10.6 ± 0.2	10.7 ± 0.1	10.2 ± 0.1	10.5 ± 0.1	0.105	0.981	

Values are mean ± SEM, sample size as per Table 1.

Differences among groups ($P<0.05$) are identified by different superscripts: capital superscript $^{X>Y}$ for maternal treatment effect.

Figure 4.1.



PN, postnatal; STZ, streptozotocin; AA, arachidonic acid

Figure 4.2.

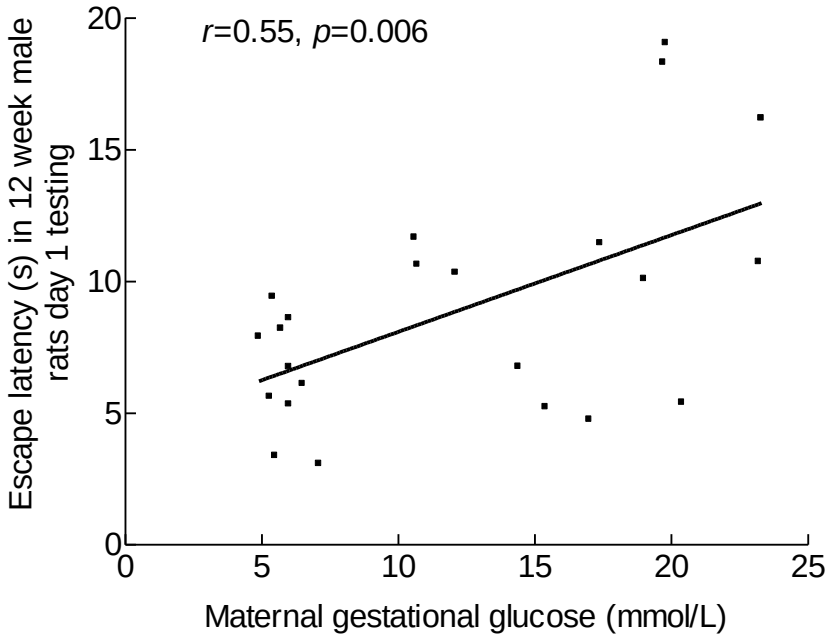

r=0.55, p=0.006
Escape latency (s) in 12 week male rats day 1 testing
Maternal gestational glucose (mmol/L)
0
5
10
15
20
25

Figure 4.3.

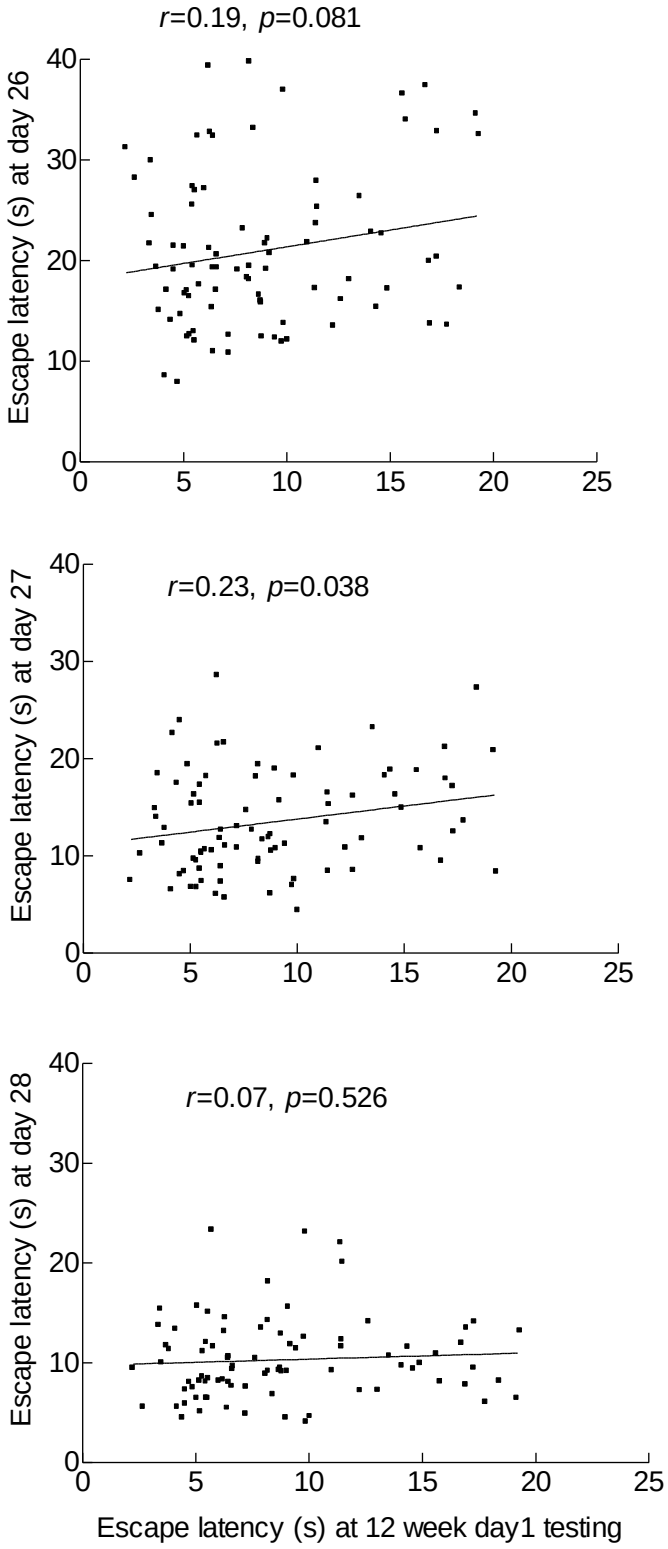
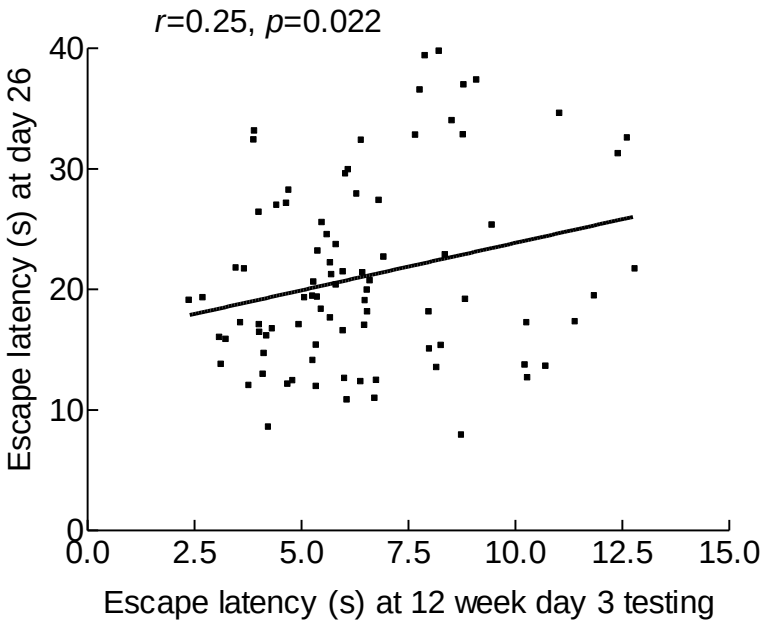

r=0.19, p=0.081
Escape latency (s) at day 26
r=0.23, p=0.038
Escape latency (s) at day 27
r=0.07, p=0.526
Escape latency (s) at day 28
Escape latency (s) at 12 week day1 testing

Figure 4.4.


r=0.25, p=0.022
Escape latency (s) at day 26
40
30
20
10
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
Escape latency (s) at 12 week day 3 testing

BRIDGE 3

The study in Chapter 4 suggested that maternal hyperglycemia has longstanding consequences to initial stages of learning in the offspring. Maternal AA supplementation and training positively influence learning outcomes. Follow-up studies also indicated higher incidence of IGT and type 2 DM in young adult offspring of diabetic mothers. In cell culture, AA is fundamental to functional integrity of the pancreatic β-cell (Dixon et al., 2004), while glucose-stimulated insulin release is dependent on plasma membrane release of AA (Konrad et al., 1994). New evidence suggests that there are linkages between glucose metabolism and bone metabolism (Confavreux et al., 2009). Newborn IDM also have compromised bone growth and mineralization (Mimouni et al., 1988), as well as reduced AA status (Ghebremeskel et al., 2004; Min et al., 2005), thus in Chapter 5 the effect of maternal diabetes and AA supplementation on offspring body composition including bone mass and glucose tolerance up to 12 weeks of age was sought.

CHAPTER 5. MANUSCRIPT 3.

LONG-TERM EFFECTS OF GESTATIONAL DIABETES ON OFFSPRING HEALTH ARE MORE PRONOUNCED IN SKELETAL GROWTH THAN BODY COMPOSITION AND GLUCOSE TOLERANCE

Jinping Zhao and Hope A. Weiler

Author affiliation:

School of Dietetics and Human Nutrition, McGill University,

Ste-Anne-de-Bellevue, Québec, Canada

*Corresponding author:

Hope A. Weiler, RD, PhD

School of Dietetics and Human Nutrition, McGill University,

Quebec, Canada H9X 3V9

Telephone: (514) 398-7905, Fax: (514) 398-7739

e-mail: hope.weiler@mcgill.ca

 Published in British Journal of Nutrition 2010;104:1641-1649.

5.1. ABSTRACT

Infants of diabetic mothers may have low arachidonic acid (AA) and develop obesity and insulin resistance in adulthood. This study tested the effect of maternal diabetes and AA supplementation on offspring body composition, bone mass and glucose tolerance from 4 to 12 weeks. Rat dams were randomized into 6 groups using a 3 × 2 design. Treatments were: Saline-Placebo, streptozotocin-induced diabetes (STZ) with glucose controlled at <13 mmol/L (STZ/GC), or poorly-controlled at 13-20 mmol/L (STZ/PC) using insulin; and fed either a Control or AA (0.5% of fat) diet throughout reproduction. Weaned offspring were fed regular chow. Measurements included offspring body composition, bone and oral glucose tolerance testing (OGTT) plus liver fatty acids of dam and offspring. Comparable to Saline-Placebo offspring, the STZ/GC offspring had greater (P<0.03) whole body and regional bone area than STZ/PC offspring. Maternal glucose negatively correlated (P<0.05) with offspring whole body bone area and mineral content at 4 weeks in all offspring and with tibia area in males at 12 weeks. Maternal liver DHA negatively (P<0.03) correlated with femur and tibia mineral content and tibia mineral density of female offspring at 12 weeks. Offspring from AA supplemented dams had higher (P=0.004) liver AA at 4 weeks. Liver AA at 4 weeks positively (P=0.05) correlated to lumbar spine mineral density in males. OGTT was not affected by maternal treatment or diet. These results suggest that maternal glucose control has long-term consequences to bone health of adult offspring. Skeletal growth appears more sensitive to maternal hyperglycemia than glucose tolerance.

Key words: Offspring of diabetic mother: Maternal arachidonic acid supplementation: Glucose tolerance test: Bone area

5.2. INTRODUCTION

The prevalence of diabetes mellitus (DM) in pregnancy is rising in part due to the sharp increase in obesity and type 2 DM (Bell et al., 2008). Fetal exposure to hyperglycemia during pregnancy can have profound and long-lasting consequences to the offspring (Freinkel, 1980). In the Northwestern University Diabetes in Pregnancy follow-up study, 40% of offspring of diabetic mothers by age 16 years had IGT defined as blood glucose 7.8 to 11.0 mmol/L 2 h after receiving a 75 g glucose load (Metzger, 2007). Furthermore, the occurrence of IGT was associated with higher insulin in amniotic fluid (Silverman et al., 1995). Based on the SEARCH Case-Control study, 30.4% of youth with type 2 DM were exposed to maternal diabetes *in utero* compared with 6.3% of nondiabetic control youth (Dabelea et al., 2008).

Whether altered maternal-fetal nutrient transfer or fetal metabolism explain the propensity for offspring of mothers with DM to develop obesity, IGT and DM is not clear. In cell culture, AA is fundamental to functional integrity of the pancreatic β-cell (Dixon et al., 2004), while glucose-stimulated insulin release is dependent on plasma membrane release of AA (Konrad et al., 1994). Furthermore, insulin sensitivity is positively associated with higher AA status in humans (Borkman et al., 1993). In rats, dietary AA supplementation lowers fasting blood glucose and insulin concentrations (Sasagawa et al., 2001), prevents high-fat diet induced insulin resistance (Wu et al., 2007) and enhances glucose disposal (Song et al., 2003). However, women with DM have lower AA in erythrocytes, particularly if obese (Min et al., 2004) and newborn infants of women with DM also have reduced AA in cord blood (Ghebremeskel et al., 2004;

Min et al., 2005; Min et al., 2006; Ortega-Senovilla et al., 2009; Thomas et al., 2005; Wijendran et al., 2000). Lower neonatal status is likely a reflection of lower maternal AA status, placental sequestration of AA, and increased fetal utilization (Ortega-Senovilla et al., 2009). Several studies have explored the possibility of maternal dietary intervention in modifying the susceptibility of offspring to adult diseases (Ibrahim et al., 2009; Korotkova et al., 2005; Siemelink et al., 2002). The differences in body weight and fasting insulin levels in adult rat offspring relate to the *n*-6 to *n*-3 fatty acid ratio in the maternal diet during the perinatal period (Korotkova et al., 2005). Similar effects have also been observed in diabetic pregnant rats and their adult offspring (Soulimane-Mokhtari et al., 2005). We thus hypothesized that maternal supplementation with AA during pregnancy and lactation would prevent subsequent development of IGT in the offspring at young adult age.

New evidence suggests that there are linkages between glucose metabolism and bone metabolism (Confavreux et al., 2009). Since newborn IDM have compromised bone growth and mineralization (Mimouni et al., 1988; Tsang et al., 1972; Verhaeghe et al., 1995), as well as reduced AA status (Ghebremeskel et al., 2004; Min et al., 2005; Min et al., 2006; Ortega-Senovilla et al., 2009; Thomas et al., 2005; Wijendran et al., 2000), we hypothesized that maternal AA supplementation may also be beneficial to offspring body composition and bone health. AA is a precursor to PGE_2 that has a biphasic effect on bone by enhancing bone formation at lower concentrations in animals, while stimulating resorption at higher concentrations (Watkins et al., 2001). Alteration in the precursor pool affects PGE_2 metabolism, while modification of dietary fatty acids leads to

parallel alterations in bone fatty acids (Weiler, 2000) and PGE_2 metabolism (Alam et al., 1993; Watkins et al., 1997). In a recent study, the serum AA was positively associated with whole body bone mass in 8-year-old children (Eriksson et al., 2009). Dietary γ-linoleic acid (GLA, a precursor to AA) supplementation enhanced bone mass in young male rats (Claassen et al., 1995) and was effective against diabetes-induced fetal bone development defects (Braddock et al., 2002). Similarly (Korotkova et al., 2005), variations in fatty acid composition in the maternal diet during the perinatal period caused changes in bone growth in adult rat offspring (Korotkova et al., 2005; Korotkova et al., 2004). Thus in this follow-up study, we sought to define the effect of maternal diabetes and AA supplementation on offspring body composition including bone mass and glucose tolerance up to 12 weeks of age.

5.3. MATERIALS AND METHODS

5.3.1. Dam STZ treatment and dietary AA supplementation

The study design, time-course and diet composition (**Table 5.1**) have been published in detail (Zhao et al., 2009). Briefly, nine-week-old female Sprague Dawley rats (University of Manitoba breeding colony) were randomized to 6 groups using a 3 × 2 design (n=5/group) and permitted 1 week adaptation while fed the Control diet. Diabetes was induced on week 1 using STZ (Sigma-Aldrich, St Louis, 60 mg/kg i.p.); control received an equivalent amount of saline denoted as Saline-Placebo. Diabetes was confirmed by blood glucose >13 mmol/L using a glucometer (One Touch® Ultra®) and blood sampling from the saphenous vein 5 d later at 16:00 h. Five dams with glucose <13 mmol/L were given a second dosage of STZ and glucose confirmed again 5 d later. One week after STZ injection

(week 2), all diabetic rats received dosages of insulin (glargine Lantus®, 0.5-10 unit/d s.c.) until postpartum week 4. Half of the diabetic rats had glucose controlled at <13 mmol/L as Good Control (STZ/GC), and the other half as Poor Control (STZ/PC) with glucose 13-20 mmol/L. Glucose prior to insulin management in the STZ/GC group ranged from 14.6 to 30.3 mmol/L, while in the STZ/PC group glucose ranged from 21.8 to 33.3 mmol/L.

