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PERSISTENT EFFECTS OF EARLY-LIFE IRON DEFICIENCY

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ABSTRACT

Iron deficiency is the leading nutrient deficiency disease in the world, and deficits in iron during early life can lead to irreversible damage in brain development if left untreated. This global health problem exists despite low dietary requirements of just milligram amounts each day to maintain good health. The World Health Organization (WHO) estimates that nearly 50% of the world's infants may suffer from iron deficiency; if there are persistent effects of early-life deficiency into adulthood, the impact on productivity and health could be profound. The two current long-term longitudinal studies in the medical literature indicate that these persistent effects can occur into the individual's second decade of life. The goal of the current rodent project is to determine the appropriate time period for iron repletion and the optimal amount of iron given during this time period to prevent the long-term consequences of early iron deficiency. Rodent models have shown that alterations in brain monoamine metabolism due to dietary iron deficiency in early life appear to be associated with changes in neurobehavioral development. Based on earlier studies, the ideal time for iron repletion in the rat appears to be after day 4 (P4) of postnatal life and sometime before P15. In this study, rats were subjected to one of three treatments: 1) iron sufficient through gestation and lactation; 2) iron deficient beginning on gestational day 5 (G5) and continuing through lactation, 3) iron deficient beginning on G5 followed by iron sufficient at P8. Although regional brain iron levels in iron repleted rats were similar to control by P21, dopamine and other monoamine levels were significantly reduced in prefrontal cortex and ventral midbrain at P21 and P90 and in striatum at P90. Monoamine-related protein levels were also disturbed in the iron supplemented group at both time points. These results suggest that iron repletion at P8 is too late to normalize changes in brain monoamine neurotransmission caused by early life iron deficiency.

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Chapter 1

INTRODUCTION

Iron deficiency is the leading nutrient deficiency disease in the world, and deficits in iron during early life can lead to irreversible damage in brain development (Beard & Connor 2003). This global health problem exists despite low dietary requirements of just milligram amounts each day to maintain good health. The World Health Organization (WHO) estimates that nearly 50% of the world's infants may suffer from iron deficiency; if there are persistent effects of early-life deficiency into adulthood, the impact on productivity and health could be profound (WHO, 2008). The two current long-term longitudinal studies in the medical literature indicate that these persistent effects can occur into the individual's second decade of life (Peirano et al. 2004, Burden et al. 2004).

Maintaining adequate body iron levels is essential to the survival of all living organisms. Iron participates in a vast number of vital biochemical processes including erythropoiesis, cellular immunity, and oxidative metabolism (Munoz et al., 2009). In the brain, iron plays key roles in neurological development and functioning. Deficits in iron have been shown to alter brain morphology, myelin formation, bioenergetics, and monoamine metabolism (Beard, Wiesinger & Conner, 2003; Burhans et al, 2005; Beard, Erickson & Jones, 2003). Furthermore, alterations in brain dopamine and neurotransmitter metabolism due to dietary iron deficiency in early life appear to be associated with changes in neurobehavioral development. Studies suggest that the effectiveness of iron repletion at correcting these alterations may depend greatly on the timing of iron repletion (Unger et al., 2007).

In several human studies, iron deficiency in young children has been associated

with developmental delays and impaired cognitive function (Lozoff 1998). To further examine the neurological effects of iron deficiency, several different animal models have been used to inform us about the human condition. Rats and humans demonstrate similar brain development patterns, specifically during the myelination period, and as a result, rats have been used extensively in this line of research. Utilizing a rodent model also allows investigators to control for experimental factors while studying the neurochemical and behavioral effects of iron deficiency. Findings from animal studies provide insight into the underlying mechanisms behind long-term observations in humans (Lozoff et al. 2004).

The goal of the current rodent project is to determine the appropriate time period for iron repletion and the optimal amount of iron given during this time period to prevent the long-term consequences of early iron deficiency. Based on earlier studies, the ideal time for iron treatment intervention seems to be after day 4 (P4) of postnatal life and sometime before P15 (Unger et al. 2007; Erikson et al. 2000). This corresponds to somewhere between 30 weeks of human neonatal gestation and 6-8 months of postnatal life in humans. Clearly, for human intervention studies, it is important to determine if we need an intervention during pregnancy, or if perhaps it can be accomplished sometime before 6 months of postnatal human life.

Through controlled diet manipulation, monitoring of blood iron levels, collection and analysis of brain regions, and data analysis, the collective study hopes to define the developmental period in which recovery from iron deficiency is most effective. The study objectives are divided into several specific aims:

1. **Specific Aim 1**: To determine the effect of iron deficiency in gestation and early lactation on regional brain iron concentrations in the prefrontal cortex (PFC), striatum (ST), hippocampus (HP), ventral midbrain (VMB), pons (PONS), and cerebellum (CB). The hypothesis is that brain iron levels will be altered by early iron deficiency and that iron repletion at postnatal day 8 will be ineffective in restoring brain iron levels.
2. **Specific Aim 2**: To determine the effects of iron deficiency in gestation and early lactation on the concentration of the monoamines dopamine (DA), serotonin (5-HT), norepinephrine (NE) and the concentrations of their major metabolites, 3,4-dihydroxyindoleacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA) in PCF, VMB, ST, and HP. The hypothesis is that brain monoamine and metabolite concentrations will be altered in early iron deficient animals and that iron repletion at postnatal day 8 will be ineffective in restoring brain

LITERATURE REVIEW

IRON IN THE BODY

Iron is essential for the survival of all mammalian cells. The average adult human body contains approximately 4g of iron, found mainly in hemoglobin and other iron-containing proteins or stored in the liver (Nadadur et al. 2008). Environmental iron exists in two thermodynamically stable oxidation states, ferrous iron (Fe2+) and ferric iron (Fe3+), making it capable of catalyzing many biochemical reactions (Anderson & Vulpe 2009). Most of the proteins that require iron play important roles in enzyme catalysis or electron transport. Some of these iron-containing proteins assist with the transport of oxygen in the body, for example, cytochromes and globin proteins (Andrews & Schmidt 2007).

Hemoproteins are an important class of iron-containing proteins that include hemoglobin, myoglobin, cytochromes, catalases, and cytochrome oxidase (Anderson &Vulpe 2009). Most iron in the body is incorporated into hemoglobin and myoglobin, which are vital oxygen-carriers in the process of cellular respiration (Wilson & Reeder 2008). Cytochromes and cytochrome oxidase are essential in electron transport and catalase the decomposition of hydrogen peroxide. Non-heme proteins that contain iron such as ribonucleotide reductase, lipoxygenases, and amino acid hydroxylases play other important roles in the cell (Anderson & Vulpe 2009).

Because it serves vital roles throughout the body, iron-dependent organisms must maintain adequate total body iron levels to avoid the functional deficits associated with iron deficiency (Andrews & Schmidt 2007). Conversely, an excess of free iron in the body is toxic. Iron's capacity to accept and donate electrons can lead to the formation of oxygen radicals, which may cause tissue damage. To provide enough iron to tissues

while preventing iron overload, organisms have developed complex homeostatic regulation mechanisms (Andrews & Schmidt 2007).

Iron Absorption

Maintaining iron balance is critical to the survival of all organisms. In animals, iron levels are regulated at the level of absorption since there are no known regulatory pathways for iron excretion. The majority of dietary iron absorption occurs in the proximal small intestine (Andrews & Schmidt 2007). Although the average human consumes 12-18mg iron each day, only 1-2mg of this is absorbed (Crichton 2002).

Mammalian organisms can acquire iron from both heme and nonheme dietary sources (Andrews & Schmidt 2007). Each form is absorbed in the duodenum via a different mechanism. Heme iron has a superior absorptive efficiency and can be transported whole across the apical membrane by heme carrier protein 1 (Shayeghi et al., 2005). Non-heme dietary iron is found primarily in the ferric (Fe+3) form and must be reduced to Fe+2 by ferrireductase enzymes prior to transport across the epithelium of the enterocyte (Gisbert & Gomollon 2009). Duodenal cytochrome b (DCYTB) is an important ferrireductase in this process (McKie et al. 2001). The reduced iron can then be transported across the apical membrane by a divalent metal transporter (DMT1) (Andrews & Schmidt, 2007). Other divalent metals such as zinc, copper, nickel, lead, cobalt, cadmium, and manganese can also be transported via DMT1 (Nadadur et al, 2008).

Within the enterocyte, iron can be utilized for metabolic processes within the cell, stored in ferritin, or exported to other tissues (Andrews & Schmidt, 2007). Stored iron never enters circulation and is eliminated from the body when intestinal cells are

sloughed off. Iron that leaves the enterocyte diffuses across the basolateral membrane through the action of the exporter protein, ferroportin, also known as iron-regulated protein 1 (IREG1) or MRP1 (Andrews & Schmidt 2007).

Almost all bodily cells express ferroportin (FPN), which facilitates the iron export into the extracellular medium. Cells with the greatest expression of FPN include macrophages and duodenal enterocytes. Both cell types have a frequent need to transfer iron across the basolateral membrane and into circulation (Anderson & Vulpe 2009). Hephaestin, a ferrooxidase, facilitates iron export by catalyzing oxidation of iron from Fe+2 to Fe+3. Upon release from the transporter protein, iron generally binds to the protein transferrin (Tf) in the plasma (Nadadur et al 2008).

Transport and Cellular Uptake of Iron

Following its release into circulation, transferrin serves as the primary iron transport protein, rapidly taking up the absorbed iron. Most of the iron acquired by cells comes from transferrin, which is capable of binding two iron atoms. Cells utilize this form, diferric Tf, most efficiently (Chua et al. 2007). Transferrin operates as a chaperone, preventing circulating iron from causing damage to tissues. In addition, it carries out the important duty of delivering iron to cells that express transferrin receptor (TfR). Other proteins like albumin also bind to circulating iron, but to a much lesser degree (Andrews & Schmidt 2007).

Transferrin has high affinity for the plasma binding protein transferrin receptor 1 (TfR1). After TfR1 binds a Tf molecule, the Tf-TfR1 complex (which contains iron bound to Tf) undergoes a conformational change and enters the cell via receptor-

mediated endocytosis through clathrin-coated pits (Aisen 2004). This results in the formation of an endocytic vesicle. Next, proton-pumping ATPase lowers the pH to approximately 5.5, causing iron to dissociate from Tf and releasing free ferrous iron (Klausner et al. 1983).

Following release from Tf, iron migrates out of the endosome and into the cytoplasm via DMT1 (Anderson & Vulpe, 2009). Transferrin remains bound to the TfR within the endosome, and the complex is recycled back to the plasma membrane. Since the delivery of iron to cells by transferrin is a process that last only 5-20 minutes, the cycle can repeat hundreds of times during the molecule's lifespan. The amount of time of iron-delivery varies based on cell type, but the half-life of each Tf molecule is approximately 8 days. Iron not bound to transferrin can also be taken up by cells, but this mechanism is not as well understood (Anderson & Vulpe, 2009).

Iron Storage and Recycling

Metabolic iron needs differ between cell types, therefore, iron management and storage are determined by cell-specific requirements. Iron that is not utilized by the cell may be stored within ferritin, an intracellular protein responsible for cellular iron management. Ferritin is comprised of 24 protein subunits in two forms, the H-subunit and L-subunit. These molecules can accumulate up to 4500 iron atoms that can be released when the cellular demand for iron increases (Koorts & Viljoen, 2007).

Importantly, ferritin synthesis can be regulated based on the available iron pool by both transcriptional and translational mechanisms. Hormones and cytokines control ferritin mRNA transcription and iron-responsive element, found in the 5'-untranslated

region of ferritin mRNA, regulates translation (Koorts & Viljoen, 2007). Post-translational regulation of the proteins involved in iron metabolism is accomplished through iron regulatory proteins (IRPs). IRPs bind to iron responsive elements (IREs), specific stem loop structures in mRNA encoding regions of proteins such as ferritin and TfR1. In times of inadequate cellular iron, IRPs bind in a manner to block translation of ferritin but allow translation of TfR1. This decreases cellular iron storage and promotes iron uptake. When cellular iron levels are above adequate, the opposite effects occur (Anderson & Vulpe 2009).

There is no efficient mechanism for iron excretion from the body, and, as a result, daily dietary iron intake accounts for less than 0.1% of total body iron. Most of the iron in the body is recycled from red blood cells in the spleen. As erythrocytes become damaged, they undergo phagocytosis by macrophages. Most of this released iron is then either stored within the macrophages of the spleen, stored within hepatocytes in the form of ferritin in the liver, or incorporated into new red blood cells (Andrews & Schmidt, 2007).

Distribution and Utilization of Iron

In mammals, iron distribution varies throughout the body based on the specific functional and developmental needs of the tissue or cell. Tissues involved in the homeostatic mechanisms of iron or oxygen such as the blood, spleen, liver, and kidney, have the highest iron concentrations (Theil & Goss, 2009). The majority of iron in the body is utilized to synthesize hemoglobin in the bone marrow (Andrews & Schmidt, 2007). Iron is assimilated into erythrocyte precursors via TfR located on the cell surface;

this is accomplished by a receptor-mediated endocytosis process. Following endocytosis, Tf and the TfR undergo conformation changes, which lead to the dissociation of free iron. The free iron exists initially as Fe+3 and must be reduced to Fe+2 prior to transport by DMT1 (Andrews & Schmidt, 2007).

