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Impact of maternal iron deficiency on cortisol levels and auditory brainstem responses in the young and adult guinea pig

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ABSTRACT

Maternal iron deficiency is a world wide and major public health issue. Despite recent researchers' interest related to this topic, its impact in the offspring still remains unclear. The aim of this study is to understand the impact of maternal iron deficiency on the auditory functions and serum cortisol levels in the young and adult guinea pig at post-natal day (PNd) 24 and PNd84, respectively. Pregnant guinea pigs were given an iron deficient (ID) or iron sufficient (IS) diet during gestation and lactation. An iron sufficient diet was provided to all pups after weaning day. No significant difference was observed in the hearing threshold and latencies in siblings from both groups at PNd24 and PNd84. However, ID offspring showed a significant higher interpeak latency I-IV at 100 dB than IS pups at PNd24. ID offspring also had significant elevated cortisol levels at PNd24 compared to IS control group. Maternal iron deficiency affects negatively the auditory functions and raises the serum cortisol levels, a biomarker of stress in the offspring.

RÉSUMÉ

La déficience en fer maternelle est un problème de santé publique mondial. Malgré l'intérêt des chercheurs lié à ce sujet, son impact sur la progéniture n'est pas clair. Le but de cette étude est de comprendre l'influence de la carence en fer maternelle sur les fonctions auditives et les taux sériques de cortisol chez le jeune cochon d'Inde jusqu'à l'âge adulte, voire au jour post-natal (PNd) 24 et 84. Les femelles enceintes ont été nourries d'une diète déficiente (ID) ou suffisante en fer (IS) pendant la gestation et la lactation. Leur progéniture a été nourrie d'une diète IS après le jour de sevrage. Aucune différence significative n'a été observée dans le seuil auditif et les latences chez la progéniture des deux groupes au jour 24 et 84. Cependant, la progéniture ID a montré une latence inter-pic (IPL) I-IV à 100 dB significativement supérieure que le groupe IS au jour 24. La progéniture ID a également montré un cortisol significativement plus élevé au jour 24 versus le groupe IS. La carence en fer maternelle affecte négativement les fonctions auditives et augmente les taux de cortisol, utilisé comme biomarqueur de stress chez la progéniture.

PREFACE

This thesis includes two articles for which I'm the first author. My meaningful contribution to the research project directed by France Rioux, Ph.D. includes the animal care technicians, the auditory brainstem response assessments, with the help of our laboratory technician, Mohamed Thabet, the cortisol and hematocrit measurements, data compilation and analysis. Sylvain Fiset, Ph.D. supervised statistical analysis.

Chapter IV The impact of maternal iron deficiency on the serum cortisol in the guinea pig offspring
This article is under review for publication in the journal of *Nutrition Research*

Chapter V The impact of maternal iron deficiency on the auditory function in the young and adult guinea pig
This article is accepted with Revisions for publication in the journal of *Nutritional Neuroscience*

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DEDICATION

This thesis is dedicated in memory of my beloved brother Jamal who always believed in me. He encouraged me during my entire life to pursue my academic studies until I reach the end of it. He always told me: “Nora, don’t give up now! You’re almost at the edge of the pyramid. Keep climbing and don’t let go! Don’t give up when you’re tired, but when you’re done!”

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LIST OF ABBREVIATION

ABR	auditory brainstem response
ACTH	adrenocorticotropic hormone
ADHD	attention deficit and hyperactivity disorder
ADNA	adrenal glands
ANOVA	analysis of variance
BW	body weight
CBC	complete blood count
CDC	centers for disease Control and prevention
CNS	central nervous system
CRH	corticotropin-releasing hormone
DALY	disability-adjusted-life-years
dB	decibel
DRIs	dietary reference intakes
Fe	iron
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
GA	gestational age
GCs	glucocorticoids
GI	gastrointestinal
Hb	hemoglobin
Hct	hematocrit
ID	iron deficient
IDA	iron deficiency anemia
IDD	iron deficient diet
IPL	interpeak latency
HPA	hypothalamic pituitary adrenal
HPC	hippocampus
IS	iron sufficient
ISD	iron sufficient diet

kHz	kilo hertz
LID	latent iron deficiency
Mb	myoglobin
MCV	mean corpuscular volume
MID	maternal iron deficiency
MSC	maternal serum cortisol
PCV	packed cell volume
PNd	post-natal day
PSM	psychosocial stress model
RDA	recommended dietary allowance
WHO	world health organization
11β-HSD2	11β-hydroxysteroid dehydrogenase type 2

CHAPTER I

INTRODUCTION

Iron deficiency anemia (IDA) is the most common nutrient disorder worldwide affecting 1-2 billion people (1,2). Pregnant women and children are adversely affected by IDA whose global prevalence rates are 38% and 43% respectively (3). Iron deficiency is the most common micronutrient deficiency in children and causes of anemia (1,2,4). Anemia increases the risk of maternal and perinatal death during pregnancy, which is a major cause of mortality, causing together between 2.5 million and 3.4 million deaths worldwide. The World Health Organization (WHO) also ranked IDA as the third leading cause of global total disability-adjusted-life-years (DALY) lost for females of childbearing age (5). In developing countries, 50% of pregnant women suffer from IDA and about 10-20% in industrialized nations (6-7). This is an important public health problem even in industrialized countries. Despite iron supplementation during pregnancy, it is estimated that 34% of pregnant US women are anemic and 30% are iron deficient in the third trimester (8-9). It was once thought that the fetus would be protected from maternal iron deficiency (MID). However, today's results show an association between maternal and neonatal iron status, especially if the mother is anemic or iron deficient (ID) (10). According to Centers for Disease Control and Prevention (CDC), vulnerable population should be encouraged to eat food rich in iron, breast-feed or use iron fortified formula for infants to prevent iron deficiency (11).

In pregnant women, the demand in iron increases due to the development of the fetus and insufficient iron storage and limited absorption from food in the woman's body (12). If the demand is not met, complications such as preterm birth, low birth weight, postpartum hemorrhage, decrease of iron storage in neonates and small neonates for gestational age may occur (13,14). Prevention and treatment of IDA are considered very important in prenatal care. Physicians often prescribe iron supplements to pregnant women to

compensate for the increased needs and inadequate iron intake, but not all pregnant women follow iron supplement guidelines because of gastrointestinal (GI) side effects such as nausea, vomiting, diarrhea, constipation, heartburn and abdominal cramps (12,15).

The human body loses about two thirds of the consumed iron through the GI tract and the rest is lost in feces (16). This accumulated loss in the long term will affect the metabolism especially during the anabolic periods. The human adult body dispenses about 0,9-1 mg of iron daily equivalent to 14 g/kg of body mass. Pregnant women should consume about 27 mg of iron daily during pregnancy (16). An amount varying from 30-60 mg/day has been proposed after the 20^{th} week of pregnancy for women with low risk of nutritional deficiency to prevent IDA (13). According to the Dietary Reference intake's (DRIs), pregnant women are recommended to consume 6.4, 18.8 and 22.4 mg of iron per day at the first, second and third trimesters respectively (17). The CDC also recommends a low iron supplement of 30 mg daily at the beginning of pregnancy to help prevent ID or IDA (11). Many women do not maintain an adequate iron status during pregnancy mainly because they don't follow the recommended nutritional guidelines as well as because iron from foods or supplements is often poorly absorbed. IDA during pregnancy occurs if the increased iron requirement is not met.

MID is likely to impair the proper functioning and development of the fetus. In fact, iron plays an important role in several biochemical processes such as transport and delivery of oxygen, growth, cellular differentiation, electro-mitochondrial transfer and numerous enzymatic systems (18,19). Iron is also an essential micronutrient involved in the synthesis of brain neurotransmitters and central nervous system myelination and is a co-factor for enzymes implicated in fatty acid metabolism (20-36). Despite the high prevalence of iron deficiency during pregnancy among Canadian women, its impact on the infant's psychological, behavioural, socio-cognitive, motor and neurophysiological development is not fully understood. Our previous study using the guinea pig as an animal model showed that the offspring from dams fed an ID diet during gestation and lactation had impaired auditory functions compared to their IS control group during

childhood (37). Furthermore, post-natal ID studies found that most children with Attention deficit hyperactivity disorder (ADHD) suffer from low iron or ID used as a strong predictor of this pathology (38-40). However, very few focused on the impact of MID in the prenatal phase on the offspring's development during childhood and adulthood.

Most pre-clinical studies have used the rat or mouse as an animal model in which to study the impact of MID in the offspring (20,27,28,32,33,35). However, brain development in rodents isn't complete at birth and reaches its optimal growth during post-natal phase. In contrast, the guinea pig has a timing of brain development pic that closely resembles that occurring in humans due to its critical development in the prenatal phase (although slightly earlier in guinea pigs) (41). Using the guinea pig seems to be an appropriate model to study the impact of moderate MID during pregnancy, reflecting the current situation in Canada, on brain development of the progeny.

Iron plays a critical role during the gestational period and the intake of iron needs to be monitored more carefully during prenatal development. Its double function in the brain by being involved in the dopaminergic system and the myelination process during development must be better understood. The aim of this study is to understand the impact of maternal iron deficiency on the auditory functions and on serum cortisol used as a biochemical stress marker in the young and adult guinea pig.

CHAPTER II

LITERATURE REVIEW

2.1 IRON

2.1.1 Background and functions

Since the beginning of time, iron has been used for its special properties in health and diseases (42). The Egyptians, Hindus, Greeks and Romans were using it for medicinal use (43,44). Later, people began using iron to treat chlorosis, characterized as a green disease, and often resulting from ID (45). In the 1930's, researchers started giving a lot more importance to iron for its nutritional properties regarding hemoglobin formation and oxygen transport (46).

Iron is an abundant element on earth and is also an essential component to all living creatures (43,47). Despite its high abundance, iron cannot be available for uptake by the body until it becomes soluble in the form of Fe^{+2} (ferrous iron) instead of the insoluble format, Fe^{+3} (ferric iron), due to its contact with oxygen forming oxides (43,48). However, the human body is composed of corresponding counterparts in which iron exists in complex forms (44). It can be either bonded with protein as heme compound (myoglobin-Mb or hemoglobin-Hb), heme enzymes or non-heme compounds (transferrin, ferritin and flavin-iron enzymes).

Iron is essential for the body partly due to its property in synthesis of oxygen transport proteins (Mb or Hb) and its formation of heme enzymes and other enzymes requiring iron involved in electron transfer and oxidation-reductions (44,49).

A healthy man of 70 kg contains about 4 grams (50 mg/kg) of iron (50). Approximately two-thirds of iron is found in hemoglobin and circulating in the form

of erythrocyte in the human body. Another 25% is contained in a readily mobilizable iron store and the last 15% in the myoglobin of muscle tissue and different enzymes playing an important role in oxidative metabolism and other cell functions. In the body, it is transported via transferrin and then stored in ferritin molecules (**Fig.1**). Once absorbed, iron isn't excreted, but mainly recycled and conserved, except during bleeding or menstruation and pregnancy. Only a small amount is lost each day (1-2 mg/day) (16,50,51).

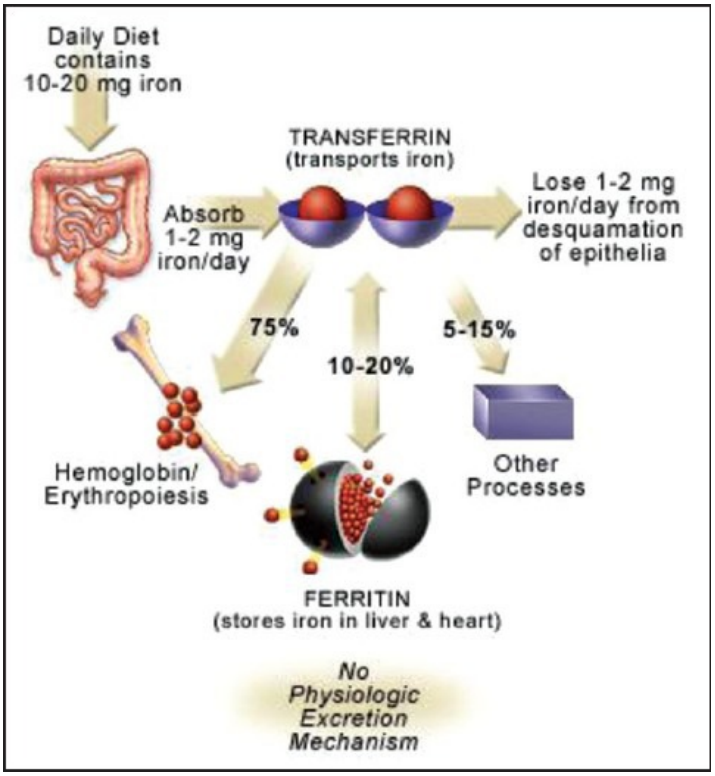

Figure 1 Schematic diagram of iron cycle in the body (51) ***Free*** **©** ***https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3999603/***

2.1.2 Iron bioavailability and metabolism

Dietary iron is found in both heme and non-heme forms. Heme is the most common form of iron found in Hb and Mb from meat, poultry and fish (50-52). Non-heme sources are obtained from cereals, legumes, fruits and vegetables. Bioavailability of heme iron (15-35%) is higher than in non-heme iron (2-20%). Only few dietary factors

will influence heme iron absorption compared to non-heme iron which is intensely influenced by other dietary intakes. However, the amount of non-heme-iron is much greater than heme-iron in the diet. Despite its low bioavailability, non-heme iron contributes more to the total dietary intake than heme-iron.

Several factors influence iron absorption and metabolism. Factors that will facilitate iron absorption are ascorbic acid, also known as Vitamin C, and muscle tissue that reduce Fe^{3+} to Fe^{2+} for uptake and absorption in the body (52). Ascorbic acid and citrate increase iron uptake by acting as weak chelators to help obtain soluble iron in the duodenum. Therefore, it is recommended to always include a source of ascorbic acid while eating a meal or a diet providing non heme iron since it is the most efficient dietary enhancer compared to other organic acids. In contrast, cooking, industrial processing and storage reduce ascorbic acid thus reducing iron absorption. Studies also showed that iron-heme food sources enhance non heme iron absorption, for instance 30 g of muscle tissue is equivalent to 25 g of ascorbic acid (51). The addition of poultry, beef and fish also increases non-heme iron absorption up to 2-3 times in the diet. Acidity of the GI also seems to enhance iron absorption in the body. In contrast, a high pH in the stomach will reduce this effect. Inhibitors of iron absorption are phytic acid, polyphenols, calcium and peptides from partially digested proteins. Phytate (myo-inositol hexakisphosphate) is the main inhibitor of iron absorption (52) in a dose dependent manner. Phytate to iron ratios should be 1:1, but 0.4:1 is preferred for iron absorption. Polyphenol food sources include tea (herbal teas), coffee (black), wine and some plant foods such as vegetables, fruits, some cereals and legumes, when combined with phytates limit iron absorption. Calcium has been shown to only limit non-heme iron absorption in single meal studies compared to multiple meal studies in which calcium has limited effect on iron absorption (53). Other studies showed that animal proteins such as milk proteins (casein and whey) and egg proteins (white) and albumin inhibit iron absorption in humans including proteins from soybean (54,55). Finally, studies suggested that some heavy metals such as lead, strontium, manganese, cobalt and zinc due to intoxication limit iron absorption, hence leading to medical

complications, especially in children (51). Lead is often found in ground water, soil and some regions, making it necessary to undergo blood tests at an early age.

2.1.3 Prevalence of iron deficiency and groups at risk

Children, adolescents and pregnant women are the most vulnerable populations at risk of suffering from iron deficiency (1-5,52). During early development, the content of iron is doubled. Among adolescents, especially in young girls because of menstruations, iron requirements are very high due to growth spurt. The average store of iron in human is around 1-3 g. Around 1 mg a day is lost through skin cells, mucosal surfaces and lining of the gastrointestinal tract. In contrast, 2 mg is lost by menstruation in premenopausal women. As much as 10-20% of pregnant women are anemic in industrialized countries compared to 50% in developing nations. In America, the highest prevalence of IDA is around 9-12% in non-Hispanic white women, 20% in black and Mexican American women including families with low income and education (56,57).

In 2012, results have shown that 13% of Canadian women aged between 12-19 years and 9% aged between 20-49 years are iron deficient (58). Although the rate of IDA in Canadian children is low (3,5-10,5%), Canadian Aboriginal children show higher prevalence with 14-50% due to high consumption of evaporated milk and cow's milk after six months of age, prolonged exclusive breastfeeding and significant load of Helicobacter pylori infection (58). Other groups of Canadian at risk of iron deficiency include children from families with low socioeconomic status, Chinese background, infants with low birth weight and children that have consumed cow's milk before 12 months of age (58).

Other risk factors such as vegetarian diets and intrauterine devices are very common in young women (59). Therefore, an adequate dietary iron intake, with respect to the age and gender (Tab.1), is required to replace the lost of iron through the skin, urine and stools and to compensate for the enhanced demand during development in order to maintain balance.

Table 1 Iron requirements of 97.5% of individuals in terms of absorbed iron[a] by age group and sex (51) ***Free © https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3999603/***

Age/sex	mg/day[b]
4–12 months	0.96
13–24 months	0.61
2–5 years	0.70
6–11 years	1.17
12–16 years (girls)	2.02
12–16 years (boys)	1.82
Adult males	
Pregnant women[c]	1.14
First trimester	0.8
Second and third trimester	6.3
Lactating women	1.31
Menstruating women	2.38
Postmenopausal women	0.96

[a] Absorbed iron is the fraction that passes from the gastrointestinal tract into the body for further use. [b] Calculated on the basis of median weight for age. [c] Requirements during pregnancy depend on the woman's iron status prior to pregnancy

2.1.4 Iron and gestation

1. Iron absorption during pregnancy

Iron absorption is at the highest after 30 weeks of pregnancy making it the greatest amount of iron transfer from the mother to the fetus (60). Iron is accumulating in the developing fetus and placenta at a high rate during the stages of pregnancy (T**able.2**). The requirement for iron significantly increases during pregnancy due to the rapid growth of the placenta and fetus including the expansion of the globular mass (50-52,56). During the first trimester, pregnant women are required to take 6.4 mg of iron daily (50). Dietary iron requirements significantly increase up to 18.8 and 22.4 mg at the second and third trimesters respectively. The upper limit of dietary iron absorption is almost 25% in the last two trimesters. Therefore, pregnant women are required to consume approximately 27 mg of iron per day (RDA-Recommended Dietary Allowance) to help meet iron needs during gestation (50,59). In total, around 1070 mg of iron is used through the entire gestation, 250 mg for basal losses, 320 mg for fetal and placental deposition and 500 mg for an increase in hemoglobin mass. At delivery, loss of iron in blood and blood trapped in the placenta is around 150-250 mg. After

delivery, maternal iron stores improve when iron is released during the breakdown of surplus red blood cells in the body and recycled (50,59).

Table 2 Estimated iron deposit in the conceptus during pregnancy (50) ***Reprinted with permission from Dietary Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Cooper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc, © 2002 by National Academy of Sciences, Courtesy of the National Academies Press, Washington, D.C.***

Stage of pregnancy	Fetus (mg)	Umbilicus and Placenta (mg)	Total (mg)
First trimester	25	5	30
Second trimester	75	25	100
Third trimester	145	45	190
Total	245	75	320

During the first trimester of pregnancy, the additional amount of iron required is low and is explained by the replenishment of the mother's iron stores due to the cessation of menstruations. In the last two trimesters, dietary iron requirements increase until the end of gestation due to the expansion of blood volume and erythrocyte mass from the mother.

IDA is usually observed in late pregnancy making iron supplementation necessary at least from the second trimester of pregnancy. A study conducted in Moncton showed that IDA was induced at the third trimester in pregnant women (60).