The dietary AA intervention started on the same day (week 2) as insulin treatment. Half of the dams continued on Control diet which was modified from AIN-93G (Reeves, 1997) with cornstarch as the sole source of carbohydrate. The other half was fed the AA diet, which was the same diet but with 0.5 g/100g of fat as AA (RBD-ARASCO: 40.6 % AA; Martek Biosciences Corp., Columbia, MD).

After 1 week adaptation to insulin treatment and diet (week 3), dams were mated and continued on their respective diets and treatments, and the pups were reared by their natural dams until postnatal (PN) week 4. On PN d 3, the pups were weighed and randomized to end-points (cull or PN week 4 or 12) then litters were culled to ≤8 pups, keeping equal numbers of each sex when possible. At PN week 4, half of the pups were euthanized with dams for measurement of fatty acid status at weaning; the other half were transferred to a regular chow (LabDiet® 5012: 23% crude protein, 6% crude fiber, 4.5% fat) and housed in same-sex groups of up to 4 per cage until 12 weeks of age (PN week 12) for a long-term assessment. Thus based on dams' treatment and diet, the 6 groups of offspring were: S+C (Saline-Placebo + Control diet); S+A (Saline-Placebo+ AA diet); STZ/GC+C (STZ/GC + Control diet); STZ/GC+A (STZ/GC + AA diet); STZ/PC+C (STZ/PC+ Control diet); STZ/PC+A (STZ/PC+ AA diet).

Throughout the entire study period, dams were housed individually or with pups, and all rats were housed in standard hanging cages with controlled temperature (21-23 ℃), humidity (55%), and 12-h light cycle and fed *ad libidum.* Dam food intake was monitored daily. The institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the University of Manitoba Protocol Management and Review Committee.

5.3.2. Offspring body composition and bone mass

Weight was measured weekly for assessment of growth using a digital scale (Mettler Toledo; SB32000, Columbus, OH, USA). At 4, 8 and 12 weeks of age, rats were anaesthetized using isofluorane gas (^{Pr}AErrane, Baxter Corp. Mississauga, ON, Canada) for measurement of bone mass including whole body, lumbar spine, femur and tibia using a small animal program and dual-energy x-ray absorptiometry (DXA, QDR 11.2, 4500A series, Hologic). Whole body composition values were obtained for lean mass, fat mass and percent fat. Whole body length from tip of nose to base of tail was measured in the anesthetized state using a nonstretchable measuring tape. All scans were performed in singlet with the rat in the posterior position with limbs extended. The DXA measurements have been validated using Hologic hardware and software for rats 130 g in weight and higher for whole body assessments and in rodents as small as in mice (Wlodarski et al., 2002) for high-resolution regional scans.

5.3.3. Offspring oral glucose tolerance test (OGTT)

OGTT was conducted at 8 and 12 weeks of age according to published methods (Kawa et al., 2003) and prior to the DXA scan. Briefly, after 10-h without food, blood was collected via saphenous vein for the 0 time point. Immediately following, rats were fed an oral dose of 70% glucose solution (1 g of glucose/kg of body weight) from a syringe (Kawa et al., 2003). Blood was collected accordingly at 7.5, 15, 30 and 60 min after administration of glucose solution and the blood glucose was determined using a glucometer (One Touch® Ultra®). Area under the curve (AUC) was calculated from OGTT results using the trapezoidal method (Purves, 1992), which has been validated for glucose tolerance testing (Allison et al., 1995).

5.3.4. Tissue collection

At 12 weeks of age, rats were anaesthetized with isoflurane gas (^{Pr}AErrane, Baxter Corp. Mississauga, ON, Canada) and followed by exsanguination via cardiac puncture. Blood was collected, stored on ice until centrifuged at 1500 rpm for 15 min at 4 ℃. Liver was collected and flash frozen, serum and liver were stored at -80 ℃ until analysis.

5.3.5. Liver fatty acid analysis

To reflect fatty acid status in response to maternal treatments, liver fatty acids were analyzed in dams (postpartum week 4) and offspring (PN week 4 and 12). Total lipids from tissues were extracted according to the method from Folch et al (Folch et al., 1957). Briefly, after adding an internal standard (C17:0), liver lipid was extracted in chloroform-methanol (2:1, by vol.) containing BHT. Lipid extracts were transmethylated in methanolic HCl at 80 ℃ for 1 h. Fatty acids

from 12 to 22 carbons were quantified (mg/g tissue) using gas chromatography (Varian Star 3400, Mississauga, Canada). Only values for AA and DHA are reported in view of their importance in maternal-fetal transfer. Additionally, DHA has a stronger effect on bone mineral accretion than EPA in growing rats (Kruger et al., 2005).

5.3.6. Statistical analysis

Data are presented as groups by maternal treatment and diet, offspring sex and age; and expressed as mean $\pm$ SEM. Main and interaction effects were identified using a mixed (repeated measurements) model procedure of the Statistical Analysis Systems program (version 9.1 SAS Institute, Cary, NC, USA) as is suitable for the randomized 3×2 factorial design and recommended by the University of Manitoba Statistical Advisory Service. Significant differences ($P<0.05$) among means of groups by maternal treatments or any interaction effects were assessed using Bonferroni *post hoc* test.

The correlations between dam mean gestational glucose (mmol/L) and offspring OGTT, DXA scan outcome measurements were detected using Pearson's correlation coefficient *r* to quantify the strength of the relationship. Correlations between liver AA and DHA (mg/g tissue) from dams and offspring of all ages with offspring OGTT, DXA scan outcome measurements of all ages were examined. Values at 4 weeks of age were averaged per litter per sex to examine longitudinal relationships with same-sex liter mates. Values at 8 and 12 weeks were available for all offspring and thus individual values used in correlation analyses.

5.4. RESULTS

5.4.1. Experimental system

Induction and control of diabetes was successful as indicated by the mean gestational glucose concentrations at targeted ranges (**Table 5.2**). No significant difference was observed among the mean number of pups per litter/group ($P>0.05$). There were no differences in feed intake between different groups at any given days from mating to delivery (range: 21 to 36 g/d) or during lactation (39 to 58 g/d) regardless of treatment and diet.

5.4.2. Offspring body composition

Total weight gain (from d 3 to 12 weeks) was greater in the STZ/GC offspring compared with STZ/PC offspring regardless of maternal diet (587.1% *v.* 499.5%, $P=0.009$), while the Saline-Placebo offspring were intermediate (543.7%, $P=0.252$). Main effects of sex and age were detected in body weight, body length (data not shown), lean mass and fat mass (**Table 5.3**, all $P<0.0001$). The sex and age effects were manifested as values in male rats being higher than female rats and values at 4 and 8 weeks less than at 12 weeks. There were no main effects (all $P>0.05$) of maternal diabetes or AA diet on offspring body weight, body length, lean mass or fat mass.

5.4.3. Offspring bone area, bone mineral content and density

Maternal diabetes and gestational glucose control had significant effects on bone area as STZ/PC offspring had smaller bone area than offspring of Saline-Placebo and STZ/GC at 4 weeks in whole body ($P\leq0.044$) and at 12 weeks in tibia ($P\leq0.028$) (**Figure 5.1**). Femur area of STZ/PC offspring at 8 weeks was less ($P=0.024$) than that of STZ/GC offspring, while there was no difference

between offspring of STZ/PC and Saline-Placebo. There was no effect of maternal treatment on offspring bone area in lumbar spine (P>0.05, data not shown). There was no effect of maternal treatment on offspring bone mineral content (BMC) and bone mineral density (BMD) at any age (P>0.05). Main effects of offspring sex and age were observed for all bone area, BMC and BMD (data not shown) measurements with values increasing over time (P<0.05). Values for all treatment groups combined were higher for males than females at 12 weeks for whole body BMC (12.09 ± 0.15) vs. (8.35 ± 0.11) g, P<0.05) and whole body BMD (0.155 ± 0.003) vs. (0.149 ± 0.002) g/cm^2, P<0.05). There was no effect of maternal diet on offspring bone area, BMC and BMD.

5.4.4. Offspring oral glucose tolerance test

No main effects (P>0.05) of maternal treatment, diet or offspring sex were detected on the OGTT tested at 8 and 12 weeks of age. However, glucose AUC during OGTT (AUC Glucose$_{0\text{-}60}$) was greater at 8 weeks than at 12 weeks (443.7 ± 4.8) vs. (385.2 ± 4.5) mmol/L × min, P<0.0001) regardless of maternal treatment and diet.

5.4.5. Liver AA and DHA

Dams from STZ/GC plus Control diet had significantly lower liver AA than dams from Saline-Placebo plus AA diet and STZ/PC plus Control diet, while those receiving STZ/GC plus AA diet and Saline-Placebo plus Control diet were not different from any group (**Table 5.4**). The STZ/GC dams had lower (P=0.04) liver DHA than Saline-Placebo dams, whereas STZ/PC dams were intermediate.

At 4 weeks, the AA diet offspring had higher (P<0.004) liver AA than Control diet offspring. No difference was observed in offspring liver DHA regardless of dams' treatment and diet.

For 12 week rats, no difference was observed in offspring liver AA regardless of dams' treatment and diet. The STZ/GC offspring had higher (P=0.023) liver DHA than offspring of STZ/PC and Saline-placebo dams. The AA diet offspring had lower (P=0.030) liver DHA than Control diet offspring.

At 4 weeks, male rats had higher liver AA and DHA than female rats, while at 12 weeks, the female rats had higher liver DHA than male rats (all P<0.04).

5.4.6. Associations between dam treatment, diet and offspring outcome measurements

Glucose AUC of OGTT (AUC $Glucose_{0\text{-}60}$) in 8 and 12 weeks offspring did not correlated to any variables that examined including dam's mean gestational glucose, dam (postpartum week 4) and offspring liver AA and DHA (4 and 12 weeks) (data not shown).

Dams' gestational glucose concentration had a significant relationship to bone mass of their offspring at 4 weeks of age. Maternal glucose was negatively correlated with whole body (male: r -0.41; P=0.039; female: r -0.51; P=0.013) and lumbar spine (female: r -0.42; P=0.047) bone area (**Figure 5.2**), as well as whole body BMC (male: r -0.41; P=0.039; female: r -0.53; P=0.010). A negative correlation appeared at 12 weeks in male (r -0.43; P=0.038) rats between maternal glucose and tibia bone area (**Figure 5.2**). In addition, dams' gestational glucose concentration was negatively correlated with 4 week offspring whole body fat

(male: *r* -0.44; *P*=0.030; and female: *r* -0.49; *P*=0.018), and body weight in female rats (*r* -0.45; *P*=0.032) but not thereafter at 8 or 12 weeks.

Maternal liver AA and DHA did not correlated with any measure of bone in 4 weeks offspring. However, in female offspring maternal DHA was negatively correlated with femur (*r* -0.48; *P*=0.030) and tibia (*r* -0.47; *P*=0.038) BMC and tibia BMD (*r* -0.50; *P*=0.026) at 12 weeks. The only relationships observed between liver fatty acids and bone of the offspring were positive correlations in males between liver AA and lumbar spine BMD (*r* 0.30; *P*=0.05) and liver DHA with lumbar spine BMD (*r* 0.30; *P*=0.049) at 4 weeks of age. In male offspring at 4 weeks, liver AA and DHA were positively correlated (*r* 0.86; *P*<0.0001). No long-term associations between offspring liver AA were observed with bone area, BMC or BMD (data not shown).

5.5. DISCUSSION AND CONCLUSIONS

The most novel observation of this study is the long-term adverse relationship between maternal hyperglycemia and skeletal size of adult offspring. Specifically, offspring from poorly-controlled diabetic dams (STZ/PC) had significantly smaller bone area of whole body, femur and tibia than offspring from diabetic dams in good-control (STZ/GC). The maternal glucose concentration during pregnancy inversely related to offspring whole body BMC and bone area at 4 weeks of age and persisted to 12 weeks for tibia area in male rats. This is particularly important in view of the fact that peak bone mass is considered well established by 12 weeks of age in rats (Sengupta et al., 2005) and therefore suggests that skeletal size is programmed by fetal exposure to maternal hyperglycemia. Similarly, human infants born to mothers with DM have low

BMC at the distal radius(Mimouni et al., 1988) and osteoblast activity (Verhaeghe et al., 1995) and BMC inversely related to maternal glucose (Mimouni et al., 1988). Furthermore, altered calcium and magnesium homeostasis exist in newborn infants of mothers with DM (Mimouni et al., 1988; Tsang et al., 1972; Verhaeghe et al., 1995) that persists beyond infancy to at least 16 weeks in rats (Bond et al., 2005) and 10 years in children (Mughal et al., 2005). While it is postulated that this is a compensatory mechanism to support BMD, it appears that cortical density of femur becomes normal while reduced trabecular density persists to 16 weeks in rats (Bond et al., 2005; Bond et al., 2005). Correspondingly, at the completion of our 12-week study, BMC and BMD of the STZ offspring were not different from control offspring, although bone architecture was not examined. The current study adds important new information that maternal hyperglycemia has long-term consequences to bone size of the offspring, implying an altered growth trajectory.

The inverse association between maternal liver DHA with tibia mineral content and density in 12 week old female offspring is also consistent with the fetal origins of disease hypothesis (Sayer et al., 2005). Even though this hypothesis considers both fetal and neonatal factors, our data suggest the observations are more closely linked to fetal events since the 12 week measures of BMC and BMD did not relate to liver DHA in neonatal offspring. The mechanism(s) by which DHA exposure *in utero* might limit BMC and BMD later in life is elusive. Although various eicosanoids act on bone, the prostaglandins seem to be the primary mediators of bone cell function (Watkins et al., 2001). PGE_2 exhibits biphasic effects on bone, enhancing formation at moderate

concentrations but inhibiting formation at both high and low concentrations (Raisz et al., 1990). There is evidence in adult guinea pigs that prostaglandin synthesis is programmed by fetal exposure to long-chain PUFA (LC-PUFA) (Aprikian et al., 2007). DHA is known to inhibit osteoclastogenesis (Poulsen et al., 2008; Yuan et al., 2010) that is required for modeling of bone during growth (Narducci et al., 2011). Since bone area was not associated with DHA, it is likely that the lower BMC and BMD are linked to altered architecture. Indeed, feeding maternal diets (linseed oil) to elevate fetal exposure to DHA compared to a balance in n-3/n-6 fatty acids (soybean oil) results in lower femur length and lower cortical cross-sectional area and cortical BMC at the mid-diaphysis in 30 week-old female offspring (Korotkova et al., 2005; Korotkova et al., 2004). Even in humans, mothers with higher RBC DHA have neonates with lower spine and femur BMC (Weiler et al., 2005). These three studies imply that higher amounts of DHA in maternal tissue, through an unidentified mechanism in fetal development, programs the fetus for lower BMC and BMD that is apparent as early as infancy in humans (Weiler et al., 2005) and continues to adulthood as evidenced in rats (Korotkova et al., 2004).

In the present study, maternal diabetes and diet did not affect glucose tolerance in the offspring when measured at 8 and 12 weeks postnatally. To mimic the clinical management of diabetes in pregnant women (Walker, 2008), insulin was used to control glucose and the offspring were nursed by their natural dams. Thamotharan et al (Thamotharan et al., 2003) has reported glucose intolerance, as well as increased food intake and obesity in d 60 and 180 STZ-offspring who were fostered to normal dams, but not in those fostered to diabetic

dams. It is possible our proven method for OGTT (Kawa et al., 2003) was not sufficient (1 g glucose/kg body weight) to detect early signs of diabetes in comparison to other studies that used higher dosages and bypassed the gastrointestinal tract (2 g/kg i.p. or i.v.) (Han et al., 2007). While it is conceivable that the normal glucose tolerance observed in our study might have been accompanied by hyperinsulinemia, a subgroup analysis using 8 week data showed that insulin was 200.0 vs. 183.4 pmol/L at 30 min into the OGTT in Saline-Placebo and STZ/GC groups. Thus hyperinsulinemia was not a factor in our study. Future studies should confirm these observations using higher dosages or more sensitive tests such as the euglycaemic-hyperinsulinaemic-clamp that readily identified glucose intolerance in 12 week old STZ-offspring reared by the biological dams (Holemans et al., 1991).