Iron Balance and Regulation

To prevent the toxic effects of excess circulating iron, the body has adapted mechanisms to keep iron concentrations within target levels to meet metabolic needs. As before mentioned, iron absorption is regulated depending on iron stores in the body. Further regulation also occurs at both the level of cellular uptake and of cellular export. During iron deficiency, the expression of TfR1 and DMT1 increase on the surface of systemic cells and on the apical membrane of enterocytes, respectively. In all bodily cells, the expression of FPN decreases in response to iron starvation. These adaptations promote the delivery and import of iron into cells and decrease iron efflux. In times of iron repletion or iron overload, the opposite events occur as expression of TfR1 and DMT1 decrease while production of FPN and ferritin increase (Anderson & Vulpe 2009).

Hepcidin, a peptide hormone produced in the liver, also contributes to iron homeostasis. When systemic iron levels are high, hepcidin synthesis in the liver is elevated. This elevation in hepcidin reduces iron efflux from intestinal cells and from hepatocytes in the liver by binding to FPN and causing its internalization and subsequent degradation. In times of heightened iron requirements, hepcidin synthesis is reduced such that sufficient ferroportin is present on the cellular membranes to release iron into circulation (Anderson & Vulpe 2009).

Brain Iron Distribution

Iron deposition into the brain significantly increases during periods of rapid brain growth (Taylor & Morgan, 1990). In the developing animal brain, iron is present in varying amounts in different brain regions with the caudate nucleus, the putamen, the globus pallidus, and the substantia nigra have the greatest levels of iron (Beard et al., 1993, Hallgren & Sourander, 1958). This difference in iron content likely corresponds to the developmental processes specific to each region. Iron distribution in the adult brain is also non-uniform, with the highest concentrations being present in the basal ganglia and the substantia nigra (Beard et al., 1993).

Iron is believed to be transported across the blood brain barrier via transferrin, which then binds to brain-specific transferrin receptors. In several different animal models, investigators have found transferrin in oligodendrocytes and, in humans, transferrin has been found in high levels in the white matter of the brain (Beard & Connor 1993).

Uptake of iron in the brain increases in times of iron starvation and decreases when an individual's iron status is high (Taylor et al. 1991). There are several mechanisms of brain iron uptake that are involved in this regulatory process. At the blood brain barrier, iron bound to transferrin enters the brain via transferrin receptors located on endothelial cells of the blood brain barrier and is then exported into the cytoplasm by DMT1 (Connor & Benkovic 1992, Fishman et al. 1987). Uptake of iron into the brain does occur in hypotransferrenemic mice that express transferrin at 1% of control levels, which indicates that there is an alternative mechanism for brain iron uptake that does not

involve transferrin. Recent evidence also suggests that iron can be exported to the central nervous system through the action of neuronal ferroportin after iron has entered the cytosol of endothelial cells. Ferroportin is assisted by the ferroxidase, ceruloplasmin, which oxidizes the released ferrous iron to ferric iron. Ferrous iron then binds to Tf in the brain interstitial fluid (Rouault & Copperman 2006).

Functions of Iron in the Brain

Three specific topics, myelination, gamma-Aminobutyric acid (GABA) metabolism, and monoamine metabolism have been the focus of most research studies on brain iron deficiency (Beard & Connor 2003). Iron serves as an enzyme cofactor in reactions involved in neurotransmitter synthesis (Beard & Connor 2003). Specifically, tryptophan hydroxylase, an enzyme involved in 5-hydroxytyptamine (serotonin) synthesis, and tyrosine hydroxylase, an enzyme involved in the synthesis of catecholamines such as dopamine, require iron (Youdim & Green 1978). Additional roles of interneuronal iron such as hydrogen peroxide reduction, amino acid metabolism, and fat desaturation, may also influence brain functioning but have received less attention (Beard & Connor 1993).

IRON DEFICIENCY

Iron deficiency is the leading single nutrient deficiency in the world (Beard & Connor 2003). The World Health Organization (WHO) estimates that 1.62 billion people, approximately 24.8% of the world's population, are affected by anemia (WHO, 2008), which includes 46-66% of children under four years old (Stoltzfus, 2004). It is believed that as many as 50% of anemia cases may be attributed to iron deficiency

(WHO, 2001). Although the prevalence of infantile iron deficiency has markedly decreased in the United States over the past three decades, iron deficiency among infants and young children continues to be a major global health concern, especially in low-income populations (Brotanek et al. 2005). Globally, it is estimated that approximately 50% of women of reproductive age are iron deficient. In Latin America, the prevalence of iron deficiency is believed to be 10-30% among reproductive-age females and 40-70% among pregnant women (Beard & Connor 2003).

Studies have shown that even in cases of moderate iron deficiency, when anemia is not present, significant alterations in cell morphology and functioning can occur (Beard et al. 1993). Maintaining adequate iron status is essential to the proper functioning of the central nervous system; therefore, a state of iron-deficiency could impact many developmental processes (Beard & Connor 2003). The neurochemical and neurophysiological effects of nutrient deficiencies, such as iron deficiency, are dependent of several key factors: the severity of the deficit, the duration of the deficit, and the timing relative to critical periods of brain growth and development (Kretchmer et al. 1996). Iron deficiency that occurs *in utero* or during early postnatal life causes significant declines in brain region iron concentrations (Kwik-Uribe et al. 2000). As a result, neurological functioning is impaired.

Neurological Effects of Iron Deficiency in Animals

In all mammals, late gestation and early postnatal life are critical periods of neurological development and differentiation. During this period, nutrient demands are elevated due to the rapid rate of brain growth. Therefore, this time frame could be

significant in the development of brain disorders (Morgane at al., 2002). Animal models have been used extensively in this line of research because they can be made experimentally iron deficient during specific periods of development that correspond to periods of human development. Furthermore, investigators have the ability to control for environmental factors that can influence iron status (Lozoff 2006, Nelson et al. 2002). Deficits in iron during gestation and early life have been shown to alter brain morphology, neurochemistry, and bioenergetics (Beard 2008). These alterations vary between brain regions based on each region's developmental need for iron at the time of the deficiency (Kretchmer et al. 1996).

Dietary iron deficient rodents display significantly lower regional brain iron concentrations when compared to iron sufficient rodents (Chen et al. 1995, Erikson et al. 1997); studies have also shown that the brain is able to rapidly regain iron during iron supplementation when iron deficiency is begun postweaning (Piñero et al. 2000, Erikson et al. 1997). Crowe and Morgan (1992) also observed rapid recovery of brain iron in early-life iron deficient rats following supplementation and associated this with increased transcytosis of iron and Tf across the blood brain barrier. According to Piñero et al. (2000), two weeks of iron repletion following early-life iron deficiency also corrected both total brain iron levels and regional brain iron concentrations in rats. This increase in iron uptake is highly selective and does not coincide with any changes in overall blood brain barrier permeability. Investigators noted that further repletion was necessary to fully normalize the concentrations of iron regulatory proteins, such as Tf and TfR, throughout the brain (Piñero et al. 2000). Several iron repletion studies have also shown that the distribution of the acquired iron is regiospecific and that distribution varies

between developmental periods (Focht et al. 1997, Connor 1994, Roskams and Connor 1994).

Since iron is essential for proper neurogenesis and differentiation, iron deficiency in early postnatal life can alter neuron morphology in multiple brain regions, most notably in the hippocampus (Rao et al., 2007) and striatum (Ward et al., 2007). In these regions, dendrite arborization is impaired, thus compromising the number and complexity of connections (Beard 2008). Furthermore, morphological alterations in oligodendrocytes during iron deficiency have been shown to decrease the amount of myelin in white brain matter, thus potentially impairing nerve conduction (Beard et al. 2003, Ortiz et al. 2004).

Iron is important in the functioning of dopamine and other monoamines and iron deficiency has been shown to alter monoamine metabolism in rodents. Studies in both cell cultures and animal models have demonstrated significant differences in dopamine and norepinephrine metabolism with iron deficiency (Beard 2008). Investigating groups have chosen different developmental periods to induce iron deficiency in rat pups. Beard et al. (2003) examined the effects of iron deficiency during lactation and found that rodents made iron deficient from postnatal day 4 to 21 that were then replenished with iron from postnatal day 21 to 49, demonstrated lower DAT, D1R, and D2R densities in the striatum compared with controls. Despite iron repletion at postnatal day 21 (P21), dopamine biology was not normalized in the rodents (Beard et al. 2003).

Several studies have examined the effects of iron deficiency earlier in development, during gestation and early lactation, on neurological functioning and behavior. Felt et al. (2006) made rodents iron deficient from gestational day 7 (G7) until

weaning. The iron deficient group was then maintained on an iron-sufficient diet until sacrifice at P35. Investigators found no significant differences in monoamines, dopamine transporter, and dopamine receptor concentrations between iron deficient and control groups. However, concentrations of serotonin transporter were reduced in the striatum of rats in the iron deficient group. In addition, behavioral testing indicated that the iron deficient rats demonstrated sensorimoter deficits, were more hesitant in novel settings, had poorer spatial water maze performance than controls; these results could be indicative of persistent hippocampal and striatal dopamine dysfunction (Felt et al. 2006).

In a subsequent study, rats were made iron deficient from mid-gestation (G15) until postnatal day 4 (P4) when the pups were cross-fostered to iron-sufficient dams. The pups were continued on iron-sufficient diets until P65. Evaluation of regional monoamines revealed normalization of monoamine concentrations and dopamine and serotonin transporter values with iron repletion at P4 (Beard et al. 2007). These results suggest that iron repletion during early lactation may prevent the effects of iron deficiency during mid-gestation. Results from these studies imply that a narrow developmental window exists during which iron repletion may reverse the effects of iron deficiency during gestation and early lactation in rodents.

Neurological Effects of Iron deficiency in Humans

In humans, iron status is measured using hemoglobin levels, hematocrit levels, mean corpuscular volume, transferrin iron saturation, and erythrocyte zinc protoporphyrin; however, alterations in these values can occur as the result of anemia caused by chronic disease or by deficiencies in nutrients other than iron. Therefore, measuring serum ferritin is the preferred method for accessing iron stores and measuring

serum transferrin receptor is the preferred method for accessing tissue iron (Cook 2005). Iron deficiency is defined as having an abnormal value for at least two of the following three indicators: serum ferritin, transferrin saturation, and free erythrocyte (CDC 2002).

In the brain, iron is essential to neurogenesis and regional differentiation. Therefore, iron deficiency during critical neural development periods, such as the first 6-18 months of postnatal life in humans, can lead to abnormalities that may permanently impair brain function (Lozoff et al., 2006). Most human studies in the literature focus on the developmental consequences of iron deficiency in infants aged 6 to 24 months, a period during which iron deficiency is most prevalent (Lozoff & Georgieff 2006). Early studies of iron deficiency in infants used developmental tests such as the Bayley Scales of Infant Development. This body of research produced mixed results with some studies showing irreversible developmental damage from iron deficiency despite supplementation and some showing partially-reversible damage. However, only half of the studies were controlled intervention trials and most did not include measures of both cognition and behavior (Beard 2008, Grantham-McGregor & Ani 2003).

Shafir et al. (2008) examined the effect of iron deficiency with and without anemia on motor skills. Subjects were full-term inner-city African-America infants, aged 9 to 10 months old, and investigators measured development using gross motor development milestones, the Peabody Developmental Motor Scale, the Infant Neurological International Battery, the motor quality factor of the Bayley Behavioral Rating Scale, and a sequential/bi-manual coordination toy retrieval task. The group found a linear association between iron status and performance on developmental tests; this relationship extended to include the iron-deficient infants that were not anemic

(Shafir et al. 2008). These results demonstrate that iron deficiency that is not severe enough to cause anemia, can still have a significant, detrimental effect on functioning. In addition to motor skills assessment, the study group also evaluated social-emotional behavior in the population group. Investigators found that infants with poor iron status were more shy, less engaged, showed less soothability, and less positive affect (Lozoff et al. 2008). These results are consistent with other studies of behavior in iron-deficient infants (Wachs et al. 2005, Beard et al. 2005, Olney et al. 2007).

Recent studies have also measured maternal iron status and evaluated mother-child interactions. In a randomized controlled intervention, IDA South African mothers were less responsive and more controlling of their infants than nonanemic mothers and the offspring of IDA mothers demonstrated developmental delays at 10 weeks of age. A subgroup of the IDA mothers was then supplemented and, at 9 months, their behavior toward their infants resembled that of the control mothers while the non-supplemented IDA mothers were significantly more negative towards their children. In both groups with mothers that were IDA during early postpartum, infants scored worse than control group infants at 9 months of age (Perez 2005). Based upon these results, it is plausible that infants born to IDA mothers may face two developmental injuries, the iron deficiency they acquire from their mothers and a lack of enriching parental interactions.

In Chile, Algarin et al. (2003) compared nerve transmission and central nervous system functioning of formerly IDA (iron deficiency anemic) children to children who were nonanemic during infancy by measuring auditory brainstem responses and visual evoked potentials. At four years old, former IDA children had lower nerve conduction velocity than the nonanemic during infancy group despite the fact that the former IDA

children had undergone years of iron treatment (Algarin et al. 2003). Such results may be attributed to the decreased myelination and alterations in monoamine metabolism associated with early-life iron deficiency.

In one of the few longitudinal studies investigating the long-term behavioral effects of early iron deficiency, Lozoff et al. (2000) followed a group of children in Costa Rica from infancy until adolescence. The hallmark finding from this study was the persistent effects of early-life iron deficiency on development. Children who had been treated for severe, chronic iron deficiency in infancy (n=48 children) continued to perform worse on arithmetic, writing achievement and motor skills tests than the children who had good iron status as infants (n=114 children). Furthermore, the early iron deficiency group demonstrated more problems with anxiety/depression, social problems and attention problems than their peers (Lozoff et al. 2000). Children in the early iron deficiency group also lagged behind in motor and cognitive development through 19 years of age (Lozoff et al. 2004). Results from these and other human studies demonstrate that iron deficiency in infancy can have significant effects on behavior and cognition even 20 years after supplementation.