2. Biomarkers of Iron deficiency

ID is marked by the depletion of iron stores and is characterized by weakness, fatigue, short attention span, poor appetite, higher risk of infection and irritability (59). In contrast, IDA is usually marked by low hemoglobin (Hb) level and has the same symptoms of ID. It also includes paleness, exhaustion and a fast heart rate.

To asses iron status in pregnant women during gestation, hemoglobin and serum ferritin are the two most commonly biochemical measures used in laboratory (59). Hb,

ferritin and packed red blood cells get diluted due to the expansion of the plasma volume in pregnant women. The level of Hb decreases in the second trimester, but somewhat increases at the third trimester. Low concentration of Hb or serum ferritin may be associated with high plasma volume (hypervolemia) compared to high levels of Hb with low plasma volume (hypovolemia). Hypovolemia is also associated with reduced fetal growth compared to hypervolemia characterizing larger newborns. The CDC indicates that concentration of Hb below the 5th percentile, during pregnancy, induces IDA: <11.0 g/dL in the first and third trimesters and <10.5 g/dL in the second. ID is also observed if serum ferritin is below 15 ng/mL. Hematocrit (Hct) is also another indicator of iron status consisting of complete blood count (CBC) and made of red blood cells (62,63). Low Hct values may indicate anemia or massive blood loss.

3. Iron transport and storage in the fetus

During pregnancy, iron is transported from the mother to the fetus via the placenta (61). The first step involves iron absorption across the maternal gut before being bonded to transferrin. Transferrin is the protein responsible for the transportation of iron and has two iron-binding sites with similar affinities for iron (60,61). Serum transferrin passes through the mother's liver and then to the placenta. It binds to the transferrin receptor on the surface of the placenta creating a complex that incorporates into vesicles and internalizes with respect to the pH due to H+-ATPase. The pH inside the vesicle is equivalent to 5.5 allowing iron to be released from transferrin. Transferrin becomes apo-transferrin (transferrin with no iron) and is only released when the pH turns back to 7.4 at the surface of the receptors of the placenta, as the complex gets recycled.

The excess of iron in the fetus is stored in the iron-store protein ferritin (61). Fetal liver regulates the storage of iron according to iron concentration. Iron in the fetus takes precedence over maternal hematocrit and iron stores respectively. If pregnant mothers meet all nutritional requirements during pregnancy, the fetus can store a 6 to 8 month of iron supply in the last two months before term (59). Low dietary iron intake

from the mother reduces fetal iron stores, increasing the risk of inducing IDA in infants.

4. Maternal iron deficiency and child's development

In early gestation, IDA increases the risk of preterm delivery and low-birth-weight in infants by two to three times (59). During pregnancy, the consequence of ID in the progene has been associated to cognitive impairment such as lower intelligence scores, language, gross motor and attention tests seen in 5 year old children. The causes of MID outcome on cognitive development in the offspring remain unclear, but may be related to decreased oxygen delivery to the placenta and fetus, increased rates of infections or adverse effects of ID in the brain (59,61).

2.2 AUDIOLOGY CONCEPTS

2.2.1 Physiological acoustics

An acoustic signal consists of a pressure wave that propagates in a gas, liquid, or solid environment (64). The sound frequency corresponds to the number of compressions and air expansion phases, which get to the ear per second. A sound cycle refers to the distance between two phases. Therefore, the sound frequency is also defined as the accumulations per number of cycles per second. This also determines the tonality of the sound, either elevated or low. The human auditory system has the capacity to perceive frequencies between 20 Hz to 20 000 Hz. The intensity corresponds to the amplitude of vibration and is expressed in decibel (dB). The more the amplitude is elevated, the more intense the sound is, and vice-versa.

2.2.2 Audiology & physiology

The ear consists of three important elements in most mammals, which are the external, middle and internal ear (**Figure2**) (64). The external part is composed of the pinna and external auditory canal. This canal connects to the tympanic membrane of the middle

ear. Its function is to conduct the sound to the middle ear. The middle ear relays mechanically the tympanum to another membrane that will allow sound amplification estimated around 20 dB. This area also allows an easier transfer of the acoustic sound from air to the liquid environment of the ear. Sound waves cause the tympanum to vibrate. This energy is in turn transferred to the ossicles located in the middle ear and makes them vibrate.

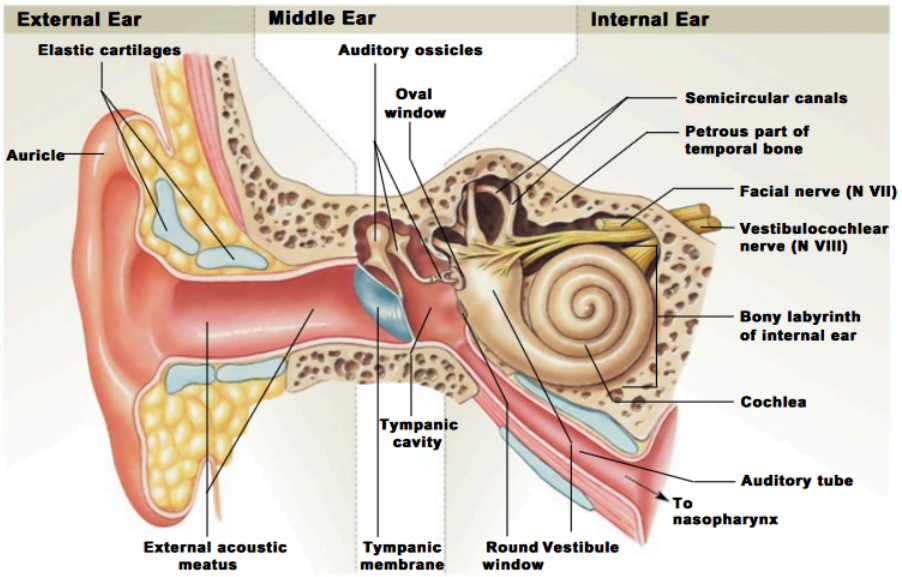

Figure 2 The Anatomy of the ear. ***MARIEB, ELAINE N.; HOEHN, KATJA N., HUMAN ANATOMY AND PHYSIOLOGY, 9th, ©2013.*** **Reprinted by permission of Pearson Education, Inc., New York, New York. (69)**

The sound signal travels in the internal ear into electrical activity also known as potential. This phenomenon is called transduction, which takes place in a sensory organ called the cochlea (64). The internal ear is also known as the labyrinth due to its sophisticated structure. It is divided into the bony and the membranous labyrinth. The bony labyrinth includes the vestibule, the cochlea and the semicircular canals. It is filled with perilymph similar to the cerebrospinal fluid that is low in K^+ and rich in Na^+. The membranous labyrinth floats in the perilymph, but also contains endolymph, which is very similar to this fluid. However, it is richer in K^+. Both fluids transmit the sound vibrations and respond to mechanical strength produced by a change of body movement and acceleration. The cochlea is a spinal bony cavity composed of the spiral organ that is the hearing receptor. The floor of the cochlea is composed of bony spiral lamina and a basilar membrane that is very flexible and fibrous. This membrane

plays an important role in sound reception. It is very thick and straight, but becomes very tiny at the edge of the cochlea. From the bony spiral organ, the auditory nerve (nerve VIII) passes over the modiolus of the cochlea before getting to the brain. This acoustic signal is then transmitted to the brain where the sound gets interpreted with respect to the environment.

2.2.3 Sound properties

Unlike the light that propagates through space at 300,000 km/s, sound is only transmitted through matter. In aqueous medium, sound travels at a velocity of 300 m/s (64). This is why it takes longer to hear the sound of thunder coming from far away than to see the lightning itself. The velocity of sound is constant in a uniform environment. The speed of sound is highest in solids, then liquids and lowest in gas or air.

Sound is a perturbation under pressure, an alternation of high and low pressure area that is caused by a vibrating object and propagated by molecules from the surrounding medium (64). By vibrating from left to right, a set of compressed zones occur which then creates a sound wave in all directions. The energy transfer is also in the direction of the sound travel and this explains why the sound that is heard is decreasing with increasing time as the sound travels away.

A sound wave has an S form like a sine wave (64). Sound has two important physical properties which are the frequency and the amplitude. The frequency is the number of waves that pass by a specific point at a certain time and the amplitude corresponds to the height of the sound wave (64). The wavelength of the wave is the distance between two troughs. The shorter the wavelength, the higher is the sound pitch heard and vice versa. Human hearing is sensitive at frequencies between 20 to 20000 Hz, especially at 1500 to 4000 Hz where it can distinguish frequency changes from 2 to 3 Hz. The amplitude or height between the highest and lowest endpoint of a peak of a sine wave indicates the intensity of the sound (64). This intensity depends on the sound energy

transmitted such as the difference in pressure between the highest (compressed) and lowest (refracted) zones.

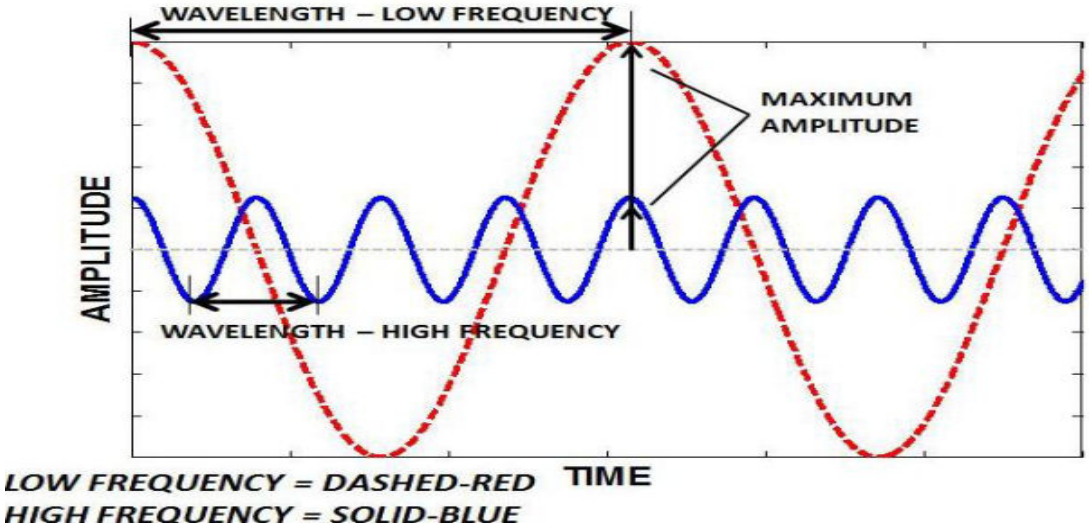

Figure 3 Sound properties: Red wave showing a lower frequency compared to blue wave with higher frequency. Wavelength (latencies) increase with decreased frequencies (72). ***Free © https://wwwnc.cdc.gov/eid/page/copyright-and-disclaimers***

The strength of a sound is a subjective interpretation of this intensity by our brain. So, a logarithmic unit called the decibel (dB) is used to measure the intensity and the strength of a sound wave (64). The threshold corresponds to the lowest intensity perceived by the person or animal, or the third peak shown in the wave. A sound of 10 dB has 10 times more energy than a sound of 0 dB. A sound of 20 dB has 100 times (10 x 10) more energy than a sound of 0 dB and so on as the scale increases in intensity. Frequent exposure to sounds higher than 90 dB can cause hearing loss. The basilar membrane's length is also responsible for perceiving the sound as mentioned above (64). The edge of the length will perceive the lowest frequency of the sound. Some people have a very sensitive hearing due to the functioning ability of this specific area. Some researchers interested in hearing functions also use, nowadays, frequencies above 8 kHz to better understand 'hearing sensitivity' at high frequencies in humans and animals (65-68).

2.2.4 Sensory electromechanical transduction

This step corresponds to coding information from an external medium by sensory receptors, multiple physical and chemical parameters and nerve impulses (64). The basilar membrane is also composed of ciliated sensory cells that are influenced by movement where the transduction of a sound stimulus occurs. An opening of the cations canals results in a configuration associated with the entry of K^+ and exit of Na^+ from the internal ciliated sensory cells and leads to a gradual depolarization (potential receptor). When these canals close, it will allow the cell to repolarize and sometimes even hyperpolarize. All these sensory cells are like efferent neurofibers that transmit messages from the brainstem to the ear. A loud low-pitched sound provokes a lower movement between the brainstem and these sensory cells and where the energy will spread in a wider zone of the basilar membrane. This will help protect some other internal ciliated sensory cells against lesions caused by strong sounds.

2.2.5 Primary auditory pathways

The ascending auditory pathways will pass the auditory information mainly by the cochlear receptors to the brainstem (64). First, the impulses from the cochlea use the afferent neurofibers that overpass the spiral ganglion of the cochlea where the sensitive bipolar auditory neurons are located. They will then get to the cochlear nucleus of the medulla oblongata. These then reach the superior olivary nucleus just between the junction of the medulla oblongata and the overpass. The axons move toward the lateral lemniscus and transit by the inferior colliculus which is the auditory center of the midbrain. This part communicates with the middle geniculated nucleus of the thalamus allowing the sound to finally get to the primary auditory pathway. Here, the sound is consciously interpreted and this is very subjective for each organism. The auditory pathway does not cross the medial line. Therefore, the auditory cortex receives the impulses from both ears.

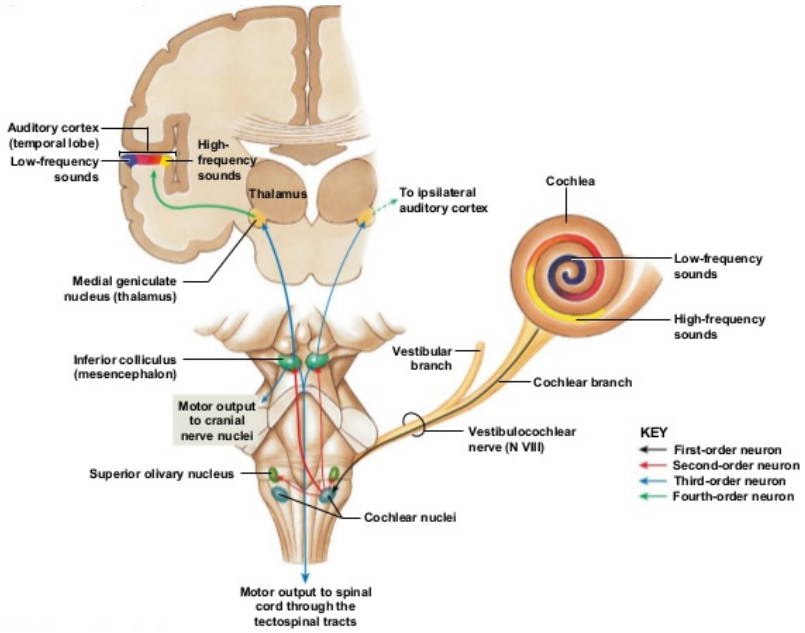

Figure 4 Pathways for auditory sensations. ***MARIEB, ELAINE N.; HOEHN, KATJA N., HUMAN ANATOMY AND PHYSIOLOGY, 9th, ©2013.*** **Reprinted by permission of Pearson Education, Inc., New York, New York. (69)**

2.2.6 Auditory brainstem responses (ABR)

The ABR allows a large specter of different physiological results. It determines the threshold also known as the auditory acuity indicated by the last peak (P) or P3 which is observed at the lowest intensity. The ABR also helps determine the origin of the hearing loss occurring from different reasons. The most common case occurs at a neurosensorial hearing loss, which implies a lesion at the cochlear area. In this case, the latency seems to be in a normal range at a high intensity, but deviates at a lower intensity compared to normal hearing in this zone.

The ABR also measures the latency or wavelength in milliseconds (ms) of a wave also known as neuronal transmission velocity and interpeak latency (IPL) between waves. It differs for every peak and frequency identified by the ABR. It also characterizes the amplitude in µV. Moreover, the amplitude reflects the number of neurons recruited in an auditory stimulation, the nucleus involved and their level of synchronization. Our previous results using the guinea pig showed that very elevated amplitudes might occur in waves showing a hyperacusis, which is a neuronal desynchronization, an

increased sensitivity to a certain frequency and intensities by the neurons (70,71). This pathology implies hyperactivity by the neurons resulting in a decreased neuronal inhibition as a result of an altered metabolism of the neurotransmitters.

Several factors might affect the ABR measures during the experiment such as technical calibration for example the intensity of the acoustic sound and its nature passing through the ear, the positioning of the electrodes through the vertex brain and next to the ears from the appropriate axes and also a magnetic field from any opened devices can all lead to increased level of artifacts. Artifacts are characterized as errors during the ABR experiment and need to be minimized during the process. Other factors derived from the subjects themselves can alter the ABR such as age: a decrease of the myelination process occurs with aging (20,24,32).

2.2.7 Involvement of iron in CNS myelination processes and transmission of ABR

Brainstem maturation is almost complete at birth in human term infants (31). As mentioned previously, iron plays an important role in the myelination processes of the central nervous system (20-30). First, the iron containing enzymes are required for the synthesis of cholesterol and lipids contained in the oligodendrocytes, the myelin forming cells. Cholesterol and lipids are key components of myelin and are also found in higher concentration in oligodendrocytes than any other cell type of the brain. The uptake of iron by the CNS olidendrocytes is also particularly high during brain development. Furthermore, iron is involved in the synthesis of neurotransmitters such as serotonin, dopamine and ϒ-amino butyric acid that all play an important role in the synaptic transmission of the cochlea and the auditory nucleus of the brainstem (32-34). Studies have demonstrated that postnatal IDA or mild ID reduces the production of neuronal myelination and modify the myelin composition of the brainstem followed by other areas in the brain (20-30). The altered CNS myelination due to limited intake of iron during development might in turn impair brain functions such as audition, cognition and behavior in the human and animal offspring (33,35,36). Finally, it was observed that iron deficiency during the prenatal period produced an abnormal

myelination of the white substance (in the cerebral cortex) in children, which can alter behaviour and the effect is possibly irreversible (20).

To evaluate the neurophysiological impact of maternal IDA on the offspring, the ABR has been used as an indicator of CNS myelination as well as to assess auditory stimulation (23-27,33). This method also evaluates the maturity of the brain. This method also helps to evaluate the maturity of the brain after birth and to differentiate any central or peripheral auditory alterations. Therefore, the ABR technique can be use to determine if maternal IDA creates a negative impact on the maturity of the brain, neuronal integrity of the auditory function and indirectly on maturation of CNS myelination in progeny.

2.2.8. Impact of postnatal ID on the audition using the ABR method

Many human and animal studies have examined the impact of ID during the postnatal phase using the ABR method (73-81). Most of the studies demonstrated that IDA impairs brain and auditory functions. Thirty three children with IDA aged between 0-60 months were compared to healthy children (control group) in the same age range. The results showed a significant ($p<0.005$) increase in latency of peak I, V and an increase in IPL III-V (74). Using the ABR as an indicator of myelination process, the researchers suggest that the results obtained might indicate a delayed maturation of myelination requiring iron or by a dysfunction of iron containing enzymes (73).

A reduction in the auditory sensitivity was observed in IDA adult rodents (79). In offspring rat at PNd40, the hearing threshold was found to be negatively affected by ID (79). An increase in latency (26,73,78) and a decrease in amplitude (74) were observed in children suffering from ID. However, very few studies have investigated the impact of maternal IDA on the offspring auditory functions.

2.2.9. Impact of maternal IDA on the offspring audition using the ABR method

The ABR was assessed 24-48 hours after birth in 23 infants with low iron stores, a stage called latent iron deficiency (LID) (serum ferritin-SF < 75 ng/ mL) and 67 healthy infants (SF > 75 ng/mL) born at >34 weeks of gestation (31). Results showed a significant increase in wave V in infants with LID compared to the control group (7,1 vs 6,6) ms $p<0.001$ and an increase in IPL III-V and I-V (2,37 vs 2,07 and 5,10 vs 4,72) ms with $p<0.06$ and $p<0.0004$, respectively. These results suggest that LID is associated with abnormal myelination in auditory pathway in late preterm and term infants. Maternal LID is believed to be a risk factor for the developing fetus due to a higher iron demand. (31). Therefore, LID occurring during the later part of pregnancy is likely to influence the development and function of auditory pathway of the infant due to the fact that the development of the brainstem is not complete at that stage.