In addition to the benefits of glucose control in the mother, many studies have postulated that dietary LC-PUFA will also confer long term benefits to the offspring. In rats inclusion of fish oil in the maternal diet during pregnancy offsets the adverse effects of a low protein diet (12%, w/w) that otherwise causes insulin resistance in the offspring at 11 month of age (Joshi et al., 2003). Feeding diabetic rat dams diets with 35% of fat as EPA and 6% as DHA limited the development of insulin resistance in 12 week old adult offspring compared to the control diet that had a n-6:n-3 ratio of 28:1 (Soulimane-Mokhtari et al., 2005). In contrast, in the OGTT test we did not observe any maternal diet effects. In addition to maternal insulin treatment, this could also be ascribed to the standard protein (20% in experimental diets and 23% in regular chow) in all groups and the lower *n*-6 to *n*-3 ratio of 7:1. In our study, the level of dietary supplementation of

AA in the dams diet (0.5% of fat) was based on Otto *et al.* (Otto et al., 2000). Our results suggest that such supplementation is safe in regard to long term growth, body composition and glucose tolerance.

In summary, this study suggests that maternal hyperglycemia in pregnancy has a long lasting adverse effect on skeletal growth in the offspring. Specifically, maternal diabetes was linked to lower tibia area. Regardless of maternal diabetes, higher maternal DHA indirectly related to lower tibia BMC and BMD in female offspring at adult ages suggesting programmed architecture of long bone. Whether the mechanism is ascribed to DHA or eicosanoid metabolism requires further study.

5.6. ACKNOWLEDGEMENTS

We would like to acknowledge the assistance of Andrea Maclntosh, Lisa Maximiuk and Sarah L. Finch on OGTT test, Gerald Nolette and Denise Borowski for providing diligent animal care and support in University Manitoba Health Sciences Centre animal facility.

Each author was involved in the study design, whether in relation to conception of the study or to method development, and was fully involved in study conduct (JZ), data analysis (JZ), and the final manuscript (all authors). All authors have no conflict of interest.

This study was supported by grants from the Canadian Institute Health Research and the Manitoba Institute of Child Health.

5.7. FIGURE LEGENDS

Figure 5.1 Bone area (cm^2) of whole body (4 weeks of age), femur (8 weeks of age) and tibia (12 weeks of age).

STZ/GC, STZ induced diabetes with glucose <13 mmol/L; STZ/PC, STZ induced diabetes with glucose 13-20 mmol/L. Values are means with their standard error means represented by vertical bars, and graph with unlike letters are significantly different ($P<0.05$). Sample size: at 4 weeks, Saline-Placebo $n=52$, STZ/GC $n=57$, STZ/PC $n=61$; at 8 and 12 weeks, Saline-Placebo $n=27$, STZ/GC $n=30$, STZ/PC $n=33$.

Figure 5.2. Correlations between mean gestational glucose concentration (mmol/L) of dams and offspring bone area (cm^2) of whole body and lumbar spine at 4 weeks and tibia at 12 weeks of age. Sample size: at 4 weeks, male=90, female=80; at 12 weeks male $n=48$, female $n=42$

Table 5.1. Major composition of the diets (g/kg)

Diet	Control	AA (0.5 g/ 100 g fat as AA)
Cornstarch	629.486	629.486
Casein (≥85%)	200.0	200.0
Soybean oil	70.000	69.138
RBD-ARASCO® (40.6% AA)	-	0.862
Fatty acid composition (% of total FA)		
Σ SFA	14.70	14.59
Σ MUFA	23.37	23.17
18:2 n-6, LA	52.21	51.93
18:3 n-3, ALA	7.70	7.76
20:4 n-6, AA	0	0.50
Σ PUFA	61.91	62.24

LA, linoleic acid; ALA, α-linolenic acid; AA, arachidonic acid.

All ingredients were purchased from Harlan Teklad (WI, USA) except for the AA which was provided in the form of RBD-ARASCO® (Martek Biosciences Corp., Columbia, MD, USA).

Table 5.2. Characteristics of dam and day 3 pup body weight, and number of rats at 4, 8 and 12 weeks

Measurements			S+C	S+A	STZ/GC+C	STZ/GC+A	STZ/PC+C	STZ/PC+A	*P*-value		
									TRT	Diet	TRT × Diet
d 5 post STZ injection glucose (mmol/L)			6.0 ±0.3^{Z}	5.8±0.3^{Z}	22.8±2.7^{Y}	21.0±4.8^{Y}	30.7±1.6^{X}	30.4±2.6^{X}	<0.0001	0.703	0.928
Gestational glucose (mmol/L)			5.5±0.2^{Z}	5.4±0.4^{Z}	11.0±1.2^{Y}	8.9±2.6^{Y}	19.0±1.3^{X}	20.1±1.3^{X}	<0.0001	0.747	0.453
Gestational insulin (unit/day)			Saline	Saline	2.6±0.8^{Y}	2.2±1.9^{Y}	5.2±1.3^{X}	6.9±0.8^{X}	0.006	0.719	0.367
Gestational weight gain (g)			150.1±8.6	133.8±10.9	115.7±8.4	129.3±19.6	127.3±17.3	113.3±8.6	0.219	0.608	0.470
N of pups born/litter			14±1	13±2	13±2	13±2	12±2	13±1	0.728	0.916	0.951
d 3 body weight (g)			7.1±0.1^{a}	7.2±0.1^{a}	6.2±0.2^{b}	6.7±0.1^{ab}	7.2±0.1^{a}	6.7±0.3^{ab}	0.0001	0.422	0.005
n of rats at 4, 8 & 12 weeks	Sex	Age (week)									
	M	4	17	8	23	9	19	14			
	F		15	12	13	12	13	15			
	M	8 & 12	9	4	12	5	10	8			
	F		8	6	7	6	7	8			

Values are means ± SEM. Mean values within a row with unlike superscript letters were significantly different (P<0 05): capital superscript $^{X}>^{Y}>^{Z}$ for main effect of maternal treatment when both diet groups are combined, the low-case superscript $^{a}>^{b}$ for diet and treatment interaction.

Table 5.3. Body composition (g) measured at 4, 8 and 12 weeks of age

	Sex	Age (week)	S+C	S+A	STZ/GC+C	STZ/GC+A	STZ/PC+C	STZ/PC+A	*P*-value			
									TRT	Diet	Sex	Age
Body wt	M	4	88.5 ± 5.0	85.0 ± 3.5	81.2 ± 2.3	82.7 ± 4.1	76.1 ± 3.5	77.7 ± 5.7	0.120	0.611	<0.0001	<0.0001
		8	335.6 ± 15.2	320.7 ± 9.5	320.9 ± 9.3	347.3 ± 15.7	329.4 ± 10.2	332.1 ± 12.8				
		12	500.0 ± 16.8	496.4 ± 18.7	468.9 ± 13.0	496.9 ± 23.4	477.8 ± 19.0	457.0 ± 14.8				
	F	4	81.7 ± 4.1	78.9 ± 3.1	73.8 ± 2.5	78.4 ± 3.2	69.0 ± 5.2	70.4 ± 3.5				
		8	204.8 ± 6.4	212.2 ± 3.0	212.3 ± 5.0	207.8 ± 7.3	187.2 ± 9.4	205.6 ± 8.3				
		12	272.4 ± 8.1	272.6 ± 8.7	277.2 ± 6.1	281.5 ± 17.8	260.2 ± 12.9	255.1 ± 8.3				
WB lean	M	4	68.3 ± 3.5	66.3 ± 3.2	64.3 ± 1.7	64.6 ± 3.4	60.3 ± 2.5	61.1 ± 4.8	0.210	0.953	<0.0001	<0.0001
		8	291.0 ± 12.0	279.2 ± 3.5	277.6 ± 7.2	294.7 ± 10.7	287.0 ± 8.0	284.8 ± 10.2				
		12	403.0 ± 11.7	409.8 ± 9.1	379.8 ± 10.2	384.0 ± 16.3	392.6 ± 14.0	368.8 ± 10.8				
	F	4	62.1 ± 2.9	61.5 ± 2.3	57.1 ± 2.0	62.7 ± 2.4	53.7 ± 3.6	54.7 ± 3.2				
		8	183.8 ± 5.1	187.1 ± 4.8	192.4 ± 3.7	189.0 ± 5.0	182.8 ± 7.1	179.3 ± 4.8				
		12	220.6 ± 3.5	229.8 ± 5.2	227.9 ± 5.0	215.4 ± 6.5	220.0 ± 9.3	211.2 ± 6.5				
WB fat	M	4	18.4 ± 1.5	16.6 ± 0.9	15.4 ± 0.8	16.3 ± 0.9	14.2 ± 1.0	14.9 ± 0.9	0.178	0.276	<0.0001	<0.0001
		8	37.5 ± 3.5	34.1 ± 6.3	36.2 ± 2.8	44.8 ± 6.5	34.9 ± 2.6	39.7 ± 3.2				
		12	84.8 ± 6.5	74.3 ± 9.4	77.2 ± 4.0	100.0 ± 22.5	73.1 ± 6.4	76.5 ± 6.0				
	F	4	17.4 ± 1.5	15.6 ± 1.0	14.8 ± 0.7	16.3 ± 1.1	13.7 ± 1.6	14.1 ± 0.5				
		8	25.4 ± 2.0	19.9 ± 1.7	20.4 ± 2.2	26.8 ± 4.0	21.0 ± 2.1	22.6 ± 1.4				
		12	48.5 ± 5.1	43.8 ± 5.4	42.1 ± 5.5	54.7 ± 10.7	44.1 ± 7.2	45.5 ± 3.4				

Values are means ± SEM. Sample size was different between 4 week and 8, 12 week as a result of tissue sampling at 4 week**.**

Table 5.4. Liver AA and DHA (mg/g tissue) measured at postpartum week 4 of dams and offspring at 4 and 12 weeks of age

	Sex	Age (week)	S+C	S+A	STZ/GC+C	STZ/GC+A	STZ/PC+C	STZ/PC+A	*P*-value			
									TRT	Diet	TRT × Diet	Sex
AA	Dam		5.6 ± 0.1^{ab}	7.0 ± 0.4^{a}	4.0 ± 0.2^{b}	6.2 ± 0.5^{ab}	6.4 ± 0.4^{a}	$5.8^{ab} \pm 0.3$	0.042	0.016	0.020	
	M	4	3.8 ± 0.3^{B}	4.6 ± 0.5^{A}	3.8 ± 0.3^{B}	5.5 ± 0.4^{A}	4.1 ± 0.2^{B}	4.6 ± 0.3^{A}	0.174	0.004	0.237	0.015
	F		3.3 ± 0.5^{B}	3.8 ± 0.5^{A}	3.8 ± 0.1^{B}	4.4 ± 0.6^{A}	3.9 ± 0.2^{B}	3.8 ± 0.4^{A}				
	M	12	6.4 ± 0.5	6.0 ± 0.2	6.9 ± 0.4	5.3 ± 1.2	5.6 ± 0.6	5.6 ± 0.4	0.211	0.196	0.232	0.725
	F		5.5 ± 0.4	5.5 ± 0.4	6.8 ± 0.3	6.1 ± 0.7	5.6 ± 0.4	5.7 ± 0.3				
DHA	Dam		2.3 ± 0.4^{X}	2.5 ± 0.3^{X}	1.3 ± 0.1^{Y}	2.0 ± 0.1^{Y}	2.1 ± 0.2^{XY}	2.0 ± 0.2^{XY}	0.007	0.115	0.145	
	M	4	1.5 ± 0.1	2.0 ± 0.3	1.5 ± 0.1	1.9 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	0.425	0.053	0.095	0.037
	F		1.2 ± 0.2	1.6 ± 0.3	1.5 ± 0.1	1.7 ± 0.2	1.5 ± 0.1	1.3 ± 0.1				
	M	12	2.5 ± 0.2^{AY}	1.9 ± 0.2^{BY}	2.6 ± 0.1^{AX}	2.2 ± 0.7^{BX}	2.0 ± 0.2^{AY}	1.8 ± 0.1^{BY}	0.023	0.030	0.222	0.0008
	F		2.6 ± 0.2^{AY}	2.1 ± 0.1^{BY}	3.0 ± 0.2^{AX}	2.8 ± 0.2^{BX}	2.5 ± 0.1^{AY}	2.6 ± 0.1^{BY}				

Values are means ± SEM. Mean values within a row with unlike superscript letters were significantly different ($P<0.05$): capital superscript $^{X>Y}$ for main effect of maternal treatment when both diet groups are combined; $^{A>B}$ for main effect of maternal diet when treatments are combined; and the low-case superscript $^{a>b}$ for diet and treatment interaction.

Figure 5.1.

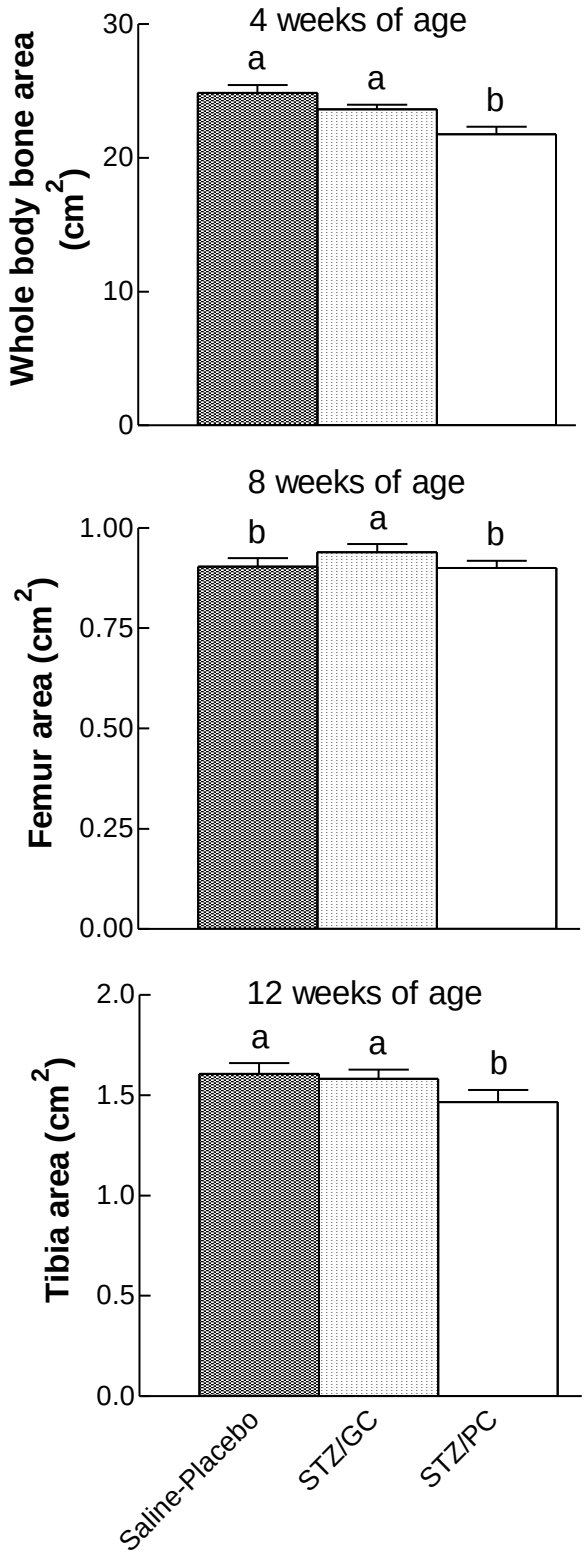
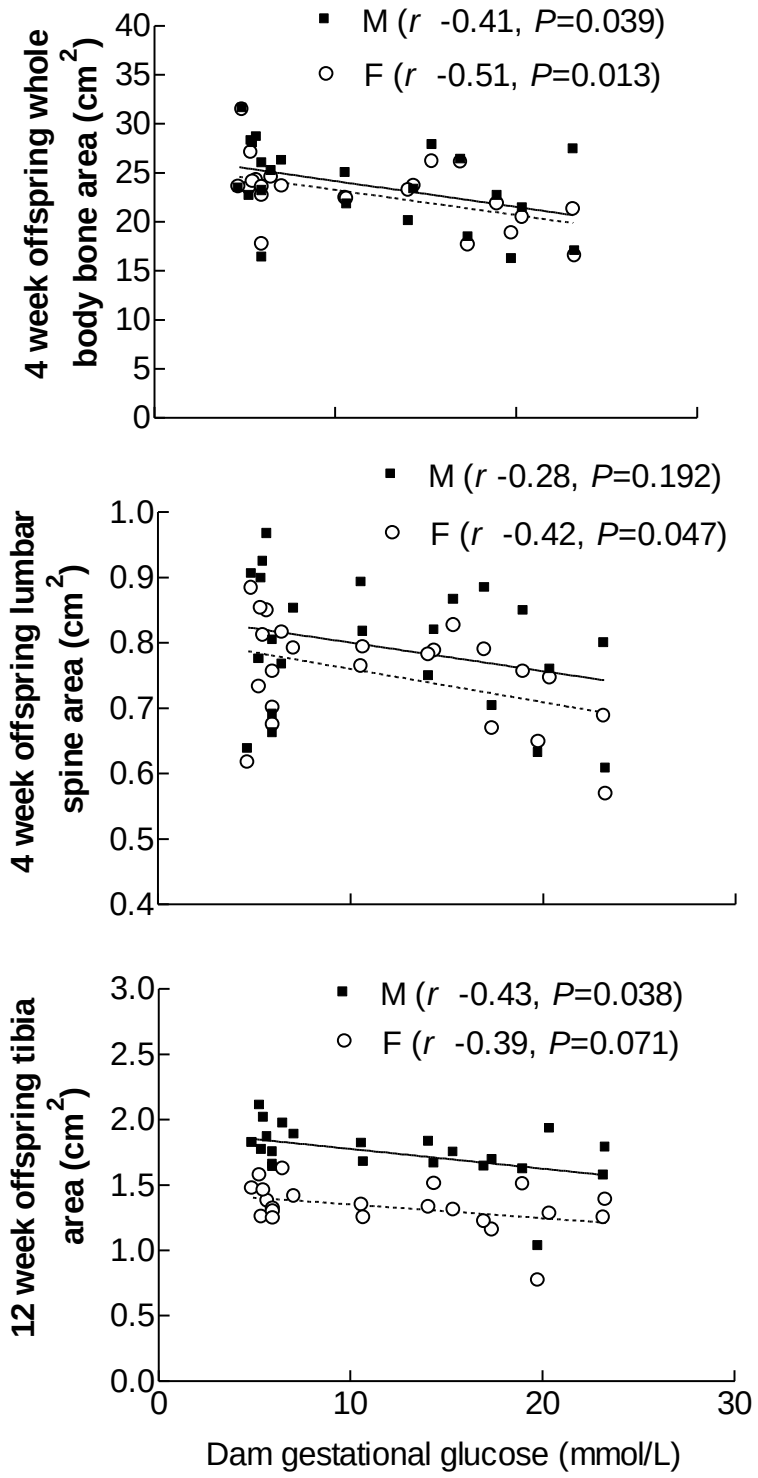

