THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOLOGY

THE EFFECTS OF EARLY IRON DEFICIENCY ON BRAIN IRON AND MONOAMINE LEVELS IN A RAT MODEL

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Spring 2012

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Biology
with honors in Biology

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ABSTRACT

The purpose of the thesis project is to examine the long-term effects of gestational and early lactational iron deficiency on brain iron and monoamine levels in a rat model of early iron deficiencies. There is existing evidence that early iron deficiency can significantly alter primary functions of rats that persist into adulthood (i.e. altered sensorimotor development and brain monoamines) even after iron supplementation at weaning (postnatal day (P) 21). Thus, the current study focuses on determining whether earlier iron repletion at P8 can prevent the long-term neurochemical outcomes associated with early iron deficiency. Assuming a 22 day gestation period, neurodevelopment of rats at P8 is the equivalent of birth in humans. In this study, rats were subjected to one of three dietary treatments: 1) iron sufficient through gestation and lactation; 2) iron deficient beginning on gestational day 5 (G5) and continuing through lactation; and 3) iron deficient beginning on G5 followed by moderate iron treatment beginning on P8. The results, attained via batch average and ANOVA, suggest that early iron deficiency in rats reduces brain iron in various regions ($p<0.05$), alters hematological markers of iron status ($p<0.05$), and has no significant effect ($p>0.05$) on brain monoamines and metabolites. Furthermore, moderate-dosed iron therapy at P8 corrects for iron deficits in the brain and blood at P21 and P90. Therefore, these findings suggest that P8 is a suitable age for iron-therapy to begin with respect to normalization of iron concentrations and the associated hematological markers.

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ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Erica Unger for putting in tremendous time and effort she put forth in properly educating me for this project. I would also like to extend my deepest gratitude to Dr. Bernhard Luscher and Dr. Gong Chen for reading my work and aiding in the production of the finished product. Finally, I would like to acknowledge Dr. Byron Jones for aiding with the secondary data analysis, particularly with respect to the appropriate statistical techniques for this particular project.

Introduction

A Brief Overview of Iron Deficiency in Humans

Iron deficiency (ID) is widely considered the most common nutrient deficiency in the world[1] and is responsible for the majority of anemia cases globally. Furthermore, ID is widespread across various demographics, with a relatively high prevalence rate in both industrialized and developing nations. Iron deficiency anemia (IDA) has effects that vary based on age of onset, but the overlapping consequences found in both children and adults include physical-performance deficits, altered immune status, and vulnerability to infections[6]. With IDA afflicting 1.6 billion people worldwide[3], efforts to better understand and treat the condition are a growing trend. However, early iron deficiency (EID) in particular appears to permanently alter the long-term livelihood of individuals, with major deficits in cognitive, motor, and behavioral faculties[2], and thus, EID will be the focus of this paper.

EID is defined by instances in which individuals suffer from ID during the pre- and/or post-natal developmental periods. EID is widespread globally, but unlike ID, EID is significantly more prevalent in developing nations, most notably Africa[5]. A recent study showed that 17.7% of preschool children and 33% of pregnant women surveyed in Zimbabwe are ID[4], with the latter group posing a risk for ID in the fetus during the pre- and postnatal stages. On a global scale, 46-66% of children under the age of four in developing countries are anemic, and EID is responsible for approximately half of these cases[11]. The demographic with the highest degree of IDA prevalence worldwide is preschool-age children, as a projected 293 million children, accounting for 47.4% of the overall population group, have IDA[5]. Additionally, the demographic with the

second greatest degree of IDA prevalence is pregnant women, accounting for 56 million IDA cases worldwide with a prevalence rate of 41.8%[5]. Given that iron-deficient preschool-age children often have IDA due to pre-natal ID through the mother's iron status, it follows that the high prevalence rates of IDA in both demographics is closely interrelated[9].

While EID and ID share the aforementioned effects on the body, EID is unique in that treatment with iron following EID does not fully ameliorate the consequences of the deficiency[2]. However, data remain mixed; while the majority of studies support the notion that iron therapy post-critical period cannot attenuate symptoms of ID, four noteworthy studies demonstrate significant improvements in numerous ID-induced deficits after iron therapy [12, 16-18]. Also, iron supplementation to mothers during pregnancy may reduce the risk of an iron deficient child[8], and thus, early identification of iron-deficient pregnant women is of great importance in reducing the prevalence rate of EID in preschool-age children. However, further study is warranted to determine an effective dose of gestational iron supplementation, as a controlled trial found no significant difference in postnatal iron status despite 30 mg gestational iron supplementation in the form of ferrous sulfate[10].

As far as the effects of EID are concerned, cognitive exam scores are consistently lower in EID children, both before and after correction of iron status[12]. In fact, cognitive scores from children with chronic, severe EID in a middle socioeconomic status household were comparable to those of healthy children raised in a low socioeconomic household[12]. The cognitive dysfunction has not proved fully treatable long-term, as group differences in cognition between control and EID were not resolved 10 years after iron supplementation and treatment, with proposed causes being alterations in neurochemistry, myelination, and energy metabolism[13]. An examination of case-control studies showed an average decline in mental development test scores by 6 to 15 points, which is indicative of iron's essential role in brain development[2].

Motor performance in children is also stunted by EID. Motor skills generally decline with age in all populations; however, EID populations start with lower motor function than their healthy counterparts, and both EID and control populations experience a similar rate of decline over the course of years[14]. Therefore, even if EID is corrected via iron supplementation, a lower base level of motor skills has already been established and the inevitable decline with age will still occur. Specifically, infants with EID average 6 to 17 points lower on motor test scores, a gap that persists into adulthood[2]. It thus follows that an iron-dependent critical period for motor development exists during infancy and perhaps earlier, a claim that is given further credence by a recent study showing that iron status and motor development and coordination follow a linear relationship[19]. While minimal evidence exists for the beginning phase of the iron-dependent motor development-specific critical period, there is evidence that the late phase of said critical period spans to at least 6 months of age, as iron supplementation during this stage improved both motor and visual development[20].

Behavioral deficits are the third major change noted in individuals with EID, and of the three major iron-deficiency-induced alterations, behavioral deficits have the most support from the scientific literature, with nearly all evidence pointing to diminished social and emotional capabilities and responses to stimuli. In a well-known Chilean study, children with EID displayed no social interaction or referencing, no protest when their toys were confiscated, and reduced motor activity when placed in an unfamiliar situation[21]. As far as behavior is concerned, the critical period for successful, long-term EID correction appears to be before the 24 month mark[15]. EID individuals who attained healthy iron status prior to that time exhibited behaviors largely similar to those of non-EID individuals. However, if chronic IDA persisted past the 2 year-mark, reductions in positive affect and frustration tolerance were observed, while delay of gratification and passive behavior became more prevalent[15]. Furthermore, the critical period may begin as

early as the late fetal stage, as children of mothers with poor iron status displayed significantly increased irritability[22].

Long-term Human Studies on Both ID and EID

Thus far, our focus has been primarily on the effects of early iron deficiency in children. Further examination of the long-term effects of EID is essential to understanding the scope and relevance of our study.

As far as long-term studies on cognitive function, one particular study found a direct correlation between hemoglobin levels (Hb) at 9 months of age and IQ score at 5 years of age. This is indicative of the fact that there is not simply a baseline level of Hb that must be attained in order to prevent iron-deficiency-induced cognitive deficits. On average, for each 10 g/L increase in Hb at 9 months of age, there was a 1.75-point increase in IQ score at 5 years of age[23]. Another study in Costa Rica standardized the subjects on a variety of factors that may lead to IQ differences. The fairly large sample size (163) underwent IQ-testing at 5 years of age and the group with moderate iron deficiency had significantly lower IQ test scores, Woodcock Johnson test scores, and motor performance[24]. The Costa Rica study spanned several years with a follow-up in early teenage years demonstrating that a gap still existed between the formerly ID and iron-sufficient groups, specifically with respect to cognitive tasks and motor function[26]. Qualitative behavioral feedback also confirmed that the EID-group had increased socio-behavioral issues even into adolescence. Finally, even more evidence for the persistent effects of EID was found in a Chilean study, whereby *formerly* iron-deficient children underwent tests at approximately age 5. The EID group was significantly outperformed by the iron-sufficient group in IQ tests, language exams, and visual-motor integration tests[15]. In conclusion, there is a strong body of evidence

suggesting that the effects of EID are persistent even if a healthy iron status is acquired following the "critical period."

The Neurophysiology of Iron-Deficiency in Humans

Data on the neurophysiological effects of ID in humans is sparse due to the ethics of invasive experimental protocols and the associated issues with running long-term controlled experiments. From the literature that does exist, one can extrapolate which brain functions and systems are being impaired by examining differences in behavior and responses to stimuli. However, please note that such extrapolations must be compared to animal studies in order to verify claims that may have credence.

To summarize the biological outcomes of ID in humans, it has been demonstrated that significant differences exist between control and ID groups with respect to neural transmission rates in the auditory system, rapid eye movement (REM) density in sleep cycles, EEG frontal asymmetry, and recognition memory with event-related potentials[2].

One means of bypassing the invasive procedures that are often necessary for neurophysiological examination is to use evoked potentials. While the role of iron in myelination is well-established, it was once unclear whether or not correction of iron status would result in proper myelination. An off-shoot of the well-known Chilean study initially concluded that infants with IDA that had not yet received iron treatment had hampered transmission speed through the auditory brainstem pathway[27]. Given myelin's role in accelerating signal transduction, the conclusion from these results was that iron deficiency had inhibited proper myelin formation. A follow-up on the iron-treated, previously-ID subjects at 4 years of age further supported this phenomenon, as absolute latencies for auditory brainstem responses continued to lag behind those without ID in infancy. In addition, the visual system also experienced longer latencies in the formerly ID group, specifically with respect to the P100 wave on visual evoked potentials[27]. The

results of EID not only had a large magnitude of effects, at approximately 1 SD, but they also appeared to persist despite correction of iron status. In conclusion, this 2003 study brought the prospect of critical-period, iron-dependent myelination to the forefront of the scientific realm, and future rat studies sought to further elucidate the mechanisms and severity of sub-optimal myelin formation as a result of EID. For the purposes of a complete literature review, it should also be mentioned that a similar study found no differences between control and ID groups with regards to neurotransmission in the auditory system[28].

As mentioned earlier, iron deficiency anemia can alter sleep patterns as well, and these effects have been demonstrated to persist long-term despite dietary corrections[29]. In one study, REM patterns were dramatically altered in the formerly iron deficient group, with differences being noted in length of REM sleep episodes, frequency of REM sleep episodes, and latency of specific REM and non-REM portions of the sleep cycle. The authors postulated that one particular cause of the shifted temporal organization of sleep was the dopamine (DA) system. ID can alter dopamine signaling in the regions of the brain implicated in REM sleep, and in doing so, it could perturb DA's vital role in sleep regulation, particularly with respect to REM sleep length and frequency[29].

While the aforementioned findings suggest possible mechanisms by which iron deficiency impairs various neurological functions, an animal model proves necessary to exert full control on the subjects throughout the experiment.

