NEUROBEHAVIORAL SEQUELAE OF EARLY IRON DEFICIENCY IN RATS

A Dissertation in
Neuroscience

by

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ABSTRACT

There is no doubt that iron is vital for proper neuronal functioning and development. Nonetheless, the effects of iron deficiency on neurological systems have not yet been thoroughly studied. A number of investigators have shown that in humans, the most salient deleterious effect of iron deficiency (ID) early in life is persistent cognitive impairment. Others have shown that early ID may cause impairment of dopamine (DA) metabolism including DA clearance, transporter density, and dopamine receptor (D1 and D2) densities. The present studies were conducted to investigate the relationship between iron deficiency early in life and cognitive functions especially attention and to elucidate the possible underlying mechanisms using an animal model. Through the use of the attention set shift paradigm we have demonstrated several novel findings regarding iron deficiency and attention in rats as well as extended our knowledge regarding the possible underlying neurobiological mechanisms and possible therapeutic strategies.

The first aim was designed to probe the interaction between early iron deficiency, during the critical window of dopamine system differentiation (PND4-PND21) and the attentional performance and stimulus control in rats. Using Attention Set Shift Testing we found that rats that were iron deficient at postnatal day-4 (PND4) to weaning (PND21) had major attention problems including sustained, selective and divided attention at the age of 45 days. These findings support our hypothesis that in rats, early ID impairs their performance on an attention-related task, an effect that also persists into adolescence and beyond in humans. These results may lead to the development of successful treatment strategies for persistent cognitive dysfunction in children and youths who suffered from ID early in infancy.
The second aim focused on the possibility of reversing the above effects induced by early iron deficiency using methylphenidate. We observed that methylphenidate treatment at 50 days old improved the performance of ID animals, especially on the easier tasks and at lower doses compared to control animals. This has potential ramifications in finding a successful treatment of persistent cognitive dysfunction characteristic of children and youths who suffered from ID early in infancy.

The third aim was designed to examine the effects of early ID on the catecholaminergic system. Therefore, we examined dopamine and norepinephrine transporter densities within certain brain areas related to attention using radioactive ligand binding technique. Our results revealed a significant age effect on DAT levels in the nucleus accumbens (NA), olfactory tubercle (OT), and substantia nigra (SN) but not in the striatum. Specifically, 21-day-old rats had greater DAT levels compared to 45-day-old rats in the NA, OT, and SN as well as in the OT compared to 75-day-old rats. Additionally, there is a significant age difference on NET levels in the dentate gyrus but not in the frontal cortex or the locus coeruleus. Specifically, NET levels were increased among 45-day-old rats compared to 75-day-old rats. However, there is no main effect for diet and no diet-age interactions on DAT and NET levels.

Overall, this work led to several novel contributions regarding the impact of iron deficiency on cognitive function. These findings are very important as they elucidate the impact of iron deficiency on catecholaminergic systems in the brain.
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Chapter 1

Introduction and Overview

1.1. Background and Significance

Iron deficiency (ID) is a concern of many health professionals and research groups including ours. ID is considered to be a global health issue as it is the most common single micronutrient deficiency worldwide. ID affects every age group, including pregnant women, infants, children and even the elderly. For instance, ID can have deleterious effects on women’s general health during child bearing years. These include impaired immune system with increased prevalence of depression (Vahdat Shariatpanaahi et al., 2007; WHO, 2001; Zimmermann and Hurrell, 2007). IDA has also been shown to alter emotion and cognition during the first postpartum year in African mothers (Beard et al., 2005b).

A wealth of evidence from animal and human research highlight the link between iron status and infant development (Beard, 2008) with possible deleterious effects during infancy, childhood and adolescence that may persist into adulthood (Andrews, 2001). Within the last decade, there has been agreement among researchers that ID during infancy leads to impairments in cognitive and behavioral performance, physical activity, immune response (Dallman, 1986, 1987), as well as impaired thermo-regulatory mechanisms (Beard et al., 1990). Moreover, ID infants usually suffer from impaired motor and language development along with impaired psychological and behavioral functions like inattention and fatigue during childhood (Andrews, 2001). There is a great debate among scientists about the link between onset of ID and the developmental and neurological effects of early ID. Many researchers assume that ID affects brain functioning only during development (Beard, 1995), especially periods of vast tissue growth.
and differentiation (Erikson et al., 1997). Nevertheless, others suggest that brain ID at any time in life may impair cognitive and behavioral functioning (Beard and Connor, 2003).

There are numerous studies in animals that address possible neurobiological mechanisms underlying the effects of early ID (Beard and Connor, 2003; Beard, 2003; 2007; Georgieff, 2008; Lozoff et al., 2006; Mohamed et al., 2011). For instance, it has been demonstrated from animal models that ID alters various brain neurotransmitters; e.g. dopamine, norepinephrine, serotonin. There is evidence to suggest that ID alters behavior (Taneja et al., 1990), decreases motor activity and learning (Youdim et al., 1987; 1989), and decreases attention and learning (Mohamed et al., 2011; Shukla et al., 1989). There is also some evidence that suggests that auditory pathway neurotransmission is impaired in ID animals (Youdim et al., 1987; 1989). Similar observations have been made in many human studies showing alterations in metabolism, myelination as well as auditory pathway neurotransmission in ID infants (Lozoff et al., 2006; Roncagliolo et al., 1998). Consequently, several research groups reported poor performance of human infants and children with IDA in various neuropsychological test batteries for motor, cognition, and emotion (Grantham-McGregor and Ani, 2001; Lozoff, 2007; McCann and Ames, 2007; Sachdev et al., 2005). Additionally these children usually show sleep-wake cycle alteration (Peirano et al., 2009).