4 weeks of age
Whole body bone area (cm2)
30
20
10
0
a
a
b
8 weeks of age
Femur area (cm2)
1.00
0.75
0.50
0.25
0.00
b
a
b
12 weeks of age
Tibia area (cm2)
2.0
1.5
1.0
0.5
0.0
a
a
b
Saline-Placebo
STZ/GC
STZ/PC

Figure 5.2.


M (r -0.41, P=0.039)
F (r -0.51, P=0.013)
4 week offspring whole body bone area (cm2)
M (r -0.28, P=0.192)
F (r -0.42, P=0.047)
4 week offspring lumbar spine area (cm2)
M (r -0.43, P=0.038)
F (r -0.39, P=0.071)
12 week offspring tibia area (cm2)
Dam gestational glucose (mmol/L)

BRIDGE 4

Although the study in Chapter 5 showed that maternal diabetes and diet did not affect glucose tolerance in the offspring when measured at 8 and 12 weeks postnatally, others have reported glucose intolerance, as well as increased food intake and obesity in STZ-offspring at week 8 and 26 postnatally (Thamotharan et al., 2003). Population-based studies also indicated higher incidence of IGT and type 2 DM in young adults, offspring of diabetic mothers (Dabelea et al., 2008; Metzger, 2007). In addition, insulin resistance is highly prevalent in youth (Lambert et al., 2004; Lee et al., 2006). The conversion of ALA to DHA is vital for providing brain DHA as in the Western diet ALA intake is about 20-30 fold greater than DHA intake (Yogev et al., 2007). The desaturase enzymes can be down-regulated in conditions such as liver disease, type 1 diabetes and ageing resulting in brain DHA deficiency (Igarashi et al., 2007). The purpose of the study reported in Chapter 6 was to investigate if brain DHA can be maintained in young insulin resistant Zucker rats on diets with and without DHA.

CHAPTER 6. Manuscript 4.

DEPOSITION OF DOCOSAHEXAENOIC ACID (DHA) IS LIMITED IN FOREBRAIN OF YOUNG OBESE *fa/fa* ZUCKER RATS FED A DIET HIGH IN α-LINOLENIC ACID BUT DEVOID OF DHA

Jinping Zhao[a], Melani E. Gillam[b], Carla G. Taylor[b], Hope A. Weiler[a,*]

Author affiliation:

[a]School of Dietetics and Human Nutrition,

McGill University, Ste-Anne-de-Bellevue, Québec, Canada

[b]Department of Human Nutritional Sciences,

University of Manitoba, Winnipeg, Manitoba, Canada

Corresponding author:

Hope A. Weiler, RD, PhD

School of Dietetics and Human Nutrition, McGill University

Ste-Anne-de-Bellevue, QC, Canada H9X 3V9

Telephone: (514) 398-7905, Fax: (514) 398-7739

e-mail: hope.weiler@mcgill.ca

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6.1. ABSTRACT

Docosahexaenoic acid (DHA) is required for neurotransmitter synthesis and learning. Conversion of α-linolenic acid (ALA) to DHA is considered adequate to support brain function in youth, but it is unknown if brain DHA can be maintained in insulin resistant states. This study investigated brain fatty acid and desaturase activities in young insulin resistant Zucker rats on diets with and without DHA. Male *fa/fa* and lean rats were fed diets enriched with flaxseed (FXO, ALA: 35.5% fatty acids), menhaden (MO, DHA: 9.2%) or safflower oil (SO, linoleic acid: 54.1%) for 9 weeks, n=8 per diet per genotype. Compared to lean, the 15 week old *fa/fa* rats were obese (56% heavier) and insulin-resistant (>18-fold in homeostasis model assessment of insulin resistance). The forebrain of *fa/fa* rats had higher palmitoleic (16:1n-7) and dihomo-γ-linolenic (20:3n-6) acids, and higher Δ9, Δ6 but lower Δ5 (all $P \leq 0.006$) desaturase indices than lean. The Δ9 and Δ6 desaturase indices correlated positively, while the Δ5 negatively (all $P \leq 0.01$) with insulin resistance. The Δ9 desaturase index positively correlated with adiposity index. The percentage of forebrain DHA of *fa/fa* rats was lower (P=0.011) than lean rats when fed FXO diet while there was no difference (P>0.05) between *fa/fa* and lean rats fed MO or SO diet. Thus, the alterations in the fatty acid and desaturase indices in the brain were consistent with inhibited forebrain synthesis of DHA in the *fa/fa* rats. ALA may not have potential to effectively serve as a precursor for synthesizing DHA for youth forebrain during insulin resistance since Δ5 desaturase activity is limited.

Key words: Hyperinsulinemia; Forebrain; DHA; α-linolenic acid; Young Zucker rats

6.2. INTRODUCTION

The rising prevalence of childhood obesity is a major public health concern worldwide in view of the associated insulin resistance (Druet et al., 2006). A US population-based study revealed a prevalence of insulin resistance of 3.1% among normal weight, 15.0% among overweight and 52.1% among obese adolescents (Lee et al., 2006). The insulin resistance syndrome is highly prevalent in Canadian youth as well (Lambert et al., 2004). Academic achievement (Krukowski et al., 2009) and intelligence (Yu et al., 2010) are lower in obese children, the cause of which is multifactorial. Lipids such as DHA are postulated to affect cognition in obesity (Morley et al., 2010). The brain is rich in DHA as a fundamental component of neural cell membranes (Salem et al., 2001) where it modulates functional properties such as fluidity, permeability for metabolite exchange, activity of membrane-bound enzymes and receptors, and electrical and humoral signal transduction (Clandinin et al., 1991). To date, whether DHA is implicated in the reduced academic achievement of children has not been addressed.

Numerous studies have reported on plasma and tissue fatty acids in adults (Sjogren et al., 2008; Warensjo et al., 2009) and youth (Decsi et al., 2000; Murakami et al., 2008; Steffen et al., 2008) with insulin resistance and related disorders. This pattern is characterized by a decrease in linoleic acid (18:2 n-6) and an increase in palmitoleic (16:1 n-7), γ-linolenic (18:3 n-6) and dihomo-γ-linolenic (20:3 n-6) acids. Since the content of these increased fatty acids is normally very small in the diet, this augmentation in plasma lipids is thus indicative of increasing endogenous desaturation of palmitic acid (16:0) by Δ9

desaturase (leading to increased 16:1 n-7) and of linoleic acid by Δ6 desaturase (increasing the proportions of 18:3 n-6 and 20:3 n-6) (Vessby, 2000). This may reflect a high intake of 16:0 and low intake of 18:2 n-6 in the diet and a concomitant increase in Δ9 and Δ6 desaturases but low Δ5 desaturase activity (Vessby, 2003; Vessby et al., 2002), the latter which is required for the synthesis of DHA. Indeed, Pima Indians, a population with the highest reported incidence of insulin resistance in the world, have low skeletal muscle DHA (Pan et al., 1995). This striking difference cannot be explained by diet since among the Australian population, even individuals with little or no discernible DHA intake had muscle DHA much higher than the Pima study group (Borkman et al., 1993; Pan et al., 1995).

Under normal physiological conditions, the adult liver has ample capacity to synthesize DHA from circulating ALA. In adult rats the estimated rate of DHA synthesis by liver is about 10× the DHA consumption rate in brain tissue (Igarashi et al., 2007). This high synthesis rate is supported by the high expression and high activity of the elongase and desaturase enzymes in the liver (Igarashi et al., 2007; Rapoport et al., 2007). However, in humans below normal blood levels of DHA are common in liver disease, diabetes and aging (Brenner, 2003; Watanabe et al., 1999). Such reductions are postulated to be associated with reduced desaturase or elongase activity and are a risk factor for brain disease in the absence of dietary DHA supplementation (Rapoport et al., 2009). It is not clear if brain DHA can be sustained in obesity and insulin resistant states but it is suspected to be inadequate since people with insulin resistance have greater cognitive decline (van Oijen et al., 2008; Young et al., 2006). In fact, insulin

activity is required elongase and desaturase enzymes in liver (Brenner, 1990). Very few studies have examined the effect of insulin resistance on brain fatty acid metabolism (Guesnet et al., 1990). Although one study in the Zucker model of insulin resistance observed no difference in brain DHA compared with non-insulin resistant rats, the diet provided preformed eicosapentaenoic acid (EPA) and DHA which bypasses Δ5 desaturase (Guesnet et al., 1990). It is well known that deposition of DHA is higher in brain when it is provided in the diet compared to ALA (Abedin et al., 1999). Thus, it is unknown if Δ5 desaturase activity is sufficient to compensate for a diet devoid of DHA. The purpose of the present study was to compare the effects of dietary n-6 PUFA (linoleic acid, 18:2 n-6), n-3 PUFA (ALA, 18:3 n-3) and n-3 long chain-PUFA (LC-PUFA: DHA, 22:6 n-3) on the fatty acid composition and desaturases activities of three brain regions in obese and hyperinsulinemic young rats. It is particularly important to study this association in young rats as brain responds to n-3 fatty acid supplementation in an age- and time-dependent manner (Barcelo-Coblijn et al., 2003; McGahon et al., 1999). This study was designed to advance understanding of the impact of hyperinsulinemia on brain fatty acid profile and the effectiveness of different dietary PUFA supplementation to prevent the postulated aberrations in fatty acid status in a youth model of insulin resistance.

6.3. METHODS AND MATERIALS

6.3.1. Animals and diets

The study design and diet composition have been published in detail (Gillam et al., 2009; Mollard et al., 2005). Briefly, 5-week-old male *fa/fa* and lean Zucker rats (Charles River Laboratories, St. Constant, QC) were randomly

assigned to diets containing 10% (w/w) fat mixtures with flaxseed oil (FXO, ALA: 35.5% fatty acids), menhaden oil (MO, DHA: 9.2%) or safflower oil (SO, linoleic acid: 54.1%) as the primary oils (n = 8 per group per genotype). To control for possible effects of the n-3 diets (FXO or MO) on body weight, there were weight-matched (WM) groups within each genotype with rats fed SO control diet in an amount to maintain body weight similar to FXO or MO groups, whichever weighed less. All diets had similar total saturated fatty acids (SFA), monounsaturated fatty acid (MUFA), and PUFA and thus the PUFA to SFA ratio was close to 2.1 for all groups. The n-6 to n-3 ratios for the MO, FXO and SO control diets were 1.0, 0.5 and 58.6, respectively (**Table 6.1**). Both the MO and FXO diets met the minimum suggested requirements for rodents according to the AIN-93 diets for growth and maintenance; ALA of at least 2 g/kg diet (Reeves, 1997), while the SO diet provided 0.9 g/kg diet. Previously diets with 0.7 and 1.1 g ALA/kg diet supported brain DHA in rats at amounts not different from diets with ALA at 2 g/kg diet (Bourre et al., 1993).

The rats were given a 1 week adaptation while fed the SO control diet, and then received their respective test diets *ad libitum*, except the WM group, for 9 weeks. The duration of 9 weeks was used since in rats 8 weeks is required for brain fatty acid status to fully respond to changes in dietary fat (McGahon et al., 1999). The diet was made fresh weekly and stored at -20 ℃ until fed. Fresh feed was provided daily and feed intake was recorded, while rats were weighed weekly. Throughout the entire study period, rats were housed individually in standard hanging cages with controlled temperature (21-23 ℃), humidity (55%), and 12-h light cycle. The experiment was conducted in accordance with the

guidelines of the Canadian Council for Animal Care and was approved by the University of Manitoba Protocol Management and Review Committee.

6.3.2. Tissue collection

At the end of the 9 week feeding trial, rats (15 weeks of age) were food deprived overnight and euthanized by CO_2 asphyxiation. Trunk blood was collected, stored on ice and centrifuged at 4 ℃ for 15 minutes. Serum for biochemical measurements was stored at -80 ℃. The brains were quickly removed, weighed and dissected on ice into forebrain, cerebellum and hippocampus. Samples were flash frozen in liquid nitrogen and stored at -80 ℃ until fatty acid analysis.

6.3.3. Brain fatty acid analyses

Total lipids were measured in forebrain, cerebellum and hippocampus since the proportion of free fatty acids and phospholipid-bound fatty acids is modified by CO_2 asphyxiation (Ledwozyw, 1991). In brief, fatty acids were extracted using chloroform: methanol 2:1 containing 0.01% butylated hydroxytoluene (BHT) according to a method (Weiler et al., 2002) adapted from Folch et al (Folch et al., 1957). After adding an internal standard, C17:0, brain tissues were homogenized and the crude lipid extracts were transmethylated in 1.2 mL of methanolic HCl (3 mol/L, Supelco, Bellefonte, PA) at 80 ℃ for 1 hour. Fatty acids were identified by comparison of retention times with standards and expressed as g/100 g of total fatty acids. For comparison of the results to similar studies (Blond et al., 1989; Guesnet et al., 1990), eight fatty acids including the most prominent peaks on gas chromatography (Varian Star 3400, Mississauga, Canada) analysis were included in the calculation: SFA (16:0 and 18:0), MUFA

(16:1 n-7 and 18:1 n-9), PUFA (18:2 n-6), LC-PUFA (20:3 n-6, 20:4 n-6 and 22:6 n-3). Fatty acids including 18:3 n-3, 18:3 n-6 and 22:5 n-6 were below detection limits. The combined composition of these fatty acids accounts for >62% of the total fatty acids of the brain.

6.3.4. Estimation of desaturase activity of brain

The product to precursor ratios of individual fatty acids in different regions of brain was calculated to estimate the activities of different desaturases as follows: 16:1 n-7/16:0 for Δ9 desaturase, 20:3 n-6/18:2 n-6 for Δ6 desaturase, and 20:4 n-6/20:3 n-6 for Δ5 desaturase (Warensjo et al., 2009).

6.3.5. Biochemical measurements

Serum glucose concentration was determined using the glucose oxidase method with a Glucose Assay Kit 510-A (Sigma Diagnostics Inc., St Louis, MO). Dilutions for serum were between 10- and 25-fold for lean and *fa/fa* Zucker rats. Absorbance was read at 450 nm (SPECTRAmax 340, Molecular Devices Corp, Sunnyvale, CA) and the concentration was adjusted for dilutions. Serum insulin concentration was determined using a rat specific radioimmunoassay kit (Linco Research Inc., St. Charles, MO). Lean Zucker rat serum was diluted by a factor of 5, while *fa/fa* Zucker rat serum was diluted by a factor of 100. All serum assays and standards were run in duplicate, and the coefficient of variation was <10%. Insulin resistance was estimated using the homeostasis model assessment of insulin resistance (HOMA-IR) with the formula: [fasting insulin (pmol/L) × fasting glucose (mmol/L)]/135 (Muellenbach et al., 2009). An adiposity index was computed for each rat as 100 × (sum of fat pad weights)/(body weight) (Noto et al., 2007).