The Role of Iron in the Brain – A Rodent Model

Recent rat studies have uncovered a variety of data on how iron is distributed throughout the brain. Rats are considered a suitable model for human subjects due to the fact that rats display great similarities to humans with respect to brain development and myelination. Studies conducted in the past decade lay proof to this phenomenon, as much like humans, rats suffering

from perinatal IDA displayed behavioral and neurochemical alterations as adults in spite of iron-repletion-therapy[30]. Much like humans, motor skills and relative cognition based on performance tests also appear to be hindered by EID in the rat model.

In the cases of EID, including the pre-natal period, rats have consistently been shown to have reduced brain iron, with iron concentrations varying greatly depending on the region of the brain being examined[2]. Our study continued in this vein by noting both brain iron and monoamine levels at various points in the life cycles of rats fed diets varying in iron content. As per the present literature, the effects of ID on the nervous system have been implicated in four primary neurophysiological alterations, with a particular emphasis on the hippocampus: brain metabolism, gene expression, myelination, and neurotransmitter systems[34]. The majority of the relevant rat experiments took a similar approach to the human observational studies by examining the effects of developmental (or critical-period) iron deficiency on various stages of the rats' life cycles.

Iron plays a vital role in brain development, primarily due to the increased oxygen demand of differentiating neurons and the need to produce monoamine neurotransmitters[2]. Therefore, it follows the brain metabolism in individuals suffering from EID will be hampered, a phenomenon that is manifested through loss-of-function phenotypes in such individuals. As a brief background, cell development is a function of genetics, with the DNA of each cell being transcribed into RNA and subsequently translated to protein structures outside of the nucleus. Iron is hypothesized to cause loss-of-function by failing to couple with synthesized proteins, resulting in loss of the encoded phenotype and, ultimately, deleterious effects[2]. One major protein that requires adequate iron concentrations is Cytochrome Oxidase C, which is the terminal enzyme in oxidative phosphorylation. The findings from a 2000 study indicate that EID significantly reduces Cytochrome Oxidase C activity in the piriform and cingulate cortices, the medial dorsal thalamic nucleus, and, most notably, all regions of the hippocampus[31]. This is not only an indication of which brain regions are most metabolically active during development, but

also points to the fact that regions most susceptible to diminished iron concentrations are those implicated in memory. Furthermore, other studies that examined the hippocampus specifically showed that the brains of rats that suffered from EID were more prone to hypoxia-induced hippocampal damage, which is yet another indication that a lack of iron during the developmental period can compromise brain metabolism[32,33]. Finally, the hippocampi of EID rodents display abnormal dendritic growth that is not ameliorated with iron-treatment at P21 or after[35]; this may contribute to altered synaptic plasticity and spatial learning in rodent models[2].

From a genetic-expression standpoint, the data is sparse and has only recently come under the spotlight. Protein scaffolding machinery, such as expression of transcripts involved in actin and tubulin production, has been demonstrated to diverge in ID rodents when compared to control subjects[38]. Additionally, one study had highly compelling results, as of the 25 genes examined in the hippocampus for ID-induced alterations, all 25 showed gene expression changes during the early periods of the rat life cycle, with some of the deviations being normalized by P65[36]. Particular changes were noted with respect to the mammalian target of rapamycin pathway and the Alzheimer's disease genetic framework. These findings were consistent with an earlier study on mRNA expression patterns in the brain, in which the researchers found that many but not all of the early (P21) genetic expression alterations were correctable at a later age (P90) in the context of an iron-sufficient diet[37]. These findings are in contrast to the behavioral, cognitive, and motor outcomes of EID, from which the symptoms of ID are not fully ameliorated in adulthood despite iron therapy. Thus, it appears that the symptomatic response to EID in mammals is not clearly correlated with the effects of ID on gene expression patterns, though further research is warranted.

The effects of EID on myelination are well-documented and seem far more in-line with the associated behavioral deficits from a time-frame perspective. One possible reason for reduced myelination in ID rats is a decrease in oligodendrocyte formation and differentiation during

development[40]. This is further supported by a study on CNPase, a marker of oligodendrocyte activity. CNPase density in the subcortical white matter of rats was found to be lower than control in a group of rats that were perinatally ID[39]. The spinal cord has also been implicated as a major region that suffers reduced myelination and signal transduction as a result of perinatal ID[42]. In slight contrast to the theory about oligodendrocyte differentiation, one rat study showed that even post-weaning ID could induce decreases in CNPase density, indicating that the critical period for myelination is broader than what one might suspect[43]. The notion that a critical period exists at all for this specific phenomenon comes from yet another experiment, which elucidated the biochemical basis for EID-induced reductions in myelination and oligodendrocyte numbers, as well as obstacles that must be overcome in order to restore proper myelination in the rat nervous system[44].

The last major neurophysiological impact of ID in the rat model, and one of the primary focuses of our study, is the effects of perinatal ID on neurotransmitter systems, monoamines, and brain iron status. As established earlier, EID can lower brain iron content in both the short- and long-term, but unlike certain behavioral deficits, timely intervention can fully normalize iron concentrations to those of control subjects. For instance, iron treatment at P21 fully restores brain iron deficits despite drops in concentration of up to 50% prior to intervention[46]. However, the iron therapy does not ameliorate associated behavioral deficits, indicating that iron therapy, like many other stimuli, has a critical period that is a function of the fact that the nervous system develops rapidly perinatally and aspects of functional development cease fairly early. Studies point to the critical period being after P4 in rats, as detrimental effects of EID on both brain monoamines and behavioral outcomes were abolished by iron treatment at P4[47], which is the developmental equivalent of the third trimester of human pregnancy[46]. As far as the latter limit of the critical period, studies done at P8, the developmental equivalent of a newborn human, show that

treatment does not ameliorate many long-term EID effects[46], though there are region-dependent monoamine changes that have been observed.

The neurotransmitter systems are subject to great endogenous regulation, and thus, disrupting certain portions of the various pathways can cause unfavorable effects in rodents. As mentioned earlier, iron is incorporated into various protein structures in the brain, including those classified as enzymes essential to neurotransmitter production, such as tryptophan and tyrosine hydroxylase, which are implicated in serotonin and dopamine/norepinephrine production, respectively[2]. Furthermore, dopamine appears to be the most likely culprit for a variety of EID-induced behavioral manifestations, as evidenced by iron's critical role in dopamine systems. In ID rats, D_1 and D_2 receptor densities are altered, dopamine, serotonin, and norepinephrine transporters appear to have altered expression, and extracellular levels of dopamine and norepinephrine are significantly above the control baseline[48,49]. The long-term outcomes of these neurochemical imbalances parallel those of behavioral symptoms: despite iron treatment following EID, D_2 receptor and SERT densities are persistently below baseline[33] and dopamine transporters are lost due to iron chelation[2]. Contributing to disrupted neurotransmitter transport and binding are plausible effects of the surface protein Thy1 on signal transduction across chemical synapses[2]. Thy1 has reduced expression in an ID-rat model[51], indicating that both the functionality of chemical synapses may be comprised in ID individuals.

As far as the neurophysiological basis for behavioral deficits is concerned, dopamine insults correlate extraordinarily well with ID symptoms. Rats with EID have displayed highly similar responses to rats with controlled dopamine deficits when placed in a water maze[2]. Likewise, rats with dopamine deficits and rats with developmental ID display abnormal behavior when placed in novel surroundings[30], a trend that is also observed in humans with EID. In addition to behavioral alterations, individuals with hampered dopaminergic functions suffer from cognitive issues, particularly with respect to a diminished attention span[52]. While a host of factors

can affect cognition, dopamine may play a role in ID-induced cognitive disorders, as methylphenidate has been shown to improve cognitive function in EID rats[53]. Lastly, ID-induced damage to nigrostriatal dopamine neurons of rats during development can result in reduced motor control and motor sequencing[2]. With dopamine insults and ID paralleling one another so closely with regards to the three major ID-symptoms observed in humans, it is not unreasonable to conclude that the long-term effects of ID implicate monoamines, with dopamine being the most well-researched.

Current Study

The goals of this project were multi-fold:

1. To elucidate the effects of EID on brain iron concentrations in the ventral midbrain (VMB), hippocampus (HPC), striatum (ST), prefrontal cortex (PFC), pons (PONS), and cerebellum (CB). We hypothesize that, like previous studies, iron therapy starting at P8 will prove largely ineffective at ameliorating brain iron deficits in the formerly ID-model.
2. To determine the effects of EID on the concentration of the monoamines dopamine (DA) and serotonin (5-HT) in the VMB, HPC, ST, and PFC. Concentrations in the same CNS tissues will also be determined for the metabolites of major brain monoamines: 3,4-dihydroxyindoleacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA). We hypothesize that both the pre- and post-iron treatment EID groups will display altered monoamine and metabolite concentrations at the ages they were analyzed.
3. To further elucidate the specificity of the critical period during which ID can result in long-term consequences on brain iron and monoamine levels, both of which, if abnormal, are a strong indication of future behavioral patterns[30].

Materials and Methods

Overview

This experiment employs a longitudinal model with cross-sectional data collected at P21 and P90. Such a design facilitates the investigation of ID's effects during early gestation through the post-weaning period, as well as allowing for examination of the effects of iron repletion during the lactation phase.

Animal Housing and Breeding

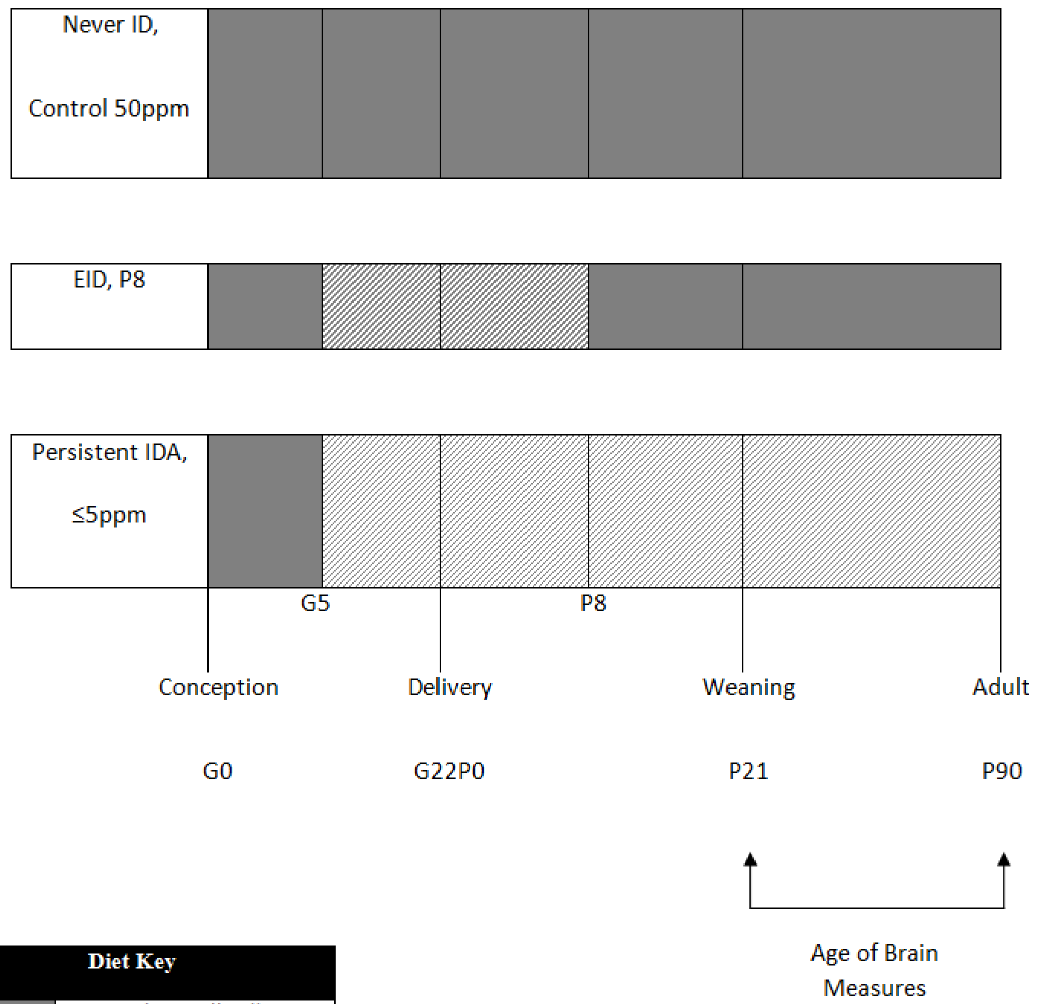
Young female and male Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN). Animals were pair-housed in clear 8" x 17" plastic cages with stainless steel lids. Female rats received an iron sufficient diet (50 ppm iron; Harlan Teklad, Madison WI) prior to mating and males received a commercial pellet diet (Purina 5001). All rats were given free access to food and deionized distilled water for 24 hours 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 experiment.