Changes in dopamine, norepinephrine and serotonin metabolism caused by iron deficiency are associated with alterations in cognitive functioning and a broad range of behaviors. These attributes may explain human disturbances in motor control, sleep/wake cycles, learning, and memory observed with iron deficiency (Lozoff et al. 2006). The current study examines these monoamine systems and helps to identify the optimal time for iron repletion to prevent long-term behavioral deficits.

Chapter 3

MATERIALS AND METHODS

Introduction

This chapter discusses the study design and the materials and methods used in the experiments. The study employs a longitudinal model with cross-sectional data being collected at weaning (P21) and P90. This design allows investigators to examine the effects of iron deficiency during early gestation through the postweaning period, and the effects of iron repletion during lactation.

Animals, Housing, and Breeding

Young female and male Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN). Animals were pair housed in clear plastic cages, 8" x 17", with stainless steel lids. Female rats received an iron adequate diet (50 ppm iron; Harlan Teklad, Madison WI) prior to mating and males received a commercial pellet diet (Purina 5001). All rats were given free access to food and deionized distilled water 24 hr each day in a temperature-controlled environment with a 12:12-hour light:dark cycle. The Pennsylvania State University Animal Care and Use Committee approved all animal procedures involved in this study.

After females reached a body weight of 200-220 grams, each was housed singly with a male rat. Males were randomly assigned across experimental groups. Conception was determined by the presence of a vaginal plug, and following conception, females were housed singly and checked daily for pregnancy status. Female body weights were collected every 5 days from G0 to G20. Day of delivery occurred between G20 and G23 and was considered P0.

Study Design

At gestational day 5 (G5), females either remained on the 50 ppm diet or were switched to a <5 ppm Fe diet (Harlan Teklab, Madison WI). Dams were then maintained on their respective diets throughout gestation and lactation. At P8, all pups were out-fostered to other lactating dams as outlined in the study design. This design eliminated the potential confounding variable of biological effects determined by dam-specific characteristics. Pups born to control or iron deficient mothers were out-fostered to other control or iron deficient mothers, respectively, to form the "never IDA" and "persistent IDA" groups, respectively. Other early iron deficient litters were out-fostered to control mothers to form the "EID" group and to satisfy the study Aims. This approach also provided EID pups with a rapid onset of iron therapy by an immediate switch from an iron-deficient to an iron adequate dam. After weaning, pups were maintained on their respective diets and were housed in pairs according by gender until sacrifice. To evaluate the acute and persistent effects of early ID, animals were sacrificed at P21 and P90, respectively. The study generated the following groups:

- CN (never IDA) -Rats fed CN diet until sacrifice
- ID (persistent IDA) -Rats fed ID diet until sacrifice
- IDP8CN -ID rats cross-fostered to CN dams on P8

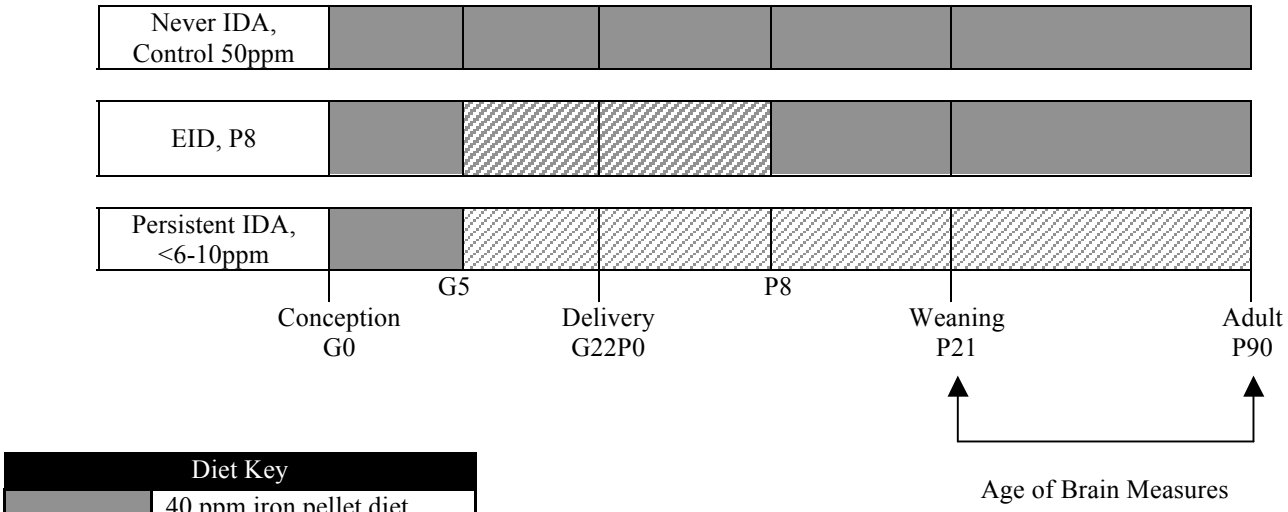


FIGURE 1: Experimental Design

Growth Measures

Maternal body weights were collected at G0, G10, G20, P0, P10, and P21 and pup body weights were collected at delivery (P0), culling, P8, and P15. At P0, P8 and P15, a total of 3 pups were chosen randomly from each litter and weighed to approximate average pup body weight. All weights were measured to the nearest 0.1 gram.

Body Iron Status

Maternal iron status was evaluated by measuring hemoglobin and hematocrit levels at G0 and after delivery at P0, P8, and P21. Blood samples of approximately 200 μl were collected by tail vein puncture for these procedures. Further, blood (5 μL) was collected from 3 randomly chosen pups from each litter at P0, P8, and P15 to assess hemoglobin levels. At the study

endpoints, P21 and P90, evaluation of offspring iron status was based on hemoglobin, hematocrit, serum iron, total iron binding capacity (TIBC), transferrin saturation (TSAT), and liver and spleen iron levels.

Hematology

Hemoglobin was measured by colorimetric methods using the cyanmethemoglobin method (Sigma Aldrich, St. Louis MO). To measure hematocrit levels, microcapillary tubes were centrifuged (13,700 x g, 5 min, RT) and the percent of red blood cells in each sample measured. Serum iron and TIBC were determined using colorimetric methods as described elsewhere (Cook 1980); the procedure was modified to use 50µL of sample. To calculate transferrin saturation, the ratio of serum iron to TIBC was calculated and converted to a percentage. Liver and spleen iron concentration values were determined by colorimetric assay using procedures described by Cook (1980), with ferrozine as the color reagent.

Euthanasia and Dissection

Rats were anesthetized by CO2 asphyxiation and then decapitated at P21 and P90. Following sacrifice, blood was collected, and brain, liver, and spleen were rapidly removed from each animal and placed on ice. Brains were divided at the midline; the right hemisphere was immediately frozen in a dry-ice/isopentane slurry and the left hemisphere was dissected into 6 regions: prefrontal cortex (PFC), striatum (ST), hippocampus (HP), ventral midbrain (VMB), pons (Pons), and cerebellum (CB). All brain samples were stored at -80ºC prior to analysis.

Brain Region Homogenization

Brain regions were thawed on ice and then homogenized in phosphate buffered saline (PBS, 9.1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 150 mM NaCl, pH 7.4) containing protease inhibitors (10mL/g tissue; Roche, Indianapolis IN). Homogenate was aliquoted into individual tubes and frozen at -80°C

Regional Brain Iron

Atomic absorption spectrometry was used to assess brain iron concentration in PFC, ST, HP, VMB, Pons, and CB using a revised version of procedures noted in Erikson et al. (1997). Briefly, a total of 10 µl homogenate was added to an equal volume of ultrapurified nitric acid in a 400µL polypropylene microfuge tube. Samples were digested for 48 hr at 50ºC and diluted 1:10 with 3.12 mmol/L nitric acid. Prepared samples were analyzed using a graphite furnace atomic absorption spectrometer (PerkinElmer AAnalyst 600, Shelton, CT). Standards were prepared by diluting a Perkin Elmer iron standard (PE#N9300126) in 0.2% ultra-pure nitric acid, and blanks prepared with digesting and diluting reagents to control for possible contamination. All standard curves exceeded $r > 0.99$.

Monoamine levels

Homogenates were added to equal volumes of cold 0.2M $HCLO_4$ and passed through a micro-Sephadex column to remove endogenous substrates (5000 x g, 4°C). Concentrations of the monoamines dopamine (DA), norepinephrine (NE), serotonin (5-HT) and the concentrations of their major metabolites, 3,4-dihydroxyindoleacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA) were determined in VMB, ST, PFC, and HP by

reverse phase, high performance liquid chromatography (HPLC) with electrochemical detection. Samples were injected by an ESA model 542 autosampler onto a 15 cm column with 2 mm bore and 3 μm, C-18 packing (ESA Model MD-150X2). The mobile phase contained 75 mM sodium phosphate, 25 μM EDTA, 7.0 μM triethylamine, and 10% v/v acetonitrile. Once separated, compounds were measured with a coulometric detector (ESA model 5014B, guard cell potential, +400 mV; working cell potentials, -174 mV and 350 mV). Concentrations were determined by comparing peak heights to known standard concentrations analyzed with each group of samples. 3,4-dihydroxybenzylamine (DHBA) was added to each sample to correct for sample-to-sample and run-to-run variations.

Statistical Analysis

All analyses were performed using the GraphPad Prism System statistical analysis package (GraphPad Software, Inc.). Values are reported as arithmetic means ± SEM. For maternal and offspring body weight and hematology data, two-way analysis of variance (ANOVA) was used to determine differences between diet treatment groups. One-way ANOVA was used to test for significant differences between groups in all other cases. Post-hoc analyses was performed using Tukey's HSD. Significance level was set at $*P<0.05$.

Chapter 4

RESULTS

Hematological values for CN and ID dams

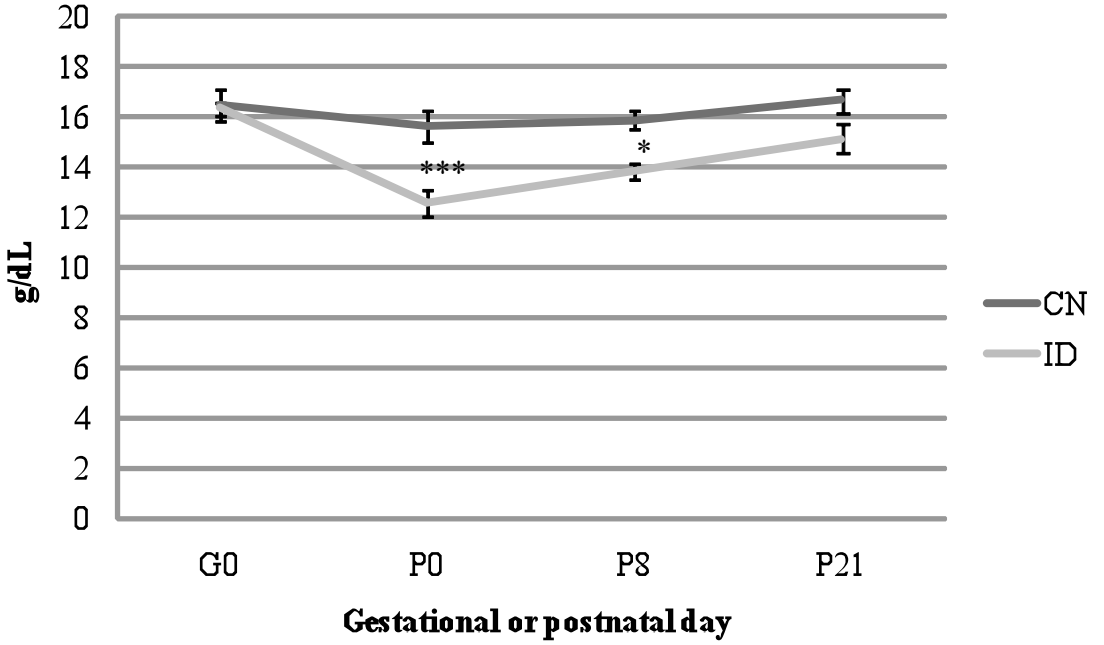
Blood was collected from dams prior to breeding and during lactation to assess iron status. Two-way ANOVA revealed a significant diet treatment x time interaction ($P<0.05$), indicating that the relationship between time and hemoglobin level was different between treatment groups. The effect of treatment was significant ($P<0.001$; Figure 2A). Specifically, significant differences in hemoglobin levels between CN and ID rats were observed at P0 ($P<0.001$) and P8 ($P<0.05$).

Statistical analysis of the hematocrit data showed that the diet treatment x time interaction was not significant ($P=0.44$). Thus, the relationship between time and hematocrit levels was similar CN and ID dams. No differences in hematocrit levels were observed between CN and ID dams (Figure 2B).