6.3.6. Data analysis

Results are expressed as the means ± SEM. Data were analyzed using a factorial ANOVA and the general linear model procedure of SAS (version 9.1, Cary, NC, USA) as is suitable for the genotype (lean, *fa/fa*) by diet (FXO, MO and SO plus WM as required) factorial design. Significant differences ($P<0.05$) among means of groups by dietary oil mixture or by regions of brain or any interaction effects were assessed using Bonferroni post-hoc t-tests. The relationships between desaturase indices of brain and insulin resistance and adiposity index were investigated using Pearson's correlation coefficient *r* to quantify the strength of the relationship.

6.4. RESULTS

6.4.1. Experimental system

Due to genotype, *fa/fa* rats were significantly heavier than lean rats (**Table 6.2**). There were no differences in body weight among the FXO, MO and SO groups, therefore, the WM group was not included in further analyses. Thus, any changes due to dietary intervention were not confounded by differences in body weight. The *fa/fa* rats consumed 41% more total feed than lean rats (**Table 6.3**). At 15 weeks of age, the body weight of *fa/fa* rats was 56% greater accompanied by 197% more visceral fat. Thus the adiposity index was significantly ($P<0.0001$) higher than leans. In addition, the *fa/fa* rats were insulin resistant as indicated by the substantially greater (>18-fold, $P<0.0001$) HOMA-IR value in *fa/fa* rats compared to lean rats. After 9 weeks SO diet consumption, both genotypes had lower (1693 ± 92 pmol/L, $P=0.020$) fasting serum insulin

concentrations compared to rats fed with FXO (2569 ± 159 pmol/L) and MO (2485 ± 142 pmol/L).

6.4.2. Brain fatty acid composition

Although there was no significant difference in hippocampus weight between genotypes (P>0.05, **Table 6.3**), the whole brains of *fa/fa* rats were 7% smaller than those of lean rats (P<0.0001). The *fa/fa* brain weight was 0.31% of body weight, while the lean brain weight was 0.52% of body weight (P<0.0001).

For SFA (16:0 and 18:0), no effect of diet, genotype or diet by genotype interaction was observed in all three studied brain regions (all P>0.05, **Table 6.4**). For 16:1 n-7 and 20:3 n-6, there were similar genotype and diet main effects. Compared to lean rats, *fa/fa* rats had higher (all $P \leq 0.0002$) 16:1 n-7 in all three brain regions and higher 20:3 n-6 in forebrain and cerebellum regardless of diet. Rats fed MO had a higher and those fed SO had a lower (all $P \leq 0.006$) proportion of 16:1 n-7 and 20:3 n-6 among the three diet groups regardless of genotype.

For forebrain 18:1 n-9, no effect of diet, genotype or diet by genotype interaction was observed (P>0.05). The high n-6 diet (SO group) effectively elevated forebrain 18:2 n-6 in both *fa/fa* and lean rats (P=0.001), while no effect of genotype or diet by genotype interaction was observed (all P>0.05). The forebrain AA was responsive to diet only, as rats from SO groups had significantly higher AA values than the rats from n-3 dietary groups (P=0.0006). For the forebrain DHA, there was a diet by genotype interaction effect (P<0.0001); high n-3 diets (FXO and MO groups) elevated DHA (P<0.0001) in lean rats, but *fa/fa* rats fed FXO were unable to achieve higher DHA than rats fed

high n-6 PUFA (SO diet). There was no genotype effect for forebrain DHA in rats fed SO diet or in rats fed MO diet.

For the cerebellum 18:1 n-9 and 18:2 n-6, no effect of diet, genotype or diet by genotype interaction was observed (all $P>0.05$, **Table 6.4**). After 9 week of consuming the MO diet, the cerebellum had significantly lower AA ($P=0.02$), but there were no differences in cerebellum AA between rats fed the FXO diet and those fed the SO diet. There was no genotype or diet by genotype interaction ($P>0.05$). For cerebellum DHA, again, there was a diet effect only; rats fed high n-6 diet (SO group) had significantly lower cerebellum DHA than rats fed n-3 PUFA rich diets (FXO and MO groups, $P=0.004$). Both lean and *fa/fa* rats from the FXO groups had similar cerebellum DHA as rats from MO groups ($P>0.05$). There were no genotype and diet by genotype interactions observed.

For the hippocampus 18:2 n-6, no effect of diet, genotype or diet by genotype interaction was observed (all $P>0.05$, **Table 6.4**). There was a diet effect for 18:1 n-9 as rats fed MO as well as FXO had significantly higher values than rats fed SO diet. Hippocampus AA and DHA responded to diet, as rats fed high n-6 diet (SO group) had significantly higher AA, but with significantly lower DHA in comparison with rats fed high n-3 diets (FXO and MO groups, $P<0.0001$). Feeding FXO and MO had equivalent effects in elevating the proportion of DHA in hippocampus of brain regardless of genotype ($P>0.05$). There was no genotype and diet by genotype interaction effect ($P>0.05$).

6.4.3. Estimated desaturase activity

There were genotype effects in all three estimated desaturase activities, as *fa/fa* rats had significantly higher Δ9 (all regions) and Δ6 (forebrain and

cerebellum) but lower Δ5 (forebrain only) desaturase activities than lean rats ($P \leq 0.002$, **Table 6.5**). There were also diet effects as rats fed with MO had the highest Δ9 ($P \leq 0.003$) and Δ6 ($P \leq 0.01$) but lowest Δ5 ($P \leq 0.004$) desaturase activities in all brain regions, while rats fed SO diet had the lowest Δ9 and Δ6 but highest Δ5 (all $P \leq 0.01$) desaturase activities in all brain regions. Rats fed FXO diet had intermediate Δ9, Δ6 and Δ5 desaturase activities across the brain regions compared with rats fed the other two diets.

6.4.4. Correlations between desaturase indices and insulin resistance and visceral fat

The Δ9 desaturase activity in all three brain regions was positively correlated with insulin resistance ($P \leq 0.022$, **Figure 6.1**). The Δ6 desaturase activity in both forebrain and cerebellum was positively ($P \leq 0.033$) correlated with insulin resistance. In forebrain only the Δ5 desaturase activity was negatively ($P = 0.013$) correlated with insulin resistance regardless of genotype. The Δ9 desaturase activity in all three brain regions was positively correlated with adiposity index ($P \leq 0.0009$, **Figure 6.2**) regardless of genotype. In cerebellum only the Δ6 desaturase activity was positively ($P = 0.019$) correlated with adiposity index regardless of genotype. In forebrain only the Δ5 desaturase activity was negatively ($P = 0.044$) correlated with adiposity index regardless of genotype.

6.5. DISCUSSION AND CONCLUSIONS

DHA is the most abundant LC-PUFA in the brain, representing roughly 15% of total fatty acids (Sinclair, 1975). Normal brain DHA can be maintained in adult rats by the liver supply of DHA through elongation and desaturation of ALA when dietary DHA is absent but ALA is sufficient (≥4.6 g/ kg diet) (Igarashi et

al., 2007). However, reducing ALA at or below 1 g/kg diet results in at least 25% lower brain DHA in many species including fish (Xu et al., 1993), rats (Ward et al., 1996) and guinea pigs (Weisinger et al., 1998) despite elevated expression and activities of Δ5 and Δ6 desaturases in response to n-3 PUFA deprivation (Demar et al., 2005). In the present study providing 0.9 g/kg ALA resulted in ~20% lower brain DHA compared to ALA at 35.5 g/kg, despite the higher Δ5 desaturase activity on the basis of n-6 PUFA synthesis in the SO group.

Conversely, desaturase enzymes can be down-regulated in conditions such as liver disease, type 1 diabetes, ageing and possibly insulin resistance resulting in brain DHA deficiency (Igarashi et al., 2007). Indeed in the present model of insulin resistance, lower forebrain DHA was observed in the *fa/fa* rats fed a diet with more than ample ALA (35.5 g/kg diet) for 9 weeks. Synthesis of DHA was likely the limiting factor rather than deposition of DHA since *fa/fa* rats fed a diet with preformed DHA had forebrain values not different from lean controls. This contrasts data from healthy animal models where lack of dietary DHA in healthy monkeys, piglets, and mice did not decrease brain DHA (Bourre et al., 1989) when sufficient quantities of ALA were in the diet (Connor et al., 1992). Even in our lean rats, those fed high ALA compared to preformed DHA had similar brain DHA. These results thus suggest that ALA (flaxseed oil in the present study) was not effective as a precursor for synthesizing DHA within young adult forebrain when accompanied by insulin resistance.

The observation that the *fa/fa* rats did not have lower DHA in cerebellum or hippocampus regardless of diet suggests that the forebrain desaturase or elongase activity is vulnerable to hyperinsulinema. This supposition is supported

by the lower Δ5 desaturase activity in *fa/fa* rat forebrain but not the other regions. Lower forebrain DHA is associated with alterations in neurotransmission pathways (Delion et al., 1994) and has also been observed in some pathological conditions associated with neurobehavioral changes, such as Alzheimer disease (Soderberg et al., 1992) and schizophrenia (Horrobin et al., 1991). The present study adds insulin resistance as one more factor that specifically affects forebrain DHA status in a model of obese youth. Obesity is also characterized by hypercortisolemia (Rocchini et al., 1989) that inhibits transcription of desaturase enzymes (Brenner, 1990). Future studies should examine relationships among brain desaturase activity, DHA, hyperinsulinemia and corticosteroids in the obese *fa/fa* Zucker rat.

Brain fatty acids have only been studied in female Zucker *fa/fa* rats (Blond et al., 1989; Guesnet et al., 1990). In the present study, all three estimated desaturase activities of forebrain were altered in male *fa/fa* rats in comparison with lean rats. The altered desaturase indices of forebrain were significantly correlated with insulin resistance (HOMA-IR). While the present study cannot clearly distinguish between reduced liver and brain conversion of ALA to DHA in the aetiology of low forebrain DHA, muscle and adipose DHA was previously reported as similar between our *fa/fa* and lean rats (Gillam et al., 2009), suggesting liver synthesis is adequate in the *fa/fa* model of insulin resistance. Thus the lower brain DHA observed herein is likely ascribed to pathophysiology localized in forebrain. However, some, but not all (Georges et al., 1993), *in vitro* studies show increased Δ9 (Wahle et al., 1977), Δ6 (Blond et al., 1989) and

decreased Δ5 (Wahle et al., 1977) desaturase activities in liver microsomes of *fa/fa* Zucker rats as compared with lean rats. Further studies are warranted.

The underlying mechanism for this regionally selective modification in DHA composition is consistent with previous studies. Mice (Carrie et al., 2000) and rats (Levant et al., 2006; Xiao et al., 2005) fed n-3 PUFA deficient diets have reduced DHA, particularly in forebrain. DHA accretion (Levant et al., 2006; Xiao et al., 2005) and uptake coefficients (Ouellet et al., 2009) are different among specific brain regions under normal physiological conditions. In our hyperinsulinemic model, the indices of desaturase activities between *fa/fa* and lean rats fed FXO were similar in cerebellum and hippocampus, but lower in forebrain resulting in lower accretion of DHA in forebrain and higher accretion in both cerebellum and hippocampus (t-test, $P<0.005$). We speculate that hyperinsulinemia is able to stimulate synthesis of DHA in cerebellum and hippocampus, but not forebrain. Indeed insulin infused into forebrain does not alter energy metabolism whereas in hippocampus phosphocreatinine was enhanced (Henneberg et al., 1994), suggesting insulin might have a greater influence in hippocampus and possibly cerebellum. Cerebellum glucose is tightly regulated and non-responsive to both hyper- and hypoglycaemia in men with type 1 diabetes whereas forebrain glucose readily reflects blood values (Heikkila et al., 2010). Future work should aim to characterise insulin metabolism in each brain region under normal and hyperinsulinemic states to confirm if desaturase enzymes are also differentially affected.

This is the first study regarding the effect of n-6 or n-3 LC-PUFA on brain desaturase activities; however, controversy has surrounded the roles of n-6 vs. n-3

fatty acids with regard to insulin resistance (Berry, 1997; Storlien et al., 1996). Even though the SO diet did not support brain DHA, the lower insulin in this group suggests that diets with n-6:n-3 ratios above 1 may improve insulin resistance and thereby also support endogenous synthesis of fatty acids (i.e. insulin stimulates synthesis). However, high amounts of n-6 PUFA but not n-3 PUFA result in the opposite as demonstrated by Mohan et al. (Mohan et al., 1991) using the same Zucker model (10 weeks old and feeding duration of 10 weeks) where serum insulin values in safflower oil (high in LA) fed obese rats were twice those of the menhaden or coconut oil fed *fa/fa* rats. Low plasma LA is a common feature in individuals with insulin resistance, and insulin sensitivity is associated with a high proportion of LA (Warensjo et al., 2006). In the population-based (*n*=895) Finnish study, men with a high proportions of LA in plasma fatty acids, indicating a high intake of dietary LA, had a lower risk of developing diabetes and showed lower increases in serum insulin and blood glucose a 4 year follow-up (Laaksonen et al., 2002). Thus low LA is likely a manifestation of insulin resistance and thus further research is required to optimize dietary LA recommendations.

The brain is an organ generally well-protected against external blood-borne influences, but the cerebral fatty acid composition can be extensively modulated by dietary lipids (Angulo-Guerrero et al., 1998). It could be argued that the cerebral fatty acid composition can also be modulated by insulin resistance. Since all three brain regions were responsive to modification by dietary LC-PUFA, it is possible that dietary DHA supplementation can be used as a preventive or treatment strategy to minimize the DHA deficiency in the brain of

insulin-resistant individuals. The possible compromised cognitive function and suboptimal learning ability would greatly affect the potential of youth at school, work, social, and family situations (Gibson et al., 2002). The possible combined burden of the obesity epidemic and insulin resistance in youth is thus concerning beyond the standard chronic disease concerns (Hebert et al., 2001). Cognitive impairment has long been thought to be irremediable and terminal; however, increasing understanding of its associations with common and modifiable conditions such as insulin resistance and DHA status will challenge these assumptions.

6.6. ACKNOWLEDGEMENTS

We would like to acknowledge the assistance of Shirley C. Fitzpatrick-Wong and Marilyn Latta for fatty acid analysis and Amy Noto on data management. This study was supported by grants from the Flax Council of Canada, Canada-Manitoba Agri-Food Research and Development Initiative and Natural Sciences and Engineering Research Council of Canada.

6.7. FIGURE LEGENDS

Figure 6.1. The relationships between HOMA-IR and Δ9, Δ6 and Δ5 desaturase indices in forebrain (A), cerebellum (B) and hippocampus (C) of *fa/fa* and lean Zucker rats fed experimental diets for 9 weeks.

HOMA-IR = fasting insulin (pmol/L) × fasting glucose (mmol/L)/135.

Δ9 desaturase:16:1 n-7/16:0; Δ6 desaturase: 20:3 n-6/18:2 n-6; Δ5 desaturase : 20:4 n-6/20:3 n-6.

Figure 6.2. The relationships between adiposity index and Δ9, Δ6 and Δ5 desaturase indices in forebrain (A), cerebellum (B) and hippocampus (C) of *fa/fa* and lean Zucker rats fed experimental diets for 9 weeks. [Adiposity index = 100 × (sum of fat pad weights)/(body weight)].

Table 6.1. Fatty acid composition of lipids in diets

Fatty acids (g/100 g)	FXO	MO	SO
Σ SFA	25.5	25.2	26.2
Σ MUFA	19.4	20.5	18.5
Σ PUFA	55.1	54.3	55.3
18:2 n-6	19.2	23.2	54.1
18:3 n-6	0	0.4	0
20:4 n-6 (AA)	0	0.5	0
Σ (n-6) PUFA	19.2	24.4	54.1
18:3 n-3 (ALA)	35.5	1.1	0.9
20:5 n-3	0	8.0	0
22:6 n-3 (DHA)	0	9.2	0
Σ (n-3) PUFA	35.5	24.3	0.9
n-6/n-3 PUFA	0.5	1.0	58.6
PUFA/SFA	2.2	2.1	2.1

FXO: flaxseed oil mixture diet; MO: menhaden oil mixture diet; SO: safflower oil mixture diet. SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid. AA: arachidonic acid (20:4 n-6); ALA: α-linolenic acid (18:3 n-3); DHA: docosahexaenoic acid (22:6 n-3).