Once the females attained an adequate bodyweight (200-220 grams), they were housed apart from one another and paired with male rats, which were randomly assigned across all groups. Conception was identified by the presence of a vaginal plug, and following conception, females were housed singly and underwent daily checks for pregnancy status. Female body weights were collected every 5 days from G0 to G20. Day of delivery (P0) occurred between G20 and G23.

Study Design

At G5, females either remained on the 50 ppm iron diet or were switched to a ≤5 ppm iron diet (Harlan Teklab, Madison WI). Dams were maintained on their respective diets throughout the gestational and lactational periods. At P8, all pups were out-fostered to other lactating dams in order to eliminate the confounding variables associated with the biological effects of dam-specific characteristics. All pups born to control mothers were out-fostered to other control mothers, while only some of the pups born to ID mothers were out-fostered to other ID mothers, thus creating the "never ID" and "persistent ID" groups, respectively. The remaining pups born to ID mothers were out-fostered to control mothers to form the "EID" group, which would aid in evaluating the efficaciousness of iron-treatment at P8. This particular transition is unique because it provides EID pups with rapid-onset iron therapy, since they immediately switch from an iron-deficient to an iron-sufficient dam. After weaning, pups were maintained on their respective diets and housed in pairs according to gender until sacrifice. To evaluate the short- and long-term effects of EID, animals were sacrificed at P21 and P90, respectively. In summary, the three major groups of subjects are:

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



Diet Key	
	50 ppm iron pellet diet (iron sufficient)
	≤5 ppm iron pellet diet (iron deficient)

FIGURE 1 - Study Design[55]

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, with all weights measured to the nearest tenth of a 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 were collected by puncturing the tail vein and extracting approximately 200 μL of blood. Furthermore, blood (5 μL) was collected from 3 randomly chosen pups from each litter at P0, P8, and P15 to approximate pup hemoglobin levels. At the study endpoints of P21 and P90, evaluation of offspring iron status was based on hemoglobin, hematocrit, spleen iron levels, liver iron levels, serum iron, total iron binding capacity (TIBC), and transferrin saturation (TSAT).

Hematology

Hemoglobin was measured via colorimetric methods by using the cyanmethemoglobin method (Sigma Aldrich, St. Louis MO). To measure hematocrit levels, microcapillary tubes were centrifuged (13,700 x g, 5 min, RT) to separate red blood cells from serum. The percent of red blood cells in each sample was measured thereafter. Serum iron and TIBC were also determined using colorimetric methods[54], and the procedure was modified to use 50 μL of sample. In order to calculate transferrin saturation, the ratio of serum iron to TIBC was calculated and put in percentage form. Liver and spleen iron concentration values were determined by colorimetric assay[54] with ferrozine as the color reagent.

Euthanasia and Dissection

Rats were put under anesthesia by means of carbon dioxide asphyxiation and then decapitated at P21 and P90. Following sacrifice of each rat, blood was collected, and the brain, liver, and spleen were rapidly removed and placed on ice. Brains were divided at the midline. The left hemisphere was dissected into 6 regions: ventral midbrain (VMB), hippocampus (HPC), prefrontal cortex (PFC), striatum (ST), pons (Pons), and cerebellum (CB). The right hemisphere was immediately frozen in a dry-ice/isopentane slurry. Brain samples from both hemispheres were stored at a temperature of -80°C prior to analysis.

Brain Region Homogenization

Brain regions were thawed on ice and subsequently 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 (10 mL/g tissue; Roche, Indianapolis IN). Also, homogenate was aliquoted into individual tubes and then frozen at -80°C.

Brain Iron Concentrations by Region

Brain iron concentrations in the VMB, HPC, PFC, ST, PONS, and CB were measured by atomic absorption spectrometry. A total of 10 μL homogenate was added to an equivalent volume of ultra-purified HNO_3 in a 400 μL polypropylene microfuge tube. Samples were digested for 48 hr at 50°C and diluted 1:10 with 3.12 mmol/L HNO_3. A graphite furnace atomic absorption spectrometer was used to analyze the prepared samples (PerkinElmer AAnalyst 600, Shelton, CT). The standards were prepared by diluting a Perkin Elmer iron standard (PE#N9300126) in 0.2% ultra-pure HNO_3; the blanks were prepared with diluting and digesting reagents to minimize possible contamination. All standard curves exceeded $r > 0.99$.

Monoamine concentrations

Homogenates were added to equal volumes of cold 0.2 M $HClO_4$ and passed through a micro-Sephadex column in order to remove any endogenous substrates (5000 x g, 4°C). Concentrations of dopamine (DA), serotonin (5-HT), 3,4-dihydroxyindoleacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA) were determined in the VMB, HPC, ST, and PFC 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 consisted of 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 that were analyzed with each group of samples. 3,4-dihydroxybenzylamine (DHBA), which was added to each sample, acted as a self-check standard by correcting for sample-to-sample and run-to-run variations.

Statistical Analysis

Statistical analyses were performed using the GraphPad Prism System statistical analysis package (GraphPad Software, Inc.) and IBM SPSS software. Representative averages of all examined dependent variables were taken for each litter. 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, with the two factors being treatment and time. One-way ANOVA was used to test for significant differences between groups in all other cases, with the sole factor being treatment, which was

compared across the examined times (P21 and P90) for all groups. Post-hoc analyses was performed using Tukey's HSD. Significance level was set at $p<0.05$.

Results and Figures

Hematological Findings for CN and ID dams

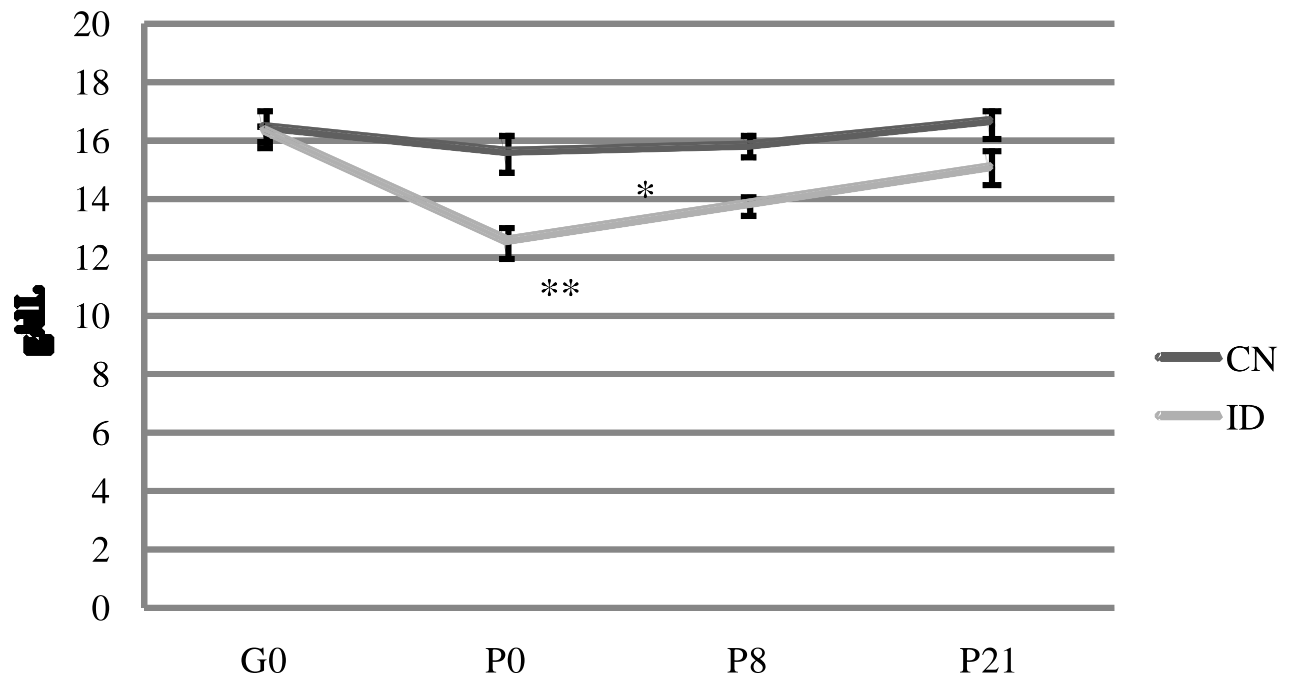
In order to assess iron status, blood was collected from dams prior to breeding and during lactation. A two-way ANOVA showed a significant treatment x time interaction ($p<0.05$), meaning that the relationship between time and hemoglobin level was different between the two treatment groups. The effect of treatment alone was also significant ($p<0.001$; Figure 2A), as significant differences in hemoglobin levels between CN and ID rats were observed at P0 ($p<0.001$) and P8 ($p<0.05$).

A two-way ANOVA of the hematocrit data showed that the diet treatment x time interaction was not significant ($p=0.44$). Therefore, the relationship between time and hematocrit levels was similar between CN and ID dams. No differences in hematocrit levels were observed between CN and ID dams (Figure 2B).