In a study using the rat, eight weeks old females were fed an ID diet (2-6 μg/Fe/g) or fed with an IS diet (240 μg/Fe/g) from two weeks prior to mating, during gestation and until the end of lactation. The offsprings were fed their corresponding dam's diet (27). The offspring of ID dams had an increase in wave 1 latency compared to the control group (IS diet) at PNd40, but not at PNd14 and PNd21. This increased latency indicates slower impulse conduction velocity and was validated by a thinner axon diameter in the ID group due to an alteration of myelin expression (24). The offspring of both groups were healthy looking with normal hematocrit values until PNd14, but showed a transient decrease at PNd21.

Cord ferritin is considered to be the best indicator of *in utero* fetal iron status in humans and was used to validate ID risk in infants. Using the ABR method, a decreased frequency and an increased latency in all three waves occurred in premature infants aged between 27-33 weeks gestational age (GA) suffering from ID (22). ABR changes were irreversible despite the correction of iron status and anemia with iron therapy between 6 to 12 month. However, a study with monkeys showed that premature infants with *in utero* LID (10 μg Fe/g via dam's diet) or postnatal ID (1.5

mg Fe via formula) had unaffected ABR compared with controls fed with an IS diet until 8 month of age (79).

In a previous study by Rioux's research group (37), maternal iron deficiency was shown to impair the CNS auditory functions in the offspring using guinea pigs as the animal model. In this study, two groups of pregnant guinea pigs were fed an iron sufficient (IS) or iron deficient (ID) diet during gestation and lactation. The offspring from both dietary groups were fed their dams' diet until weaning. All pups received the IS diet after weaning (PND9). The ABR was assessed in the offspring during childhood at PNd24. The ID offspring group had a higher threshold than the control group at different intensities from 32, 16, 8 and 4 kHz with a difference of 15 dB (37). In other words, the deficient group was less able to perceive low intensity (dB) sound meaning that these pups had an altered auditory acuity. The ID group also presented an abnormal increase in peak III latency from 100 to 50 dB at 32, 16 and 2 kHz compared to the IS group that had values in a normal range. Compared to the control offspring, the ID group also showed a delay in peak 1 at 100 and 80 dB followed by higher amplitude between 35 and 50 dB, a sign of hyperacusis, although IDA and IS pups presented Hct values in normal range (0.36 ± 0.01 and 0.35 ± 0.01) at PNd24. However, in this study using the guinea pigs, auditory functions were not assessed during adulthood when the brain development is completed. To our knowledge, no study has investigated the long-term impact of maternal ID on auditory functions later in life.

2.3 CORTISOL LEVELS, STRESS AND HYPERACTIVITY

2.3.1 Cortisol secretion and functions

Cortisol, also known as hydrocortisone or compound F, is a steroid hormone that is synthesized from cholesterol. It is the dominant glucocorticoid stress hormone in primates. It is produced by the adrenal glands and is released upon activation of the hypothalamic pituitary adrenal axis (HPA) (**Figure 6**) (82-86). Several biochemical

tests can measure the cortisol levels either by evaluation of plasma cortisol, saliva cortisol, ACTH measurement, etc (87).

In response to the perception of a stressor, the hypothalamus secretes corticotropin-releasing hormone (CRH) and sends it to the pituitary gland which in turn will secrete adrenocorticotropic hormone (ACTH). This hormone will cause adrenal glands to release cortisol into the bloodstream. This raise of blood cortisol, among other functions, will in turn increase blood pressure and heart rate (84). Its level increases during the anabolic phases such as during pregnancy for the developing fetus and for the production of energy required by the mother (86).

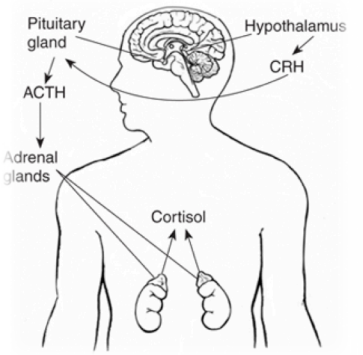

Figure 5 Cortisol secretion and process: In response to stress, the hypothalamus secretes and sends CRH to the pituitary gland which in turn will secrete ACTH. This hormone will cause adrenal glands to release cortisol into the bloodstream (88) ***Free © https://www.niddk.nih.gov/copyright***

Cortisol is also affected directly by the social environment (89). In other words, if a person lives in a stressful or poor environment, the cortisol levels tend to increase with time. This could eventually alter the endocrine and immune systems. Studies have shown that cortisol serum will not only increase during pregnancy, but will be more elevated during a stressful event for the mother and this cortisol gets transmitted across the placenta to the fetus and thus can affect fetal development (90). Several stress factors also affect cortisol levels such as light, temperature, food, etc. On the immunological side, cortisol plays an important anti-inflammatory function and plays a role in hypersensitivity, immune suppression and resistance to disease (89,90). Indeed, it allows the body to cope with stressful situations that require a lot of energy,

especially during anabolic phases. Abnormal cortisol levels were found in people suffering from cancers and certain mental illnesses such as depression, headaches, fatigue, anxiety, behavioural changes etc.

In rodents, such as rats, the concentration of corticosterone in the blood is the main glucocorticoid involved in the stress response (91). In humans as well as guinea pigs, cortisol is the primary stress hormone. Indeed, plasma cortisol levels may be used as an indicator of stress in the guinea pig. In rats, corticosterone is a more suitable biomarker for chronic stress than cortisol, but cortisol has a faster response during severe acute stress (91,92).

2.3.2 Cortisol level during development

The maternal serum cortisol (MSC) increases two to four times during normal gestation (93,94). It readily crosses the placental barrier of the embryo and fetus. This crossing process is regulated by a placental enzyme, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which partially allows MSC, in its active form, to pass through the blood placenta barrier to the fetus (95,96). Therefore, MSC and fetus cortisol levels are significantly correlated.

During gestation, 11β-HSD2 oxidizes cortisol to its inactive form of cortisone (97,98). This will act as a barrier and will somewhat protect the fetus during critical stages of development. As pregnancy advances, the levels of 11β-HSD2 also increase. However, the level of this placental enzyme decreases at the third trimester allowing a higher level of MSC reaching the fetus. This mechanism will ensure that the fetus is exposed to sufficient amounts of cortisol, among other functions, which is optimal for the development of the lungs and preparing the fetus for delivery (99,100).

During gestation, glucocorticoids (GCs) receptors in the CNS of the fetus will allow the entry of GC to the blood brain barrier, which is critical in normal brain development (101-104). Previous results in rodents showed that elevated and moderate

levels of GCs, during early development, improve physiological and behavioral stress response followed by an increase of neural plasticity and persisting improvements in cognitive tasks assessing learning and memory (105,106). In contrast, high levels of GCs, during critical periods, seem to be toxic for fetal brain development. Results in animal studies receiving synthetic GCs in the prenatal phase showed an increase in stress reactivity, poorer performance on cognitive tasks and reduced hippocampal volume (107-111).

Prenatal maternal stress is also associated with increased exposure to cortisol in the fetus which can affect the programming of the HPA axis and thus lead to the offspring's behavioural, neuroendocrine, neuropsychological and immunological problems (112-115). Maternal psychosocial stress can also reduce the length of pregnancy, which will cause preterm delivery (90). However, very little is known about a link of MID and its outcome on the offspring's serum cortisol, known as the major stress hormone.

Some results have shown that pregnant guinea pigs have higher cortisol levels than non-pregnant females as the pregnant female needs more energy (116). As energy requirements increase during pregnancy, so does the level of cortisol. The cortisol level subsequently declines due to an adaptation of the HPA (85,92,108,117). Researchers believe that HPA adaptation could be used to protect against other new stressful events. In general, newborns have low cortisol levels at birth, but this level rises in the encounter of an event not previously experienced.

For children, the cortisol levels reach their highest level 45-60 minutes immediately after waking up (118). Cortisol levels also vary with age (86). Indeed, it's level and reactivity have been found to decrease with age (118). In addition, the cortisol concentration is 25% higher in first born babies compared to a child born of a multiparous pregnancy (119,120).

The sex of the individual is also important as females tend to have higher cortisol levels than males, but both sexes have comparable levels after a sustained period of stress (121). However, male guinea pigs have higher serum cortisol than females, especially when exposed to an aggressive context (116).

2.3.2.1 Cortisol levels and anxiety during pregnancy and its impact on the children cortisol level and development

A prospective study in 125 children investigated for the first time the relationship between cortisol concentration in the amniotic fluid, infant cortisol and infant mother-attachment in response to stress (115). Although no association was made between amniotic fluid and infant-parent attachment, authors concluded that there is a significant effect of amniotic fluid and infant cortisol in response to stress (115). In addition, the results of a study in 125 pregnant women suggest that children exposed to high levels of cortisol in amniotic fluid had higher cortisol levels that decreased slighty over time (121). Also, research has found a link between anxiety during pregnancy and higher cortisol levels at sunrise in preadolescents (122). A similar study found an association between prenatal anxiety and increased cortisol response to stress related to the first year of life in children (123).

2.3.3 Cortisol levels and attention deficit hyperactivity disorder (ADHD)

Research results suggest that hyperactivity and impulsivity in children with Attention deficit hyperactivity disorder (ADHD) may be associated with the dysfunction of the HPA axis (82,120). In a study using the ringing of an alarm clock as a stressor, the cortisol ratio was found to be lower in the ADHD (n=102) compared to the control children (n=146), (7.5 nmol / L vs. 13.1 nmol / L) during the day and 30 minutes after awakening (15.8 nmol / L vs. 21.5 nmol / L) (124). The cortisol levels in both groups were equivalent by night time. Similarly, the cortisol levels were consistently lower at sunrise (10.5 nmol / L vs. 15.5 nmol / L), 30 minutes after awakening (15.8 nmol / L vs. 21.8 nmol / L) and bedtime (3.3 nmol / L vs 3.1 nmol / L) in children with ADHD

(n= 197) compared to the control group (n=221), respectively (125). In a smaller study, conducted among 13 children with ADHD, the cortisol levels were also found to be lower 30 minutes after waking up and throughout the day compared to the control group (82). In addition, the ADHD group showed more anxiety before and after in a dental examination than the control group (82). The lower levels of cortisol in ADHD children may be explained by a dysfunction of the HPA axis over the years (82,120).

In contrast, a study observed that ADHD children (n=12) had higher cortisol levels compared with the control group (n=21) while performing AMPS (Assessment of motor and process skills-measure of functional task performance and physiological responses) (126). Since cortisol is required in cognitive processing, the AMPS helped determine a child's ability to complete meaningful tasks. Researchers suggested that an adequate level of cortisol is required to pass the AMPS tasks and that lower or higher cortisol levels may impair the AMPS results as might be the case in children with ADHD. These conflicting results may be justified in part by several factors that may influence cortisol production such as genetic factors, the type of stressor experienced and other environmental factors that may impact the development of the HPA axis (82,126).

2.3.4 Impact of ID on plasma cortisol levels

Very little research has been conducted on the association between IDA and cortisol levels in humans or in animal models. A postnatal study investigated stress responses in children with IDA at 6 and 12 months of age in response to a venipuncture (120). No significant difference was found in terms of plasma cortisol between the ID children and control groups at the time of the puncture. However, a significant elevation in plasma cortisol levels was found in ID compared to control children aged 12 months at 30 minutes (97.8 mg / dl vs 43.4 mg / dl) and 45 minutes (90.1 mg / dl vs 36.4 mg / dl) after venipuncture. (120).

Youdim et al. (21) found that there was a 15-20% reduction in the activity of the enzyme monoamine oxidase (MAOA) in the brains of ID rats resulting in an increase in noradrenaline (21). This increase consequently caused an increase in the production of glucocorticoids (21). The MAOA protein in humans degrades monoamines and affects the behaviour (21).

Weinberg et al. measured the corticosterone levels in rats (10 males and 10 females) fed with a diet containing low iron (91). In contrast to previous studies, it was found that the ID rats had corticosterone levels lower compared to the control group, before and after, the exposure to ether or a cardiac puncture used as stress factors (91). Some researchers have explained these results from the viewpoint of an adaptation of the HPA axis in continuously stressful period such as pubertal development. They also suggest that this may be due to hormonal changes and increased psychosocial demands followed by reacting to novelty with impulsiveness (124-128). However, a study conducted in young rhesus monkeys (n = 34) did not observe any significant difference in blood cortisol levels in the ID monkeys compared to the control group (n = 57) when separated from their home cage to a novel environment and introducing them to a new object and the venipuncture (130). Another rat study also suggests that ID diet induced stress in male rats by increasing the level of cortisol (131). The concentration of cortisol in the 94 rats was 72, 43, 44 and 46 mg compared to the control group 25, 26, 22 and 14 mg at days 10, 20, 30 and 40, respectively after ID exposure (131).

2.3.5 Involvement of iron in the pathophysiology of hyperactivity

ADHD is the most neuropsychiatric disorder of childhood which characterized by inappropriate impulsivity, over-activity, inattention and altered executive functions (38-40). It also consists of two symptom domains: hyperactivity/impulsivity and inattentiveness (38). For this study, the guinea pig was chosen as an animal model because it's an appropriate model for the purpose of social and behavioural experiments (131).

Several studies have tried to illustrate the mechanism by which iron deficiency is linked with ADHD, although the process seemed unclear. First, iron is a coenzyme of tyrosine hydroxylase, which is critical for the synthesis of dopamine. (38-40,124,125,132). Dopamine is a key neurotransmitter in the pathophysiology of ADHD (38). A decrease in dopamine transporter function in the dopamine receptor is observed in patients with ADHD causing a dopamine dysfunction, resulting in an increased extracellular dopamine, as well as reduced dopamine receptors in the striatum which coordinates cognitive functions, motor and action planning and food anticipatory behavior. Iron deficiency may also impair basal ganglia which is composed of the stratum and cause an imbalance between inhibitory/excitatory neurotransmitters such as GABA(γ-aminobutyric acid) playing an important role in ADHD. Low iron levels in the thalamus were also found in children with ADHD compared to controls when using MRI helping explain the etiology of ADHD. Furthermore, since iron deficiency alters dopamine receptor density and activity, results suggest that brain iron stores may influence dopamine-dependent functions. Low serum ferritin levels have been associated in children with ADHD compared healthy controls. Some researchers have suggested that postnatal ID in children can be associated to the symptoms associated with ADHD due to the impaired dopaminergic neurotransmission caused by insufficient iron level (39).

2.3.5.1 Impact of postnatal ID on hyperactivity

Some studies have shown that a low level of ferritin in children is associated with behavioural problems and ADHD (38-40). A study in 2957 children and adolescents with iron deficiency concluded that those with iron deficiency have a greater chance of having mental retardation and developmental delays relative to healthy children (133). These iron deficient children and adolescents are more susceptible to have ADHD (2.8% vs 1.8%) compared to children with adequate iron status group (117). It was also observed that behavioural problems increased with lower iron stores (38-40).

Indeed, children and adolescents with ADHD with comorbidity (intellectual disability and learning disorders, anxiety disorder, tic disorder, elimination disorders and conduct disorder) had a mean hemoglobin and a mean corpuscular volume (MCV) lower than subjects with ADHD only (38).

A research conducted in Paris among children and adolescents aged 4-14 years (n = 53) with ADHD, observed that the mean serum ferritin was lower in subjects with ADHD relative to the control group (23 ng / ml vs 44 ng / ml) (39). The lower level of serum ferritin in children was associated with lower cognitive and higher hyperactivity scores, both of which were assessed with the form "Conners' Parent Rating Scale (CPRS)" (39). The authors are suggesting that children suffering from iron deficiency are more inattentive, distracted and have learning difficulties (39). In addition, children who were severely iron deficient were suffering from increased motor restlessness related to the restless legs syndrome (39). However, the level of serum iron, hemoglobin and hematocrit were within the normal range for both groups (39). These results suggest that low levels of ferritin may impair dopaminergic activity in children and contribute to symptoms of ADHD (39).

Another research conducted in rhesus monkeys supports our previous results. This study used 8 to 9 month old rhesus monkeys and showed that monkeys suffering from moderate anemia were more distracted and less focused on new objects with different shapes and colors containing treats when introduced in their cage and that was made during cognitive tests (134). A depleted iron diet (10 mg Fe / g) was given during the prenatal period (n = 14) or during very early postpartum period (n = 12) to assess the difference in behavior of these animals compared to the control group (n = 12) (100 mg Fe / g) (134). Iron deficiency during the prenatal period caused a more impulsive behaviour and lack of prudence facing danger in the progeny. The postnatal group proved to be very different, as they demonstrated arousal, irritation and a cognitive delay (134). This study also suggest that less fearful and more impulsive behaviours were linked to prenatal iron insufficiency and more tense and withdrawn behaviours

were related to postnatal iron deprivation (134).

2.3.5.2 Impact of prenatal IDA on the offspring hyperactivity

To date only few studies have investigated the impact of IDA during pregnancy on symptoms associated with hyperactivity in the progeny. Leblanc et al (135) have observed that guinea pigs offspring-born from IDA mothers seem to be more active than their control counterparts (135).

In this study, iron deficiency induced during pregnancy and lactation resulted in moderate ID in dams similar to what is usually observed in women from industrialized countries (136). The offsprings born from dams fed an ID diet but fed an iron sufficient diet after weaning were ID at weaning and had reached normal Hb status after PNd24. The ID group had significantly higher mean number on central and external square crossings and mean number of sequence movements at both time periods in an open task at PNd24 and 40. These results showed clearly that the offspring born to ID dams were significantly more active.

In contrast, studies using the rat have showed that the adult offspring born from dams fed an ID diet during gestation and lactation were similarly active, showed little behavioural differences and no significant difference in locomotor activity compared to offspring born from dam fed an IS diet (130,137). Other rat studies have also shown that both young (at PNd8 and 10) and older offsprings (12 weeks) born to ID dams showed greater levels of motor activity compared to rats born to IS dams (35,138,139).

This discrepancy between the rat and guinea pig studies could be attributed to the time at which the testing was performed (adulthood vs childhood) and the difference in animal model. Indeed, the peak of brain development in guinea pigs occurs during the prenatal period whereas rapid brain development is mainly postnatally in the rat (116).

CHAPTER III

RESEARCH HYPOTHESIS AND OBJECTIVES

3.1 Aim

This research proposal aims to determine the impact of maternal IDA on the hearing response and serum cortisol levels of the offspring. In order to verify our hypothesis, we will study two distinct dietary groups. The first group of pregnant females guinea pigs will be fed a control diet containing sufficient iron and the other group will be fed an iron deficient diet during the entire pregnancy and lactation. After weaning, pups from both groups are going to be fed the control diet.

3.2 Research hypothesis

1. Maternal IDA during gestation and lactation will have long-term adverse effects on the auditory functions such as higher auditory threshold and delayed latencies in the offspring using the ABR.

2. Maternal IDA during gestation and lactation will increase blood cortisol levels in the offspring and dams, an indicator of stress/hyperactivity.

3.3 General objectives

To determine the impact of mild maternal iron deficiency (MID) during gestation and lactation on:

1. The auditory brainstem response (ABR) in the offspring 15 days after weaning (PNd24) and during early adulthood when the brain is fully developed (PNd84).

2. The blood cortisol levels in the offspring at PNd24 and PNd84 and in dams during pregnancy.

3.4 Specific Objectives

1. To assess hematocrit as an indicator of iron status in the pregnant females at every trimester during gestation and in the offspring at PNd24 and 84.