Table 6.2. Final body weight (g) of *fa/fa* and lean Zucker rats fed experimental diets for 9 weeks

Genotype	Diet				*P* value		
	FXO	MO	SO	WM	Diet	Geno	Diet × Geno
fa/fa	615 ± 11^{X}	606 ± 18^{X}	616 ± 13^{X}	574 ± 14^{X}	0.060	<0.001	0.739
lean	385 ± 4^{Y}	399 ± 6^{Y}	394 ± 10^{Y}	366 ± 16^{Y}			

Values are means ± SEM, *n*=8 per group. Different capital superscripts ^{X} and ^{Y} indicate significant main effect of genotype ($P<0.05$). fa: *fa/fa* Zucker rat; ln: lean Zucker rat; Geno: genotype. WM: weight-matched fed with safflower oil mixture diet.

Table 6.3. Characteristics of *fa/fa* and lean Zucker rats at 15 week of age

Measurement	*fa/fa*	lean	*P* value
Total feed intake (g/rat)	1790 ± 22	1267 ± 13	<0.0001
Final body wt (g)	613.4 ± 8.3	393.4 ± 4.2	<0.0001
Visceral fat pad wt (g)[1]	40.4 ± 1.0	13.6 ± 0.5	<0.0001
Adiposity index	6.6 ± 0.4	3.5 ± 0.1	<0.0001
Fasting insulin (pmol/L)	4253.9 ± 418.3	243.5 ± 33.4	<0.0001
Fasting glucose (mmol/L)	7.2 ± 0.2	6.9 ± 0.3	0.163
HOMA-IR	226.9 ± 28.4	12.4 ± 1.8	<0.0001
Brain wt (g)	1.88 ± 0.03	2.01 ± 0.04	<0.0001
Relative brain wt (g/100 g body wt)	0.3 ± 0.01	0.5 ± 0.01	<0.0001
Hippocampus wt (mg)	77.5 ± 2.7	77.0 ± 6.0	0.460
Relative hippocampus wt (mg/100 g body wt)	12.6 ± 1.1	19.6 ± 1.4	<0.0001

Values are means ± SEM, *n*=32 per genotype.

[1]Visceral fat = epididymal + peri-renal fat pads.

Adiposity index was computed as 100 × (sum of fat pad weights)/(body weight).

HOMA-IR: homeostasis model assessment of insulin resistance: [fasting insulin (pmol/L) × fasting glucose (mmol/L)]/135.

Table 6.4. Fatty acid profile in brain regions of *fa/fa* and lean Zucker rats fed experimental diets for 9 weeks

Fatty acids (g/100g)	fa+FXO	ln+FXO	fa+MO	ln+MO	fa+SO	ln+SO	*P* value		
							Diet	Geno	Diet × Geno
Forebrain									
16:0	17.9 ± 0.6	17.2 ± 1.6	18.7 ± 0.6	19.2 ± 0.2	19.4 ± 0.4	18.6 ± 0.2	0.124	0.580	0.630
16:1n-7	0.6 ± 0.0^{BX}	0.4 ± 0.0^{BY}	0.6 ± 0.0^{AX}	0.5 ± 0.0^{AY}	0.6 ± 0.0^{BX}	0.4 ± 0.0^{BY}	0.006	<0.0001	0.416
18:0	18.8 ± 0.6	17.6 ± 1.8	19.2 ± 0.3	19.7 ± 0.1	19.2 ± 0.2	19.2 ± 0.2	0.216	0.687	0.514
18:1n-9	15.8 ± 0.6	15.5 ± 0.8	15.5 ± 0.6	14.9 ± 0.1	14.0 ± 0.2	14.8 ± 0.2	0.063	0.990	0.375
18:2n-6	0.8 ± 0.0^{B}	0.7 ± 0.1^{B}	0.7 ± 0.0^{B}	0.7 ± 0.0^{B}	0.9 ± 0.0^{A}	0.8 ± 0.0^{A}	0.001	0.145	0.335
20:3n-6	0.4 ± 0.0^{BX}	0.3 ± 0.0^{BY}	0.5 ± 0.0^{AX}	0.4 ± 0.0^{AY}	0.3 ± 0.0^{CX}	0.3 ± 0.0^{CY}	<0.0001	0.0002	0.213
20:4n-6	8.4 ± 0.5^{B}	8.3 ± 0.8^{B}	8.1 ± 0.2^{B}	8.7 ± 0.1^{B}	10.1 ± 0.1^{A}	10.0 ± 0.2^{A}	0.0006	0.645	0.637
22:6n-3	11.3 ± 0.4^{b}	13.6 ± 0.1^{a}	15.1 ± 0.7^{a}	15.1 ± 0.3^{a}	11.4 ± 0.3^{b}	11.1 ± 0.3^{b}	0.0001	0.011	0.0001
Cerebellum									
16:0	16.7 ± 1.6	15.2 ± 0.3	14.3 ± 0.8	15.2 ± 0.5	14.3 ± 0.6	14.0 ± 0.6	0.130	0.655	0.406
16:1n-7	0.5 ± 0.1^{AX}	0.3 ± 0.0^{AY}	0.4 ± 0.0^{AX}	0.4 ± 0.0^{AY}	0.4 ± 0.0^{BX}	0.3 ± 0.0^{BY}	0.004	0.0001	0.125
18:0	17.3 ± 1.3	17.2 ± 0.3	15.7 ± 0.5	17.0 ± 0.7	16.0 ± 0.4	15.7 ± 0.4	0.157	0.626	0.462
18:1n-9	16.2 ± 0.9	17.5 ± 0.3	18.0 ± 0.7	17.1 ± 0.3	16.5 ± 0.5	16.9 ± 0.2	0.331	0.654	0.208
18:2n-6	0.8 ± 0.0	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.0	0.9 ± 0.1	0.9 ± 0.1	0.559	0.296	0.841
20:3n-6	0.5 ± 0.0^{BX}	0.4 ± 0.0^{BY}	0.5 ± 0.0^{AX}	0.4 ± 0.0^{AY}	0.4 ± 0.0^{CX}	0.3 ± 0.0^{CY}	0.0006	<0.0001	0.717

Table 6.4. *(Continued)*

Fatty acids (g/100g)	fa+FXO	ln+FXO	fa+MO	ln+MO	fa+SO	ln+SO	*P* value		
							Diet	Geno	Diet × Geno
20:4n-6	7.7 ± 1.0^{A}	6.8 ± 0.1^{A}	5.6 ± 0.2^{B}	6.2 ± 0.1^{B}	7.1 ± 0.3^{A}	6.8 ± 0.2^{A}	0.016	0.602	0.334
22:6n-3	13.6 ± 0.6^{A}	11.0 ± 0.5^{A}	11.1 ± 1.3^{A}	12.2 ± 0.5^{A}	8.3 ± 0.9^{B}	7.9 ± 0.5^{B}	0.004	0.860	0.512
Hippocampus									
16:0	19.1 ± 0.2	18.7 ± 0.1	18.5 ± 0.1	19.0 ± 0.2	19.2 ± 0.2	18.9 ± 0.2	0.151	0.693	0.055
16:1n-7	0.5 ± 0.0^{AX}	0.4 ± 0.0^{AY}	0.5 ± 0.0^{AX}	0.4 ± 0.0^{AY}	0.4 ± 0.0^{BX}	0.3 ± 0.0^{BY}	0.005	<0.0001	0.191
18:0	19.6 ± 0.2	19.5 ± 0.4	20.0 ± 0.3	19.7 ± 0.2	19.5 ± 0.2	20.1 ± 0.2	0.550	0.779	0.226
18:1n-9	15.0 ± 0.2^{A}	15.3 ± 0.2^{A}	15.0 ± 0.3^{A}	15.1 ± 0.3^{A}	13.7 ± 0.1^{B}	13.9 ± 0.2^{B}	<0.0001	0.368	0.837
18:2n-6	0.6 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.259	0.988	0.228
20:3n-6	0.4 ± 0.0^{A}	0.4 ± 0.0^{A}	0.4 ± 0.1^{A}	0.4 ± 0.0^{A}	0.3 ± 0.0^{B}	0.2 ± 0.0^{B}	<0.0001	0.165	0.721
20:4n-6	10.8 ± 0.1^{B}	10.7 ± 0.2^{B}	10.4 ± 0.5^{B}	10.2 ± 0.2^{B}	12.1 ± 0.1^{A}	12.2 ± 0.3^{A}	<0.0001	0.941	0.907
22:6n-3	13.3 ± 0.1^{A}	12.3 ± 0.1^{A}	14.0 ± 0.1^{A}	13.8 ± 0.2^{A}	10.3 ± 0.1^{B}	10.1 ± 0.3^{B}	<0.0001	0.394	0.216

Values are means ± SEM, *n*=8 per group.

Differences among groups ($P<0.05$) are identified by different superscripts: capital superscripts $^{A>B>C}$ for main effect of diet, $^{X>Y}$ for genotype effect, and the low-case superscripts $^{a>b}$ for diet and genotype interaction.

Table 6.5. Desaturase indices in brain regions of *fa/fa* and lean Zucker rats fed experimental diets for 9 weeks

Desaturase indices							*P* value		
	fa+FXO	ln+FXO	fa+MO	ln+MO	fa+SO	ln+SO	Diet	Geno	Diet × Geno
Forebrain									
Δ9 16:1/16:0×100	3.4 ± 0.1^{BX}	2.3 ± 0.1^{BY}	3.5 ± 0.1^{AX}	2.7 ± 0.1^{AY}	3.0 ± 0.0^{CX}	2.0 ± 0.1^{CY}	<0.0001	<0.0001	0.225
Δ6 20:3/18:2	0.58 ± 0.03^{BX}	0.46 ± 0.01^{BY}	0.65 ± 0.03^{AX}	0.54 ± 0.03^{AY}	0.34 ± 0.02^{CX}	0.33 ± 0.02^{CY}	<0.0001	0.0009	0.065
Δ5 20:4/20:3	19.3 ± 1.7^{BY}	25.7 ± 1.7^{BX}	17.4 ± 0.9^{CY}	21.9 ± 0.6^{CX}	32.3 ± 1.6^{AY}	36.5 ± 0.4^{AX}	<0.0001	<0.0001	0.676
Cerebellum									
Δ9 16:1/16:0×100	3.2 ± 0.2^{AX}	2.2 ± 0.0^{AY}	3.1 ± 0.1^{AX}	2.5 ± 0.2^{AY}	2.6 ± 0.1^{BX}	1.8 ± 0.1^{BY}	<0.0001	<0.0001	0.214
Δ6 20:3/18:2	0.60 ± 0.04^{BX}	0.46 ± 0.04^{BY}	0.66 ± 0.05^{AX}	0.50 ± 0.03^{AY}	0.48 ± 0.05^{BX}	0.40 ± 0.05^{BY}	0.0147	0.002	0.646
Δ5 20:4/20:3	17.0 ± 3.2^{A}	17.7 ± 0.6^{A}	10.9 ± 0.5^{B}	14.8 ± 0.4^{B}	17.7 ± 1.4^{A}	20.8 ± 2.5^{A}	0.0041	0.086	0.623
Hippocampus									
Δ9 16:1/16:0×100	2.7 ± 0.0^{AX}	2.1 ± 0.0^{AY}	2.6 ± 0.2^{AX}	2.2 ± 0.1^{AY}	2.2 ± 0.1^{BX}	1.7 ± 0.0^{BY}	0.0033	0.0002	0.406
Δ6 20:3/18:2	0.65 ± 0.03^{B}	0.57 ± 0.00^{B}	0.71 ± 0.08^{A}	0.73 ± 0.05^{A}	0.47 ± 0.02^{C}	0.39 ± 0.02^{C}	<0.0001	0.242	0.513
Δ5 20:4/20:3	27.4 ± 0.9^{B}	29.1 ± 1.0^{B}	26.3 ± 5.5^{B}	24.7 ± 1.8^{B}	41.2 ± 0.9^{A}	50.8 ± 0.6^{A}	<0.0001	0.192	0.172

Values are means ± SEM, *n*=8 per group.

Differences among groups ($P<0.05$) are identified by different superscripts: $^{A>B>C}$ for main effect of diet, $^{X>Y}$ for genotype effect.

Δ9 (16:1 n-7/16:0×100), Δ6 (20:3 n-6/18:2 n-6) and Δ5 (20:4 n-6/20:3 n-6).

Figure 6.1.

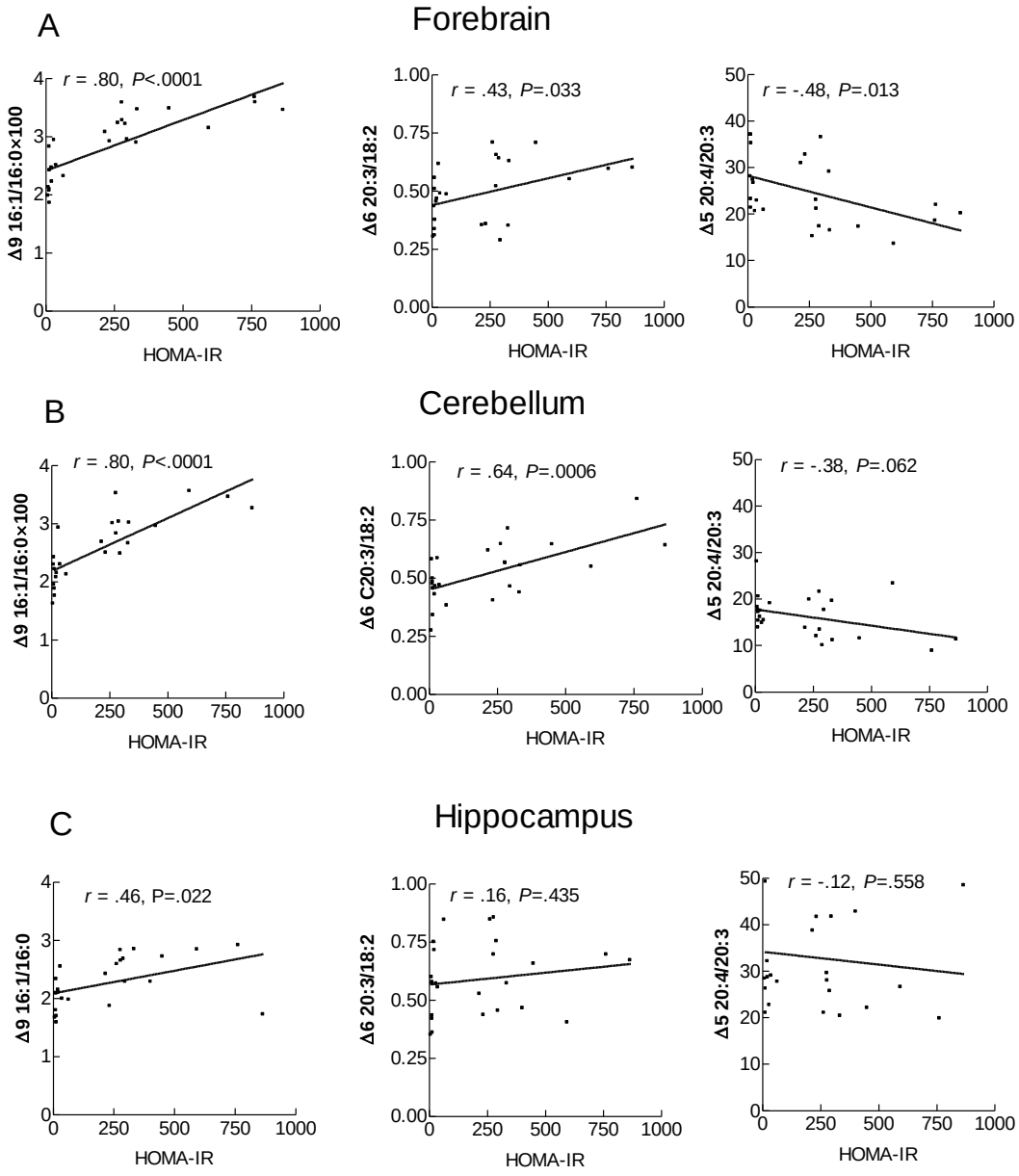
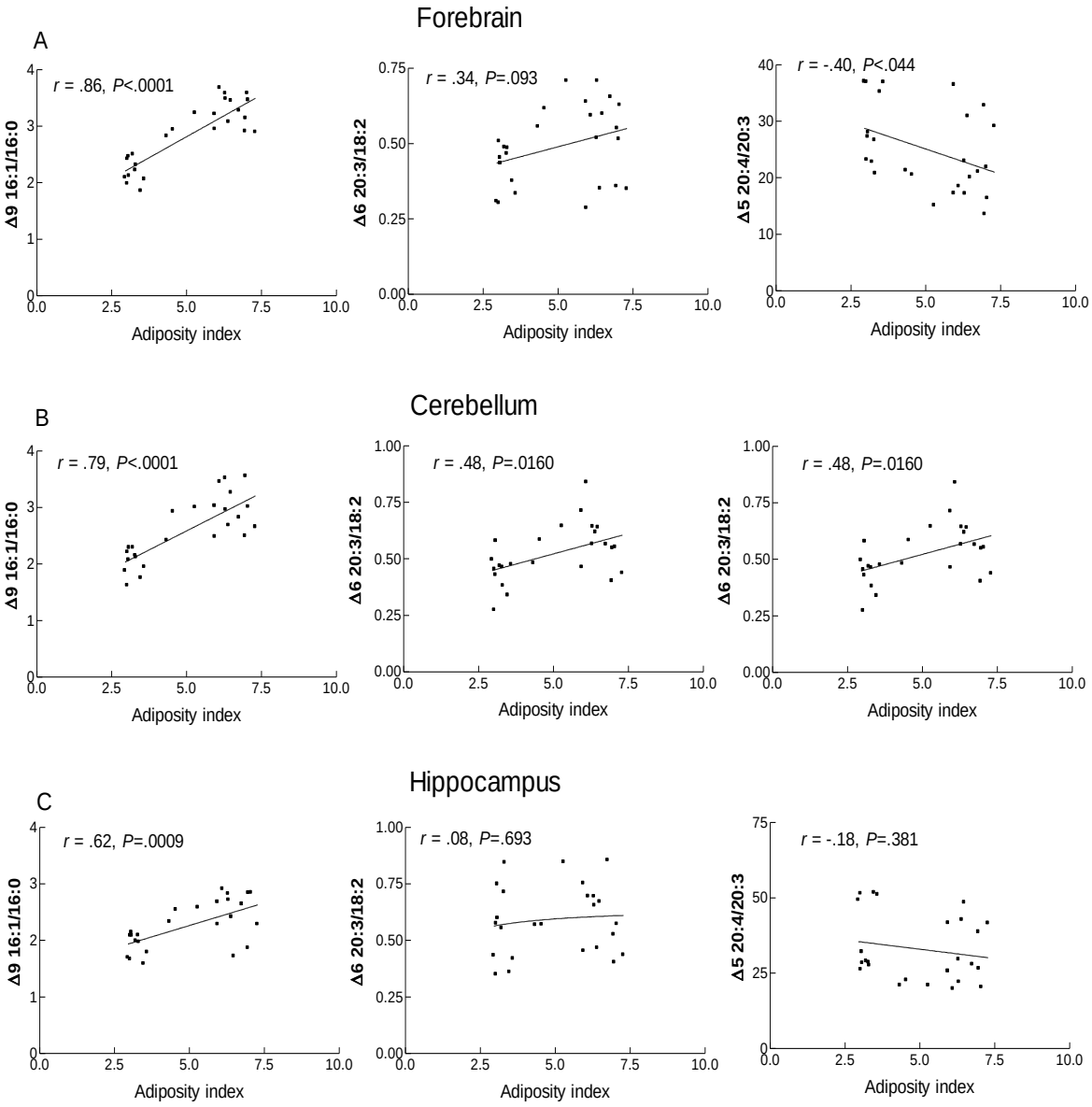