Gender Differences Among Offspring

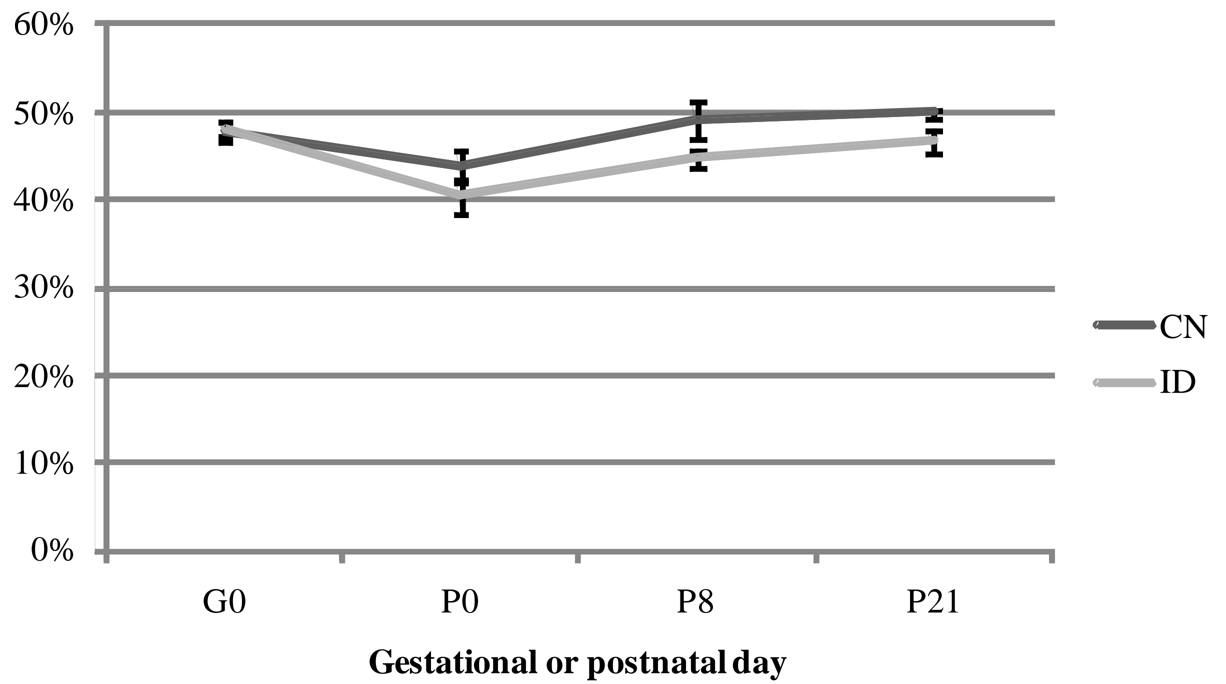
In order to determine if gender played a confounding role in the relevant variables, representative males and females were taken from each litter by averaging the results for each dependent variable by gender within the litter. Two two-way ANOVAs were run for gender x treatment and gender x age. There was a significant difference in bodyweight noted at P21 and P90 for gender x treatment. All other variables were not significantly gender-dependent and were analyzed together.

FIGURE 2A - Maternal Hemoglobin at G0, P0, P8 and P21[55] [α]



[α]Values are means ± SEM; ***p<0.001, *p<0.05 compared to CN

FIGURE 2B - Maternal Hematocrit at G0, P0, P8 and P21[55] $^{\alpha}$



$^{\alpha}$Values are means ± SEM

Hemoglobin, Hematocrit, and Body weights of Offspring

In order to assess iron status and physical growth outcomes in each treatment group, hemoglobin levels and body weights were measured for rat offspring at P21 and P90. A two-way ANOVA revealed an insignificant treatment x time interaction ($p=0.346$), meaning that the relationship between diet and time was not different between treatment groups (Table 1A). However, the effects of treatment and time individually were highly significant ($P<0.001$). A two-tailed T-test for significance found that hemoglobin at P21 was significantly lower in the persistent-ID pups versus never-ID pups ($p<0.005$). Similarly, the hemoglobin of persistent-ID pups was significantly ($p<0.005$) lower than that of IDP8CN pups, indicating that iron therapy at P8 had at least partially corrected for iron deficits induced prior to P8 (Table 2A and 2B; Figure 3A and #B).

For hematocrit percentage, a two-tailed T-test found that hematocrit at P21 was significantly lower in the persistent ID pups versus control pups ($p<0.005$). Similarly, the hematocrit of persistent-ID pups was significantly ($p<0.005$) lower than that of IDP8CN pups, meaning that iron therapy at P8 had ameliorated EID-induced hematological deficits. A two-way ANOVA of the hematocrit data showed that the treatment x time interaction was highly significant ($p<0.001$), as were the effects of treatment and time alone ($p<0.001$) (Table 1C).

A two-tailed T-test found no significant differences in body weight versus control for both P21 and P90. A two-way ANOVA of bodyweight data showed that the treatment x time interaction was not significant ($p=0.155$), but the effect of treatment was significant ($p<0.05$) and the effect of age was highly significant ($p<0.001$) (Table 1B). In summary, these statistical conclusions indicate a state of iron-deficiency in the pups that did not receive iron-intervention at P8, but the differences in hemoglobin were not strong enough to alter bodyweight independently of increases caused by age.

Table 1A – Two-Way ANOVA for Hemoglobin with Factors of Treatment and Age (P21 and P90)

Tests of Between-Subjects Effects

Dependent Variable:hemoglobinUGDL

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	558.758[a]	5	111.752	33.345	.000
Intercept	6005.217	1	6005.217	1791.875	.000
DIET_NUM	69.786	2	34.893	10.412	.000
AGENUM	488.155	1	488.155	145.659	.000
DIET_NUM * AGENUM	7.291	2	3.645	1.088	.346
Error	144.108	43	3.351		
Total	6699.820	49			
Corrected Total	702.866	48			

a. R Squared = .795 (Adjusted R Squared = .771)

Table 1B – Two-Way ANOVA for Bodyweight with Factors of Treatment and Age (P21 and P90)

Tests of Between-Subjects Effects

Dependent Variable:Weight

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	852444.475	5	170488.895	328.746	.000
Intercept	1524111.97	1	1524111.97	2938.875	.000
DIET_NUM	4295.820	2	2147.910	4.142	.023
AGENUM	826964.717	1	826964.717	1594.598	.000
DIET_NUM * AGENUM	2021.613	2	1010.806	1.949	.155
Error	22299.964	43	518.604		
Total	2216204.77	49			
Corrected Total	874744.438	48			

a. R Squared = .975 (Adjusted R Squared = .972)

Table 1C – Two-Way ANOVA for Hematocrit with Factors of Treatment and Age (P21 and P90)

Tests of Between-Subjects Effects

Dependent Variable:Hemocrit_Percent

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3426.125[a]	5	685.225	52.443	.000
Intercept	61264.376	1	61264.376	4688.788	.000
DIET_NUM	388.015	2	194.007	14.848	.000
AGENUM	2635.281	1	2635.281	201.688	.000
DIET_NUM * AGENUM	246.684	2	123.342	9.440	.000
Error	509.580	39	13.066		
Total	66842.510	45			
Corrected Total	3935.704	44			

a. R Squared = .871 (Adjusted R Squared = .854)

Other Hematology, Spleen Iron, and Liver Iron

Three other major hematological factors that were considered were serum iron, total iron binding capacity (TIBC), and transferrin saturation percentage. A two-way ANOVA for serum

iron revealed an insignificant treatment x time interaction ($p=0.329$), meaning that the relationship between diet and time was not different between treatment groups (Table 3A). However, the effect of treatment alone was highly significant ($p<0.001$). Furthermore, the persistent-ID rats showed statistically significant decreases in serum iron at both P21 ($p<0.001$) and P90 ($p<0.05$) when compared to rats fed an iron-sufficient diet. The persistent-ID rats also showed significant decreases in serum iron at P21 ($p<0.05$) versus IDP8CN rats. TIBC at P21 was significantly lower for both the persistent-ID group and the IDP8CN group versus never-ID rats ($p<0.005$), indicating a lag-time between iron-intervention and TIBC amelioration. Lastly, transferrin saturation percentage was significantly lower for persistent-ID rats versus never-ID rats at P21 ($p<0.05$) (Table 2A).

Spleen and liver iron concentrations were also measured at P21 and P90 and subjected to subsequent statistical analysis. A two-way ANOVA for spleen iron revealed an insignificant treatment x time interaction ($p=0.099$); thus, the relationship between diet and time was not different between treatment groups (Table 3B). However, the effect of time alone was highly significant ($p<0.001$). A two-tailed T-test found no significant differences in spleen iron among all groups at P21 and P90. The two-way ANOVA for liver iron showed a statistically significant treatment x time interaction ($p=0<0.001$), and there was also a significant effect from dietary treatments across P21 and P90 ($p<0.05$) (Table 3C). Furthermore, there was a highly significant effect from time on liver iron across dietary treatment groups ($p<0.001$). At P90, the persistent-ID group displayed a statistically significant decrease in liver iron versus never-ID rats of the same age ($p=0.005$) (Table 2B).

TABLE 2A

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

	CN	ID	IDP8CN
Body Weight (g)	45.2±2.4 (12)	44.2±2.7 (8)	52.5±3.8 (7)
Hematocrit (%)	35.2±0.005 (12)	22.8±0.025 (8) [a,b]	35.8±0.012 (7)
Hemoglobin (g/dL)	9.5±0.2 (12)	5.9±0.7 (8) [a,b]	8.8±0.3 (7)
Serum Iron (μg/dL)	376.4±41.0 (12)	144.0±25.6 (8) [a,b]	302.7±48.2 (7)
TIBC (μg/dL)	861.8±54.5 (12)	631.6±35.3 (8) [a]	618.0±34.5 (7)
Transferrin Saturation (%)	44.9±4.8 (12)	24.1±4.7 (8) [a]	50.1±7.5 (7)
Liver Iron (μg/dL)	39.2±8.3 (12)	58.0±16.0 (8)	57.5±11.3 (7)
Spleen Iron (μg/dL)	127.2±23.0 (12)	130.5±17.5 (8)	138.7±26.5 (7)

[1] Values are means ± SEM (n); [a]$p < 0.05$, relative to CN values; [b]$p<0.05$ relative to IDP8CN values

TABLE 2B

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

	CN	ID	IDP8CN
Body Weight (g)	293.8±15.4 (8)	309.5±8.0 (7)	332.4±10.5 (7)
Hematocrit (%)	47.3±0.7 (8)	45.8±0.7 (5)	46.8±0.8 (5)
Hemoglobin (g/dL)	15.1±0.3 (8)	13.3±1.3 (7)	15.1±0.8 (7)
Serum Iron (µg/dL)	303.9±21.7 (8)	227.5±15.6 (5) [a]	275.6±15.3 (5)
TIBC (µg/dL)	644.7±48.1 (8)	636.0±24.6 (5)	703.7±77.9 (5)
Transferrin Saturation (%)	49.1±4.1 (8)	35.7±1.8 (5)	41.5±4.8 (5)
Liver Iron (µg/dL)	206.4±22.5 (8)	101.9±21.3 (7) [a]	147.0±17.1 (7)
Spleen Iron (µg/dL)	945.2±168.3 (8)	537.1±122.6 (7)	866.1±152.7 (7)

[1] Values are means ± SEM (n); [a]$p < 0.05$ relative to CN values

Table 3A – Two-Way ANOVA for Serum Iron with Factors of Treatment and Age (P21 and P90)

Tests of Between-Subjects Effects

Dependent Variable:plasmaIron

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	393087.284	5	78617.457	5.844	.000
Intercept	2899755.05	1	2899755.05	215.555	.000
DIET_NUM	294336.816	2	147168.408	10.940	.000
AGENUM	33525.791	1	33525.791	2.492	.122
DIET_NUM * AGENUM	30713.414	2	15356.707	1.142	.329
Error	578456.453	43	13452.476		
Total	4281188.31	49			
Corrected Total	971543.737	48			

a. R Squared = .405 (Adjusted R Squared = .335)