2. To determine the neurophysiological impact of MID during gestation and lactation on the ABR in the offspring by evaluating the threshold and latency at 100 and 80 dB at PNd24 and 84.

3. To analyze the serum cortisol levels obtained by using Elisa cortisol technique as a biomarker of stress in the offspring at PNd24 and 84 and in the dams at every trimester of pregnancy and before sacrificed.

4. To monitor the body weights of pregnant females and offspring on a regular basis.

3.5 Rationale

The links between IDA during pregnancy and the offspring's health and development have not yet been well established.

IDA is the most common nutritional deficiency that occurs during pregnancy, the prevalence is high even among Canadian women. IDA during pregnancy induced by a low iron intake can potentially have a negative impact on the infant's health and development. Because iron is involved in CNS development, it's reasonable to believe that IDA during pregnancy may have behavioral and neurophysiological impacts in the

offspring. This study focuses mainly on the impact of gestational IDA on the auditory functions and blood cortisol levels in the offspring.

This study involves the nutritional, auditory and psychology fields providing a comprehensive interdisciplinary approach to the topic. This research project will contribute to the understanding of the impact of MID on hearing function and hyperactivity in the offspring. The outcome of this project is to eventually help prevent adverse effects in children by developing efficient prevention strategies targeting women of childbearing age.

1. Animal model

   For this project, the guinea pig was used as an animal model for the following reasons; as in humans, cortisol is the primary glucocorticoid hormone involved in the stress response and generally varies according to stressors and increases faster in severe stress encounters or when faced with a previously unknown event (116,140,141). Similar to humans, the reproductive cycle is divided into three trimesters. During iron deficiency anemia, a decrease in hematocrit, an indicator of iron status, is observed mainly during the third trimester similar to that observed in humans with comparable biomedical values (116). Contrary to other rodents, rapid growth of the guinea pig's brain occurs during gestation which closely resembles that occurring in humans (141). Also, the guinea pig is an excellent model for the purpose of social and behavioural experiments. For these similarities with humans, the guinea pig is a suitable animal model for the proposed experimental study.

2. Methodology

   Animals, diet and design

   In this study, we used 24 Hartley guinea-pig females and 2 males (Cavia porcellus, 13-18 weeks old), purchased from Charles River Laboratories (St

Constant, QC, Canada). The animals were housed in a controlled environment of ~ 22 ° C in the Animal Care Unit at University of Ottawa. They were housed on a 12-12hr light-dark cycle with lights on at 0700. On their arrival, females were randomly assigned to their respective diets; iron sufficient (IS: 114 mg/kg iron) or iron deficient diet (ID: 11.7 mg/kg iron) as purified pellets (Harlan Teklad, Madison, WI, USA). Both males received the IS diet. Fresh water was provided (*ad libitum*) daily. Body weights were measured every two days from arrival at the animal unit.

Three weeks were allocated to all the animals for their adaptation to the new environment and diets. After the third week of the acclimation period, one male was placed with six females in a large mating cage for 28 days, for each dietary group. The same number of females per dietary group were mated with the same male; the latter was crossed from one group of females to another new group of females housed 1 month after the arrival of the first group to avoid any genetic confounding factor. After successful mating confirmed by Vaginal Smears technique, three (3) females were placed in separate cages and fed their respective diet throughout gestation and lactation. The same applies for the other pups in the same cage until the weaning day. The PNd1, day of birth in offspring was determined when parturition appeared within 24 hours and by the detection of sperm in vaginal smears. ID and IS dams gave birth to 16 and 25 pups at PNd24 and 11 and 27 pups at PNd84, respectively.

After weaning (PNd 9), pups from the same litter (n=2) were placed in a cage and were fed the IS diet to limit the treatment effect to maternal IDA (i.e. the combined gestation and lactation time periods).

The research protocol was approved by the University of Ottawa of the Animal care committee and was in compliance with the National Institutes of Health and National Research Council ‘Guide for the Care and Use of Laboratory Animals’.

The Vaginal Smears Technique

The vaginal smears technique was used to determine if the female guinea pigs was pregnant. The first step involves visual inspection of the external vaginal area to see if any whitish color is present. If yes, a first check mark on the spreadsheet is made to indicate the likelihood of intercourse between female and male.

The next step is to do a simple clean cotton swab (sterile from lab) humidified with a normal saline solution (NaCl 0.9%) to the vaginal area. It was brushed gently to prevent any risk of infection. The cotton swab was removed with a drop of sterile water on a microscope slide and then cover slipped.

The slides were put under the objective lenses of the microscope (Binocular Microscope) at 10 x, or 20 x or 40 x. If intercourse occurred, sperms were seen clearly by using all of the above objective lenses. If few sperms were present, another small check mark was written demonstrating a possible pregnancy. If many sperms appeared, a big check mark was indicated showing a high probability of pregnancy in the female.

These steps are repeated for 7 days to confirm the analyses. Therefore, dates of each of the trimesters and delivery of every female were predicted from the estimated first day of pregnancy.

Blood collection & biochemical analysis

In order to measure the Hematocrits (Hct), blood punctures were done on the right ear of each animal after swabbing it with a 70% alcohol solution in dams during each trimester of pregnancy and just before sacrifice (PNd16) and in the offspring at PNd24 and 84. Approximately 60 mm of blood was collected into microcapillary tubes (Heparinized, pre-calibrated 100/Vial) provided from Globe SCIENTIFIC INC.

To estimate the Hct, the microcapillary tubes were centrifuged at 10 000 rpm using a HAEMATOKRIT 210-Hettich for 3 minutes before taking a reading. Since the gestational (Gd) period is 75 days, the Hct was assessed at Gd24, Gd48, Gd65 to determine at which trimester IDA occurred during pregnancy. Hct was also assessed the day of sacrifice in dams (PNd16) and in offspring (PNd24 and 84).

In order to measure the cortisol, a drop of blood from the microcapillary tubes was deposited on filter paper (Human bloodstain ID-card Whatman) for 24 hours in a room to open air and then frozen at -80 ° C. Each paper was properly labeled.

Serum cortisol-Enzo Cortisol Elisa kit

Only 24 females and their respective offsprings were used for the cortisol since the protocol was conducted in the second year, whereas all animals were used for the ABR procedure. The procedure involves two main stages. We had 134 samples duplicate for a total of 268 wells as described by the Cortisol Elisa kit. Each kit contains 96 wells in a plate. Sample Whatman cards were thawed from -80°C and cortisol levels from blood droplets were determined using a commercially available ELISA kit (Cortisol EIA kit, Enzo Life Sciences, Cat. No. ADI-901-097). Briefly, a 3.0 mm diameter circle of each drop sample (n = 8 per group per blood collection interval) was punched from the blood stain cards using a Gem Hole Punch (McGill Inc., Marengo, IL), and placed in labeled glass tubes containing 280 µl of assay buffer diluted in dH_20 at 1:10 concentration to stabilize the solution. The tubes were covered with parafilm and shaken on the Belly Dancer® Laboratory shaker (Structure Probe Inc., West Chester, PA) for 24 h at RT prior to the ELISA procedure. On the next day, 214.5 µl of each blood sample and 5.5 µl of steroid displacement reagent (SDR) were mixed in labeled aliquots and vortexed. Standards and samples were prepared as recommended by the manufacturer. CORT concentrations were determined in a PowerWave^{TM} XS2 Microplate Spectrophotometer (BioTek, Winooski, VT). Resulting CORT

concentrations were calculated using a four-parameter equation that relied on constant values determined from the standard curve and quantified in units of pg/ml of corticosterone per punch. The analytic range of the assay was 32 – 20,000 pg/ml.

Auditory brainstem response (ABR)

The auditory brain stem response (ABR) was tested in pups at PNd24 and PNd 84. Each animal received an intra muscular injection (2.3 ml/kg) of an anesthetic solution of ketamine/xylasine (2:1) before data recording. Ketamine alone has been reported not to alter ABR thresholds and allows an excellent recording quality. When sedated, animals were placed on a water circulating heating pad (T/Pump 500®, Gaymar Industries Inc., Orchard Park, NY) to maintain normothermia during the experiment. Due to the fact that temperature can influence the ABR rectal temperature was monitored (Data Therm™, Geratherm Medical AG, Geschwenda) every 2 minutes, and corporal thermal fluctuations of less than ± 0.5°C was avoided by regulating the circulating fluid temperature during all the experimentation. Brainstem responses to auditory stimulations were recorded in an electrically shielded double wall acoustic chamber between two subdermal platinum FE-2 needle electrodes (Grass Product Group of Astro-Med Inc., West Warwick, RI) placed on the vertex site (active electrode – non inverting – C_z) and on the mastoid, below the left ear (reference electrode – inverting – A_1). A third electrode placed below the right ear was used as the ground. Inter-electrodes impedance was kept ≤ 1 kΩ (Opti-Amp 8002 Power Transmitter, IHSystems, Miami, FL). Bioelectrical activity recorded at C_z site was amplified 100 000 times and filtered on a digital bandpass of 300 - 3000 Hz. When physiological responses exceeded a voltage of + 8 μV, a potential artefact was eliminated from recording. The time window of this latter was 12.8 ms from pure tone onset. The acquisition system (SmartEP-IHSystems) was recorded 256 responses, fifteen minutes after anaesthesia and files generated at the end of this acquisition was saved on a specific computer for subsequent off-line analysis. An

insert earphone (Etymotic Research Inc., Elk Grove Village, IL) connected to the brass insert of a high frequency transducer (IHSystems) delivered the acoustic stimulation to the ipsilateral ear with a series of trapezoidal envelopes pure tones. The tone pip rise/fall time was set at 500 μs, with a 10 000 μs plateau and a repetition rate of 21.1/s; polarity was established on alternating phase. Orderings presentation of frequencies was submitted in a decreasing order, from 32 kHz, 16 kHz, 8 kHz, 4 kHz and 2 kHz. Animals were exposed to a range of stimulus intensities starting from 100 dB peSPL (decibel peak-equivalent Sound Pressure Level) followed by a descending order from 80 to 20 dB peSPL, with 10 dB peSPL steps, well above the threshold and with 5 dB peSPL steps near the supra-threshold. Some animals went until 5 dB. At the end of the automated procedure, every frequency was reviewed and new traces (ranging from 2 to 4) were collected with 2 to 3 dB peSPL changes in the intensity around threshold, in order to establish this latter with more precision. ABR measures, thresholds as well as peaks and nadirs positioning were processed blinded, i.e., without knowledge of animal treatment group. A second experimenter was involved in a cross-check, in order to confirm the data reliability. Thresholds and latencies for peaks I-IV were measured in this condition.

ABR latencies (neural transmission times) and Peaks I-IV (brain transmission time)

The ABR reflects a succession of series of action potentials and postsynaptic relays along the 8th nerve and brainstem auditory pathway called neurogenerators identified as waves or peaks (P). The peaks I to IV are often identified at the beginning of every wave and follow each other simultaneously between 1 to 5 ms. This methodology of identifying peaks was used from the study of Wada & al. (1996). For example, a wave at 100 dB showing the first peak between 1 and 2 ms would be considered as peak 1. Peak 2 would be the next one between 2 and 3 ms and so on for peaks 3 and 4. Peak 3 usually has the highest amplitude. Peak 1 would identify the origin of the auditory nerve (nerve VIII or cocleovestibular

nerve). Peak 2 corresponds to the cochlear nucleus. Peak 3 represents the middle portion of the superior olivary complex (SOC) and the lateral lemniscus. Finally, peak 4 would contribute to the SOC and minimally to the trapezoidal body.

Anesthesia procedure

At the last day of the experiment, each animal received an intra muscular injection (2.3 ml/kg) of an anesthetic solution of ketamine/xylasine (2:1). After the ABR procedure, all animals were brought to the necropsy room where tissues were collected after decapitation by guillotine and scapular tools before being frozen into the liquid nitrogen.

Organ collection

The organs were rapidly extracted and weighed, and wet weight values normalized using each guinea pig's body weight. The weight of the hippocampus, heart, kidneys and adrenal glands, including adrenal gland length were recorded. After being submerged in liquid nitrogen, tissues samples were frozen at -80˚C for future analysis.

3. Statistical analysis

This study used a quantitative research method design. Specifically, we propose to assess the contribution of six independent and seven dependent variables to the observed variation in biomarkers in the study population.

The independent variables were the Group (ID, IS), Gender of all the guinea pigs (male or female), ABR Days (24 or 84 for the pups and 16 for the dams), the three Trimesters of pregnancy including the last day of the experiment (4 times), dB (100 or 80) and the Frequency (2-32 kHz). The dependent variables were the Body weight (g), organs Weight-hippocampus, Heart, Kidneys and Adrenal

glands (g) including the length of the Adrenal glands (cm), Threshold (dB), the Latency (ms), Body temperature during ABR (˚C), the Cortisol level (pg/mL) and the Hematocrit (%).

To reach this objective, we performed a series of linear mixed models on the pups' auditory threshold and latencies. Additional linear mixed models were also performed on pups' weight, body temperature and hematocrit level. All linear mixed models were estimated with an uncorrelated covariance structure and the final models were all fit by maximum likelihood (ML).

Regarding the weight of the dams, two linear models were fit. In the first model, we examined the relationship between the weight and the beginning and the end of the habituation period. In the second model, we examined the same relationship but for the gestation period. In both models, diet and period were treated as categorical fixed effects and the animal was integrated into the model as a random intercept effect (as confirmed by two independent likelihood ratio tests, $p<0.001$ and $p<0.05$, respectively).

Given that the weight of the pups that were sacrificed at Day 24 could not be measured at Day 84, two linear mixed models were run on pups' weight. In one model, the weights of the groups that were sacrificed at Day 24 were compared on Day 1 and Day 24. In the second model, the weights of the groups that were sacrificed at Day 84 were compared on Day 1, Day 24 and Day 84. In both models, Diet and Day were treated as categorical fixed factors and a random intercept was included.

For the hematocrit data, two different linear mixed models were performed, one for the dams and one for the pups. The linear mixed model performed on the dams' hematocrit included the diet and the periods of measurement as categorical fixed factor and a random intercept effect on the dams was added to the model, which was supported by a significant likelihood ratio test ($p<0.001$). The linear

mixed model performed on the pups' hematocrit included diet and day as categorical fixed factor. A random intercept effect on the pups was also included into the model, as supported by a significant likelihood ratio test ($p<0.001$).
A multivariate analysis was used for the weight of the kidneys and adrenal glands including the length of adrenal glands, to a strong correlation between the measures of the two weights (left and right) of organs in this case, for dams and pups. Dams and pups were compared with the respective diets (ID/IS) at the last day of the experiment. Bivariate analysis is also used with a Pearson correlation to help determine any correlation between both organs and if significant difference occurred in both groups. Separated analyses were also performed for the weight of the hippocampus and heart, used as dependent variables, in a t-test and two-way ANOVA in dams and in pups respectively in both dietary groups to see if any significant differences appeared.

Regarding the cortisol levels, our instrument was not able to detect cortisol levels over 84000 pg/ml. Consequently, 5 females out of 10 in the IS group and 2 females out of 10 in the IDA group had censured data. Thus, 35% of our sample presented censured over 84000 pg/ml. To perform multiple imputations of the censured data, we used R (142) and the package Amelia (143). We hypothesized that the missing values were random and normally distributed over a range between 84000 and 150000 (99% confidence interval). Our imputation model also included the three trimesters during the gestation period. As the observed individual trend of the cortisol for each animal as a function of the trimesters was linear, we imputed the data with a linear model. In addition to the intra variability of the cortisol for each animal, the model included the variable diet. For each animal, 50 imputations were performed, which is higher than the usual recommendation for this kind of imputation process. In addition, the relative efficiency values for each coefficient were all over 0.99, which suggested that our imputation model was very reliable in estimating the regression coefficients of our mixed model. Stata 14.0 (144) was used to analyze the imputed data set. More specifically, for both diets (IS and IDA), we used a linear mixed model to predict

the dams' cortisol as a function of the Period. In the model, trimester and diet were treated as a categorical fixed effect. To consider the variability associated with each dam, a random intercept for each dam was integrated into the model. The level of cortisol at the postpartum period for the IS and the IDA groups was compared via an independent t-test.

We were not able to get a reliable measure of cortisol for 2 pups (one IS and on IDA pup) and those pups were excluded from the analysis. To analyze the pups' cortisol levels, an ANOVA diet (2) x day (2). Due to small number of pups in the IDA groups at both PNd24 and PN84, we could not include gender in the interaction term.

Regarding the ABR, for safety reasons, we did not test the pups' threshold over 100 dB. Due to this procedure, 8.9% of our data for the threshold were censured over 100 dB. We also used R and the package Amelia to impute the censured data. To impute the data, we hypothesized that the missing values were random, normally distributed and over 100 dB. As the observed individual trend of the threshold for each animal as a function of the frequencies was clearly cubic, we imputed the data with the variable frequency elevated to the 3^{rd} degree. In addition to the intra variability of the threshold for each animal, the model included the variables Sex, Age, Diet and dams' id. For each animal, 50 imputations were performed, which is higher than the fraction of missing information (FMI) estimated for each coefficient of the model. This is over the usual recommendation for this kind of imputation process. In addition, the relative efficiency values for each coefficient were all over 0.99, which suggested that our imputation model was very reliable in estimating the regression coefficients of our mixed model.

Stata 14.0 was also used to analyze the imputed data using a linear mixed model. More specifically, for both diets (IS and IDA), we used a linear mixed model to predict the pups' auditory threshold as a function of frequency (from 2 to 32 kHz)

and day (24 and 84 days of age). In the model, frequency was treated as a continuous fixed effect and diet and day were treated as categorical fixed effects. To facilitate the interpretation of the coefficients, frequency was transformed on a scale from 1 to 5, which represented the increasing values of the different octaves from 2 kHz. In addition, to fit the predicted values of the model to the observed data, frequency was elevated to the third polynomial degree (cubic relationship). To consider the variability associated with each pup, a random intercept for each pup was integrated into the model. In a previous model, the variable frequency was also included in the model as random slope effect, allowing us to consider the individual slope of each pup as a function of frequency. However, the addition of this random slope effect was not significant (Likelihood test, $p > 0.05$) and it was dropped from the final model.

To determine if the body temperature changed as a function of the diets, day and frequency, a linear mixed model was performed on temperature. In this model, diet and day were treated as categorical fixed factors and the frequency was treated as a continuous fixed factor. In addition, a random effect on the intercept and a random effect on the slope were added to the model, which was supported by a significant likelihood ratio test ($p < 0.001$).

Finally, two different linear mixed models were performed on the pups' latency. One model was run at 80 dB and a second was run at 100 dB. At 80 dB, 96.52% of the data were missing at a frequency of 2 kHz. We therefore removed this frequency from the analysis and performed the mixed model using frequencies 32, 16, 8 and 4 kHz. At 100 dB, the percentage of missing data was less drastic and we kept all frequencies (32 to 2 kHz) in the model. For both models, we imputed the missing data using the same approach as the one described for the threshold values. In both models, diet, day and peak were treated as categorical fixed effects and frequency was treated as a continuous fixed effect. In addition, as supported by a likelihood ratio test ($p<0.001$), both models included a random intercept effect on the pups and a random slope effects on the frequency.

The criterion of $p<0.05$ was be used to reject the null hypothesis and two sided tests were applied to compare the results of the two groups (ID, IS). And all linear mixed models were estimated with an uncorrelated covariance structure and the final models were fit by maximum likelihood (ML).

This research study expects the ID group pups to have the highest cortisol level and less hearing ability. In this research project, the IS group is expected to have a lower cortisol level and better hearing ability than the ID group due to full nutritional requirements compared to pups born from IDA dams. More precisely, we expect the ID pups to have a higher cortisol level than the IS at PNd24, but somewhat similar at PNd84 since an IS diet will be given after weaning day to all pups. We also expect the ID siblings to have a higher threshold and increased latencies at all frequencies both periods than the control group. As mentioned previously, the pups of each group will be divided into two different groups. Each one will be assessed 24 days after birth or 84 days after birth and all chosen randomly.