A
Forebrain
r = .80, P<.0001
Δ9 16:1/16:0×100
r = .43, P=.033
Δ6 20:3/18:2
r = -.48, P=.013
Δ5 20:4/20:3
HOMA-IR
B
Cerebellum
r = .80, P<.0001
Δ9 16:1/16:0×100
r = .64, P=.0006
Δ6 C20:3/18:2
r = -.38, P=.062
Δ5 20:4/20:3
HOMA-IR
C
Hippocampus
r = .46, P=.022
Δ9 16:1/16:0
r = .16, P=.435
Δ6 20:3/18:2
r = -.12, P=.558
Δ5 20:4/20:3
HOMA-IR

Figure 6.2.


Forebrain
A
r = .86, P<.0001
r = .34, P=.093
r = -.40, P<.044
Δ9 16:1/16:0
Δ6 20:3/18:2
Δ5 20:4/20:3
Adiposity index
Cerebellum
B
r = .79, P<.0001
r = .48, P=.0160
r = .48, P=.0160
Hippocampus
C
r = .62, P=.0009
r = .08, P=.693
r = -.18, P=.381

BRIDGE 5

Endogenous conversion of precursors is vital in providing AA and DHA into tissues, because in the Western diet LA intake is about 20-fold greater than that of AA, and ALA intake is about 20-30 fold greater than DHA intake (Yogev et al., 2007). Normal brain AA and DHA is required for optimal brain function; this is guaranteed by the ample capacity in synthesizing AA and DHA in adult liver. However, in humans below normal blood levels of DHA are common in liver disease, diabetes and aging (Brenner, 2003; Watanabe et al., 1999). The study reported in Chapter 6 revealed that forebrain DHA was lower (P=0.011) in the *fa/fa* young rats when fed flaxseed but not different when fed menhaden or safflower oil diets, even with lower Δ5 ($P \leq 0.006$) desaturase indices. If the lower forebrain DHA in this study is a general phenomenon in obese insulin-resistant individuals, this would be a significant public health concern. The possible compromised cognitive function and suboptimal learning ability would greatly affect the potential of youth (Gibson et al., 2002). Cognitive impairment has long been thought to be irremediable and terminal; however, increasing understanding of its associations with common and modifiable conditions such as insulin resistance and DHA status will challenge these assumptions.

CHAPTER 7. SUMMARY AND CONCLUSION

Four main hypotheses are proposed in this thesis. The first hypothesis was that offspring of diabetic rat dams have lower AA status that is associated with neurobehavioral deficit. Maternal AA supplementation improves offspring neurodevelopment. A rodent study thus has been carried out to investigate the effect of maternal diabetes and AA supplementation on offspring neurodevelopment.

The first manuscript (**Chapter 3**) reported the study result up to post-natal day 29 (Zhao et al., 2009). In this study, although the dams underwent insulin treatment, the pups from poorly-controlled diabetic dams had significantly lower liver AA than pups from good-controlled diabetic dams at day 14 of age. This novel finding expands knowledge of the effect of experimental diabetes on AA metabolism as not only do diabetic-rats have greatly depressed AA status in a number of organs and serum (Brenner, 2003; Brenner et al., 2000; Clark et al., 1983; Dang et al., 1988; Faas et al., 1983; Gudbjarnason et al., 1987; Holman et al., 1983; Hu et al., 1994; Hurtado de Catalfo et al., 1998; Lin et al., 1985; Ramsammy et al., 1993), but such reductions in AA status are also observed in the offspring. This observation aligns with those of a growing number of human studies reporting that maternal diabetes during pregnancy reduces newborn AA status (Ghebremeskel et al., 2004; Min et al., 2005; Thomas et al., 2006). In these studies, in women with type 1 diabetes with nearly optimal glycemic control (A1C 4-6%), the cord plasma AA was decreased. Thus both mother and infant

under the diabetic scenario are at risk for lower AA status even with a high degree of blood glucose control. In this rat study, maternal dietary AA (0.5% fat) supplementation during pregnancy and lactation, however, elevated AA status in all offspring at days 14 and 29 of age, indicating enhanced maternal transfer from conception to weaning despite our low amount of dietary AA.

A treatment effect was observed in day 14 liver AA, as STZ-PC offspring had lower liver AA than STZ-GC offspring. There was no difference in brain AA and DHA among groups at any tested time. In some ways this data relates to that of others in humans and animals. For example, fetal adipose tissue contains much higher AA and DHA than maternal adipose tissue; and 50 times more LC-PUFA is stored in the adipose tissue than is deposited in the fetal brain during *in utero* life. Adipose was not harvested in this thesis research and would be required in new studies to establish if whole body LC-PUFA is compromised in IDM. For the pregnant women, the reserved LC-PUFA in the adipose tissue can be mobilized for supply to the fetus during pregnancy (Bakewell et al., 2006). For the newborns, the adipose store of LC-PUFA plays a big role to support brain and retinal development during the critical first months of postnatal life since the capacity of synthesis is low. This is also true in rats, although in rats the development of adipose tissue occurs after birth, in contrast to the active intrauterine development of adipose tissue occurring in humans (Herrera et al., 2000). Lefkowitz (Lefkowitz et al., 2005) reported that 8-d-old rat pups were fed formula with deuterium-labeled ALA (d5-ALA) as the only source of n-3 fatty acids. At 8 d, the rat brains contained ~1.6 mg of DHA. After 3 wk of feeding, the brain contained an additional 4.0 mg of DHA (5.6 mg total DHA), including

2.4 mg (60%) deuterium labeled and 1.6 mg (40%) unlabeled. This study suggests that body stores had been an important source of brain DHA in rats resulting in preservation of brain. Future studies in IDM should examine other tissues for DHA content to establish if mobilization of DHA from stores is adequate to support brain DHA.

As for lower liver AA vs. brain AA status, Lefkowith et al (Lefkowith et al., 1985) suggested that the liver supplies AA to other tissues when the animal is deprived of essential fatty acids. Thus the delay in the reduction in levels of AA in the brain, kidney, heart, testis, and spleen might be caused, in part, by the secondary effects of lipid modification in the liver. They proposed that three types of tissues can be identified in terms of their ability to retain AA. The first type shows a rapid decline in the level of AA, for example, liver. The second type retains relatively high levels of AA, for example, heart, kidney and testis. The third type retains AA to a greater extent under AA deficient conditions, for example brain. This hypothesis would help explain the lower liver but not brain AA at d14 and d29 of STZ-PC offspring vs. STZ-GC offspring. The ability of synthesis AA is significantly increased along with rats grow up, thus there is no difference in 12 week rats between treatment and dietary groups.

The most significant finding of this study was that AA supplementation in both healthy and poorly-controlled diabetic dams improved neurobehavioral performances in the offspring, particularly in the Morris water maze test. Without the supplement, the offspring of the poorly-controlled diabetic dams had neurodevelopmental delay as indicated by the 57% reduction in performance compared to the offspring of Saline-Placebo dams in the day 18 rota rod standard

test. Additionally, they took over twice the time to complete the negative geotaxis on day 7.

A concern regarding supplementing AA alone is that it might affect the maternal and/or offspring DHA status (Weiler et al., 2002). Such concern is not supported however by this study since the dams receiving dietary AA supplementation during pregnancy improved liver DHA in day 3 pups of the good-controlled diabetic dams and day 14 pups of the poorly-controlled diabetic dams. Otherwise there was no significant difference in DHA status of liver and brain between AA supplemented and non-supplemented groups in pups and dams. Thus, this study provides evidence that maternal AA supplementation alone at low dose is feasible and effective in promoting the offspring developmental outcomes dependent on brain function, at least in an experimental model.

Dietay supmentation of AA is feasible as studies have shown AA is well absorbed by infants, young women and men. Low-dose AA supplementation (80 mg/d, equivalent to the amount in one egg) for 3 weeks increased AA, but did not decrease DHA, in RBC and plasma phospholipids in young women (Hirota et al., 2010). Similarly AA supplementation (838 mg/d) for 2 week in men increased serum AA content by 142% for 4 weeks without affecting serum DHA content (Kusumoto et al., 2007).

To evaluate fatty acids absorption, a 3 day metabolic balance study was conducted in 3 week old term and preterm (34.2 weeks) infants on different formulas (Moya et al., 2001). The intestinal absorption of fatty acids is high and is comparable in terms and preterms (92.0% vs. 93.0%). LA and ALA were absorbed at 94%; AA at 75.0 %; and DHA at 62.3%. Similar results have also

been observed in Carnielli's study, as the absorption of AA was 82.1% and DHA was 82.4% in 4 week old preterm (28.2 weeks) infants (Carnielli et al., 1998).

LC-PUFA, mainly n-3 LC-PUFA, has been used in type 1 and type 2 DM (Hendrich, 2010). Garg et al reported (Garg et al., 2007) that consumption an n-3 LC-PUFA enriched dip significantly increased plasma DHA by 80%. AA is easier than DHA to be absorbed as longer acids were less well absorbed than shorter acids (Moya et al., 2001). It shouldn't be a problem for the newborn absorption of AA since AA and DHA has been added in infant formula (Koletzko et al., 2008).

In this study, alterations in AA status were observed in conjunction with compromised performance in neurodevelopmental tests such negative geotaxis in 7 day old pups and rota rod in 18 day old pups. This is similar to humans whereby infants of mothers with diabetes prior to pregnancy have significantly lower scores on the Bayley Scales of Mental (91.0 vs. 98.5, $P<0.05$) Development Index and Psychomotor (85.2 vs. 95.5, $P<0.05$) Development Index than do infants of mothers in the nondiabetic group at 1 year of age (Levy-Shiff et al., 2002). Suboptimal performances on neurodevelopmental tests, and lower psychomotor performance score and IQ have been reported in other studies of infants (Rizzo et al., 1994) and children (Rizzo et al., 1997; Yamashita et al., 1996) of diabetic mothers. It is therefore suitable that the first hypothesis be accepted; the imbalance between maternal supply and fetal demand for AA during the perinatal period may contribute to the cognitive impairment and/or behavioral disorders in children of diabetic mothers (Ghebremeskel et al., 2004). Maternal AA (0.5% of fat) supplementation through pregnancy and lactation improved

offspring neurodevelopment. These findings are significant in light of the challenges in achieving optimal control of glucose.

The second hypothesis was that the neurobehavioral deficit in the offspring of diabetic dams persists to young adult age, and maternal AA supplementation has a long-lasting beneficial effect on offspring neurodevelopment. For the short-term study, half of the offspring of diabetic rats were euthanized for weaning fatty acid analysis, the other half were transferred to a regular chow for a long-term assessment for up to 12 weeks of age. The manuscript (**Chapter 4**) reported the results (Zhao et al., 2011).

A novel finding of this study was the positive correlation between dams' gestational glucose concentration and the initial water maze escape latency of young adult offspring. Adult (12 week) male and female offspring prenatally exposed to hyperglycemia at 13 to 20 mmol/L (poorly-controlled rat dam) required more time to find the hidden-platform than did control offspring on the first day of testing, while the offspring exposed to hyperglycemia <13 mmol/L (good-controlled rat dam) were intermediate. Escape latency in the water maze is generally considered to be a parameter reflecting the spatial learning and cognitive capacity (Morris et al., 1982). Poor performance during initial exposure to the problem suggests a deficient search or problem solving capacity. These data are consistent with previous reports that rats prenatally exposed to cocaine are capable of exhibiting a variety of learned behaviours, ample training provided (Heyser et al., 1995). This is also consistent with infant studies (Riggins et al., 2009) as impairments occurred at the initial stage of memory processing when cognitive demands were high; when cognitive demands were moderate, there

were no observable differences in performance of controls and infants of diabetic mothers. The dysfunction appears to be modulated by the nature and extent of the demands placed on the circuitry of memory (de Haan et al., 2006). The hippocampus, which is central to recognition and recall memory function, undergoes a growth spurt with a significant amount of LC-PUFA deposition during late fetal life and is particularly vulnerable to metabolic abnormalities (Georgieff, 2008).

The other key observations of this study were the long-lasting benefits of maternal AA supplementation on offspring neurodevelopmental test performance regardless of diabetes. In rota rod and water maze tests, the offspring of dams fed an AA supplemented diet during reproduction performed consistently better. This is similar to previous studies where AA supplementation prevented the age-related AA decline (~20% decrease compared to the young control) in the hippocampus (McGahon et al., 1997) and improved the performance in a water maze test in aged rats (Kotani et al., 2003). Mice fed very high DHA without AA during both prenatal and postnatal periods showed a significantly longer latency to escape on water maze cue trial at 11 weeks of age; this adverse effect is overcome by adding AA in the diet (Wainwright et al., 1997).

Diabetes in pregnancy creates a suboptimal AA supply for fetal development, along with other diabetes-related disturbances (Siddappa et al., 2004). Like DHA, AA is vital to brain development and the consequence of the disturbances in the supply of AA during development is significant as major developmental changes of neurobiological processes occur during prenatal life (Hadders-Algra et al., 2007). The short-term study has revealed that maternal AA

supplementation improves neurodevelopment of offspring (up to day 29) of poorly-controlled (glucose > 13 mmol/L) diabetic dams (Zhao et al., 2009). This long-term study extends our previous observation of the beneficial effect of maternal AA supplementation on offspring neurodevelopment from weaning (Zhao et al., 2009) to young adulthood, in the absence of dietary effects in brain fatty acid composition. These changes are significant and consistent with typical tissue changes of newborns in low AA status.