Table 3B - Two-Way ANOVA for Spleen Iron with Factors of Treatment and Age (P21 and P90)

Tests of Between-Subjects Effects

Dependent Variable:SpleenIron

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5946323.28	5	1189264.65	15.443	.000
Intercept	9888751.00	1	9888751.00	128.412	.000
DIET_NUM	373832.379	2	186916.189	2.427	.100
AGENUM	5000941.04	1	5000941.04	64.940	.000
DIET_NUM * AGENUM	375311.182	2	187655.591	2.437	.099
Error	3311352.34	43	77008.194		
Total	18194281.9	49			
Corrected Total	9257675.63	48			

a. R Squared = .642 (Adjusted R Squared = .601)

Table 3C - Two-Way ANOVA for Liver Iron with Factors of Treatment and Age (P21 and P90)

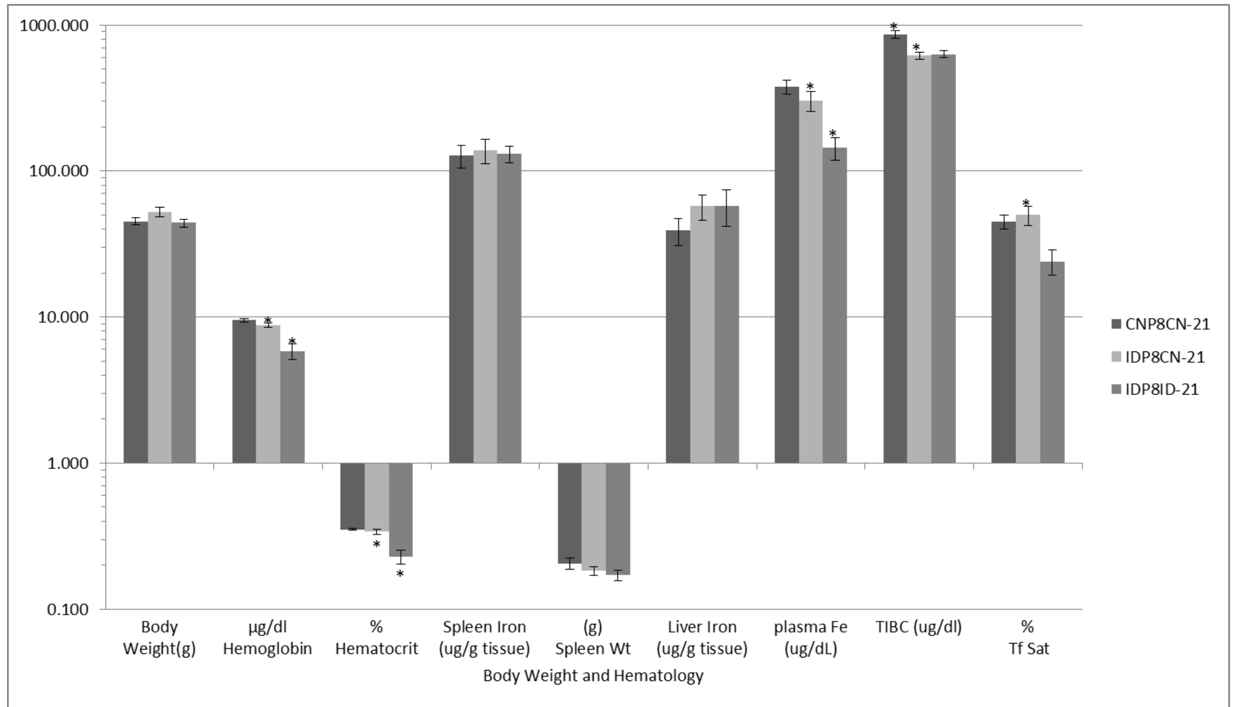
Tests of Between-Subjects Effects

Dependent Variable:tissueiron

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	176599.477	5	35319.895	17.159	.000
Intercept	488575.326	1	488575.326	237.365	.000
DIET_NUM	15449.674	2	7724.837	3.753	.031
AGENUM	118600.415	1	118600.415	57.620	.000
DIET_NUM * AGENUM	33337.121	2	16668.561	8.098	.001
Error	88508.228	43	2058.331		
Total	721948.515	49			
Corrected Total	265107.705	48			

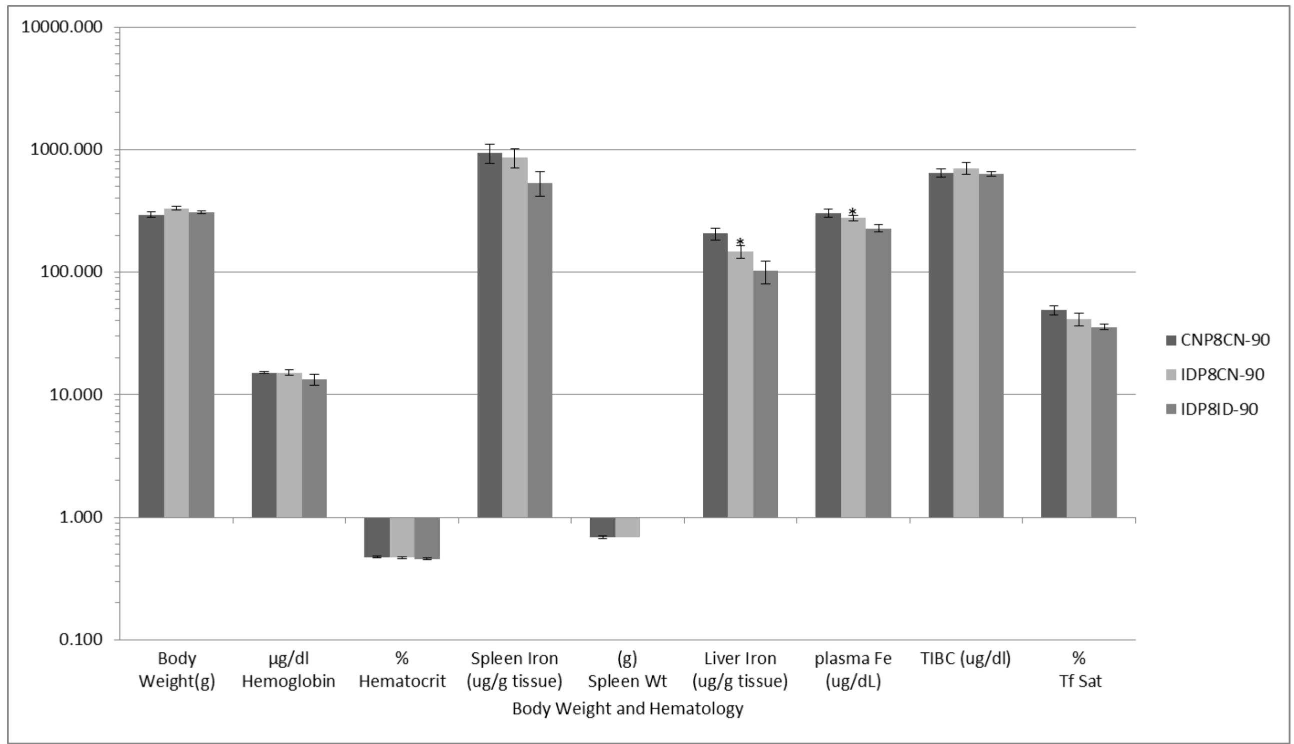
a. R Squared = .666 (Adjusted R Squared = .627)

FIGURE 3A – Logarithmic Bar Graph of Two-Tailed T-Tests for Body Weight and Hematology at P21[α]



[α]Asterisks indicate statistically significant results

FIGURE 3B – Logarithmic Bar Graph of Two-Tailed T-Tests for Body Weight and Hematology at P90[α]



[α]Asterisks indicate statistically significant results

Brain Iron Concentrations

Results of Two-Tailed T-tests:

Regional brain iron content was analyzed for significance across ages and treatment groups. The VMB and PFC had no statistically significant differences in brain iron content across age groups and treatment protocols ($p>0.05$). The CB had significantly lower iron concentrations in the persistent-ID group in comparison to never-ID rats at both P21 and P90 ($p<0.05$). Additionally, the CB of IDP8CN rats had significantly lower concentrations of iron than that of never-ID rats at P21 but not P90, an indication that iron therapy-induced repletion in the CB is progressive and ultimately successful ($p<0.05$). The HPC had significantly lower brain iron ($p<0.05$) in persistent-ID rats versus never-ID rats at P21. Persistent-ID rats also had lower HPC iron concentrations than IDP8CN rats ($p<0.05$), indicating rapid amelioration of iron deficits in that particular tissue. In the striatum, IDP8CN rats had significantly lower iron concentrations ($p<0.05$) and persistent-ID rats were trending on significance ($p=0.059$), both in comparison to never-ID rats. Finally, both IDP8CN and persistent-rats had significantly lower iron concentrations in the PONS relative to never-ID rats at P90 (Figures 4A and 4B). This is an indication that EID may have uncorrectable long-term effects on PONS iron-concentration.

Results of One-Way ANOVA:

P21: A one-way ANOVA run for the factor “treatment” across P21 groups found statistically significant differences in iron concentrations in the ST and CB ($p<0.05$) but not in other examined brain regions.

P90: A one-way ANOVA run for the factor “treatment” across P90 groups found statistically significant differences in iron concentrations in the PONS and CB ($p<0.05$) but not in other examined brain regions. The dual occurrence of CB across age groups may indicate that it is particularly susceptible to disrupted iron concentrations due to EID.

Dopamine Concentrations in the STR, PFC, VMB, and HPC

Results of Two-Tailed T-tests:

No significant differences were observed for dopamine concentrations across all examined brain regions, ages of examination, and treatment protocols ($p>0.05$).

Results of One-Way ANOVA:

One-way ANOVAs with the factor "treatment" for both P21 and P90 found no statistically significant differences in dopamine concentrations across all examined brain regions ($p>0.05$).

Serotonin Concentrations in the STR, PFC, VMB, and HPC

Results of Two-Tailed T-tests:

No significant differences were observed for serotonin concentrations across all examined brain regions, ages of examination, and treatment protocols ($p>0.05$).

Results of One-Way ANOVA:

One-way ANOVAs with the factor "treatment" for both P21 and P90 found no statistically significant differences in serotonin concentrations across all examined brain regions ($p>0.05$).

Metabolite (DOPAC, 5-HIAA, HVA) Concentrations in STR, PFC, VMB, and HPC

Results of Two-Tailed T-tests:

No significant differences were observed for all analyzed metabolite concentrations across all examined brain regions, ages of examination, and treatment protocols ($p>0.05$) (Figures 4C and 4D).

Results of One-Way ANOVA:

One-way ANOVAs with the factor “treatment” for both P21 and P90 found no statistically significant differences in any metabolite concentrations across all examined brain regions. Some metabolites trended towards significance but did not achieve it ($p>0.05$).

See Table 4A for One-Way ANOVA results with factor “treatment” for all relevant dependent variables at P21.
See Table 4B for One-Way ANOVA results with factor “treatment” for all relevant dependent variables at P90.