CHAPTER IV

IMPACT OF MATERNAL IRON DEFICIENCY ON SERUM CORTISOL IN THE GUINEA PIG OFFSPRING

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Abstract

Iron deficiency (ID) has been reported as a risk factor in the pathology of ADHD, although the mechanisms seem unclear. In 2015, ADHD mean prevalence is 5,9%. We hypothesized that maternal iron deficiency (MID) has an impact on ID offspring hyperactivity validated by serum cortisol used as a biomarker of stress at postnatal day (PNd) 24 and PNd84. 12 female guinea pigs per group were fed an iron sufficient (IS) diet (114 mg/kg) or ID diet (11.7 mg/kg) during the gestational and lactational periods. Pups in both groups were weaned at PNd9 and given an IS diet. Hematocrit (hct) and cortisol levels were measured at every trimester and at the day of sacrifice in dams and at PNd24 and 84 in pups, on sacrifice day. The weight of dams and pups' hippocampus, heart, kidneys and adrenal glands including the length of adrenal glands was recorded. The body weight in every animal was also recorded every second day until the day of sacrifice. Cortisol values in ID pups were significantly ($p<0.05$) higher than IS pups at PNd24. However, both groups of offspring had similar cortisol at PNd84 ($p>0.05$). Dams in both groups showed no significant ($p>0.05$) difference in cortisol during gestation. No significant difference was observed in organ weight or adrenal gland length. The present study suggests that MID seems to have a reduced impact on cortisol in the ID offspring when entering adulthood. Further investigations are suggested to validate MID as an internal stressor causing hyperactivity in the offspring.

Keywords

Hyperactivity; Offspring; Maternal iron deficiency; Serum cortisol; Hematocrit

Abbreviations

ADHD, attention deficit and hyperactivity disorder; ADNA, adrenal glands; Hct, hematocrit; ID, iron deficiency; IDA, iron deficiency anemia; IDD, iron deficient diet; IS, iron sufficient; ISD, iron sufficient diet; MID, maternal iron deficiency; MSC, maternal serum cortisol; PNd, post-natal day; PSM, psychosocial stress model

1. Introduction

Iron deficiency anemia (IDA) is the most common nutrient disorder worldwide affecting 1-2 billion people and is a major public concern [1, 2]. Pregnant women and children are adversely affected by IDA whose global prevalence rates are 38% and 43% [3]. In developing countries, 50% of pregnant women suffer from IDA and about 10-20% in industrialized nations [4, 5].

Due to the development of the fetus, the insufficient iron storage and the limited absorption from food in the woman's body, the demand for iron increases considerably in pregnant women [6]. If the requirement demands are not met, well-known complications may occur such as preterm birth, low birth weight, small neonates for gestational age, postpartum hemorrhage, and decrease of iron storage in neonates [7, 8]. Physicians often prescribe iron supplements for pregnant women to help them to compensate for the increased needs and inadequate iron intake, but not all pregnant women follow iron supplement guidelines because of gastrointestinal (GI) side effects complications such as nausea, vomiting, diarrhea, constipation, heartburn and abdominal cramp [6, 9].

Several studies have tried to illustrate the mechanism of iron deficiency in Attention-deficit/hyperactivity disorder (ADHD). ADHD is the most common childhood

neuropsychiatric disorder with a mean prevalence of 5.9% [10]. Iron deficiency has been reported as a risk factor in the pathology of ADHD, although the process seemed unclear. First, iron is a coenzyme of tyrosine hydroxylase, which is critical for the formation of dopamine. Iron is an essential micronutrient, involved in the synthesis of neurotransmitters such as dopamine [11-13]. Dopamine is a key neurotransmitter involved in the pathophysiology of ADHD [12]. A decrease in dopamine transporter and activity in the dopamine receptor is observed in patients with ADHD causing a dopamine dysfunction, resulting in an increase extracellular dopamine, as well as reduced dopamine receptors in the striatum. Iron deficiency may also cause impair basal ganglia and cause an imbalance between inhibitory/excitatory neurotransmitters such as GABA (γ-aminobutyric acid) playing an important role in ADHD and being also low. Low iron levels in the thalamus were also found in children with ADHD compared to controls when using MRI helping explain the etiology of ADHD. Furthermore, since iron deficiency alters dopamine receptor density and activity, results suggest that brain iron stores may influence dopamine-dependent functions. Low serum ferritin levels have been associated in children with ADHD compared healthy controls. Some researchers have suggested that postnatal ID in children can be associated to the symptoms associated with ADHD due to the impaired dopaminergic neurotransmission caused by insufficient iron level [11].

In fact, hyperactivity, inattention, anxiety and even fatigue are common features in children with iron deficiency anemia and in ADHD [11]. One study interested in stress response found that infant suffering from postnatal IDA at 12 months of age have higher plasma cortisol level than the control group at 30 and 45 minutes after a vein puncture [14]. Another study with postnatal ID rats found that an ID diet causes internal physiological stress that is characterized by an increase in cortisol level from PNd10 to PNd40 than rats fed an IS diet [15]. Most results show that postnatal ID children with ADHD have lower serum iron and ferritin level than the control group [12, 11, 16].

Cortisol is a stress hormone produced by the adrenal glands and is released upon activation of the hypothalamic pituitary adrenal (HPA) axis [17, 18]. It is secreted

mainly during periods requiring energy or anabolic phases like pregnancy, child development and adolescence growth including stressful factors and events. Cortisol is used as a biomarker of stress due to its 20 minutes reactivity to a stress response [17]. Sustained elevated cortisol is associated with hippocampal atrophy followed by cognitive impairment, atherosclerosis, Cushing's disease and enlargement in adrenal glands in depressed patients [19, 20]. Although several studies have shown a link between postnatal ID in children with ADHD and other diseases such as Restless syndrome, to our knowledge, no study has specifically investigated the possible impact of ID during the prenatal phase on the progeny's stress and hyperactivity levels.

Our research group, however, has observed that guinea pigs offspring born from IDA mothers seem to be more hyperactive than the control group [21]. After reaching a normal hemoglobin (Hb) level at PNd24 with an IS diet fed at weaning, prenatal ID pups showed more movements than the IS group at PNd24 and PNd40 when tested in an open field. ID offspring were significantly more active with respect to higher central, external and peripheral square crossing including the time spent by movement than the control group. At PNd40, a decrease in number of movements and time spent per square was observed in both groups, but ID offspring still showed significantly higher level of activity than the control group. However, a research conducted in rhesus monkeys supports our guinea pig results. A depleted iron diet (10 mg Fe / g) was given during the prenatal period (n = 14) or during very early postpartum period (n = 12) to assess the difference in behavior of these animals compared to the control group (n = 12) (100 mg Fe /g) [22]. Iron deficiency during the prenatal period caused a more impulsive behaviour and lack of prudence facing danger in the progeny [22]. The postnatal group proved to be very different, as they demonstrated arousal, irritation and a cognitive delay. This study also suggests that less fearful and more impulsive behaviours were linked to prenatal iron insufficiency and more tense and withdrawn behaviours were related to postnatal iron deprivation [22].

For this project, we hypothesized that maternal iron deficiency (MID) has an impact on ID offspring hyperactivity, which will be validated by serum cortisol used as a biomarker

of stress at postnatal day (PNd) 24 and PNd84. The main objective was to evaluate the impact of MID during pregnancy on stress response in the offspring by measuring the serum cortisol levels as a biomarker of stress in the guinea pig.

2. Materials and methods

2.1 Animals and experimental design

The guinea pig was used as an animal model for various reasons. As in humans, cortisol is the primary glucocorticoid hormone involved in the guinea pig's stress response and it generally fluctuates according to various stressors [23, 24]. The guinea pig presents a decrease of hematocrit levels - a strong indicator of iron status - during the third trimester of its reproductive cycle with values similar to those observed in humans [25]. Contrary to other rodents, the guinea pig has a period of cerebral development that occurs prenatally just like in humans [26]. Also, the guinea pig is an excellent model for the purpose of social and behavioural experiments [27].

24 Hartley guinea-pig females and 2 males (Cavia porcellus, 13-18 weeks old) were purchased from Charles River Laboratories (St Constant, QC, Canada). All animals were housed in a controlled environment of ~ 22 ° C at the animal Unit They and on a 12-12hr light-dark cycle with lights on at 0700 at University of Ottawa. At their first day of arrival, females were randomly assigned to their respective diets; iron sufficient (IS: 114 mg/kg iron) or iron deficient diet (ID: 11.7 mg/kg iron) as purified pellets (Harlan Teklad, Madison, WI, USA) and were provided fresh water (*ad libitum*) daily. In contrast, both males received an IS diet during the entire study. Animals' body weights were measured every two days from arrival at the animal unit until the last day of the experiment.

Upon arrival, three weeks were allocated to all animals for their adaptation to the new environment and diets. After the third week of acclimation period, one male was placed

with six females equivalently in a large mating cage for 28 days for both dietary groups. The latter was crossed from one group of females to a new batch of females housed 1 month after the arrival of the first group to avoid any genetic confounding factor. Vaginal Smears technique confirmed successful mating between males and females. PNd1, day of birth in offspring, was determined when parturition appeared within 24 hours.

Once the pregnancy was confirmed by Vaginal Smears technique, the pregnant females were then placed in a separate cage of 3 for the period of gestation and lactation and fed their respective diets and the same applies for their offspring in the same cage until the weaning day. After weaning (PNd9), pups from the same litter (n=2) were placed in a cage and were fed the IS diet to limit the treatment effect to maternal IDA (i.e. the combined gestation and lactation time periods) until the last day of the experiment. The duration of the entire study was conducted in a one-year timeline. The research protocol was approved by the University of Ottawa of the Animal care committee and was in compliance with the National. Institutes of Health and National Research Council 'Guide for the Care and Use of Laboratory Animals'.

2.2 Organ and blood collection.

Tissue samples were collected on PNd16 for the dams and on PNd24 and 84 for the offspring. The organs were rapidly extracted and weighed, and wet weight values normalized using each guinea pig's body weight before statistical analysis. The weight of the hippocampus, heart, kidneys and adrenal glands (ADNA), including adrenal gland length were recorded. Droplets of blood were collected from each animal through a small puncture of the right ear, after swabbing with a 70% alcohol solution. Blood - approximately 60 mm of blood was collected into microcapillary tubes (Heparinized, pre-calibrated 100/Vial; Globe Scientific Inc) in pregnant females during each trimester and on the day of euthanasia (PNd16), and on PNd24 and 84 in the pups.

2.3 Hematocrit

To evaluate the hematocrit concentrations (an indicator of iron status), the microcapillary tubes were centrifuged at 10 000 rpm for 3 min using a HAEMATOKRIT 210-Hettich before taking a reading using the micro-scale method. Since the gestational (Gd) period last 75 days, hematocrit (Hct) were measured at Gd24, Gd48, and Gd65 to assess trimestral IDA. Hct was also determined at PNd24 and 84 in all offspring and at PNd16 in dams. In order to measure cortisol, a drop of blood from the microcapillary tubes samples were deposited for each stated time interval on Schleicher and Schuell specimen collection paper (Whatman International Ltd., Maidstone,UK) and let to dry for a 24 h period prior to freezing at -80 °C until further processes.

2.4 Cortisol Immunoassay

Sample Whatman cards were thawed from -80°C and cortisol levels from blood droplets were determined in duplicates using a commercially available ELISA kit (Cortisol EIA kit, Enzo Life Sciences, Cat. No. ADI-901-097). Briefly, a 3.0 mm diameter circle of each drop sample (n = 8 per group per blood collection interval) was punched from the blood stain cards using a Gem Hole Punch (McGill Inc., Marengo, IL), and placed in labeled glass tubes containing 280 µl of a solution consisting of assay buffer diluted in dH_20 at 1:10 concentration. The tubes were covered with parafilm and shaken on the Belly Dancer® Laboratory shaker (Structure Probe Inc., West Chester, PA) for 24 h at RT prior to the ELISA procedure. On the next day, 214.5 µl of each blood sample and 5.5 µl of steroid displacement reagent (SDR) were mixed in labeled aliquots and vortexed. Standards and samples were prepared as recommended by the manufacturer. Cortisol concentrations were determined in a PowerWave^{TM} XS2 Microplate Spectrophotometer (BioTek, Winooski, VT). Resulting CORT concentrations were calculated using a four-parameter equation that relied on constant values determined from the standard curve and quantified in units of pg/ml of corticosterone per punch. The analytic range of the assay was 32 – 20,000 pg/ml.

2.5 Data analysis

A series of linear mixed models were performed to take into consideration the variability associated with each animal. Regarding the weight of the dams, two linear models were fit. In the first model, we examined the relationship between the weight and the beginning and the end of the habituation period. In the second model, we examined the same relationship but for the gestation period. In both models, diet and period were treated as categorical fixed effects and the animal was integrated into the model as a random intercept effect (as confirmed by two independent likelihood ratio tests, $p<0.001$ and $p<0.05$, respectively).

Given that some pups were sacrificed on Day 24 and others were sacrificed on Day 84, we had an unbalanced design regarding the weight of the pups, which was measured on Day 1, Day 24 and Day 84. Consequently, we run two linear mixed models. In a first model, we compared the weights of the groups that were sacrificed on Day 24 whereas on a second model, we compared the weights of the groups that were sacrificed on Day 84.

A multivariate analysis was used for the weight of the kidneys and ADNA including the length of adrenal glands, to a strong correlation between the measures of the two weights (left and right) of organs in this case, for dams and pups. Dams and pups were compared with the respective diets (ID/IS) at the last day of the experiment. Bivariate analysis is also used with a Pearson correlation to help determine if any significant difference occurred in both groups. Separated analysis were also performed for the weight of the hippocampus and heart, used as dependent variables, in a t-test and two-way ANOVA in dams and in pups respectively in both dietary groups to see if any significant difference appeared.

Two different mixed linear models were performed for the Hct data, one for the dams and one for the pups. The linear mixed model performed on the dams' hematocrit included the diet and the periods of measurement as categorical fixed factor and a random intercept effect on the dams was added to the model, which was supported by a significant likelihood ratio test ($p<0.001$). The linear mixed model performed on the

pups' hematocrit included diet and day as categorical fixed factor. A random intercept effect on the pups was also included into the model, as supported by a significant likelihood ratio test ($p<0.001$).

Regarding the cortisol levels, our instrument was not able to detect cortisol levels over 84000 pg/ml. Consequently, 5 females out of 10 in the IS group and 2 females out of 10 in the IDA group had censured data. Thus, 35% of our sample presented censured over 84000 pg/ml. To perform multiple imputations of the censured data, we used R [29] and the package Amelia [30]. We hypothesized that the missing values were at random and normally distributed over a range between 84000 and 150000 (99% confidence interval). Our imputation model also included the three trimesters during the gestation period. As the observed individual trend of the cortisol for each animal as a function of the trimesters was linear, we imputed the data with a linear model. In addition to the intra variability of the cortisol for each animal, the model included the variable diet. For each animal, 50 imputations were performed, which is higher than the usual recommendation for this kind of imputation process. In addition, the relative efficiency values for each coefficient were all over 0.99, which suggested that our imputation model was very reliable in estimating the regression coefficients of our mixed model. Stata 14.0 [31] was used to analyze the imputed data set. More specifically, for both diets (IS and IDA), we used a mixed linear model to predict the dams' cortisol as a function of the period 3 trimesters. In the model, trimester and diet were treated as a categorical fixed effect. To consider the variability associated with each dam, a random intercept for each dam was integrated into the model. The level of cortisol at the postpartum period for the IS and the IDA groups was compared via an independent t-test (two-way).

We were not able to get a reliable measure of cortisol for 2 pups (one IS and on IDA pup) and those pups were excluded from the analysis. To analyze the pups' cortisol levels, an ANOVA diet (2) x day (2) with sex as covariate was performed. Due to small number of pups in the IDA groups at both PNd24 and PNd84, we could not include gender in the interaction term.

The criterion of $p<0.05$ was be used to reject the null hypothesis and two sided tests were

applied to compare the results of the two groups (ID, IS). And all mixed linear models were estimated with an uncorrelated covariance structure and the final models were fit by maximum likelihood (ML).

3. RESULTS

3.1 Body weight data - Dams

The mixed linear model showed an inter class correlation (ICC) of 0.611, which revealed that 61% of the variability of the weight was attributable to the individual dams. During the habituation period, there was no effect of diet or interaction between diet and period. However, there was a significant effect of period ($p<0.001$), indicating that both groups of dams gained weight during the habituation period. During the gestational period, 30% of the variance was attributable to the variability among the dams (ICC=0.299). The mixed model also revealed a significant interaction between diet and period ($p<0.001$). The IDA dams had a significant lower gestational weight gain than IS dams during the gestational period (477.29 ± 22.45 and 587.75 ± 27.96, respectively).

3.2 Body weight data - Pups

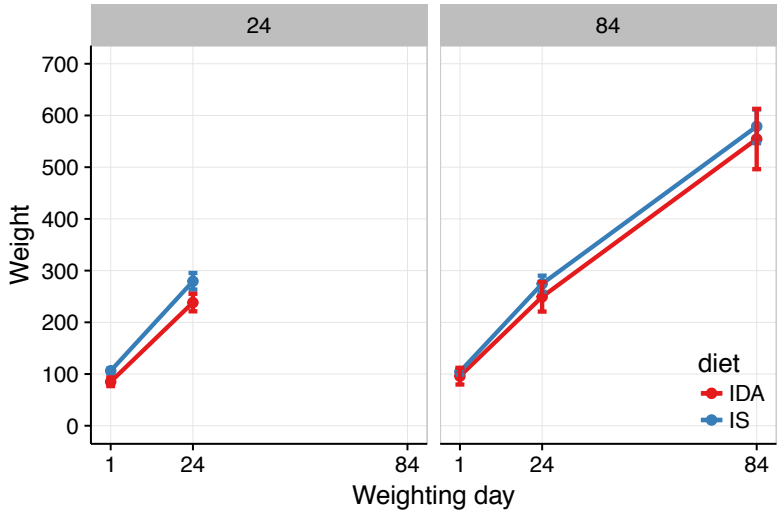

Figure 1 a) and b) Pups' body weight at PNd1, PNd24 and PNd84 in both dietary groups.

Figure 1 a) Pups' body weight of each dietary group as function of the day they were weighted at PNd1 and PNd24.

Figure 1 b) Pups' body weight of each of the 2 diet groups as a function of the day the pups were weighted at PNd1, PNd24 and PNd84.

Data are means, n=12 IDA (n=7 at PNd24 and n=5 at PNd84) and n=26 IS (n=13 at PNd24 and n= 13 at PNd84) for the pups. The error bars represent the 95% confidence intervals.

A first mixed model performed on the 24-day diet groups revealed an inter class correlation (ICC) of 0.319, which indicate that 32% of the variation of weight was the result of the random effects attributed to the individual pups (**Fig. 1a**). The inclusion of this random effect was supported by a significant likelihood ratio test (p=0.04). The mixed effects model revealed that the pups' weight of the IDA diet group was significantly lower than the one of the IS diet group (p<0.001) which may be linked with lower gestational weight in IDA dams. Pups' weights also increased from Day 1 to Day 24, which was expected. No interaction was observed between the diet groups and the day of the weighting.

A second mixed model performed on the 84-day diet groups showed an ICC of 0.385 (**Fig. 1b**). Thus, about 39% of the weight variation was associated with the individual variation of each pup. The addition of the random intercept effect was significant

(p<0.001). There was no difference between the two diets (p=0.14) and no interaction between the diets and the weighting days (p=0.486). Unsurprisingly, the weight increased from Day1 to Day84.