The fetus depends on maternal supply of AA and DHA and accretes high amounts of AA and DHA in the brain, as well as in the adipose tissue (50 times more AA and DHA in adipose tissue) (Haggarty, 2010), thus the newborn pups of untreated diabetic dams should have lower AA status than control pups. In this model, daily insulin treatment was used. Thus, their pups may have compromised AA status but perhaps not as low as those pups from untreated dams. In addition, after birth without maternal AA and DHA supply, there is a decline of AA and DHA status in the infant. Indeed, Leaf et al (Leaf et al., 1992) have observed a one-third reduction in the relative proportion of AA despite a three-fold increase in the precursor LA in plasma of neonates between birth and three weeks of age. This may help explain why there is not lower liver AA at day 3 but a lower liver AA at day 14. As for lower liver AA vs. brain AA status, Lefkowith et al (Lefkowith et al., 1985) suggested that the liver supplies AA to other tissues when the animal is deprived of essential fatty acids. Thus, the delay in the reduction in levels of AA in the brain, kidney, heart, testis, and spleen might be caused, in part, by the secondary effects of lipid modification in the liver. They proposed that three types of tissues can be identified in terms of their ability to retain AA.

The first type shows a rapid decline in the level of AA, for example, liver. The second type retains relatively high levels of AA, for example, heart, kidney and testis. The third type retains AA to a greater extent under AA deficient conditions, for example brain. This hypothesis would help explain the lower liver but not brain AA at day 14 and day 29 of STZ-PC offspring vs. STZ-GC offspring. The ability of synthesizing AA is significantly increased with age (Makrides et al., 1994), thus there is no difference in AA of 12 week rats among the maternal treatment and dietary groups. It is also possible that STZ-PC offspring may have lower brain AA than STZ-GC offspring at day 14 of age in specific phospholipids or brain regions but it was not observed as only total fatty acids rather than phospholipids were measured and in a small sub-group to enable the longer term assessments.

AA has been extensively studied for its role in growth and development (Aaes-Jorgensen, 1961), recently, experts (Bazinet, 2009; Hadders-Algra, 2008) have proposed that the importance of AA in the brain may be overlooked. Early studies of the role of PUFA have been largely thought to be structural, increasing the fluidity of cellular membranes (Hansen et al., 1946), and mainly focused on DHA, because autopsy analyses have found lower DHA but not AA in the brain of infants who had been fed formula without LC-PUFA (Farquharson et al., 1992; Makrides et al., 1994). In addition, the higher concentration of DHA in brains of breast-fed infants may explain the superior neurodevelopment reported in breast-fed compared with formula-fed infants (Makrides et al., 1994). However, less is known about the effect of the infant's neonatal fatty acid status on neurodevelopmental outcome. Even though both AA and DHA are important to

neurodevelopment (Saste et al., 1998), brain accretion of AA exceeds that of DHA during gestation (Hadders-Algra, 2008), a period that is crucial to brain development. Recent evidence indicates that AA status, peri-natal in particular, is related to short and possibly long-term neurologic outcome (Groen et al., 2005; Hadders-Algra, 2008; Hadders-Algra et al., 2007). Infants with lower AA (13% vs.12%, *P*=0.03) at birth in cord blood had mildly abnormal movements at 3 months (Bouwstra et al., 2006) and persisted to 12 weeks of age (van Goor et al., 2010). The occurrence of this abnormal neurologic outcome was related to maternal supplementation of DHA but was not seen when DHA was combined with AA during pregnancy and lactation (van Goor et al., 2010). The presence of the mildly abnormal movements in infants is associated with an increased prevalence of minor neurologic dysfunction and attention deficit at school age (Groen et al., 2005). This could thus imply that during neonatal life, a period of rapid proliferation of the glial populations and formation of neuronal synapses (Svennerholm et al., 1989), the supply of AA to the fetus is more critical than the supply of DHA. It has been proposed that AA might have critical developmental implications early in fetal development whereas DHA would have continued effects on brain. Currently, brain AA has been intensively studied in neonates and young children (Hadders-Algra, 2008), while optimal brain DHA are of more concern in infancy, older children, adults and seniors.

In addition, several major breakthroughs, including the discovery of prostaglandins and the role of AA as a secondary messenger in signal transduction, have advanced our understanding of the importance of AA in the brain. AA is a precursor to a host of signaling molecules known as eicosanoids,

which include thromboxanes, leukotrienes, prostacyclins, and prostaglandins (Stroud et al., 2009). AA and its end-products are involved in physiological processes in the central nervous system (Bazan, 2005; Maekawa et al., 2009). Altered brain AA metabolism has been associated with a number of neurological, neurodegenerative, and psychiatric disorders (McCown et al., 1997).

Thus, the second hypothesis that the neurobehavioral deficit in the offspring of diabetic rats persisted to young adult age and maternal AA supplementation have long lasting benefit is also accepted.

The third hypothesis was that young adult offspring of diabetic rat dams have higher incidence of IGT and compromised bone growth. Maternal glucose control and AA supplementation were beneficial to offspring glucose tolerance and bone health. For this hypothesis the results are reported in manuscript 3 (**Chapter 5**) (Zhao et al., 2010).

The most novel observation of this study was the long-term adverse relationship between maternal hyperglycemia and skeletal size of adult offspring. Those offspring from poorly-controlled diabetic dams had significantly smaller bone areas, synonymous with bone size, of whole body, femur and tibia than offspring from diabetic dams in good-control. The maternal glucose concentration during pregnancy inversely related to offspring whole body bone mineral content and bone area at 4 weeks of age and persisted to 12 weeks for tibia area in male rats. This is particularly important in view of the fact that peak bone mass is considered well established by 12 weeks of age in rats (Sengupta et al., 2005) and therefore suggests that skeletal size is programmed by fetal exposure to maternal hyperglycemia. Similarly, human infants born to mothers

with diabetes have low bone mineral content at the distal radius (Mimouni et al., 1988) and osteoblast activity (Verhaeghe et al., 1995) and bone mineral content inversely related to maternal glucose (Mimouni et al., 1988). Furthermore, altered calcium and magnesium homeostasis exists in newborn infants of mothers with diabetes (Mimouni et al., 1988; Tsang et al., 1972; Verhaeghe et al., 1995) that persists beyond infancy to at least 16 weeks in rats (Bond et al., 2005) and 10 years in children (Mughal et al., 2005). The current study added important new information that maternal hyperglycemia has long-term consequences to bone size of the offspring, implying an altered growth trajectory.

Offspring from AA-supplemented dams had higher liver AA at 4 weeks, while liver AA at 4 weeks was positively correlated with lumbar spine mineral density in males, but this association did not persist to 12 weeks.

In this long-term study, maternal diabetes and diet did not affect glucose tolerance in the offspring when measured at 8 and 12 weeks postnatally. Thus, the third hypothesis that maternal glucose control and AA supplementation were beneficial to offspring bone health is also accepted. But the results did not support the hypothesis on glucose tolerance as maternal diabetes and diet did not affect glucose tolerance in the offspring when measured at 8 and 12 weeks postnatally.

The fourth hypothesis was that forebrain DHA can be maintained by liver when DHA was absent from the diet but adequate in ALA in obese insulin-resistant young rats. In the manuscript 4 (**Chapter 6**) (Zhao et al., 2010), after feeding the experimental diet for 9 weeks, the 15-week-old *fa*/*fa* rats was a

suitable model for study obesity and insulin resistance (56% heavier and >18-fold in HOMA-IR than lean).

The main novel finding of this study was that lower forebrain DHA was observed in obese insulin-resistant young rats fed a diet with ample ALA (35.5 g/kg diet) but without DHA for 9 weeks, accompanied by lower forebrain Δ5 desaturase activities than lean rats. In contrast, normal brain DHA has been reported in healthy adult rats fed a diet with much lower ALA (4.6 g/ kg) in the absence of DHA (Igarashi et al., 2007), supported by high synthesizing capacity as the rate of DHA synthesis by liver was about 10 × the DHA consumption rate in brain tissue (Igarashi et al., 2007; Rapoport et al., 2010).

Endogenous conversion of precursors is vital in providing AA and DHA into tissues, because in the Western diet LA intake is about 20-fold greater than that of AA, and ALA intake is about 20-30 fold greater than DHA intake (Yogev et al., 2007). Normal brain AA and DHA is required for optimal brain function; this is guaranteed by the ample capacity in synthesizing AA and DHA in adult liver. However, in humans below normal blood levels of DHA are common in liver disease, diabetes and aging (Brenner, 2003; Watanabe et al., 1999). Such reductions are postulated to be associated with reduced desaturase or elongase activity and are a risk factor for brain disease in the absence of dietary DHA supplementation (Rapoport et al., 2009). If the lower forebrain DHA in this study is a general phenomenon in obese insulin-resistant individuals, this would be a significant public health concern.

As overt AA and DHA deficiency is a rare and extreme condition, now much of the work on LC-PUFA in nutrition has focused on relatively subtle

effects on clinical outcomes such as intelligence quotient and academic achievement (Krukowski et al., 2009). The possible compromised cognitive function and suboptimal learning ability would greatly affect the potential of youth at school, work, social, and family situations (Gibson et al., 2002). The possible combined burden of the obesity epidemic and insulin resistance in youth is thus concerning beyond the standard chronic disease concerns (Hebert et al., 2001). Cognitive impairment has long been thought to be irremediable and terminal; however, increasing understanding of its associations with common and modifiable conditions such as insulin resistance and DHA status will challenge these assumptions. Thus, the 4th hypothesis was accepted that the ALA may not have potential to effectively serve as a precursor for synthesizing DHA for youth forebrain during insulin resistance since Δ5 desaturase activity is limited.

Although convincing studies, including both human and rats, reported that offspring of diabetic mothers have higher rates of IGT (Dabelea et al., 2008; Han et al., 2007; Holemans et al., 1991; Metzger, 2007; Thamotharan et al., 2003), no difference was observed in the glucose tolerance testing in the offspring between Saline-Placebo dams and diabetic dams. As mentioned in Chapter 4 (Paper 3), it is possible that the proven method for OGTT (Kawa et al., 2003) was not sufficient (1 g glucose/kg body weight) to detect early signs of diabetes in comparison to other studies that used higher dosages and bypassed the gastrointestinal tract (2 g/kg i.p. or i.v.) (Han et al., 2007). Future studies needed to confirm these observations using higher dosages or more sensitive tests such as the euglycaemic-hyperinsulinaemic-clamp that readily identified glucose intolerance in 12 week old STZ-offspring reared by the biological dams

(Holemans et al., 1991). It might need longer duration (>12 weeks) or larger sample size.

In this thesis the AA dietary intervention study was carried out in STZ-induced diabetes which resembles mainly type 1 diabetes. The increasing prevalence of type 2 DM in women of childbearing age leads to an increasing number of pregnant women with type 2 DM. Pregnancy complicated by type 2 DM is a high-risk pregnancy, associated with birth defects and high perinatal mortality to the same extent as in type 1 diabetes (Hieronimus et al., 2004). Thus, similar intervention studies may be needed in a type 2 diabetic rat dam model. Although the results of this study are promising, there are many questions that need to be addressed before applying these results to humans. Compared with animal studies, there is a wider variation in humans in terms of dietary intake of LA and AA, treatment options and age, and complications caused by diabetes or pregnancy. Thus target groups need to be determined. In the current study, 0.5% of fat as AA improved development in offspring of dams with and without diabetes, while the dosage may not be suitable to humans although studies shown this dosage was safe to both mother and offspring. Furthermore, it is very similar to the amount used in a human study (Otto et al., 2000). In that study, 0.26 g of AA and 0.57 g of DHA were provided daily to pregnant women and assuming women consume 67 g fat daily, the proportion of AA was ~0.4% of the fat. This 4 week supplement (started in the 2nd trimester) proves to be effective in increasing the DHA levels in both plasma and erythrocytes without a concomitant decline of AA. Furthermore, prenatal LC-PUFA availability, which can be influenced by maternal dietary DHA intake during pregnancy, can have an effect

on quality of movement in later life (Kruger et al., 2010). However, the dosage for the potential study in human with diabetes would be higher as these diabetic women have lower endogenous synthesis, compromised body stores and inability to increase the conversion rate during the 3rd trimester.

It would also be worthwhile to consider other nutrients altered in the course of diabetes during pregnancy and possible nutrient-nutrient-disease interactions. For example, in a pilot study, the STZ/PC offspring had higher hemoglobin than Saline-Placebo offspring (postnatal day 3: 16.79 vs.4.49; day 14: 19.07 vs.10.12; day 29: 14.46 vs.11.52 g/dL). This is in accordance with the hypothesis that offspring of diabetic mothers are at risk for perinatal brain iron deficiency. Pedersen hypothesized that maternal hyperglycemia results in fetal hyperglycemia (Macfarlane et al., 1988). Chronic fetal hyperglycemia and hyperinsulinemia increase fetal basal metabolic rate and oxygen consumption by up to 30% in a relatively hypoxic intrauterine environment. To increase oxygen-carrying capacity, the hypoxia stimulates compensatory fetal erythropoiesis and thus expands the fetal red blood cell mass. It has been found that erythropoietin was elevated in umbilical plasma at delivery of IDM (Widness et al., 1981). Polycythemia, defined as central hemoglobin concentrations more than 20 g/dL and hematocrit level more than 65%, is present in 20% to 30% of infants of diabetic mothers, at birth (Nold et al., 2004).

There are studies regarding the effect of iron deficiency on neurobehavioral tests, and their results have been used as mechanic explanations for the effect of diabetic pregnancy on offspring neurodevelopment. These studies, however, were conducted in an iron deficiency model rather than a

diabetic model. Such study is needed in exploring the possible role that iron deficiency might play in the compromised offspring neurodevelopment of diabetic pregnancy in the STZ-diabetic model. This experimental study will clarify the role of AA and iron in offspring of diabetic mothers and as the groundwork leading to future intervention trials in humans.

Children of diabetic mothers have higher risk of obesity, insulin resistance and type 2 DM. Academic achievement (Krukowski et al., 2009) and intelligence (Yu et al., 2010) are lower in obese children. In response to this crisis, the Zucker rat study was conducted. In this Zucker model, forebrain DHA was lower than lean rats. Further research is warranted using the STZ-diabetic rat dam model, may be through a diet manipulation model to yield offspring that are obese and insulin resistance.

In this thesis a series of novel findings were made regarding effect of diabetic pregnancy on neurobehavior, glucose tolerance, and bone growth on the offspring. Elucidation of the mechanism(s) is needed through further study. DHA is a major component of neuronal membranes. In rats, low brain levels of LC-PUFA during development produce alterations in the mesocortical and mesolimbic dopamine systems, which is consistent with the frontolimbic dysfunction believed to occur in schizophrenia. Of note, such animals exhibit decreased densities of dopamine-immunoreactive vesicles, the vesicular monoamine transporter, and the dopamine receptor in frontal cortex suggesting hypoactivity of the mesocortical projection (Levant et al., 2004). It has reported that suboptimal fetal/neonatal AA and DHA supply alters the genomic expression, neurometabolism and electrophysiology of the hippocampus during the period of

infancies and, strikingly, in adulthood (Georgieff et al., 2005). These areas require further study.

Determination of bone biomarkers such as plasma osteocalcin, insulin-like growth factor 1 (IGF-1) and parathyroid hormone (PTH) concentrations or urinary N-telopeptide excretion are required to explore the physiological mechanisms that explain the small bone size in offspring of STZ/PC offspring. Cellular mechanisms such as osteoblast and osteoclast proliferation by which LC-PUFA affect bone should also be sought (Kruger et al., 2010). In addition, bone strength assessments are also important in exploring the effect of diabetic pregnancy on offspring bone health.

In conclusion, the studies in this thesis demonstrated that offspring of diabetic rat dams have lower AA status even though dams were treated daily with insulin throughout pregnancy and lactation. Maternal hyperglycemia has longstanding consequences to neurobehavioral development in both sensory-cognitive and psychomotor functions in the offspring. Maternal AA supplementation and training positively influence learning outcomes. Maternal glucose control has long-term consequences to bone health of adult offspring; their skeletal status appears more sensitive to maternal hyperglycemia than glucose tolerance. For the obese insulin-resistant young rats, dietary DHA may be required in order to maintain normal forebrain DHA as its synthesis from precursors was limited by impairement in $\Delta 5$ desaturase activity. The STZ study has some limitations including the negative finding on the glucose tolerance testing in the offspring of STZ-diabetic dams, further study is warranted.

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