Table 4A - One-Way ANOVA for All Relevant Variables at P21

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Weight	Between Groups	308.274	2	154.137	2.118	.142
	Within Groups	1746.872	24	72.786		
	Total	2055.147	26			
hemoglobinUGDL	Between Groups	67.289	2	33.645	20.070	.000
	Within Groups	40.233	24	1.676		
	Total	107.522	26			
SpleenIron	Between Groups	584.914	2	292.457	.060	.942
	Within Groups	116233.227	24	4843.051		
	Total	116818.141	26			
SpleenWeight	Between Groups	.034	2	.017	1.616	.220
	Within Groups	.252	24	.010		
	Total	.286	26			
tissueiron	Between Groups	2304.962	2	1152.481	.962	.396
	Within Groups	28744.908	24	1197.705		
	Total	31049.870	26			
plasmaIron	Between Groups	261344.346	2	130672.173	8.799	.001
	Within Groups	356409.517	24	14850.397		
	Total	617753.863	26			
TIBC	Between Groups	373753.253	2	186876.626	8.748	.001
	Within Groups	512684.857	24	21361.869		
	Total	886438.110	26			
TfSatPercent	Between Groups	3039.052	2	1519.526	5.489	.011
	Within Groups	6644.022	24	276.834		
	Total	9683.074	26			
VMB	Between Groups	110.329	2	55.164	1.187	.322
	Within Groups	1115.339	24	46.472		
	Total	1225.667	26			
Hpc	Between Groups	176.177	2	88.088	2.725	.086
	Within Groups	775.724	24	32.322		
	Total	951.901	26			
ST	Between Groups	159.617	2	79.808	3.786	.037
	Within Groups	505.930	24	21.080		
	Total	665.547	26			
PFC	Between Groups	73.681	2	36.840	1.423	.261
	Within Groups	621.226	24	25.884		
	Total	694.907	26			
Pons	Between Groups	85.088	2	42.544	1.828	.182
	Within Groups	558.497	24	23.271		
	Total	643.585	26			
CB	Between Groups	189.948	2	94.974	3.949	.033
	Within Groups	577.170	24	24.049		
	Total	767.119	26			

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
DHBA	Between Groups	368.905	2	184.452	.994	.385
	Within Groups	4455.219	24	185.634		
	Total	4824.124	26			
DOPAC	Between Groups	262.627	2	131.313	2.481	.105
	Within Groups	1270.212	24	52.925		
	Total	1532.839	26			
DA	Between Groups	172.230	2	86.115	1.494	.245
	Within Groups	1383.710	24	57.655		
	Total	1555.940	26			
fiveHIAA	Between Groups	81.039	2	40.520	1.175	.326
	Within Groups	827.787	24	34.491		
	Total	908.826	26			
HVA	Between Groups	4.124	2	2.062	.542	.589
	Within Groups	91.340	24	3.806		
	Total	95.464	26			
FiveHT	Between Groups	.751	2	.376	.463	.635
	Within Groups	19.478	24	.812		
	Total	20.229	26			
Pfc_DA	Between Groups	6.459	2	3.230	.218	.806
	Within Groups	355.988	24	14.833		
	Total	362.448	26			
Pfc_fiveHIAA	Between Groups	65.906	2	32.953	1.030	.372
	Within Groups	767.892	24	31.996		
	Total	833.798	26			
Pfc_HVA	Between Groups	.298	2	.149	.177	.839
	Within Groups	20.214	24	.842		
	Total	20.512	26			
Pfc_fiveHT	Between Groups	1.084	2	.542	.353	.706
	Within Groups	36.877	24	1.537		
	Total	37.961	26			
VMB_DHBA	Between Groups	628.804	2	314.402	.557	.580
	Within Groups	13534.937	24	563.956		
	Total	14163.740	26			
VMB_DOPAC	Between Groups	30.279	2	15.139	.953	.400
	Within Groups	381.122	24	15.880		
	Total	411.401	26			
VMB_DA	Between Groups	8.714	2	4.357	.352	.707
	Within Groups	297.364	24	12.390		
	Total	306.078	26			
VMB_fiveHIAA	Between Groups	53.204	2	26.602	.596	.559
	Within Groups	1072.102	24	44.671		
	Total	1125.306	26			

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
VMB_HVA	Between Groups	.064	2	.032	.019	.981
	Within Groups	39.740	24	1.656		
	Total	39.804	26			
VMB_fiveHT	Between Groups	2.599	2	1.299	.254	.778
	Within Groups	122.795	24	5.116		
	Total	125.394	26			
HPc_DHBA	Between Groups	185.756	2	92.878	.828	.449
	Within Groups	2693.072	24	112.211		
	Total	2878.827	26			
HPC_DOPAC	Between Groups	57.498	2	28.749	.956	.399
	Within Groups	721.618	24	30.067		
	Total	779.116	26			
HPC_DA	Between Groups	6.292	2	3.146	.283	.756
	Within Groups	267.164	24	11.132		
	Total	273.456	26			
HPC_fiveHIAA	Between Groups	49.742	2	24.871	.585	.565
	Within Groups	1020.300	24	42.513		
	Total	1070.042	26			
HPC_HVA	Between Groups	1.314	2	.657	.992	.385
	Within Groups	15.885	24	.662		
	Total	17.199	26			
HPC_fiveHT	Between Groups	1.267	2	.634	.307	.739
	Within Groups	49.569	24	2.065		
	Total	50.837	26			
Hemo_Percent	Between Groups	790.809	2	395.404	20.227	.000
	Within Groups	449.602	23	19.548		
	Total	1240.411	25			

Table 4B - One-Way ANOVA for All Relevant Variables at P90

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Litter	Between Groups	1283.864	2	641.932	124.456	.000
	Within Groups	98.000	19	5.158		
	Total	1381.864	21			
Weight	Between Groups	5574.652	2	2787.326	2.577	.102
	Within Groups	20553.091	19	1081.742		
	Total	26127.744	21			
hemoglobinUGDL	Between Groups	15.660	2	7.830	1.432	.263
	Within Groups	103.875	19	5.467		
	Total	119.535	21			
SpleenIron	Between Groups	680965.585	2	340482.792	2.025	.160
	Within Groups	3195119.11	19	168164.164		
	Total	3876084.70	21			
SpleenWeight	Between Groups	.253	2	.126	1.866	.182
	Within Groups	1.288	19	.068		
	Total	1.541	21			
tissueiron	Between Groups	41308.190	2	20654.095	6.566	.007
	Within Groups	59763.320	19	3145.438		
	Total	101071.510	21			
plasmaIron	Between Groups	82742.117	2	41371.059	3.540	.049
	Within Groups	222046.936	19	11686.681		
	Total	304789.053	21			
TIBC	Between Groups	148831.357	2	74415.678	.883	.430
	Within Groups	1601755.39	19	84302.915		
	Total	1750586.75	21			
TfSatPercent	Between Groups	2416.864	2	1208.432	3.851	.039
	Within Groups	5962.186	19	313.799		
	Total	8379.050	21			
VMB	Between Groups	40.464	2	20.232	1.927	.173
	Within Groups	199.449	19	10.497		
	Total	239.913	21			
Hpc	Between Groups	.230	2	.115	.028	.972
	Within Groups	77.234	19	4.065		
	Total	77.463	21			
ST	Between Groups	23.841	2	11.921	1.301	.295
	Within Groups	174.104	19	9.163		
	Total	197.945	21			
PFC	Between Groups	9.225	2	4.613	.395	.679
	Within Groups	221.756	19	11.671		
	Total	230.981	21			
Pons	Between Groups	44.149	2	22.075	4.165	.032
	Within Groups	100.707	19	5.300		
	Total	144.856	21			

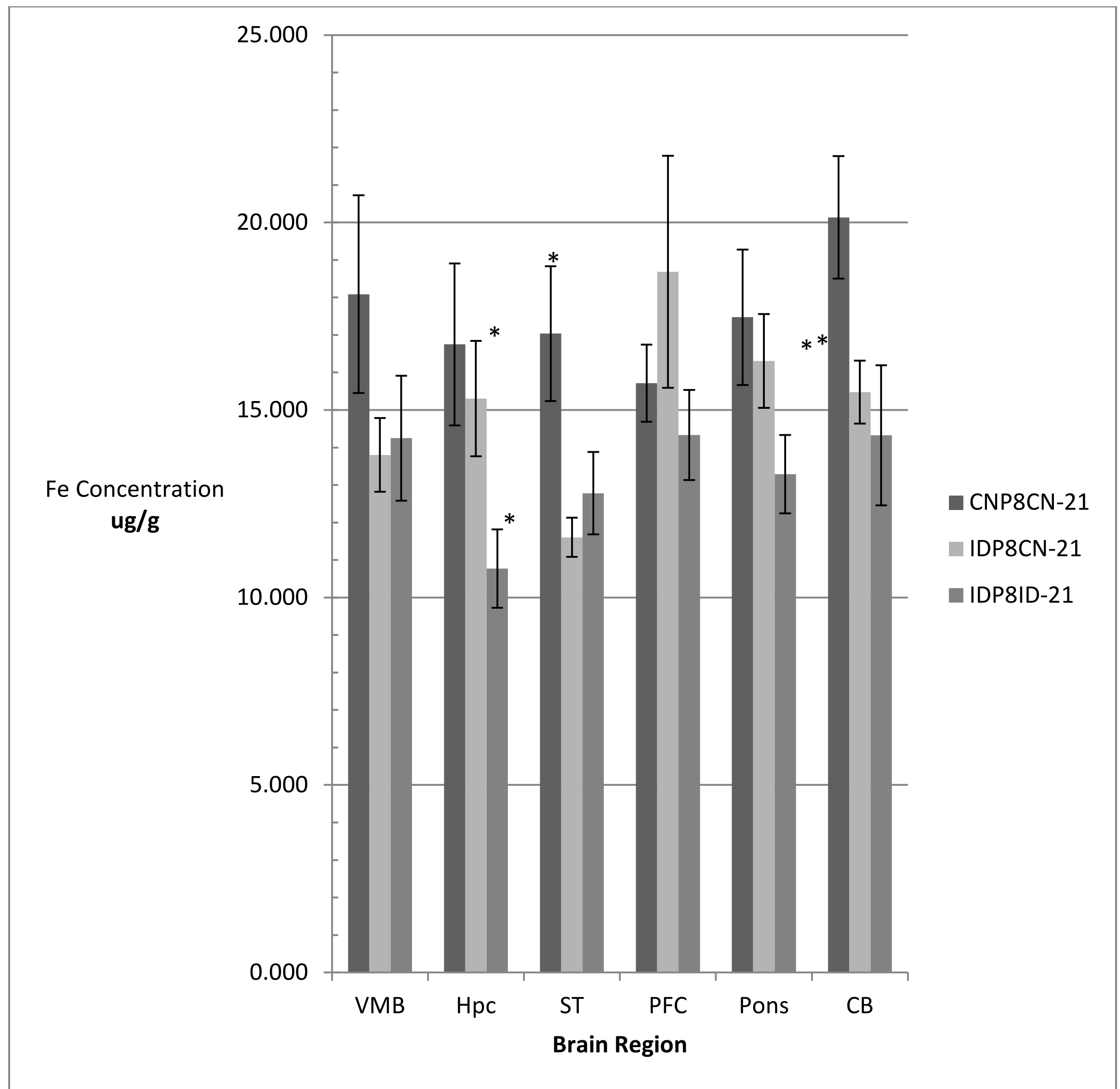
ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
CB	Between Groups	109.947	2	54.974	5.662	.012
	Within Groups	184.486	19	9.710		
	Total	294.433	21			
DHBA	Between Groups	145.678	2	72.839	.479	.627
	Within Groups	2890.996	19	152.158		
	Total	3036.674	21			
DOPAC	Between Groups	83.973	2	41.987	.117	.890
	Within Groups	6834.377	19	359.704		
	Total	6918.350	21			
DA	Between Groups	74.135	2	37.068	.147	.864
	Within Groups	4788.767	19	252.040		
	Total	4862.903	21			
fiveHIAA	Between Groups	5.461	2	2.730	.155	.857
	Within Groups	334.287	19	17.594		
	Total	339.748	21			
HVA	Between Groups	5.559	2	2.780	.757	.483
	Within Groups	69.801	19	3.674		
	Total	75.360	21			
FiveHT	Between Groups	.098	2	.049	.089	.915
	Within Groups	10.387	19	.547		
	Total	10.485	21			
Pfc_DA	Between Groups	10.875	2	5.438	.277	.761
	Within Groups	373.094	19	19.637		
	Total	383.969	21			
Pfc_fiveHIAA	Between Groups	.138	2	.069	.007	.993
	Within Groups	193.662	19	10.193		
	Total	193.801	21			
Pfc_HVA	Between Groups	.247	2	.123	.420	.663
	Within Groups	5.588	19	.294		
	Total	5.835	21			
Pfc_fiveHT	Between Groups	.622	2	.311	.379	.689
	Within Groups	15.579	19	.820		
	Total	16.201	21			
VMB_DHBA	Between Groups	34.338	2	17.169	.182	.835
	Within Groups	1795.499	19	94.500		
	Total	1829.837	21			
VMB_DOPAC	Between Groups	3.300	2	1.650	.135	.875
	Within Groups	232.406	19	12.232		
	Total	235.706	21			
VMB_DA	Between Groups	13.372	2	6.686	.400	.676
	Within Groups	317.886	19	16.731		
	Total	331.258	21			