3.3 Organs weight and length data

Among the dams, the weight of the hippocampus (0.36 ± 0.02 g [IS n=23] vs 0.37 ± 0.01 g [IDA n=22]), heart (2.64 ± 0.12 g [IS n=12] vs 2.52 ± 0.10 g [IDA n=12), left (L) and right (R) kidneys and adrenal glands (ADNA) (**Table 1**) were not significantly different between the two dietary groups. In addition, the lengths of adrenal glands were also comparable in both groups (**Table 1**).

Table 1 Weight of both kidneys and adrenal glands and length of adrenal glands of dams and pups at the day of sacrifice in the two dietary groups[1]

Kidneys and Adrenal glands	**IS**	**IDA**	**P-value**
Dams			
Weight (g)			
R Kidney	2.50 ± 0.11	2.33 ± 0.06	0.175
L Kidney	2.66 ± 0.13	2.40 ± 0.06	0.095
R ADNA	0.22 ± 0.02	0.23 ± 0.03	0.813
L ADNA	0.27 ± 0.02	0.22 ± 0.02	0.127
Length (cm)			
R ADNA	1.10 ± 0.03	1.07 ± 0.03	0.546
L ADNA	1.14 ± 0.04	1.23 ± 0.05	0.203
Siblings, PNd24			
Weight (g)			
R Kidney	1.26 ± 0.10	1.00 ± 0.07	0.075
L Kidney	1.24 ± 0.11	1.01 ± 0.07	0.147
R ADNA	0.06 ± 0.01	0.04 ± 0.00	0.031*
L ADNA	0.07 ± 0.01	0.05 ± 0.00	0.049*

Length (cm)			
R ADNA	0.73 ± 0.03	0.66 ± 0.04	0.118
L ADNA	0.73 ± 0.02	0.64 ± 0.04	0.037*
Siblings, PNd84			
Weight (g)			
R Kidney	1.85 ± 0.09	1.68 ± 0.15	0.335
L Kidney	1.85 ± 0.11	1.66 ± 0.16	0.364
R ADNA	0.11 ± 0.01	0.11 ± 0.00	0.616
L ADNA	0.12 ± 0.01	0.12 ± 0.01	0.934
Length (cm)			
R ADNA	0.88 ± 0.03	0.96 ± 0.02	0.206
L ADNA	0.93 ± 0.03	0.92 ± 0.02	0.795

[1] Data are means ± SEM, n=12 (IDA) and 12 (IS) for the dams or n=12 IDA (n=7 at PNd24 and n=5 at PNd84) and n=26 IS (n=13 at PNd24 and n= 13 at PNd84) for the pups.

Among the pups at PNd24, the weight of the hippocampus (0.25 ± 0.02 g [IS n=25] vs 0.24 ± 0.02 g [IDA, n=15]), heart (1.25 ± 0.10 g [IS n=13] vs 1.11 ± 0.13 g [IDA, n=7), kidneys (R and L) and adrenal gland length (R) (**Table 1**) were comparable. Despite the weight of both adrenal glands and ADNA length (L) in IDA pups being significantly reduced versus the IS group, no significant correlation or even nearing significance between both ADNA weight (r=0.0490, p=0.9376) was observed. This is mainly due to the reduced number of pups in the IDA group versus to the IS group and possibly to a low variability among the pups, which may be due to a non-accurate measure.

Similarly at PNd84, the weight of the organs and length of adrenal glands were comparable between both dietary groups; hippocampus (0.29 ± 0.03 g [IS n=27] vs 0.27 ± 0.03 g (IDA n=11), heart (1.92 ± 0.17 g [IS n=13] vs 1.98 ± 0.16 g [IDA, n=5), kidneys and adrenal glands (**Table 1**) including the length of adrenal glands. However, as expected, all pups' organs weight and adrenal glands' length increased from PNd24 to PNd84 ($P<0.05$).

3.4 Hematological data

The mixed linear model revealed a significant interaction between diet and periods ($p<0.001$). The IS dams (n=24) maintained normal Hct (44.34 ± 0.57) throughout gestation (**Fig. 2**). However, the IDA dams, had significantly lower Hct at the trimester 2 and 3 compared to the IS group ($P<0.001$) (**Fig. 2**). In addition, the mean Hct value among IDA females was below the normal range (0.37-0.48) [22] at the trimester 3. During the postpartum phase, (PNd16), the dams's mean Hct values (**Fig. 2**) increased in both groups. However, the IDA dams still showed Hct values below normal and significantly lower than those observed in the IS group ($P<0.001$).

The mixed linear model performed on the pups' hematocrit levels showed a significant interaction between diet and day ($p<0.001$). At PNd24 and PNd84, the pups Hct values were in the normal range in both dietary groups. However, offspring born from IDA dams showed significant slightly lower Hct values compared to those born from IS group at PNd24 (42.33 ± 0.69 and 44.68 ± 0.54, respectively, $P<0.05$). At PNd84, the effect of maternal ID on the pups Hct levels could not be observed: the IDA pups had the same Hct levels than those of the IS siblings (50.67 ± 0.86 and 48.50 ± 0.52).

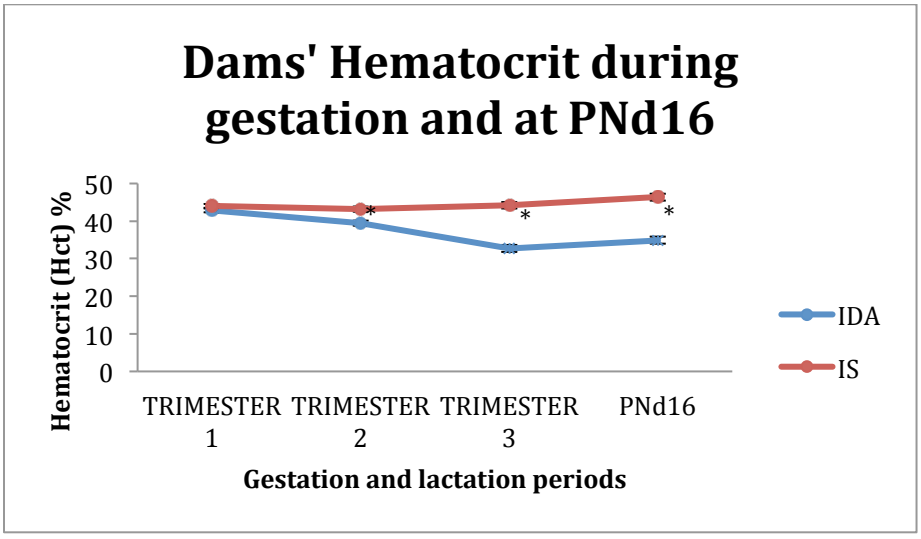

Figure 2 **Dams' hematocrit values during first, second and third trimesters and at PNd16 in the IDA and IS groups.**

Hct of dams guinea pigs fed an IDD or ISD during gestation and at the last day of the experiment. Data are means ± SEM, n=23 (IDA) or 24 (IS). *Different from IS, $P \leq 0.001$.

3.5 Serum cortisol

Our mixed linear model with imputed data showed that dams' cortisol values increased from trimester 1 to 2 (t=3.49, p=0.00) and from trimester 1 to 3 (t=7.78, p=0.000) in both dietary groups. These results show that the levels of MSC increase progressively in anabolic phase. However, there was no interaction and the dams had comparable cortisol values in both dietary groups during pregnancy (trimester 1, 2 and 3) and the postpartum period.

The ANOVA diet x day with sex as covariate revealed a significant interaction ($p < 0.01$) and a significant effect of sex ($p < 0.005$). A series of a post hoc contrasts revealed that IDA offspring showed significantly higher cortisol levels (p=0.008) (**Fig. 3**) compared to IS pups only at PNd24. At PNd84, the levels of cortisol were comparable in both dietary groups. Pups' cortisol values decreased from PNd24 to PNd84 in both groups. However, this cortisol reduction was statistically significant (p=0.001) only for the IDA group.

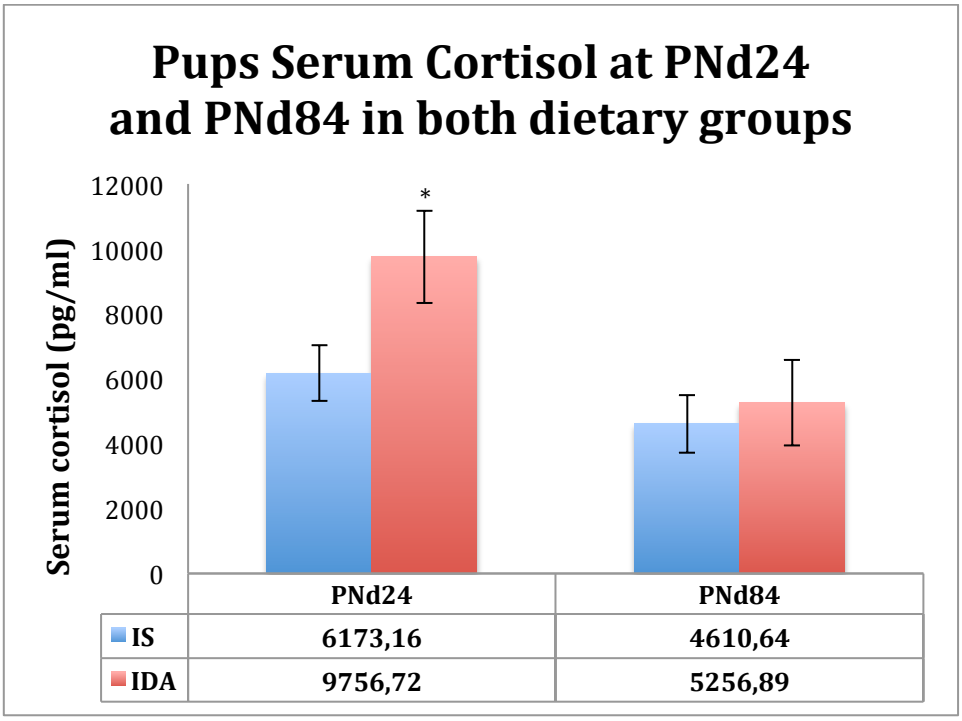

Figure 3 Pups serum cortisol values at PNd24 and PNd84 in both diet groups

[1] Data are means ± SEM, n=11 IDA (7 PNd24 and 4 PNd84) and n=25 IS (13 PNd24 and 12 PNd84) in pups.

[2] Significant difference (p=0.008*) by diet at PNd24

[3] No significant difference (p=0.590) between PNd24 and 84 among IS siblings

[4] Significant difference (p=0.001*) between PNd24 and 84 among IDA siblings

* Different from IS, $P \leq 0.05$

4. Discussion

The aim of this study was to determine if MID during pregnancy had an impact on the cortisol secretion in the guinea pig offspring. We also assessed cortisol in the dams at distinct time intervals during pregnancy.

Our findings confirmed effects of the ID diet in inducing a mild maternal IDA in the pregnant dams. This condition is common in pregnant women in industrialized countries. Here, we report that the dams fed the ID diet had lower Hct values compared to those fed

the IS diet during the trimester 2 and 3, which were below normal range values during the third trimester. These observations are consistent with studies supporting Hct and Hb values below the normal range in the third trimester in MID pregnant women from developed countries [32, 33]. Our results also confirmed that iron deficiency in the ID group remains in a mild form as mean Hct values stayed above 30% of anemic values [34]. Following weaning, the pups from both prenatal dietary groups were fed the IS diet; our findings indicate that this led Hct values to reach the normal range for all pups at PNd24 and PNd84, although Hct counts appeared slightly reduced in IDA pups compared to IS siblings at the PNd24 interval. Most results support an association between maternal Hct's status and that of their offspring [3, 4, 8, 9]. Nonetheless, low Hct values, during early development, in offspring as well as in children are physiologically normal and is due to the anabolic period of life [35-37].

The IDD induced weight loss in IDA dams compared to the control group during gestation and lactation. Over the course of pregnancy, IDA dams showed a reduced body weight compared to IS dams. Similarly, IDA weighed less than their IS control group siblings at birth and PNd24. These findings are consistent with studies showing that IDD led to reduced weight gain accompanied by reduced energy levels [8, 9].

Our findings support that despite the fact that dams' gestational cortisol levels were similar among ID and IS groups, IDA pups showed increased cortisol secretion at PNd24 compared to IS counterparts. These values were normalized by PNd84, suggesting that the effects might be transient. Similarly, IDA rats showed higher serum corticosterone values (72, 43, 44 and 46 mg) compare to non-anemic rats (25, 26, 22 et 14 mg) when levels were measured at 10, 20, 30 et 40 post-natal days. Our findings also suggest a reduced impact of IDA on corticosterone secretion as time elapsed [15]. In support of these findings, one study showed that 12-month old babies suffering from IDA had significantly elevated cortisol secretion versus non-anemic babies 30 and 45 min following venipuncture [14]. Together, these results suggest that iron deficiency represents an internal stressor that could increase cortisol secretion. These findings are consistent with previous results from our lab showing that pups born from ID dams were

significantly more active than the IS control group at PNd24 [21]. At PNd40, movements and time spent per square decreased in pups from both groups, but IDA pups still showed significantly higher score than IS control group. These results suggest that maternal ID may alter the level of stress or arousal through an elevation serum cortisol affecting activity level in the offspring [17, 18]. Of interest, recent studies have proposed a relationship between ID and symptoms of ADHD in children [10-12]. For instance, a study assessing children and adolescents aged between 4-14 years with ADHD found that the level of serum ferritin was correlated with ADHD symptom-severity measured using the "Conners' Parent Rating Scale (CPRS): cognitive subscore and tended toward a correlation with the hyperactivity subscore. These findings suggest that children suffering from iron deficiency are more inattentive, easily distracted and experienced learning difficulties, and that low ferritin levels likely contribute to discrete ADHD symptoms. In juvenile and adult rats, postnatal ID has been associated with increased anxiety [38].

Very few studies have investigated the impact of maternal ID on levels of stress and hyperactivity in the offspring. With the exception of our previous study focusing on the impact of MID on hyperactivity in the guinea pig offspring, another research conducted in rhesus monkeys supports similar results [22]. In this later study, prenatal ID animals showed heightened impulsivity and lack of prudence when facing a dangerous situation compared to IS monkeys. They also showed increased arousal, irritation and cognitive deficit. In this context, another study found that guinea pig offspring born to ID dams showed reduced memory and increased anxiety and nervousness when exposed to an open field test and Morris-Water-Maze to measure locomotor behavior [39].

Our study did not show lasting effects of MID on the weight of gross measure of the hippocampus, heart, kidneys and adrenal glands. Other findings showed reduced cross-sectional area volume using stereological approach in perinatal ID rats hippocampus compared to IS group when entering adulthood despite the correction of brain iron deficiency [40]. In rodents, stressors have been associated with reduced kidney volume [41]. In our findings, the weight of the kidneys and adrenal glands were correlated in the absence of significant effects, demonstrating that their size were not affected by the diet.

Adverse consequences of severe or chronic stress also include adrenal gland hypertrophy [42]. The fact that the impact on cortisol secretion was no longer present at PNd84 may explain the absence of such changes. To date, very few studies have investigated the impact of MID on these organs weight and/or size. Increased number of ID pups to reach comparable numbers as the IS group may have helped better discriminate the groups on these variables. Some researchers showed the link between iron deficiency and heart failures, but no study has demonstrated a link between ID and heart size [43].

5. CONCLUSION

Our study is the first to investigate the impact of maternal iron deficiency in the young and adult guinea pig using serum cortisol as a biomarker of stress. Our findings suggest that increased hyperactivity in the open field exploration task observed in pups born to ID dams is coincident with elevated of cortisol levels in the pups likely affecting arousal levels in this task. Further studies measuring precisely the hyperactivity in our animal model with a larger sample size are needed to support our hypothesis that iron deficiency during pregnancy is leading to hyperactivity in the offspring. Our findings emphasize the importance of preventing iron deficiency during pregnancy in order to protect the offspring from potential detrimental cognitive, affective and endocrine outcomes.

Because of the long-term impact of MID on hyperactivity in the offspring, its outcome also seems to be paradoxical. Some people suffering with ID may feel tired due to the lack of energy and others may feel hyperactive. By using the psychosocial stress model (PSM), etiology of hyperactivity would be better explained with the concept of stress by using iron deficiency as an internal stressor. Meanwhile, it is recommended to better understand coping mechanisms with the guinea pig as an animal model by using the PSM and to see if this outcome occurs also in elderly guinea pig.

Acknowledgment

N.A., M.E.S., S.F., and F.M.R., were involved in the experimental design; N.A & M.T. conducted research; N.A. and S.F. performed statistical calculations; N.A. and S.F. analyzed the data; N.A. wrote the paper; and N.A., S.F., H.P. and F.M.R., contributed to the review and editing of the manuscript. All authors read and approved the final manuscript. This research project was supported by the Canadian Institute of Health Research (grant number#114908).

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CHAPTER V

IMPACT OF MATERNAL IRON DEFICIENCY ON THE AUDITORY FUNCTIONS IN THE YOUNG AND ADULT GUINEA PIG

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ABSTRACT

Objectives: The aim of this study was to evaluate the hearing function in guinea pig offspring at postnatal day (PNd) 24 and PNd84 born from dams suffering from iron deficiency during pregnancy and lactation by using the auditory brainstem response (ABR).

Method: Female guinea pigs (*n*= 12 per dietary group) were fed an iron sufficient (IS) diet (114 mg/kg) or an iron deficient (ID) diet (11.7 mg/kg) during the gestation and lactation periods. Pups in both groups were weaned at PNd9 and given the IS diet. The hematocrit level was measured at every trimester of pregnancy and at the day of sacrifice in dams and at PNd24 and PNd84 in pups. The animal body weight was measured on every second day until the day of sacrifice. The ABR was used in pups to measure the hearing threshold using a broad range of stimulus intensities and latency at 100 and 80 dB in response to 2, 4, 8, 16 and 32 kHz tone pips at PNd24 and 84.

Results and discussion: No significant difference between dietary groups was measured in hearing threshold and absolute latencies in pups at PNd24 and PNd84. Although the ID offspring (n=16) did not differ in brainstem transmission times (BTTs) at 80 dB compare to the IS siblings (n=25) at PNd24, they showed significant delayed interpeak latency (IPL) I-IV at 100 dB suggesting a delayed BTT. At PNd84, the latency of all peaks including IPL I-IV at 80 and 100 dB significantly decreased and was also similar in pups

from both dietary groups suggesting a better brain maturation. This is the first study investigating the long term impact of maternal iron deficiency on the auditory functions in the guinea pig offspring during early development to adulthood.

Keywords

Auditory brainstem response; Threshold; Latency; Brainstem transmission times; Offspring; Maternal iron deficiency; Hematocrit.

Introduction

Iron deficiency anemia (IDA) is the most common nutrient disorder worldwide affecting 1-2 billion people.[1,2] Pregnant women and children are adversely affected by IDA whose global prevalence rates are 38% and 43%, respectively.[3] In developing countries, as much as 50% of pregnant women suffer from IDA and about 10-20% in industrialized nations.[4,5]

Physicians often prescribe iron supplements to pregnant women in order to compensate for the inadequate iron intake in response to the elevated demand due to the development of the fetus and insufficient iron storage and limited absorption from food in the woman's body, but not all pregnant women follow the iron supplement prescription due to gastrointestinal (GI) complications (nausea, vomiting, diarrhea, constipation, heartburn and abdominal cramps).[6,7] Negative health consequences for the infant may be important during this anabolic period if pregnant women's diets do not meet the increased iron requirement for the development of the fetus.[6,8]

To date, few researchers have demonstrated the impact of maternal iron deficiency (MID) on the development of the fetus and the infant to be born. Iron is actively involved in the central nervous system myelination.[9-10] Furthermore, this essential micronutrient is implicated in the synthesis of neurotransmitters such as serotonin, dopamine and ϒ-amino butyric that all play an important role in the synaptic transmission of the cochlea

(inner ear) and the auditory *nuclei* of the brainstem[10] whose maturation is almost complete at birth in human term infants.[11] Iron also plays an important role in several biochemical processes such as transport and delivery of oxygen, growth, cellular differentiation, electro-mitochondrial transfer and numerous enzymatic systems.[12-17]

Given the major role of iron in early brain development, IDA during pregnancy is likely to impair the infant's brain functioning.[11] To date, the consequences of maternal IDA on the infant brain's neurophysiological development are not fully understood.