Hemoglobin and Body Weights of Offspring

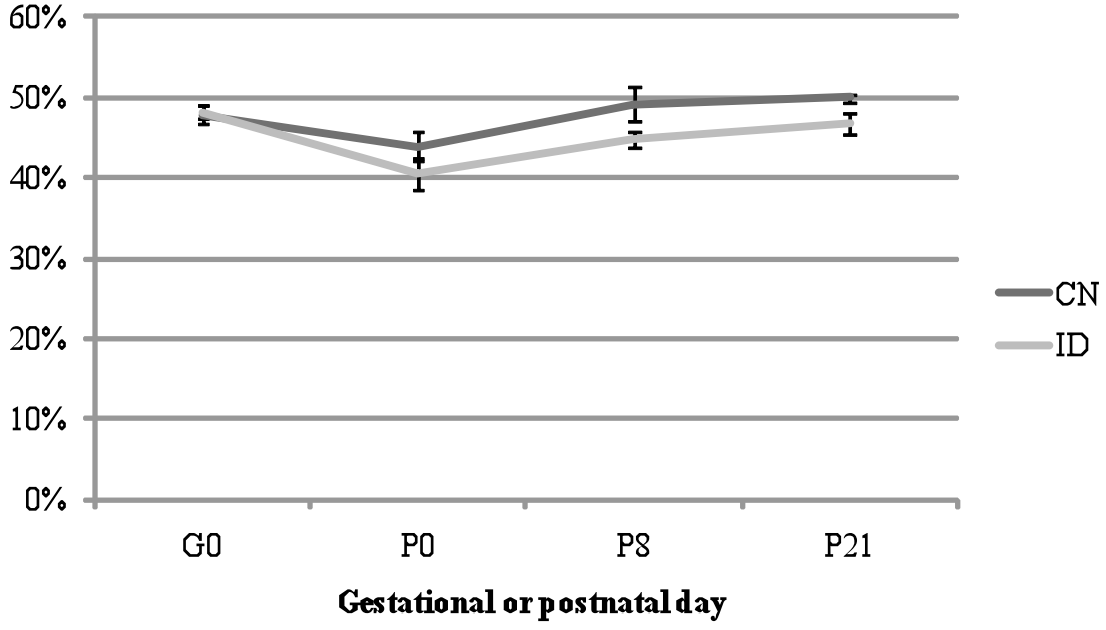
Hemoglobin levels and body weights of rat offspring were measured at P0, P8, P15 and P21 to assess iron status and growth in each treatment group. Statistical analysis revealed a highly significant diet treatment x time interaction ($P<0.001$), thus the relationship between diet and time was different between treatment groups. The effect of treatment was also highly significant ($P<0.001$). Specifically, significant differences between CN and ID pups were observed at P8, P15 and P21 ($P<0.01$ for all except P21, $P<0.001$, Figure 2C). Hemoglobin levels in CN and IDP8CN pups were not significantly different, although there was a trend toward reduced hemoglobin at P0 and P8 in IDP8CN rats compared to CN. Growth of pups,

FIGURE 2A Maternal Hemoglobin at G0, P0, P8 and P21[1]



[1] Values are means ± SEM, n = 8/diet treatment; ***P<0.001, *P<0.05 compared to CN

FIGURE 2B Maternal Hematocrit at G0, P0, P8 and P21[1]



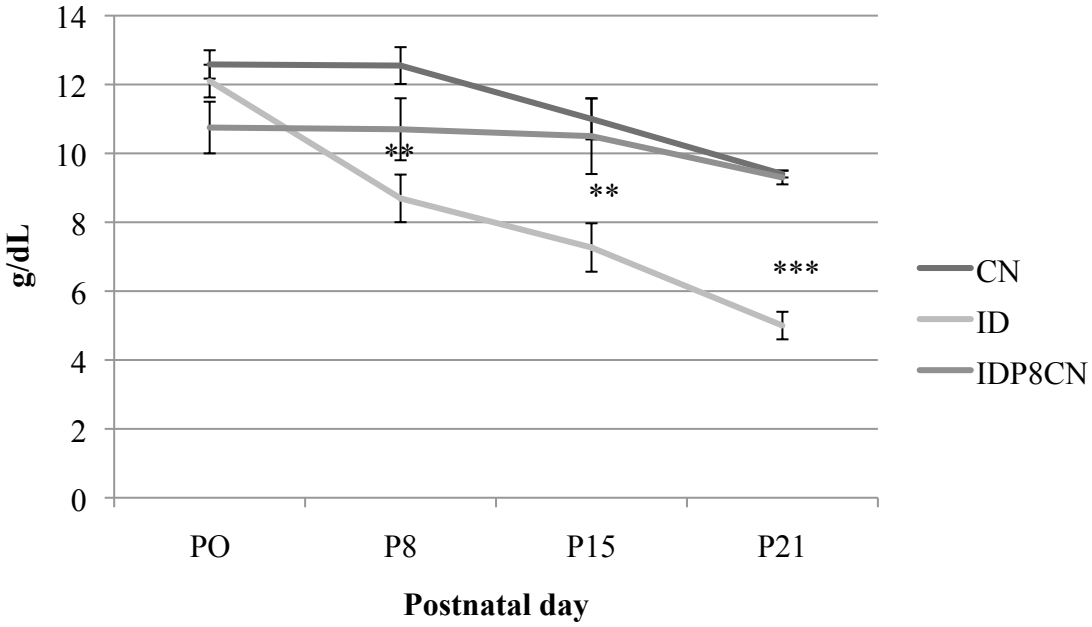
[1] Values are means ± SEM, n = 8/diet treatment

measured as body weight, was not different between treatment groups. Two-way ANOVA of body weight data indicated that the diet treatment x time interaction was not significant (P=0.99) and the effect of treatment also did not reach significance (P=0.70). Overall, these data show that ID pups were iron deficient during lactation and that IDP8CN pups had an iron status similar to CN at P15 and P21. These changes in hemoglobin were not severe enough to affect body weight.

Body Weight, Hematology, and Liver and Spleen Iron

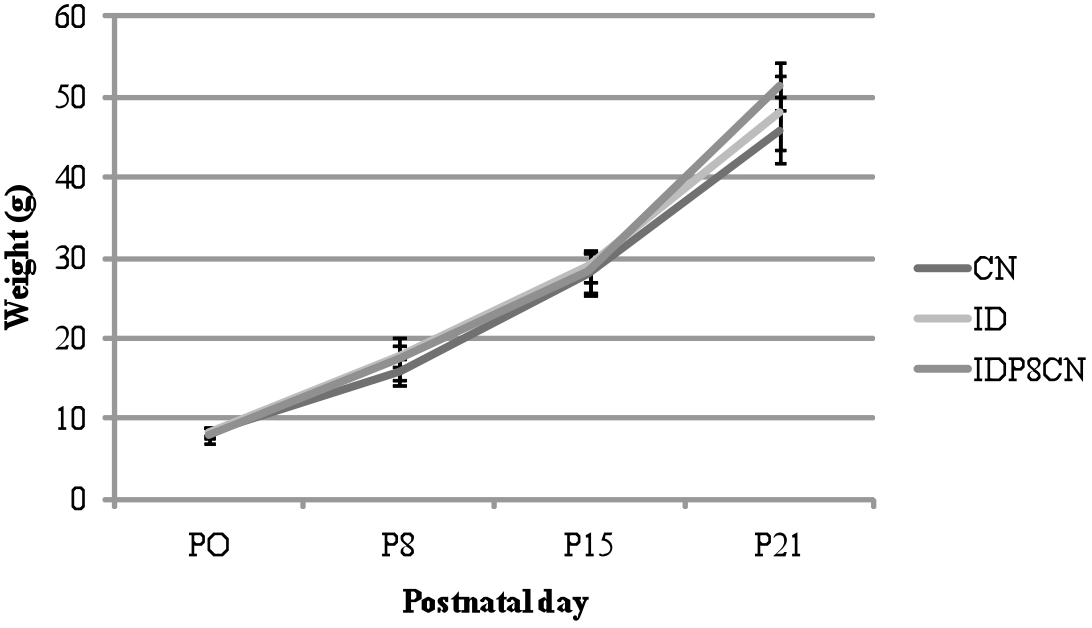
At P21, body weight differences between dietary treatment groups were observed in female rats ($P<0.05$), but not male rats (P=0.41). ID female rats weighed significantly less than IDP8CN female rats ($P<0.05$), but not CN rats at this time point. For all other analyses, significant differences between sexes were not observed, and therefore, male and female data were analyzed together. One-way ANOVA revealed that hemoglobin and hematocrit levels were different between dietary treatment groups at P21 ($P<0.001$ for hemoglobin and hematocrit). The data revealed that ID rats had significantly lower hematocrit and hemoglobin levels compared to CN and IDP8CN rats on P21 ($p<0.001$ for all), but no significant differences were observed between the CN and the IDP8CN treatment groups (Table 2A). Overall differences between treatment groups were also observed for serum iron ($P<0.001$) and transferrin saturation ($P<0.001$). Specifically, serum iron and transferrin saturation were reduced in ID rats compared to CN and IDP8CN rats ($P<0.001$ for all; Table 2A), but no differences in these measurements were observed between CN and IDP8CN rats. There were no significant effects of dietary treatment on TIBC (P=0.11), liver iron (P=0.29), or spleen iron (P=0.10) levels. Overall, these data show that ID rats out-fostered to iron-sufficient moms at P8 (IDP8CN) have similar hematology and non-heme iron levels as CN rats by P21.

FIGURE 2C Hemoglobin for P0, P8, P15 and P21 Rats[1]



[1] Values are means ± SEM, n = 2-6/diet treatment; **P<0.01 compared to CN; ***P<0.001 compared to CN

FIGURE 2D Body Weights for P0, P8, P15 and P21 Rats[1]



[1] Values are means ± SEM, n = 2-6/diet treatment

On P90, one-way ANOVA revealed significant differences between dietary treatment groups for female body weight ($P<0.05$), hemoglobin ($P<0.01$), transferrin saturation ($P<0.05$), and liver iron ($P<0.01$). IDP8CN female rats weighed significantly more than CN female rats ($P<0.05$; Table 2B). Moreover, IDP8CN rats had elevated hemoglobin levels compared to CN rats. No other differences were observed between these two dietary treatment groups. In ID rats, serum iron, transferrin saturation and liver iron were all reduced compared to CN ($P<0.01$ for all; Table 2B). Thus, with the exception of elevated hemoglobin levels and female body weight, early ID rats (IDP8CN) appear similar to CN rats.

Brain iron concentrations

For brain iron analysis, significant differences were observed in most regions between sexes, and therefore, male and female data were analyzed separately. At P21, regional brain iron concentration differences between dietary treatment groups were observed in female rats in VMB ($P<0.05$) or PFC ($P<0.01$), but not in HP ($P=0.55$), ST ($P=0.80$), Pons ($P=0.38$) and CB levels than CN female rats ($P<0.05$), but not IDP8CN rats. A significant difference was also observed in PFC with IDP8CN female rats showing elevated iron levels compared to both CN female rats ($P<0.01$) and ID rats ($P<0.01$, Figure 2E). In male rats, significant differences were also observed in Pons ($P<0.05$) but not VMB ($P=0.92$), HP ($P=0.56$), PFC ($P=0.69$), ST ($P=0.18$), and CB ($P=0.77$). Male ID rats had significantly lower Pons iron levels than IDP8CN rats ($P<0.05$, Figure 2F).

TABLE 2A
Body Weight, Hematology, and Liver and Spleen Iron for P21 Rats[1,2]

	CN	ID	IDP8CN
Body Weight Females (g)	41.8±2.6 (18)	37.3±1.4 (9)[b]	52.3±5.3 (7)
Body Weight Males (g)	47.5±3.3 (9)	43.3±2.6 (15)	48.7±3.6 (8)
Hematocrit (%)	34.9±0.4 (27)	20.9±0.7 (24)[a,b]	35.8±1.1 (15)
Hemoglobin (g/dL)	9.4±0.1 (27)	5.0±0.4 (23)[a,b]	9.3±0.2 (15)
Serum Iron (µg/dL)	388.6±31.0 (26)	109.3±20.6 (24)[a,b]	366.0±44.1 (15)
TIBC (µg/dL)	718.6±36.6 (27)	639.1±18.0 (23)	652.7±19.5 (15)
Transferrin Saturation (%)	53.0±3.4 (26)	17.9±3.4 (21)[a,b]	54.9±5.8 (15)
Liver Iron (µg/dL)	85.4±12.9 (27)	58.3±12.8 (24)	65.9±13.7 (15)
Spleen Iron (µg/dL)	255.4±54.8 (26)	157.5±13.3 (24)	140.5±28.0 (15)

[1] Values are means ± SEM (n); [a]P < 0.001, relative to CN values; [b]P<0.001 relative to IDP8CN values
[2] Abbreviations used; TIBC, Total iron binding capacity

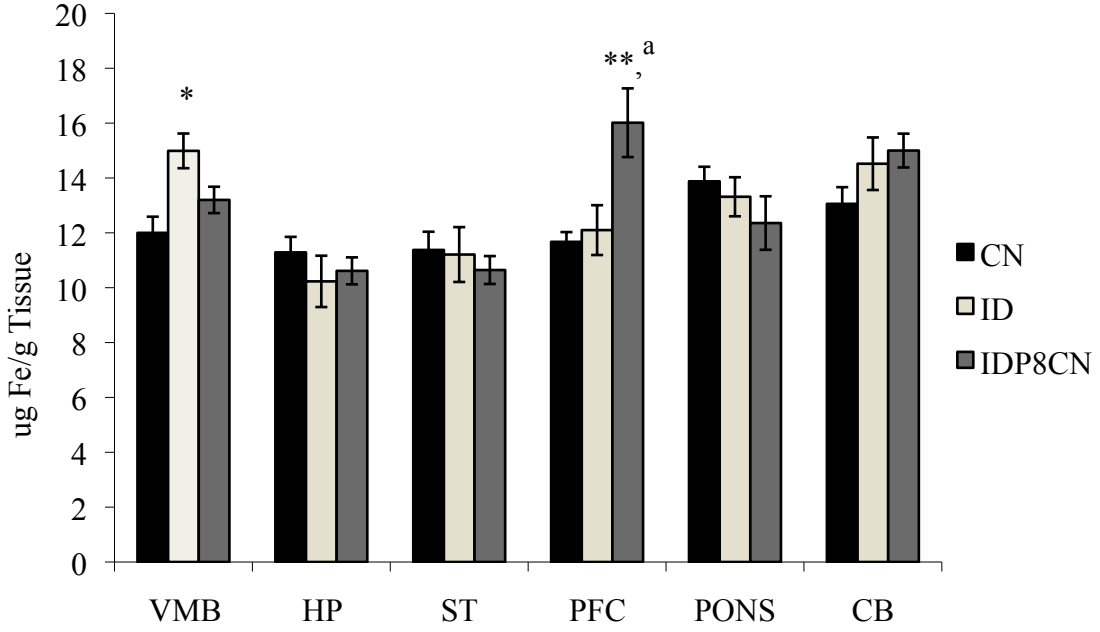
TABLE 2B
Body Weight, Hematology, and Liver and Spleen Iron for P90 Rats[1,2]