ANOVA

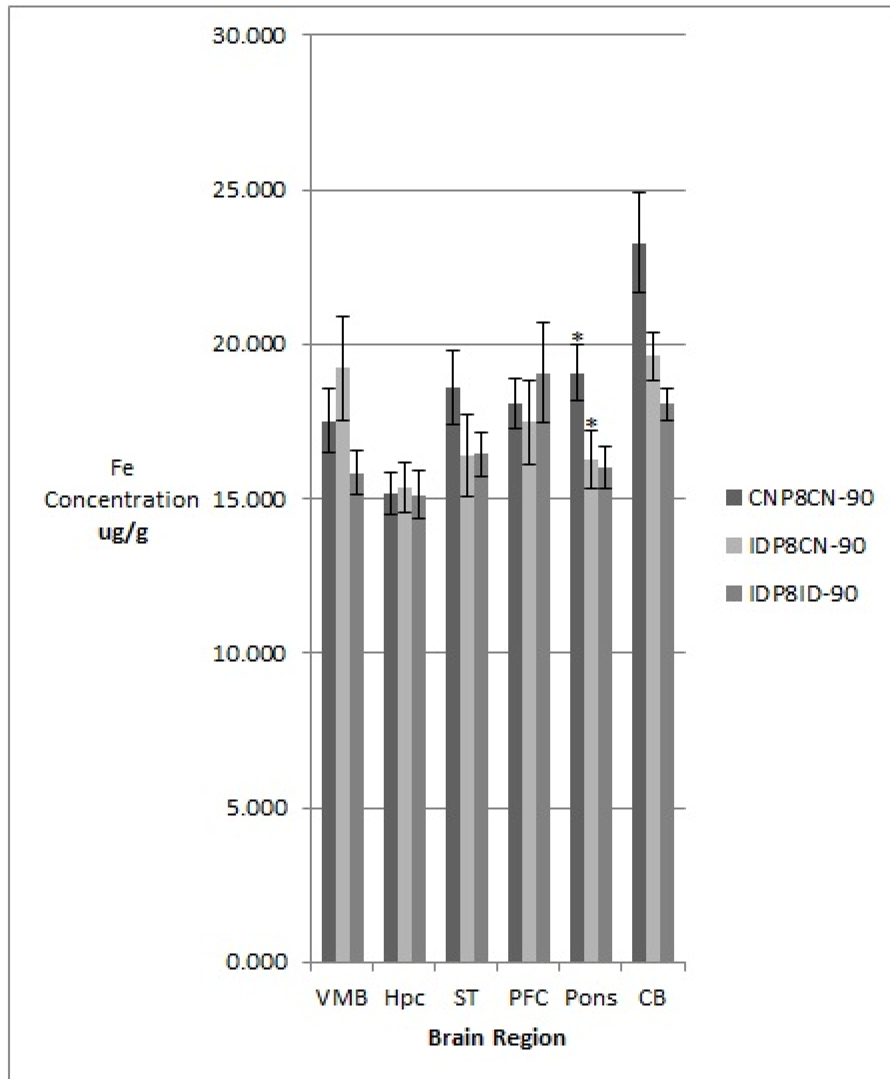
		Sum of Squares	df	Mean Square	F	Sig.
VMB_fiveHIAA	Between Groups	6.438	2	3.219	.242	.788
	Within Groups	252.817	19	13.306		
	Total	259.255	21			
VMB_HVA	Between Groups	.574	2	.287	1.236	.313
	Within Groups	4.414	19	.232		
	Total	4.988	21			
VMB_fiveHT	Between Groups	1.145	2	.572	.314	.734
	Within Groups	34.580	19	1.820		
	Total	35.725	21			
HPc_DHBA	Between Groups	92.113	2	46.057	.363	.700
	Within Groups	2412.126	19	126.954		
	Total	2504.239	21			
HPC_DOPAC	Between Groups	48.086	2	24.043	.518	.604
	Within Groups	882.255	19	46.434		
	Total	930.342	21			
HPC_DA	Between Groups	135.076	2	67.538	.612	.553
	Within Groups	2096.510	19	110.343		
	Total	2231.586	21			
HPC_fiveHIAA	Between Groups	15.308	2	7.654	.306	.740
	Within Groups	474.943	19	24.997		
	Total	490.251	21			
HPC_HVA	Between Groups	.061	2	.031	.113	.894
	Within Groups	5.134	19	.270		
	Total	5.195	21			
HPC_fiveHT	Between Groups	.802	2	.401	.362	.701
	Within Groups	21.070	19	1.109		
	Total	21.872	21			
Hemo_Percent	Between Groups	6.736	2	3.368	.855	.445
	Within Groups	59.120	15	3.941		
	Total	65.856	17			

FIGURE 4A – Iron Concentration as a Function of Brain Region for Rats at P21[α]



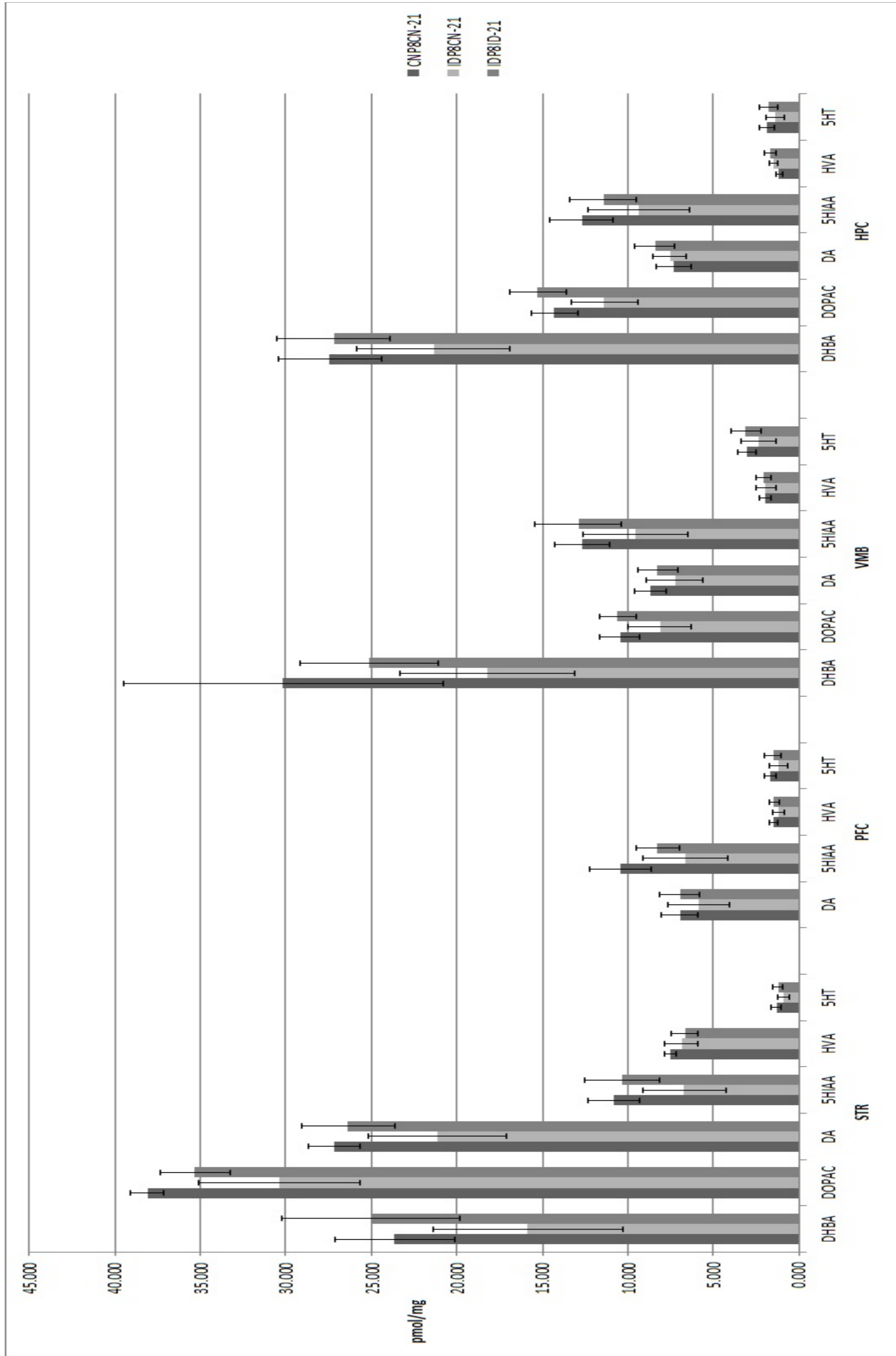
[α]Asterisks indicate statistically significant results relative to never-ID subjects

FIGURE 4B – Iron Concentration as a Function of Brain Region for Rats at P90[α]