To evaluate the neurophysiological function of the brain, a clinical tool known as auditory brainstem response (ABR) is used to assess brain responses to auditory stimulations in human and animal models. It is a noninvasive method that is based on sensory-evoked potential extracted from an electroencephalogram. This test evaluates the maturity of the brain, the neural integrity including auditory function and is also used as an indicator of the myelination process.[11] ABR also measures the auditory acuity, also known as the threshold, indicated by the lowest stimulus intensity to evoke a reliable response, as determined by visual inspection. More precisely, auditory thresholds reflect the lowest signal that a person or animal can hear against the background noise that is present in an environment.[18]The ABR also provides measures of the brainstem transmission times (BTTs); an indicator of brain maturation which are measured by the latency between peaks of a wave known as interpeak latency (IPL). Indeed, the BTT characterizes a neuronal transmission velocity between waves. The ABR also characterizes the amplitude, which reflects the number of neurons recruited in an auditory stimulation, the nucleus involved and their level of synchronization. It also allows the detection, quantification and differentiation of peripheral and central hearing disorders.[19]

Several human and animal studies have examined the impact of ID during the postnatal phase using the ABR method.[11,19,20] Results demonstrated that post-natal IDA impairs brain and auditory functions with an increase in absolute peak I, III and IV and I-IV interpeak latencies, suggesting an abnormal auditory neural maturation in early development. which is irreversible in some cases despite iron therapy.[11,19,20]

Other results not using the ABR have also shown that postnatal ID during early development in mice impairs brain lipid content by a decrease in myelination and alters its composition, reduces the proliferation of glial precursor cells and damages oligodendrocytes generation.[21] Results in rodents suffering from IDA showed an alteration in auditory acuity including a sensorineural hearing loss (SNHL) and cellular damages[22], whereas others showed no significant difference by using the ABR.[23]

To date, very few studies have investigated the impact of maternal IDA on the offspring auditory functions, during middle term development. In one study, IDA induced in late pregnancy in sheep resulted in delayed auditory nerve velocity as measured by an increase in absolute latencies I-IV in unsedated newborns compared to unsedated adults.[20] The authors of this study suggested using the increase in latency to recommend a red blood cell transfusion. However, more studies using different animal models are recommended to validate the impact of low ID on latency and red blood cell transfusion. Another study in rhesus monkeys found that MID did not impair the ABR but induced changes in the juvenile's behaviour.[24] Our research group has previously observed that mild iron deficiency during gestation and lactation impairs the central nervous system (CNS) auditory functions in the guinea pig offspring during childhood.[25-27] Two groups of guinea pigs were fed an iron sufficient (IS) or iron deficient (ID) diet; from PNd9 onward, the ISD was given to both groups of weaned offspring. ABR was assessed in the offspring on PNd24. The results showed elevated thresholds (hearing loss) associated to SNHL[25], prolonged Peak I latencies (delayed peripheral nerve transmission time) and increased PIII-NIII amplitudes (hyperacusis) in siblings from ID dams compared to the control group[26]. In contrast, the brainstem transmission times were unaltered[26]. Another study using the same animal model also found hearing impairment in offspring born from ID dams.[28] Our previous research group using the same animal model also found an alteration in the auditory acuity in offspring born from ID dams during childhood.[25] However, our previous studies did not examine the middle term consequences of gestational ID on the offspring's auditory functions, more precisely when entering adulthood. Hence, very little is known about the consequences of MID on auditory functions in the offspring later in life. Studies still need to evaluate if the impact of MID

on CNS auditory functions becomes more important, disappears or stays stable in the offspring.

Thus, the main objective of this study is to determine the impact of a mild iron deficiency during gestation and lactation in the guinea pig offspring at PNd24 and later at PNd84 when the guinea pig has reached is adult stage by using the ABR, an efficient tool for postnatal brain maturation.

Methods and Materials

Animals and experimental design

In this study, the guinea pig was used as an animal model for several reasons. Similarly to humans, pregnant female guinea-pig have a significant decrease in their hematocrit (Hct) level during the 3rd human trimester of gestation.[29] Furthermore, contrary to other rodents, the guinea pig has a period of rapid cerebral development that occurs in the prenatal phase similar in humans.[30]

In this study, we used 24 Hartley guinea-pig females and 2 males (*Cavia porcellus*, 13-18 weeks old), purchased from Charles River Laboratories (St Constant, QC, Canada). The animals were housed in a controlled environment of ~ +22°C in the Animal Care Unit at University of Ottawa. They were housed on a 12-12hr light-dark cycle with lights on at 0700. On their arrival, females were randomly assigned to their respective diets; iron sufficient (IS: 114 mg/kg iron) or iron deficient diet (ID: 11.7 mg/kg iron) as purified pellets (Harlan Teklad, Madison, WI, USA). Both males received the IS diet. Fresh water was provided (*ad libitum*) daily. Body weights were measured every two days from arrival at the animal unit.

Three weeks were allocated to all animals for their adaptation to the new environment and diets. After the third week of acclimation period, one male was mated with six

females in a large mating cage for 28 days, for each dietary group. The same number of females per dietary group were mated with the same male; the latter was crossed from one group of females to another new group of females housed 1 month after the arrival of the first group to avoid any genetic confounding factor. After successful mating confirmed by Vaginal Smears technique, three (3) females were placed in separate cages and fed their respective diet throughout gestation and lactation. The same applies for their siblings which were in the same cage until PNd9 (weaning day). PNd1 (day of birth) was determined when parturition appeared within 24 hours. ID and IS dams gave birth to 16 and 25 pups at PNd24 and 11 and 27 pups at PNd84, respectively.

At weaning (PNd9), pups from the same litter (n=2) were housed in a cage and both dietary groups of siblings were fed the IS diet to limit the treatment effect to maternal IDA (*i.e.* the combined gestation and lactation time periods). The duration of the entire study was conducted in a two-year timeline.

The research protocol was approved by the University of Ottawa Animal care committee and was in compliance with the National Institutes of Health and National Research Council 'Guide for the Care and Use of Laboratory Animals'.[31]

Hematocrit samples

Dams Hct was assessed during each equivalent trimester of pregnancy and just before sacrifice (PNd16). A blood puncture was done on the right ear of each animal after swabbing it with a 70% alcohol solution. Since the gestational period is approximately 75 days, Hct was measured at gestational day (Gd) 24, Gd48, Gd65 to determine at which equivalent trimester of pregnancy the IDA was induced. Hct was also assessed at PNd24 and 84 in all offspring. Approximately 60 mm of blood was collected into heparinized, and pre-calibrated 100/Vial microcapillary tubes (Globe Scientific Inc). The microcapillary tubes were then centrifuged at 10 000 rpm using a HAEMATOKRIT 210- (Hettich ZentriFugen), for 3 minutes before taking a reading in a microhematocrit reader.

Auditory brainstem response (ABR)

All pups were sedated and tested at PNd24 and PNd84. Each animal received an intra muscular injection (2.3 mL/kg) of an anesthetic solution of ketamine/xylasine (2:1) before data recording. Ketamine alone does not alter ABR thresholds and allows an excellent recording quality.[32] When sedated, animals were placed on a circulating water heating pad to maintain normothermia during the experiment. Since temperature can influence the ABR, rectal temperature was monitored every 2 minutes, and corporal thermic fluctuations of more than ± 0.5°C were avoided by regulating the circulating fluid temperature during all the experimentation.[25-27]

Frequencies were submitted in a decreasing order, from 32, 16, 8, 4 and 2 kHz. Animals were exposed to a range of stimulus intensities starting from 100 dB SPL (decibel peak-equivalent Sound Pressure Level) followed by a descending order from 80 to 20 dB SPL, with 10 dB SPL steps, well above the threshold and with 5 dB SPL steps near the supra-threshold. Two animals had a hearing threshold that reached until 5 dB. At the end of the automated procedure, every frequency was reviewed and new traces (ranging from 2 to 4) were collected with 2 to 3 dB peSPL changes in the intensity around threshold, to establish this latter with more precision.

ABR measures, thresholds as well as positioning of peaks and nadirs were processed by 2 investigators, unaware of animal treatment group. A second experimenter was involved in a cross-check, to confirm the data reliability. Thresholds and latencies for peaks I-IV were measured in this condition.

ABR latencies (neural transmission times) and Peaks I-IV (Brain transmission time)

The ABR reflects a succession of series' of action potentials and postsynaptic relays along the 8th nerve and brainstem auditory pathway called neurogenerators identified as waves or peaks (P) and *Nadirs* (N).

Firstly, absolute PI, II, III and IV were identified at the beginning of every wave and follow each other simultaneously between 1 to 5 ms. However, these peaks were not always available for 80 dB at 2 kHz. This methodology of identifying peaks was from the study of Wada & al.[33] For example, a wave at 100 dB showing the first peak between 1 and 2 ms would be considered as PI. Next, PII would be the one between 2 and 3 ms and so on for PIII and PIV. PIII usually has the highest amplitude.

Secondly, absolute PI, PII, III and IV latencies were analyzed as dependent variables for all tone pips frequencies (32, 16, 8, 4 and 2 kHz) at 100 and 80 dB intensities. The I-IV IPLs were also assessed as separate dependent variables to measure the BTT along the brainstem portion (central) of the auditory pathway. The I-IV IPLs were measured in response to 100 kHz tone pips (waves at low frequencies-2 and 4 kHz were not clearly identifiable at the 80 dB intensity). Absolute latencies and IPLs are effective tools to differentially diagnose impaired transmission times in the peripheral and/or the central part of the conductive pathway.

Data analysis

In all analyses, our main objective was to examine the impact of maternal iron deficiency anemia (IS diet *vs* IDA diet) on the progeny at the age of 24 and 84 days. To reach this objective, we performed a series of linear mixed models on the pups' auditory threshold, absolute latencies and inter-peak latencies. Additional linear mixed models were also performed on pups' weight, body temperatures and hematocrit levels. All linear mixed models were estimated with an uncorrelated covariance structure and the final models were all fit by maximum likelihood (ML).

To avoid the possibility of damaging the pup's hearing organ, we did not test the pups' threshold over 100 dB. Due to this decision, 8.9% of our data for the threshold were censured over 100 dB (especially at 2, 4 and 8 kHz). We used R[34] and the package Amelia[35] to impute the censured data. To impute the data, we hypothesized that for each frequency, the missing values were at random, normally distributed and over 100 dB. As

the observed individual trend of the threshold for each animal as a function of the frequencies was clearly cubic, we imputed the data with the variable frequency elevated to the 3rd degree. In addition to the intra variability of the threshold for each animal, the model included the variables sex, age, diet and dams' id. For each animal, 50 imputations were performed, which is higher than the fraction of missing information (FMI) estimated for each coefficient of the model. This is over the usual recommendation for this kind of imputation process. In addition, the relative efficiency values for each coefficient were all over 0.99, which suggested that our imputation model was very reliable in estimating the regression coefficients of our mixed model.

Stata 14.0[36] was used to analyze the imputed data using a linear mixed model. More specifically, for both diets (IS and IDA), we used a linear mixed model to predict the pups' auditory threshold as a function of frequency (from 2 to 32 kHz) and day (24 and 84 days of age). In the model, frequency was treated as a continuous fixed effect and diet and day were treated as categorical fixed effects. To facilitate the interpretation of the coefficients, frequency was transformed on a scale from 1 to 5, which represented the increasing values of the different octaves from 2 kHz. In addition, to fit the predicted values of the model to the observed data, frequency was elevated to the third polynomial degree (cubic relationship). To consider the variability associated with each pup, a random intercept for each pup was integrated into the model. In a previous model, the variable frequency was also included in the model as random slope effect, allowing us to consider the individual slope of each pup as a function of frequency. However, the addition of this random slope effect was not significant (Likelihood test, $p > 0.05$) and it was dropped from the final model.

Given that the weight of the pups that were sacrificed at Day 24 could not be measured at Day 84, two linear mixed models were run on pups' weight. In one model, the weights of the groups that were sacrificed at Day 24 were compared on Day 1 and Day 24. In the second model, the weights of the groups that were sacrificed at Day 84 were compared on Day 1, Day 24 and Day 84. In both models, diet and day were treated as categorical fixed factors and a random intercept was included.

To determine if the body temperature changed as a function of the diets, day and frequency, a linear mixed model was performed on temperature. In this model, diet and day were treated as categorical fixed factors and the frequency was treated as a continuous fixed factor. In addition, a random effect on the intercept and a random effect on the slope were added to the model, which was supported by a significant likelihood ratio test ($p < 0.001$).

For the hematocrit data, two different linear mixed models were performed, one for the dams and one for the pups. The linear mixed model performed on the dams' hematocrit included the diet and the periods of measurement as categorical fixed factor and a random intercept effect on the dams was added to the model, which was supported by a significant likelihood ratio test ($p<0.001$). The linear mixed model performed on the pups' hematocrit included diet and day as categorical fixed factor. A random intercept effect on the pups was also included into the model, as supported by a significant likelihood ratio test ($p<0.001$).

Finally, two different linear mixed models were performed on the pups' latency. One model was run at 80 dB and a second was run at 100 dB. At 80 dB, 96.52% of the data were missing at a frequency of 2 kHz. We therefore removed this frequency from the analysis and performed the mixed model using frequencies 32, 16, 8 and 4 kHz. At 100 dB, the percentage of missing data was less drastic (e.g. 42.95% at 2 kHz, 16.35% at 4 kHz, 6.01% at 8 kHz, down to 1.06% at 32 kHz) and we kept all frequencies (32 to 2 kHz) in the model. For both models, we imputed the missing data using the same approach as the one described for the threshold values. In both models, diet, day and peak were treated as categorical fixed effects and frequency was treated as a continuous fixed effect. In addition, as supported by a likelihood ratio test ($p<0.001$), both models included a random intercept effect on the pups and a random slope effects on the frequency.

RESULTS

Weight

A first mixed model performed on the 24-day diet groups revealed an inter class correlation (ICC) of 0.319, which indicate that 32% of the variation of weight was the result of the random effects attributed to the individual pups. The inclusion of this random effect was supported by a significant likelihood ratio test ($p=0.04$). The mixed effects model revealed that the weight of the IDA offspring was significantly lower than the one of the IS diet group ($p<0.001$) and that pups' weight increased from Day 1 to Day 24, which was expected. No interaction was observed between the diet groups and the day of the weighting.

At PNd84, a second mixed model showed an ICC of 0.385 in pups from both diet groups. Thus, about 39% of the weight variation was associated with the individual variation of each pup. The addition of the random intercept effect was significant ($p<0.001$). No significant difference was shown between the two diets ($p=0.14$) and no interaction between the diets and the weighting days ($p=0.486$). Unsurprisingly, the weight increased from Day1 to Day84.

Hematological data

The linear mixed model revealed a significant interaction between diet and periods ($p<0.001$). The IS dams (n=24) maintained an adequate Hct (44.34 ± 0.57) throughout gestation (**Tab.1**) based on normal Hct values for guinea pig. At the second and third trimester, the IDA dams had significantly lower Hct compared to the IS group ($P<0.001$). Furthermore, the mean Hct value among IDA females was below the normal range (0.37-0.48)[22] at the 3rd trimester (**Tab.1**). During the postpartum phase (PNd16), the mean Hct values in all dams increased in both groups (**Tab.1**). However, the IDA dams still showed mean Hct values below normal and significantly lower than those observed in the IS group ($P<0.001$).

Table 3 Dams' hematocrit values during first, second and third trimesters and at PNd16 in the IDA and IS groups.

Gestation and lactation periods	IDA	IS	*P* value
TRIMESTER 1	43.44 ± 0.636	43.88 ± 0.622	0.623
TRIMESTER 2	39.61 ± 0.639	43.04 ± 0.625	0.000*
TRIMESTER 3	33.65 ± 0.944	44.21 ± 0.925	0.000*
PNd16	35.35 ± 0.895	46.25 ± 0.877	0.000*

Hct of guinea pig dams fed an IDD or ISD during gestation and at the last day of the experiment. Data are means ± SEM, n=23 (IDA) or 24 (IS). *Different from IS, $P \leq 0.001$

The linear mixed model performed on the pups' Hct levels showed a significant interaction between diet and day ($p<0.001$). During both periods, the pups' Hct values were in the normal range in both dietary groups. However, IDA offspring showed significant slightly lower Hct values compared to those born from IS group at PNd24 (42.33 ± 0.69 and 44.68 ± 0.54, respectively, $P<0.05$). At PNd84, no impact of maternal IDA on the pups Hct levels was observed: the IDA pups had the same Hct levels than those of the IS siblings (50.67 ± 0.86 and 48.50 ± 0.52).

Body temperature data

Figure 1 illustrates the mean body temperature of pups during the ABR procedure for each diet group as a function of frequency and days.

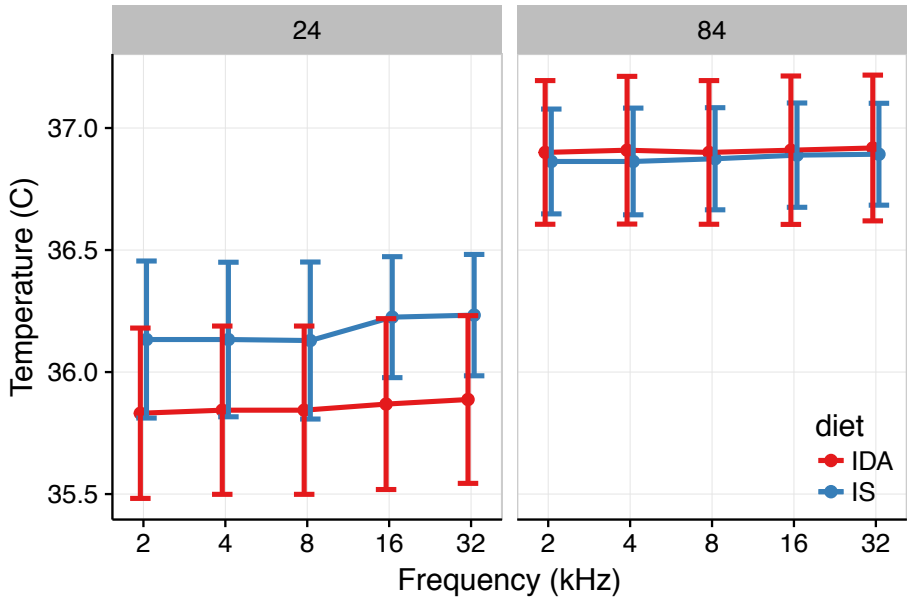

Figure 1. Pups' body temperature during the ABR procedure in both dietary groups at PNd24 and PNd84. Values are means. The error bars represent the 95% of confidence intervals; n=27 IDA (n=16 at PNd24 and n=11 at PNd84) and n=52 IS (n=25 at PNd24 and n= 27 at PNd84).

The linear mixed model revealed that the two groups that were sacrificed on Day 24 had a lower body temperature than those sacrificed on Day 84. This was expected because body temperature is usually correlated with body weight. In addition, the factor frequency was significant (P=0.035). This indicates that the body temperature increased as a function of the frequency. This effect can possibly be explained by the sequential order in which the pups were tested during the ABR procedure. Finally, there were no effects of diet and no interaction.