	CN	ID	IDP8CN
Body Weights Female (g)	236.9±2.8 (13)	247.5±8.6 (10)	261.3±8.6 (6)[b]
Body Weight Males (g)	386.1±3.4 (8)	372.4±5.1 (14)	385.6±11.4 (8)
Hematocrit (%)	46.7±0.6 (21)	45.8±0.6 (24)	47.2±0.8 (14)
Hemoglobin (g/dL)	14.9±0.4 (21)	15.2±0.3 (24)	16.7±0.5 (14)[b]
Serum Iron (µg/dL)	319.1±29.3 (20)	224.6±15.3 (21)[a]	279.6±21.7 (10)
TIBC (µg/dL)	573.3±29.9 (21)	632.1±16.9 (24)	624.4±32.6 (10)
Transferrin Saturation (%)	55.7±4.7 (20)	34.5±2.1 (21)[a]	46.1±4.6 (10)
Liver Iron (µg/dL)	268.4±37.6 (21)	131.0±14.1 (24)[a]	194.5±33.5 (14)
Spleen Iron (µg/dL)	714.5±179.5 (21)	606.8±101.1 (22)	841.0±238.4 (13)

[1] Values are means ± SEM (n); [a]P < 0.01, relative to CN values; [b]P<0.05 relative to CN values
[2] Abbreviations used; TIBC, Total iron binding capacity

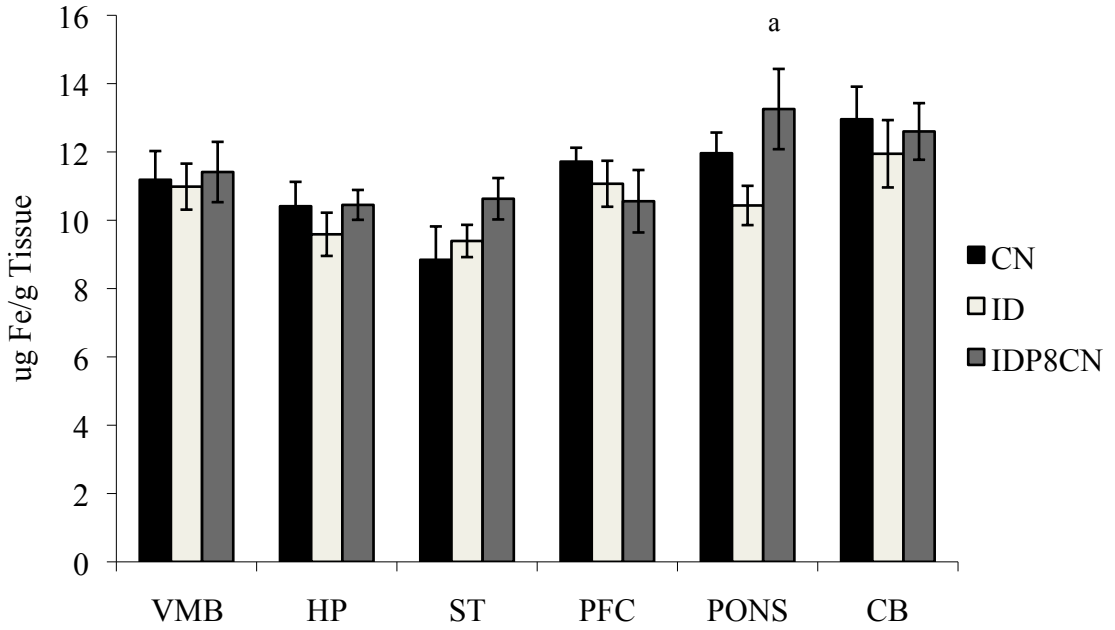
On P90, one-way ANOVA revealed significant differences in regional brain iron concentration in female rats in HP ($P<0.05$) and CB ($P<0.01$), but not in VMB ($P=0.08$), PFC ($P=0.99$), ST ($P=0.40$) and Pons ($P=0.50$). Female ID rats had significantly lower brain iron than CN rats in HP ($P<0.05$; Figure 2G). In CB, brain iron levels were reduced in both ID rats and IDP8CN rats compared to CN rats ($P<0.01$). In males, significant differences were observed in VMB ($P<0.05$), HP ($P<0.05$), ST ($P<0.01$), Pons ($P<0.001$) and CB ($P<0.001$) but not PFC ($P=0.56$). IDP8CN male rats had significantly higher VMB ($P<0.05$) and ST ($P<0.01$) iron levels than ID rats (Figure 2H); IDP8CN male rats also had higher iron levels than CN ($P<0.05$) in HP. There were significant differences observed in comparing ID and IDP8CN male rats to CN male rats in Pons and CB. Specifically, both ID and IDP8CN male rats had significantly reduced iron concentrations in Pons and CB ($P<0.001$ for all except Pons IDP8CN, $P<0.01$; Figure 2H). Thus, there were differences in regional brain iron concentrations between treatment groups at both P21 and P90, but observed trends varied between the two time points.

FIGURE 2E Brain iron concentrations in Female P21 Rats[1,2]



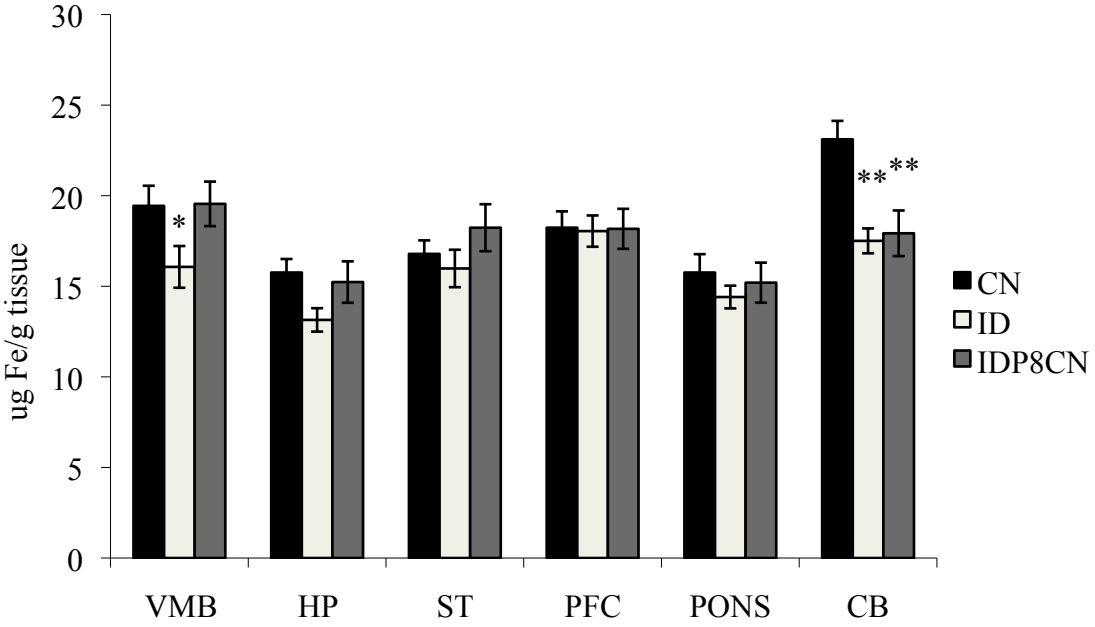
[1] Values are means ± SEM, n = 10-17 rats; *P < 0.05, relative to CN values; **P<0.01 relative to CN values; a P<0.01 relative to ID values.
[2] Abbreviations used; VMB, ventral midbrain; HP, hippocampus; ST, striatum; PFC, prefrontal cortex; CB, cerebellum

FIGURE 2F Brain iron concentrations in Male P21 Rats



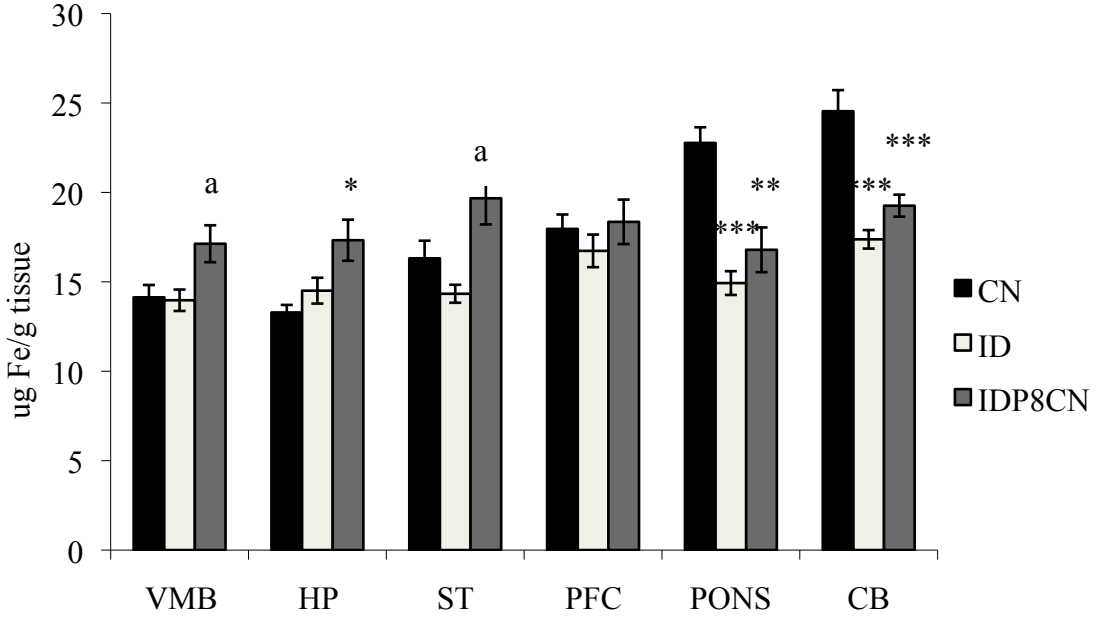
[1] Values are means ± SEM, n = 9-19 rats; a P<0.05 relative to ID values.
[2] Abbreviations used; VMB, ventral midbrain; HP, hippocampus; ST, striatum; PFC, prefrontal cortex; CB, cerebellum

FIGURE 2G Brain iron concentrations in Female P90 Rats[1,2]



[1] Values are means ± SEM, n = 6-15 rats; *P < 0.05 relative to CN values; **P < 0.01 relative to CN values
[2] Abbreviations used; VMB, ventral midbrain; HP, hippocampus; ST, striatum; PFC, prefrontal cortex; CB, cerebellum

FIGURE 2H Brain iron concentrations in Male P90 Rats[1,2]



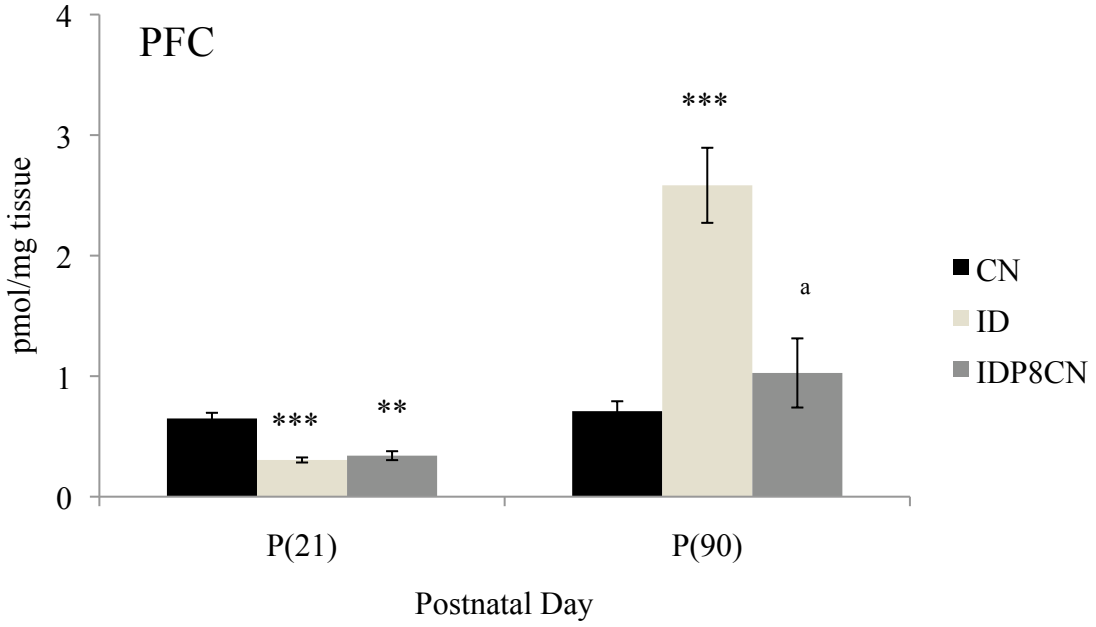
[1] Values are means ± SEM, n = 5-19 rats; *P < 0.05, relative to CN values; **P<0.01 relative to CN values; ***P<0.001 relative to CN values; a P<0.05 relative to ID values
[2] Abbreviations used; VMB, ventral midbrain; HP, hippocampus; ST, striatum; PFC, prefrontal cortex; CB, cerebellum

Dopamine concentrations in PFC, VMB, and ST

On P21, one-way ANOVA revealed significant differences in dopamine concentration between treatment groups for PFC and VMB ($P<0.001$, for both), but not for ST ($P=0.13$). Specifically, PFC dopamine was reduced in ID rats ($P<0.001$) and IDP8CN rats ($P<0.01$) compared to CN (Figure 2I). In VMB, no significant difference was observed between IDP8CN rats and CN rats but a significant elevation in dopamine was observed in ID rats compared to CN rats ($P<0.05$) and compared to IDP8CN rats ($P<0.001$).