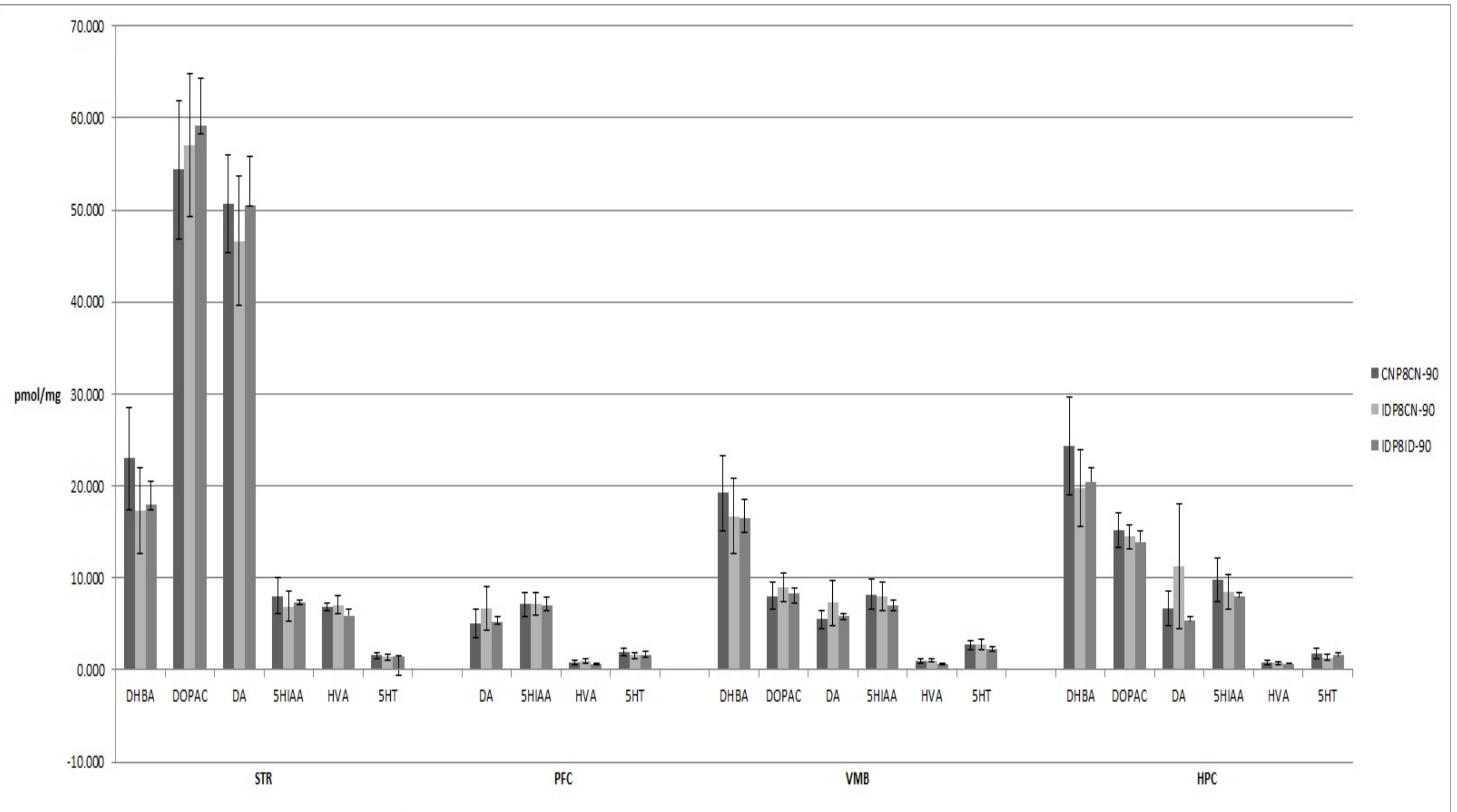
[α]Asterisks indicate statistically significant results relative to never-ID subjects

FIGURE 4C – Concentrations of Brain Monoamines and Metabolites in the STR, PFC, VMB, and HPC for Rats at P21 (next page)



CNP8CN-21
IDP8CN-21
IDP8ID-21
pmol/mg
45.000
40.000
35.000
30.000
25.000
20.000
15.000
10.000
5.000
0.000
DHBA
DOPAC
DA
5HIAA
HVA
5HT
STR
PFC
VMB
HPC

FIGURE 4D – Concentrations of Brain Monoamines and Metabolites in the STR, PFC, VMB, and HPC for Rats at P90 (next page)


pmol/mg
70.000
60.000
50.000
40.000
30.000
20.000
10.000
0.000
-10.000
DHBA
DOPAC
DA
5HIAA
HVA
5HT
STR
PFC
VMB
HPC
CNP8CN-90
IDP8CN-90
IDP8ID-90

Discussion

The purpose of this study was to examine the long-term effects of gestational and early-lactational iron deficiency on brain iron and monoamine levels in a rat model of early iron deficiencies. By reintroducing iron into the diets of ID rats at P8, we also elucidated the degree to which abnormal iron and monoamine levels can be normalized at P21 and P90 following iron treatment. The results of the study are valuable in understanding the neurochemistry of IDA-induced alterations and elucidating possible mechanisms by which behavioral outcomes can become compromised. With iron deficiency being the largest nutritional deficiency worldwide, the findings have implications for future attempts to properly time iron therapy in humans, as well as giving researchers a biochemical basis for which to synthesize and apply certain drugs that could exploit the potential pathways that are affected by EID.

To date, the majority of ID studies in animal models have used iron treatments of 50-200 μg/g by weaning onto iron-sufficient diets or using cross-fostering[46], two techniques that were used in our present study as well. In previous studies, iron treatment at P4 appeared to ameliorate most brain abnormalities with respect to monoamines and iron, while treatment at P21 has shown mixed results with several studies showing long-term deficits through adulthood[46]. In parallel with a recent study that also examined dietary iron-treatment at P8 in EID rats, we found that at P21, 13 days after ensuing iron treatment, brain iron was significantly improved in all but one region (STR). By P90, all brain regions except for one (PONS) continued to mirror the brain iron content found in never-ID rats and were trending towards even further normalization. This is a strong indication that iron-repletion therapy is successful in ameliorating brain iron deficits induced by gestational and early-lactational iron-deficiency. Whether or not the successful intervention translates into improved behavioral outcomes is not clear since this experiment did

not test for behavior, but a prior study showed that despite normalizing brain iron following treatment at P8, abnormal behavioral outcomes persisted[46]. Finally, persistent-ID rats at P21 contained significantly lower iron concentrations in the HPC than either IDP8CN or never-ID rats did in the same tissue at P21. Previous studies found that 7 weeks of iron-therapy, starting on P8, was enough to ameliorate EID[56], but this study shows that IDP8CN rats did not have significantly lower HPC iron concentrations than never-ID rats, meaning that only 13 days of iron-therapy was enough to normalize HPC iron concentrations. Thus, the critical period for HPC iron-repletion appears to extend to or beyond P8, while the appropriate length of therapy and dose of iron are at least 13 days and a moderate-iron diet, respectively.

The hematology data also revealed numerous trends that the literature seems to support. In comparison to the persistent-ID group, the IDP8CN group had significantly higher hemoglobin, hematocrit, and serum iron. Furthermore, only one hematological marker (TIBC) in IDP8CN was found to differ with that of the never-ID group (P21), and by P90, even that deficit was ameliorated. This is indicative of the fact that putting previously EID rats on an iron-sufficient diet can correct hematological markers of iron within just 13 days of initiation of treatment. The sole lingering variable, TIBC, took longer to correct. Transferrin levels were not evaluated, but since transferrin saturation percentage had no significant difference between never-ID and IDP8CN rats, it is reasonable to conclude that iron has a lag-time before being fully-incorporated into the cardiopulmonary system, a phenomenon which was manifested by the longer-term elevation in TIBC. On the other hand, please note that TIBC is often a stronger predictor of ID than serum iron or hematological saturation markers[50], so behavioral outcomes of the IDP8CN group may have represented those of an ID-model had they been tested for[46]. Lastly, the long-term effects (P90) noted between persistent-ID and never-ID groups were differences in serum iron and liver iron concentrations. While the former was quite predictable given past studies, the drop in liver iron was highly significant. One likely reason that low liver iron did not

manifest itself at P21 is that rats have a high preponderance for liver iron storage during early life, thus resulting in increased uptake at this specific tissue[7].

In both the IDP8CN and persistent-ID groups, long-term (P90) brain iron deficits were found in the PONS, an indication that early iron intervention may provide temporary increases in PONS iron, but that levels relative to never-ID rats will remain low in adulthood. In contrast to the results seen with the PONS, early-iron intervention was enough to normalize HPC iron content to the point that the IDP8CN group had significantly higher iron concentrations than the persistent-ID group at P21. Thus, the concept of tissues having altered tendencies to take up iron from the blood during the rat aging process is worth further investigation. Brain metabolism during development stages has been observed to be different from that of adulthood, so the preferential uptake of iron into various tissues is certainly a strong possibility. This is, of course, dependent on metabolic demand of those tissues, which as stated in the *Introduction*, is a function of enzymes that incorporate iron, such as those involved in major steps of oxidative phosphorylation. The system is more complex than metabolic demand, however, as crucial regulators such as the enzyme transferrin can determine differential and overall tissue uptake[25]. Furthermore, iron is one of the most tightly regulated minerals in the mammalian system, so an unusually large number of proteins, transporters, and ferroxidases may be implicated in iron transport and metabolism[45], leading to even more confounding variables in tissue-iron distribution patterns.

Perhaps the most intriguing results of this study were that both two-tailed T-tests and one-way ANOVA statistical analyses showed no significant differences in monoamine and metabolite concentrations in the four examined brain regions. This may be due to sources of error, particularly from a mathematical standpoint. While the sample size of rats was quite large, an “average of an average” approach was taken to get a representative value for each treatment/age duo. In other words, an average of litter variables was taken followed by an average

of all litters that corresponded to that particular treatment and age. Had the litters all been of the same size, this would not be confounding; however, several litters had population sizes as small as 1 rat while others were in excess of 8. While a litter analysis is more specific and often more precise, this particular study may benefit from a simple, aggregate mean of all rats in a given treatment and age of sacrifice category. Another possible confounding factor is that EID can induce losses in brain mass and volume[56], and since this study used monoamine and metabolite *concentrations*, the concentrations of these compounds in the tissue may well have been similar to those of control rats, but the overall levels of monoamines and metabolites may have been lower in the given tissue.

That said, the results are indicative of the fact that iron-intervention trends towards further normalized monoamine and metabolite levels, but since the difference in levels between iron-deficient and control rats never reached statistical significance at any stage, statistically significant amelioration was not a feasible outcome. The metabolites, in particular, appeared to trend more strongly towards normalization following treatment, which may mean that regulatory and counter-regulatory feedback loops played an increased role due to adequate concentrations of the substrate coupled with enhanced gene expression of the candidate genes mentioned in the *Introduction*. All in all, the aforementioned results closely parallel the findings of a study from 1977[41], which found that despite significant reductions in brain iron due to an early ID-diet, several monoamine levels, including those of dopamine, were either barely altered or unaltered altogether.

In conclusion, our findings indicate that iron-intervention at P8 can ameliorate deficits in iron-related hematology and brain iron concentrations in various regions of the brain. In some tissues, normalization is rapid, while in others, repletion is slower and may even persist into adulthood (P90). We suggest that further studies investigate ID-induced neurophysiological

outcomes of *both* brain metabolism *and* monoamine systems in order to further elucidate the inter-relationship between the two phenomena with respect to development and iron demand.

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