Threshold data (auditory acuity)

The linear mixed model showed an interclass-correlation (ICC) of 0.629, which indicated that about 63% of the variability of the threshold was attributable to the individual pups. **Figure 2** illustrates (1) the predicted values (thresholds) of the linear model as a function of the diets and frequencies and (2) the observed values. As one can see, there was a cubic relationship between the hearing threshold and the frequencies, but there was no difference between the two diets at PNd24 and at PNd84. The regression coefficients (*b*)

of the model were compared with the following reference bases: diet IS and PNd 24. The hearing threshold presented a significant cubic relationship with frequency ($p<0.001$). **Figure 2** shows that the threshold was higher at lower frequencies (2 and 4 kHz), that gradually decreased at higher frequency (8 and 16 kHz) and that slightly increased at the highest frequencies (32 kHz). All other effects (main and interaction effects) were not significant ($p > 0.05$). Consequently, no significant difference in the hearing threshold was observed between the IDA and IS siblings at PNd24 and at PNd84, and the auditory acuity was also similar among animals in both age groups at PNd24 and PNd84.

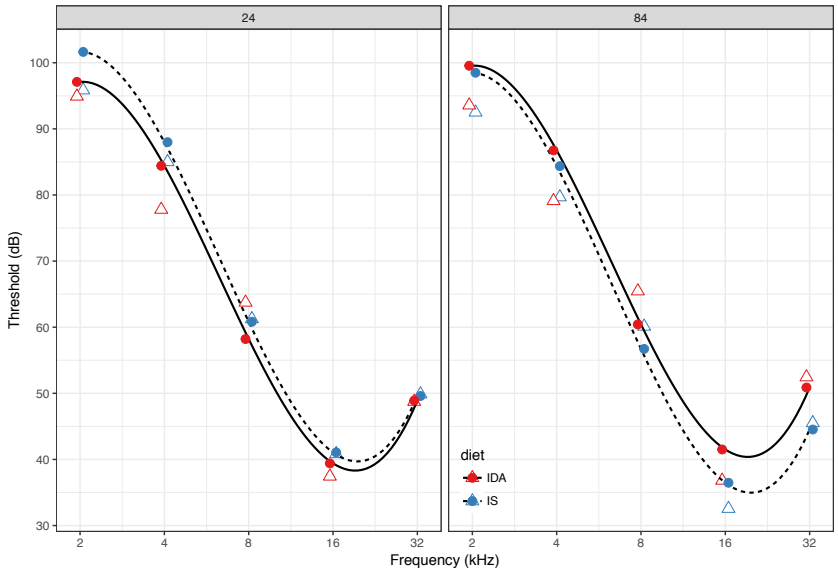

Figure 2. Mean threshold (dB) of both diets as a function of day and frequency. Fill dots represent the imputed mean values and the open dots represent the observed means. The solid lines represent the predicted data for the IDA diet groups and the dashed lines represent the predicted data for the IS diet groups; n=27 IDA (n=16 at PNd24 and n=11 at PNd84) and n=52 IS (n=25 at PNd24 and n= 27 at PNd84).

Peak Latencies

The regression coefficients (*b*) of the models were compared with the following reference bases: diet IS, day 24 and peak I. The linear mixed model showed no significant difference ($P>0.05$) in pups' latencies at 100 and 80 dB from both dietary groups at PNd24 and PNd84 (group x day). **Table 2** shows the latency at 100 dB in pups from both

dietary groups at PNd24 and PNd84. Without any surprise, the latency significantly increased from Peak I to Peak IV ($p < 0.001$).

Table 2 Absolute peak Latencies (ms) at 100 dB in pups, as a function of dietary groups and days[1]

	Day and diet			
	24		84	
Peak	IDA	IS	IDA	IS
I	2.048	2.088	2.008	2.005
II	2.958	2.917	2.831	2.856
III	3.693	3.633	3.467	3.452
IV	4.897	4.801	4.592	4.591

[1] Data are imputed means, n=27 IDA (n=16 at PNd24 and n=11 at PNd84) and n=52 IS (n=25 at PNd24 and n= 27 at PNd84) for the pups.

Interpeak latencies

At 100 dB, but not at 80 dB, the linear mixed model revealed a significant interaction between diet and peak on PNd24. More specifically, IDA siblings showed a significantly ($p=0.032$) higher I-IV IPL of 0.13 ms than the IS group at 100 dB (**Fig.3**).

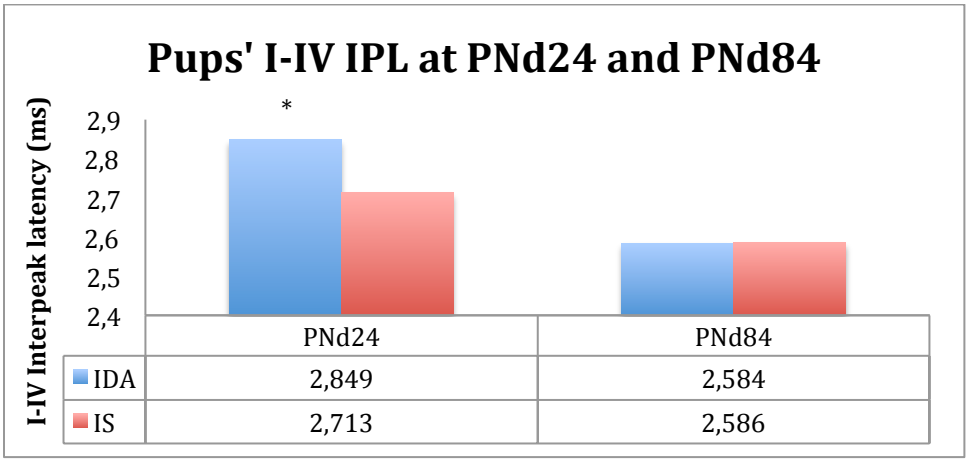

Figure 3. Pups' I-IV interpeak latency (ms) at 100 dB at PNd24 and PNd84 in both dietary groups.[1]

[1] Data are imputed means, n=27 IDA (n=16 at PNd24 and n=11 at PNd84) and n=52 IS (n=25 at PNd24 and n= 27 at PNd84) for the pups.
* Significant difference ($p=0.032$) for diet, at PNd24

At PNd84, no significant difference showed in pups from both dietary groups at 100 and 80 dB ($p>0.05$). However, I-IV IPL significantly ($p=0.001$) decreased as a function of frequency ($b=-0.1292$, $p<0.001$) from PNd24 to PNd84 in all siblings at 100 dB.

DISCUSSION

The aim of this study was to determine if MID had an impact on the auditory functions in the young (PNd24) and adult (PNd84) guinea pig offspring. We assessed the ABR absolute and inter-peak latencies at 100 and 80 dB, as well as thresholds using 5 tone pip frequencies in offsprings.

Our objective was to induce mild maternal IDA by reducing the dam's dietary content of iron to mimic this common clinical condition in pregnant women in industrialized countries. We report that the dams fed the ID diet had significant lower Hct values compared to those fed the IS diet during the second and third equivalent trimester of pregnancy; their Hct were also below normal range values during the third equivalent trimester. These results confirm that mild IDA during pregnancy was induced by the iron deficient diet; dams were still anemic at PNd16. These observations are consistent with studies supporting Hct and Hb values below the normal range in the third trimester in MID pregnant women from developed countries.[37,38] Our results also confirmed that iron deficiency in the ID group remains in a mild form as mean Hct values stayed above 30% of anemic results, but were still below the normal range of 37-42%.[22,39] Following weaning, the pups from both dietary groups were fed the IS diet in order to restore their iron status; our findings indicate that this led Hct values to reach the normal range for all pups at PNd24 and PNd84, although Hct counts appeared slightly reduced in IDA pups compared to IS siblings at the PNd24 interval. Several studies support an association between maternal and offspring Hct's status, in humans [3,4,7,8] and animal models.[25-27]

The interest of assessing the ABR's threshold (auditory acuity) is that it helps to determine if there is a dysfunction in the cochlear region (auditory inner ear), area responsible for the transduction phenomena (transformation of the sound into neural messages).[40] Very few studies focused on the influence of MID on the offspring's

auditory thresholds. The present study did not find any significant difference in the ABR's threshold between IDA and IS siblings at PNd24 and PNd84. A post-natal ID human study interested in analyzing the outer hair cell activity of the cochlea did not find a significant difference in ABR threshold when comparing IDA adults to IS patients.[41] In fact, no relationship was observed between IDA and the auditory dysfunction based on distortion product otoacoustic emission (DPOAE). The latter reflects the inner hair cells integrity in the cochlea.[41] Our group of research using the same animal model and diets previously showed that IDA siblings had a significantly higher threshold of 15 dB compare to IS siblings at PNd24, indicating that maternal IDA has a negative impact on the auditory acuity of the progeny.[25] The reason why we did not obtain the same results is still unknown, but may be due to a slight difference in the ABR procedures and a different physical environment. In this present study, we performed a similar calibration in the transducers, but we had a longer insert earphone tube (20 cm, instead of 34 cm) and a bandpass of 100-3000 Hz (300-3000 Hz in our previous study[25-27]). We also used different statistical analyses such as linear mixed models to help interpret ABR results. However, we previously demonstrated that the adverse effects of the IDA during pregnancy were diminished in siblings, when the moderate maternal IDA was countered at weaning with an IS diet containing LC-PUFA.[27]

The ABR latency-intensity curves of the major peaks (PI, II, III, IV) helps determine the nature of the potential hearing loss and distinctively diagnose either a sensorineural hearing loss (SNHL; caused by damage to the sensory cells and/or nerve fibers of the inner ear) or a conductive hearing loss (CHL; impairment of the sound-conducting apparatus of the middle ear).[25] In our findings, no significant difference was shown in peaks latency between pups from both dietary groups during childhood and adulthood. In contrast, we previously found that a moderate maternal IDA altered the absolute Peak I latency (prolonged PI latency) and induced a SNHL (altered PIII L-I curves) in IDA siblings compared to IS siblings, at PNd24.[25] However, Jougleux et al[25] mentioned not knowing if the SNHL is permanent or temporary in the young guinea pig. In fact, Jougleux et al did not perform the auditory hearing tests in the young guinea pig when entering young adulthood.

These findings led our research group to conduct a longer term study into adulthood. Although no SNHL appeared during diagnosis during childhood and young adulthood in this current study, ID offspring can still have increased risk for presbycusis (age-related hearing-loss) and other morbidities in older age characterized by increased IPL when using the ABR.[42]

This study also showed that the IDA offspring presented an increase in I-IV IPL (BTT) at 100 dB compared to the IS group at PNd24. However, this difference was no longer observed in the adult animals. These results suggest that MID has a limited short term impact on the maturity of the peripheral auditory system. Our results are in agreement with a previous study on rats where prenatal ID showed an increase in IPLs, but without the ABR threshold being affected by using the ABR at PNd40 and PNd45.[43] During postnatal IDA infants, clinical studies found an increase in IPL despite iron therapy supplementation following long-term impact on permanent myelogenesis.[44-46] However, other post-natal ID studies on humans did not find altered IPLs.[47,48]

MID also seems to have an influence on reducing thyroid hormone (TH) levels in humans which in turn, can reduce cortical myelin processes.[49,50] Consequences can include deafness, active transduction deficits and alterations of the integrity of the outer hair cells resulting in impaired auditory threshold.[50,51] Since studies have shown a link between MID and thyroid hormone, future research is also recommended to assess this relationship.

In the future, it would be of interest to investigate the impact of MID during aging later in life since age-related auditory dysfunctions may reappear differently during development in MID individuals. Therefore, further studies are suggested to evaluate the impact of MID on the auditory functions at an elderly age.

CONCLUSION

This is the first study investigating the impact of maternal iron deficiency on the auditory functions from childhood to adulthood in the guinea pig. Our findings showed that MID alters the level of BTT in IDA siblings during childhood. However, this auditory impairment was no longer observed in older offsprings entering adulthood. Although no differences in hearing thresholds, ABR absolute latencies of the early peaks (PI, II, III, IV) and IPLs were observed in ID offspring versus IS siblings during adulthood, auditory dysfunction (presbycusis) can still appear in old age.

This study involves the nutrition sciences and auditory fields providing a comprehensive interdisciplinary approach. This research will help contribute to the understanding of the impact of MID on hearing function in the offspring. The outcome of this project is to eventually help prevent adverse circumstances in children by developing efficient prevention strategies targeting women of childbearing age.

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CHAPTER IV

GENERAL DISCUSSION AND CONCLUSION

In this study, the outcome of mild MID on serum cortisol and auditory functions were examined in the young and adult guinea pig. Most studies examine the impact of ID on brain development using the rat as an animal model. However, the CNS in rats is developing mainly during the post-natal phase. This research study used the guinea pig as an animal model because similar to the humans, a brain growth spurt takes place before birth. Hence, the guinea pig is also susceptible of brain impairment resulting from MID. The iron deficient diet also induced mild ID in dams which mimics the observation in women from industrialized nations.

ID dams and their respective offspring body weights were affected by the ID diet during pregnancy, childhood and adulthood, respectively. Previous results also showed a link between MID anemia and low body weight. Our previous results also showed that offspring from ID dams achieved normal Hb and Hct level at PNd24 and PNd40 when an IS diet was given after weaning day 9. Our current results showed no significant difference in Hct levels in pups from both dietary groups at PNd24 and also PNd84 when an IS diet was also given after weaning. Our findings confirmed effects of the ID diet in inducing a mild maternal IDA in the pregnant dams. This condition is common in pregnant women in industrialized countries. Here, we report that the dams fed the ID diet had lower Hct values compared to those fed the IS diet during the trimester 2 and 3, which were below normal range values during the third trimester. In summary, these results suggest that the guinea pig is an appropriate animal model to asses the outcome of MID during gestation and offspring development until adulthood.

Although no significant differences were found between IDA and IS dams during pregnancy and the last day of experiment, the serum cortisol was affected in the ID

offspring compared to the IS control group during childhood. During adulthood, MID seemed to have a smaller influence on serum cortisol levels seen in ID pups and which were also similar to their IS counterparts group. This also suggests that serum cortisol levels were normalized in ID siblings when entering young adulthood with a transient effect.

Our findings coincide with a previous rat study which found similar results in IDA siblings that showed higher serum corticosterone values compare to non-anemic rats when levels were measured as time elapses. Another infant study supported our findings and showed that 12-month old babies with IDA had significantly elevated cortisol secretion than non-anemic babies 30 and 45 min following venipuncture (120). These findings are also consistent from our previous results showing that pups born from ID dams were significantly more active than the IS control group at PNd24 (135). At PNd 40, movements and time spent per square decreased in pups from both groups, but IDA pups still showed significantly higher score than IS control group. These results suggest that maternal ID may alter the level of stress or arousal through an elevated serum cortisol affecting activity level in the offspring.

Very few studies have investigated the impact of maternal ID on levels of stress and hyperactivity in the offspring. With the exception of our previous study focusing on the impact of MID on hyperactivity in the guinea pig offspring, another research conducted in rhesus monkeys supports similar results in which prenatal ID animals showed heightened impulsivity and lack of prudence when facing a dangerous situation compared to their IS counterparts (134).

Although our study did not show lasting effects of MID on the gross weight of the hippocampus, heart, kidneys and adrenal glands, other findings showed reduced cross-sectional area volume in perinatal ID rats hippocampus compare to IS group when entering adulthood despite the correction of iron deficiency (145). To date, very few studies have investigated the impact of MID on organ gross weight and/or size. Increased number of ID pups to reach comparable numbers to the IS group may have helped better

discriminate the groups. Also the use of cross-sectional measures per organ would have been a more precise option.

The other interest of this study focused on the consequences of MID on the auditory functions in the young and adult guinea pig. We assessed the ABR latencies at 100 and 80 dB and threshold using 5 tone pip frequencies in offspring at PNd24 and PNd84.

The interest of assessing the ABR's threshold (auditory acuity) helps determine if there is any dysfunction in the cochlear region. Very few studies focused on the influence of MID on the offspring's threshold. In this study, no significant difference was shown in the ABR's threshold between IDA and IS pups during childhood and adulthood. Another post-natal ID human study also did not find a significant difference in ABR threshold when comparing IDA to IS patients (147).

Our previous study using the same animal model and dietary treatments showed that IDA offspring had a significant higher threshold of 15 dB compare to their IS counterparts at PNd24 (37). These findings suggest that maternal IDA has a negative effect on the auditory acuity of the offspring likely by altering areas of the cochlear. The reason why we did not obtain the same results is unknown. However, this may be due to a slight difference in the ABR procedures and different physical environment. We also used different statistical analyses such as mixed linear models to help interpret ABR results compare to ANOVA analysis used in our previous study.

The ABR latency of the major peaks (PI, II, III, IV) helps determine the nature of the potential hearing loss and distinctively diagnose either a sensorineural hearing loss (SNHL) (37,70,71). In our findings, no significant difference was shown in absolute peaks latency between pups from both dietary groups during childhood and adulthood. In contrast, Jougleux et al found that maternal ID can cause SNHL in young guinea pigs shown by a significant increase in the latency of P-I in ID siblings compared to IS pups at PNd24 (70). However, the results did not confirm if SNHL is a temporary or permanent

effect in the offspring during development. Jougleux et al did not perform the auditory hearing tests in the guinea pig offspring when entering young adulthood.

These findings led our research group to conduct a longer term study during adulthood. Although no SNHL appeared during childhood and young adulthood in this current study, ID offspring can still have increased risk for presbycusis (age-related hearing-loss) and other morbidities in older age characterized by increased IPL when using the ABR.

Although no significant difference was shown on absolute latencies at PNd24 and PNd84, our results showed that the ID offspring presented an increase in I-IV IPL (BTT) at 100 dB compared to the IS group at PNd24. This difference was no longer observed during adulthood. These results suggest that MID has a short-term impact on brain development maturity observed only in the young guinea pig.

This study has several limits such small number of animals in the ID offspring. During the experiment, the survival rate of ID pups was very low versus the IS group, although we followed the same protocol as our previous study groups. The reason for this low survival rate in the ID offspring is still unknown. Furthermore, ID still needs to be evaluated as an internal stressor validating hyperactivity since cortisol is used as a biomarker and not an indicator of stress. Therefore, by using the psychosocial stress model (PSM), etiology of hyperactivity would be better explained with the concept of stress by using iron deficiency as an internal stressor. It is also recommended to better understand the coping mechanisms with the guinea pig as an animal model by using the PSM and to see if any detrimental outcome is still observed in elderly guinea pig. The serum cortisol was only measured in the second year in our study since it was experimented as a pilot study. Future studies with a greater number of offspring are recommended to evaluate the impact of MID on serum cortisol in the progeny. It would be of interest to investigate the influence of MID during aging later on in life since auditory dysfunctions may reappear. Therefore, further studies are suggested to evaluate the impact of MID on the auditory functions at elderly age.

One of the strengths of this research project lies in the choice of the animal model. The guinea pig is a good model for social and behavioural tasks, its brain experienced a peak of development during gestation similar to that in humans, the cortisol as in humans is a primary glucocorticoid and the guinea pig's hct values during gestation is very similar to the humans. A key strength of this study is that the result obtained on the pups' blood cortisol levels is in agreement with our previous results by Leblanc et al in which ID offspring showed more movements and time spent per square when exposed to an open field task at PNd24. This is also the first study evaluating the impact of MID on serum cortisol levels and on the auditory functions in the ID offspring during childhood and adulthood. Finally, this project mimicked the mild ID condition observed in pregnant women in industrialized countries and used an interdisciplinary approach by combining nutritional, psychology and audiology sciences that allowed us to investigate outcomes from a broader perspective.

This research project should emphasize the importance of preventing the adverse circumstances of gestational iron deficiency in the children by developing efficient strategies to provide adequate and available dietary iron to the pregnant women.

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