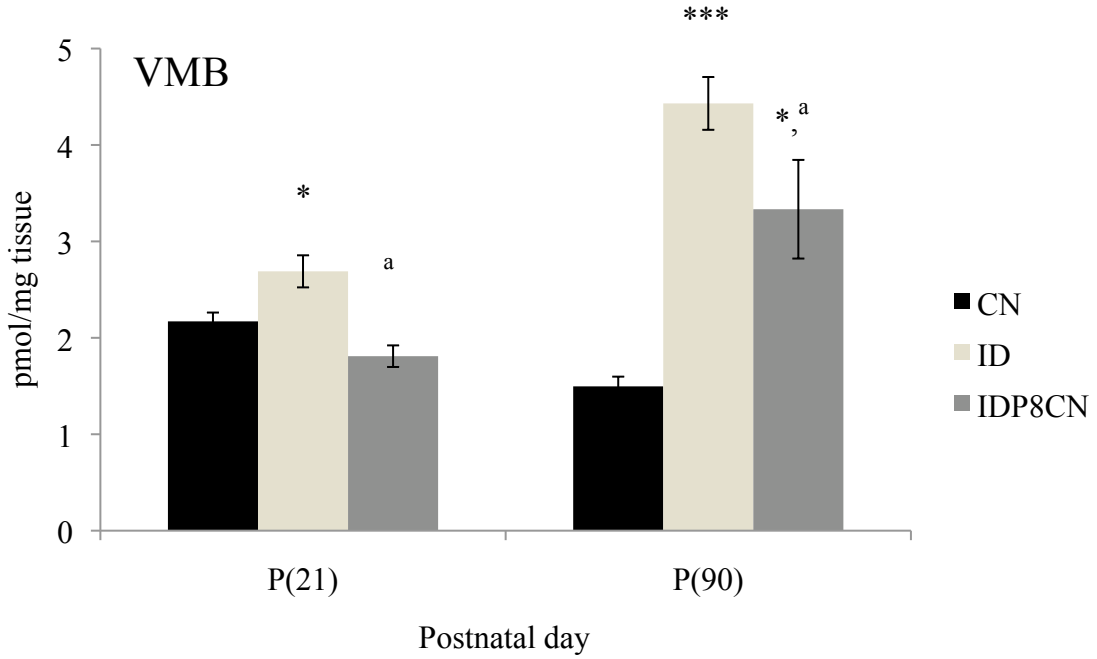
On P90, significant differences in regional dopamine concentration between groups were observed in all three brain regions, PFC ($P<0.001$), VMB ($P<0.001$) and ST ($P<0.05$). ID rats had significantly elevated dopamine compared to CN rats in PFC ($P<0.001$, Figure 2I) and compared to CN and IDP8CN rats in VMB ($P<0.001$ and $P<0.05$, respectively, Figure 2J). In PFC, dopamine in ID rats was also significantly higher than in IDP8CN rats ($P<0.001$). Further, ID rats had reduced dopamine concentration in ST compared to CN rats ($P<0.05$, Figure 2K), but no IDP8CN rats.

FIGURE 2I Dopamine in PFC for CN, ID and IDP8CN Rats[1,2]



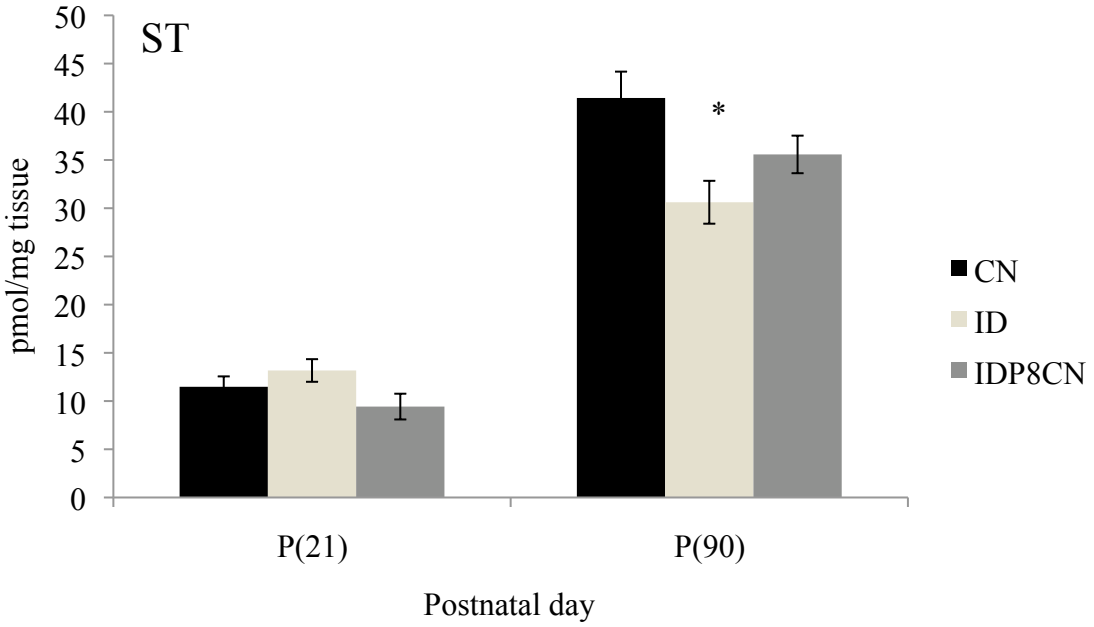
[1] Values are means ± SEM n = 7-20 rats; **P < 0.01, relative to CN values; ***P<0.001 relative to CN values; a P.05 relative to ID values.
[2] Abbreviations used; PFC, prefrontal cortex

FIGURE 2J Dopamine in VMB for CN, ID AND IDP8CN Rats[1,2]



[1] Values are means ± SEM, n = 12-16 rats; *P < 0.05, relative to CN values; ***P<0.001 relative to CN values, a P<0.001 relative to ID values
[2] Abbreviations used; VMB, ventral midbrain

FIGURE 2K Dopamine in ST for CN, ID and IDP8CN Rats[1,2]



[1] Values are means ± SEM, n = 11-16 rats; *P < 0.05, relative to CN values
[2] Abbreviations used; ST, striatum

Dopamine to metabolite ratio

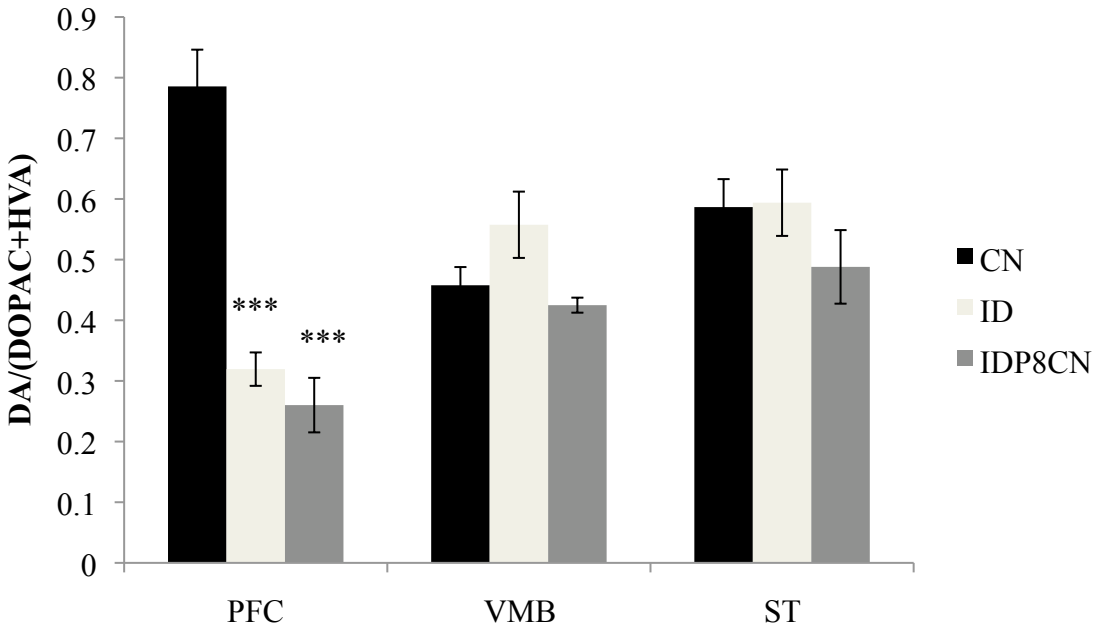
Dopamine to metabolite ratios were calculated to estimate dopamine turnover in PFC, VMB and Str. On P21, PFC dopamine to metabolite ratios were reduced in ID and IDP8CN compared to CN ($P<0.001$, for both, Figure 2L), but no significant differences were observed in VMB ($P=0.09$) or ST ($P=0.07$). On P90, one-way ANOVA again revealed significant differences in dopamine to metabolite ratio between treatment groups for PFC ($P<0.001$), but not for VMB ($P=0.62$) and ST ($P=0.09$). Specifically, PFC ratios were significantly higher in ID rats than CN rats ($P<0.01$, Figure 2M) and IDP8CN rats ($P<0.001$).

Norepinephrine concentration in PFC, VMB and ST

On P21, one-way ANOVA revealed significant differences in norepinephrine (NE) concentrations between treatment groups for VMB ($P<0.001$) and ST ($P<0.01$), but not PFC ($P=0.09$). Specifically, in VMB, IDP8CN rats had significantly reduced NE levels compared to CN rats ($P<0.001$) and ID rats ($P<0.05$). It was also observed that in VMB, ID rats had significantly reduced NE levels compared to CN rats ($P<0.001$, Figure 2N). In ST, IDP8CN rats had significantly lower NE levels in ST than ID rats ($P<0.01$).

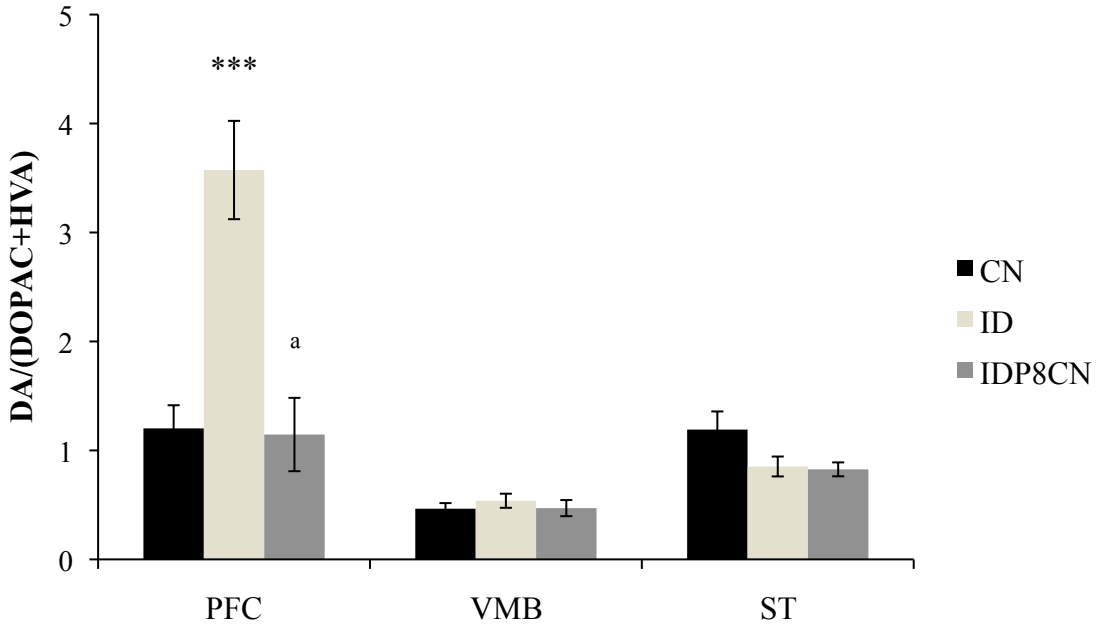
On P90, there were significant differences in PFC ($P<0.01$) NE concentration, but not in VMB ($P=0.84$) and ST ($P=0.68$). NE was reduced in the PFC of ID rats compared to CN rats ($P<0.01$) and IDP8CN rats ($P<0.05$, Figure 2O). Overall, it was observed that NE concentration in ID and IDP8CN rats deviated the most from CN rats at P21, especially in VMB and ST. By P90, NE levels were mostly normalized across diet treatment groups.

FIGURE 2L P21 Dopamine to metabolite ratios in PFC, VMB and ST for CN, ID and IDP8CN Rats[1,2]



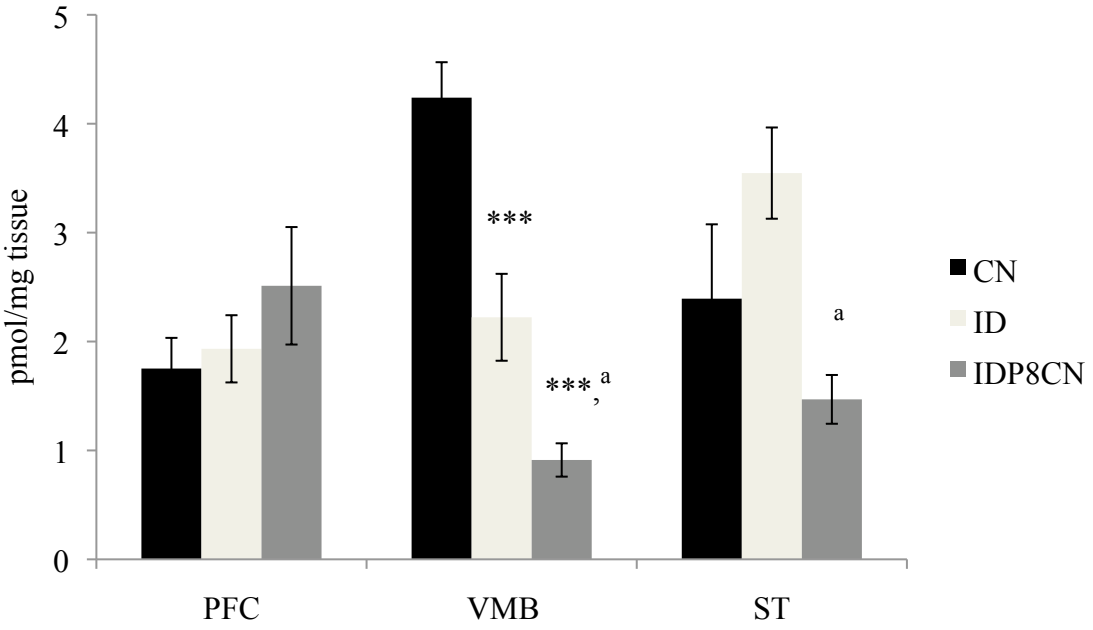
[1] Values are means ± SEM, n = 10-20 rats; ***P<0.001 relative to CN values
[2] Abbreviations used; PFC, prefrontal cortex; VMB, ventral midbrain; ST, striatum

FIGURE 2M P90 Dopamine to metabolite ratios in PFC, VMB and ST for CN, ID and IDP8CN Rats[1,2]



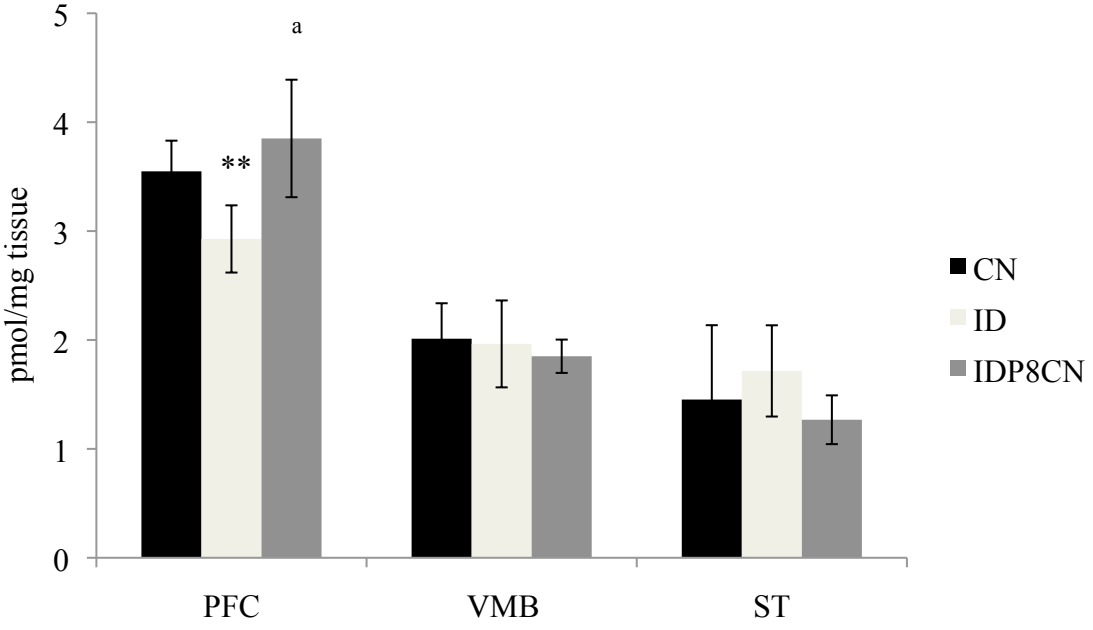
[1] Values are means ± SEM, n = 7-13 rats; ***P < 0.001, relative to CN values; a P<0.001 relative to ID values.
[2] Abbreviations used; PFC, prefrontal cortex; VMB, ventral midbrain; ST, striatum

FIGURE 2N P21 Norepinephrine in PFC, VMB and ST for CN, ID and IDP8CN Rats[1,2]



[1] Values are means ± SEM, n = 8-14 rats; **P < 0.01, relative to CN values; ***P<0.001 relative to CN values; a P<0.05 relative to ID rats.
[2] Abbreviations used; PFC, prefrontal cortex; VMB, ventral midbrain; ST, striatum

FIGURE 2O P90 Norepinephrine in PFC, VMB and ST for CN, ID and IDP8CN Rats[1,2]



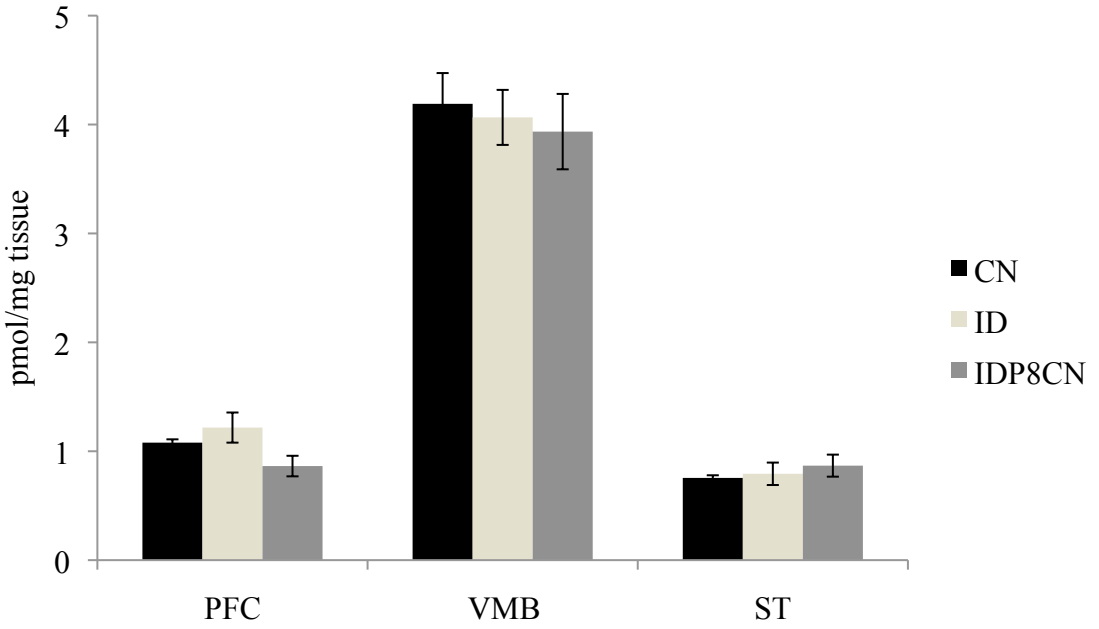
[1] Values are means ± SEM, n = 7-16 rats; **P<0.01 relative to CN values; a P<0.05 relative to ID rats.
[2] Abbreviations used; PFC, prefrontal cortex; VMB, ventral midbrain; ST, striatum

Serotonin concentration in PFC, VMB and ST

On P21, one-way ANOVA revealed no significant differences for serotonin (5-HT) concentrations between treatment groups for PFC ($P=0.09$), VMB ($P=0.83$) , and ST ($P=0.89$, Figure 2P).

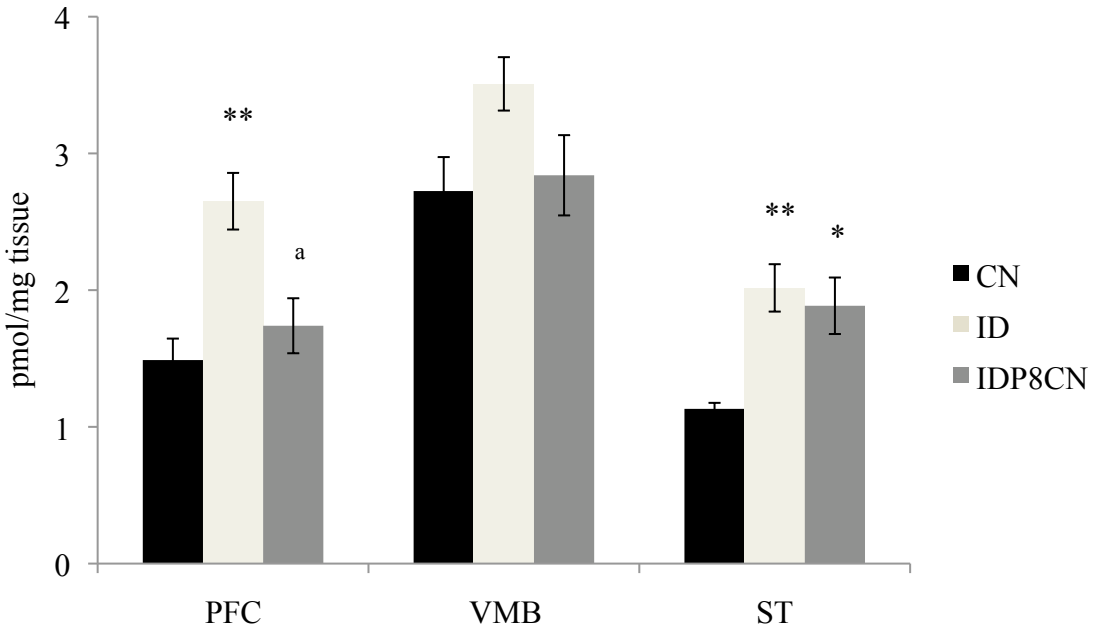
Significant differences were observed between treatment groups on P90 for PFC and ST ($P<0.01$, for both), but not for VMB ($P=0.10$). 5-HT was elevated in the PFC of ID rats compared to CN rats ($P<0.01$) and IDP8CN rats ($P<0.05$, Figure 2Q). Serotonin was also significantly higher in the ST of ID rats ($P<0.01$) and IDP8CN rats ($P<0.05$) compared to CN rats (Figure 2Q).

FIGURE 2P: P21 Serotonin levels in PFC, VMB and ST for CN, ID and IDP8CN Rats[1,2]



[1] Values are means ± SEM, n = 9-20 rats
[2] Abbreviations used; PFC, prefrontal cortex; VMB, ventral midbrain; ST, striatum

FIGURE 2Q: P90 Serotonin levels in PFC, VMB and ST for CN, ID and IDP8CN Rats[1,2]



[1] Values are means ± SEM, n = 8-17 rats; $^{*}P < 0.05$, relative to CN values; $^{**}P<0.01$ relative to CN values; a $P<0.05$ relative to ID values.
[2] Abbreviations used; PFC, prefrontal cortex; VMB, ventral midbrain; ST, striatum

Monoamine Metabolite (DOPAC, 5-HIAA, HVA) Concentrations in PFC, VMB, and ST

On P21, one-way ANOVA revealed no significant differences between treatment groups in DOPAC concentrations in PFC ($P=0.14$), VMB ($P=0.51$) and ST ($P=0.19$, Table 2C). Significant differences were observed in 5-HIAA concentrations in VMB ($P<0.01$), but not in PFC ($P=0.97$) and ST ($P=0.32$). In VMB, ID rats had higher 5-HIAA levels than CN rats ($P<0.05$) and IDP8CN rats ($P<0.01$, Table 2C)). HVA levels did not significantly differ in PFC ($P=0.72$), VMB ($P=0.24$) and ST ($P=0.21$).

On P90, significant differences in DOPAC concentration were observed in PFC ($P<0.010$) and VMB ($P<0.01$), but not in ST ($P=0.99$). Specifically, elevated PFC DOPAC levels were observed in ID ($P<0.05$) and IDP8CN rats ($P<0.01$, Table 2D) compared to CN rats. In VMB, DOPAC levels were higher in ID rats ($P<0.01$) than in CN rats. No significant differences were observed in 5-HIAA levels in PFC ($P=0.39$), VMB ($P=0.44$) and ST ($P=0.29$) nor in HVA levels in PFC ($P=0.63$) and ST ($P=0.18$). A significant difference was observed in HVA levels in VMB at P90 ($P<0.05$); ID rats and IDP8CN rats had significantly higher HVA levels than CN rats ($P<0.01$, for both, Table 2D).

TABLE 2C
P21 Monoamine Metabolite concentrations in PFC, VMB, and ST for CN, ID, and IDP8CN Rats[1,2]

		CN	ID	IDP8CN
PFC	DOPAC	0.62±0.11 (20)	0.70±0.16 (16)	1.04±0.18 (11)
	5-HIAA	0.43±0.04 (20)	0.40±0.20 (15)	0.46±0.22 (11)
	HVA	0.34±0.04 (20)	0.38±0.04 (13)	0.39±0.07 (13)
ST	DOPAC	18.50±1.11 (16)	5.19±1.17 (16)	22.05±1.74 (16)
	5-HIAA	0.65±0.04 (16)	0.31±0.34 (16)[a]	0.92±0.29 (18)
	HVA	1.89±0.08 (18)	1.65±0.11 (16)	2.38±0.48 (17)
VMB	DOPAC	2.12±0.13 (14)	2.65±0.33 (17)	2.48±0.23 (17)
	5-HIAA	1.50±0.15 (15)	2.17±0.30 (15)*	1.14±0.12 (17)[a]
	HVA	2.47±0.20 (14)	2.60±0.21 (16)	2.13±0.21 (17)

[1] Values are means ± SEM (n); *P < 0.05 relative to CN values; [a]P < 0.05 relative to ID values.
[2] Abbreviations used; PFC, prefrontal cortex; VMB, ventral midbrain; ST, striatum; [a]P < 0.05

TABLE 2D
P90 Monoamine Metabolite concentrations in PFC, VMB, and ST for CN, ID, and IDP8CN Rats[1,2]

		CN	ID	IDP8CN
PFC	DOPAC	0.32±0.08 (9)	0.47±0.03 (13)*	0.54±0.08 (8)**
	5-HIAA	0.53±0.06 (11)	0.53±0.25 (13)	0.40±0.06 (7)
	HVA	0.33±0.07 (11)	0.34±0.09 (13)	0.46±0.09 (8)
ST	DOPAC	35.65±3.50 (11)	35.78±2.61 (13)	36.15±1.11 (10)
	5-HIAA	0.47±0.06 (12)	0.98±0.39 (14)	0.47±0.10 (10)
	HVA	1.85±0.10 (12)	1.81±0.17 (14)	2.19±0.18 (12)
VMB	DOPAC	2.24±0.20 (8)	5.37±0.47 (17)***	4.29±0.63 (14)
	5-HIAA	1.66±0.45 (12)	1.56±0.46 (17)	1.07±0.15 (15)
	HVA	1.20±0.14 (12)	2.48±0.26 (16)*	2.38±0.42 (14)*

[1] Values are means ± SEM (n), *P < 0.05, relative to CN values; **P<0.01 relative to CN values; ***P<0.001 relative to CN values
[2] Abbreviations used; PFC, prefrontal cortex; VMB, ventral midbrain; ST, striatum

Chapter 5

DISCUSSION

The objective of this study was to examine the effects of iron deficiency during gestation and early lactation on brain iron and monoamine metabolism. Further, we investigated whether iron recovery at postnatal day 8 was able to correct any ID-related alterations. Biochemical variables were examined on P21 to investigate early life effects and P90 to determine whether there were any persistent outcomes of ID after supplementation. These data have clinical implications in light of recent data indicating that the behavioral outcomes associated with early life iron deficiency in humans extend into adolescence despite iron repletion fairly early in childhood (Lozoff 2004, Grantham-McGregor & Ani 2001).

Previous studies have demonstrated that iron deficiency causes a rapid depletion in brain iron concentrations (Chen et al. 1995, Erikson et al. 1997, Piñero et al. 2000). These changes in brain iron were observed in rats made iron deficient either during lactation or at weaning. Piñero et al. (2000) also showed that two weeks of iron repletion post weaning was sufficient to correct regional brain iron concentrations in the iron deficient rats. Aim 1 of our study examined the effects of two dietary conditions: (1) persistent iron deficiency beginning on gestational day 5 (G5) until sacrifice at P21 or P90, and (2) iron deficiency from G5 to P8 followed by adequate dietary iron from P8 to sacrifice at P21 or P90 on regional brain iron levels.

In our study rats were grouped by sex for analysis of the brain iron data to identify notable differences in regional iron distribution between male and female rats at both P21 and P90.. Brain iron distribution patterns showed almost no consistency between female and male rats at P21. Compared to controls, male and female ID rats did not show many changes in brain iron levels at P21. Iron was slightly reduced in female ID rats in HP and Pons (Figure 2E), and

male ID rats had slightly reduced levels in HP, PFC, Pons and CB (Figure 2F). Also at P21, regional brain iron concentrations of IDP8CN rats were higher than corresponding control values in PFC of female rats and in Pons of male rats. Similar trends were noted in several brain regions in IDP8CN rats

By P90, brain iron concentrations of ID rats were lower than corresponding CN values in several brain regions. In both female and male ID rats, reduced iron levels were observed in ST, Pons, and CB compared to CN. Male IDP8CN rats had higher iron levels than corresponding controls in several brain regions at P90, specifically in VMB, HP, and ST. However, this pattern was not seen in female IDP8CN rats; female IDP8CN brain iron concentrations had normalized in most regions by P90. In CB, both female and male IDP8CN rats had reduced iron levels compared with controls, a trend also observed in ID rats. The same pattern was also present in pons in male rats.

These findings, of elevated iron levels for IDP8CN rats at both P21 and P90, support the results of previous studies (Crowe & Morgan 1992, Piñero et al. 2000, Erikson et al. 1997) and suggests that the brain rapidly regains iron during iron supplementation following early-life iron deficiency, and that the pattern of this hyperaccumulation of iron may vary between brain regions. Moreover, several studies have found that the distribution of supplemented iron following iron deficiency is regio-specific, and that the distribution of iron among brain regions varies during different periods of development (Focht et al. 1997, Connor 1994, Roskams and Connor 1994). The elevated PFC iron concentrations at P21 in IDP8CN female rats may indicate that iron was preferentially taken up by this region because of an elevated iron requirement sometime between P8-P21. These data also suggest that this hyperaccumulation continues after weaning given the heightened levels of iron in VMB, HP and STR at P90.

IDP8CN rats, both male and female, had reduced iron concentrations in Pons and CB at P90 compared to CN rats. This pattern was also seen in male and female ID rats at P90. These results were all significant with the exception of female Pons, where ID and IDP8CN iron levels were only slightly reduced compared to controls. These results may indicate that iron handling was altered in these regions as a result of iron deficiency during gestation and early lactation and that these deficits cannot be reversed by iron repletion at P8. Further, these data suggest that an earlier repletion (prior to P8) may be necessary to completely ameliorate the effects of gestational/early lactational ID.

Aim 2 of this study examined the effects of iron deficiency and iron deficiency followed by iron sufficiency beginning at P8 on monoamine metabolism. The effects of iron deficiency on dopamine metabolism are not fully understood. Many studies have investigated whether ID alters the activity of enzymes involved in dopamine metabolism, specifically tyrosine hydroxylase and monoamine oxidases. Results have been inconclusive to date with some studies showing a decrease in enzyme activity, some showing an increase in enzyme activity, and others showing no change in enzyme activity (Youdim et al. 1983). Perhaps more important to dopamine metabolism is the hypothesis that alterations in dopamine levels with ID may be a result of dysfunction in the uptake, utilization or catabolism of DA (Beard et al. 2003).

Previous investigations of dopamine handling during iron deficiency have found that dopamine uptake is depressed when the nutritional insult occurs post-weaning (Erikson 2000, Nelson et al. 1997). Beard et al. (2003) found differences in dopamine biology with iron deficiency from P4-21 followed by repletion from P21-49 as evidenced by altered striatal DAT, D1R and D2R densities. Felt et al. (2006) induced iron deficiency during gestation (G7) and until weaning followed by repletion from weaning to P35 and did not observe any significant

differences in monoamine, dopamine transporter or dopamine transporter concentrations. However, the repleted rats did demonstrate altered behaviors that investigators linked with dopamine dysfunction (Felt et al. 2006). Reversibility of this dysfunction was observed in an experiment by Beard et al. (2007) in which rats were iron deficient from G15 to P4 followed by iron repletion from P4 to P65. In these rats, dopamine transporter values were normalized by P65 (Beard et al. 2007).

Our experiment found indicators of dopamine dysfunction consistent with the results of Beard et al. (2003). As expected, significant alterations in dopamine levels were observed in ID rats compared to controls at P21 and P90 in PFC and VMB and at P90 in ST. Dopamine levels were also found to be significantly reduced in the PFC of IDP8CN rats on P21 compared to controls. By P90, PFC dopamine levels in IDP8CN rats had surpassed CN, although this difference was not significant. In VMB, a similar pattern was observed as dopamine for IDP8CN rats was slightly reduced at P21 compared to CN (not a significant result), and was significantly elevated at P90. Striatal dopamine of ID and IDP8CN rats appeared to be normalized on P21 and IDP8CN rats had only a slight, non-significant reduction in dopamine in this region at P90 . These results seem to indicate that PFC and VMB were more sensitive to nutritional insult during gestation and early lactation than ST at P21, although it also appears that the dopamine neurotransmitter system is greatly affected between P21 and P90. This period (P21 to P90) occurs after the major developmental period of the dopamine system, but as other studies have indicated (see above), the dopamine neurotransmitter system is prone to changes in iron status after P21. The upregulation of dopamine levels in PFD and VMB, but not STR at P90 in ID rats is a clear indication that iron deficiency begun early in gestation alters dopamine metabolism. Furthermore, P8 iron repletion does not appear to completely correct these long term deficits.

Dopamine to metabolite ratios are an indication of dopamine turnover. On P21, dopamine to metabolite ratios were significantly reduced in PFC for ID and IDP8CN rats compared to controls. By P90, ratios had normalized in IDP8CN rats but not in ID rats. Significantly elevated dopamine to metabolite ratio levels were observed in PFC for ID rats at P90.Despite normalization of dopamine to metabolite ratio in IDP8CN rats, significantly altered DOPAC levels were observed in IDP8CN rats at P90. Significant variations between dietary groups were not seen in VMB and ST for either time point. These results seem to indicate that persistent iron deficiency altered dopamine release in the PFC, but that iron repletion at P8 was sufficient to mostly correct this disturbance by P90.

In addition to dopamine, our study also examined the effects of early-life iron deficiency on other monoamines, specifically norepinephrine and serotonin, and found significant differences between treatment groups. Several investigators have previously examined the relationship between iron deficiency and serotonin with mixed results. Some groups have observed a decline in brain 5-HT (Youdim et al. 1979), others found 5-HT to be elevated (Mackler et al. 1978), and still others observed no differences in 5-HT levels with iron deficiency (Nelson et al. 1997). More recently, Beard et al. (2007) repleted iron deficient rats at P4 and found that dopamine, epinephrine, and serotonin concentrations in the caudate putamen and dopamine and serotonin in the prefrontal cortex were all significantly lower in ID rats compared to controls. The group also found that concentrations of dopamine, serotonin, and 5-HIAA were lower in the VMB of iron deficient rats (Beard et al. 2007).

Our group observed slightly elevated serotonin levels in the PFC of ID rats compared to controls at P21 that became significant at P90. Serotonin levels were also significantly elevated in ST of ID rats and IDP8CN rats on P90. These results suggest the iron deficiency during

gestation and early lactation may cause alterations in striatal serotonin handling that are not corrected by iron repletion at P8. Unlike serotonin, early alterations in NE in ID rats appear to be corrected by P90

While these results are not completely consistent with past studies, they do support the hypothesis that iron deficiency alters monoamine metabolism in the rat. Furthermore, these results suggest that the behavioral consequences of early iron deficiency may be profound and may not be corrected with P8 iron repletion

Chapter 6

OVERVIEW AND CONCLUSIONS

Previous studies have established that iron deficiency can impair many developmental processes and that the nature and magnitude of these effects are dependent on the severity of the deficit, the duration of the deficit, and the timing relative to critical periods of growth and development (Beard & Connor 2003, Kretchmer et al. 1996, Piñero et al. 2000, Kwik-Uribe et al. 2000). The main objectives of this study were to determine the effects of iron deficiency in gestation and early lactation on regional brain iron concentrations and on monoamine and metabolite concentrations.

Rats that were iron deficient during gestation and early lactation then repleted at P8 had altered brain iron distribution patterns at P90; however, these results were inconsistent between sexes and between brain regions. In some regions, hyperaccumulation of iron was observed following repletion at P8, while in other regions iron levels were reduced compared to controls at P90. Examination of dopamine handling in brain regions revealed significant alterations in ID rats and IDP8CN rats compared to controls, especially in PFC and VMB. These results suggest that dopamine handling may be altered in these regions as a result of iron deficiency and that repletion at P8 did not normalize dopamine metabolism.

Based on earlier studies, the ideal time for iron treatment intervention seems to be after day 4 (P4) of postnatal life and sometime before P15 (Unger et al. 2007; Erikson et al. 2000). Overall, the results of our study seem to indicate that iron repletion at P8 may be too late to completely correct regional brain iron and regional monoamine concentrations in early iron deficient rats. These iron deficiency studies are ongoing, and the data presented in this thesis are only a portion of the proposed work. Further investigations into the effects of iron deficiency

and iron repletion on enzyme levels (tyrosine hydroxylase, phosphorylated tyrosine hydroxylase) and on transporter levels and activities (dopamine transporter, norepinephrine transporter, serotonin transporter) have yet to be completed, but will provide a more complete analysis. Based on these findings presented here, the behavioral consequences of early iron deficiency may be profound and may not be corrected with P8 iron repletion. Future studies with this study design are aimed at examining the cognitive and behavioral outcomes of early iron deficiency. The relationships between iron and monoamine deficits in our early iron deficiency model and behavior will provide significant insights into the timing of iron supplementation in humans that is appropriate to inhibit long-term deficiencies.

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Presentations/Activities

Oral presenter at Experimental Biology 2010 Conference

Medical Volunteer work in Honduras

Secretary for Global Medical Brigades, Penn State Chapter