The aim of the research presented in this dissertation is to provide the scientific community with an up-to-date review of the deleterious effects of early iron deficiency. The dissertation is organized in five chapters as follows. In chapter one, I discuss the prevalence of ID worldwide with a comment about the exact definition of ID. In chapter two, I will provide an up-to-date literature review to highlight the essence of the experimental work. Within this chapter, I will begin with the importance of iron for normal brain functions, and then move to discuss brain iron homeostasis. Then I will underscore the neurochemical effects of ID with the expected consequences of ID on brain monoamine neurotransmitters specifically dopamine and
norepinephrine. Finally, I will end this chapter by addressing the possible relationship between ID and cognitive impairments as shown from animal and human studies.

In chapter three, I report the results of our published paper: “Methylphenidate improves cognitive deficits produced by infantile iron deficiency in rats” (Mohamed et al., 2011). Through this work I will show that early ID in rats produces attention deficits. I will then show that methylphenidate treatment at 50 days old can effectively treat the attention deficit in these animals. In chapter four, I present the results of our neurochemical studies that tested the possible relation between early ID and dopaminergic and noradrenergic transporters. I end with chapter five which is dedicated to general conclusions and possible future directions.

1.2. Iron Deficiency Prevalence and Epidemiology

A significant part of the population in nearly every country shows nutritional iron deficiency (DeMaeyer and Adiels-Tegman, 1985). It is prevalent in developing as well as developed countries i.e., 40% of preschool children in developed countries have IDA compared to 80% in developing countries (WHO/FAO, 2006). Altogether, over 2-3 billion people have iron deficiency worldwide (Stoltzfus, 2001; Stoltzfus et al., 2004; WHO, 2001). Pregnant women and children are the most affected; anemia contributes to 20% of all maternal deaths and 40% in preschool children (Mason et al., 2005; UNICEF, 2004). Accordingly iron deficiency was identified as one of the priority areas for World Health Organization (WHO) (WHO, 2004).

Iron deficiency is a significant health problem in Egypt as well as most of the Mediterranean countries. According to El-Sahn and colleagues (2000), the prevalence of ID nationwide is approximately 47% among adolescents with an inverse relationship between ID and age from early childhood until adolescence (El-Sahn et al., 2000). In Canada, the prevalence of anemia is estimated to be 42.5% among non-pregnant Inuit women aged 18-74 in Nunavik,
Canada, 35.6% of them have IDA (Plante et al., 2011). In The United States, it has been reported that iron deficiency among poor and minority children is relatively high. For instance, 5% of poor black infants have IDA and 12-18% of Mexican American infants (Brotanek et al., 2005; McLoyd and Lozoff, 1998). In 1997, Looker and colleagues (1997) showed that the prevalence of iron deficiency among full-term toddlers aged 1 to 2 years in the United States is about 9% (approximately 700,000 toddlers) with 3% of them having iron deficiency anemia. These children have impaired psychomotor development, coordination and scholastic achievement as well as impaired performance in language and motor skills with overall deficits in IQ equivalent to 5 to 10 points (WHO, 2001). Furthermore, Stoltzfus et al., (2004) conducted a meta-analysis for comparative quantification of health risks for iron deficiency anemia and found that for each 1.0 g/dl decrease in hemoglobin, IQ was lower by 1.73 points.

1.3. Overlap between Anemia and Iron deficiency

Eventually IDA should not be considered just a hematologic alteration, because it is associated with broader systemic effects. There is agreement about the underlying biology of iron deficiency which includes a long-term negative iron balance manifested by a decrease in transferrin saturation with subsequent up-regulation of transferrin receptors in the circulation and on the surface of the cells (WHO, 2001). This is why WHO defined iron deficiency as a medical condition in which there are signs of a decreased iron supply to the tissues as well as decreased serum ferritin level without any compensatory mobilization of iron from its stores to the affected tissues (WHO, 2001). Additionally, the overlap between ID and iron deficiency anemia varies greatly from one population to another and depends on the selected sample gender and age (Yip, 1989). An example of such overlap comes from data collected in US national surveys that show
30-40% of children less than 5 years of age, and women of childbearing age who had ID, were also anemic (Dallman et al., 1984).

The etiology of anemia is multi-factorial; however, iron deficiency is considered to be the main cause of anemia (Jamieson and Kuhnlein, 2008; WHO, 2001). There is some confusion in definition of iron deficiency anemia and anemia. Anemia; the term popularly used to describe decreased availability of oxygen to tissues may be caused by conditions other than iron deficiency including for example hemolytic blood disorders like malaria. Mechanistically speaking this raises the question of whether the effects of iron deficiency are attributed to lack of iron or to diminished oxygenation of target tissues. It is likely that iron deficiency *per se* and not anemia is responsible for the deleterious effects of IDA on neurological functioning (Youdim et al., 1989).

The exact mechanism(s) underlying ID-related impact on cognitive functions is unknown at this time; however, we believe that catecholamine functioning in the mesolimbic system and nigrostriatal system are reasonable places to start.

1.4. Research Questions and Hypotheses

The main goal of this dissertation is to explore the impact of early iron deficiency on attention and on central monoamines, especially dopamine. The current work addresses the following important research questions:

- Is there any causal relationship between early ID and attention?
- Does dopamine play a major role in attention deficit?
- Is it possible to remedy attentional deficits in those animals that were iron deficient in early life?
To answer the above questions, we tested the following hypotheses using a well established model of ID in rats:

1- Severe iron deficiency in early life (during the period of rapid dopamine system differentiation) impairs attention.

2- Severe iron deficiency early in life will decrease dopamine transporter density in brain areas related to attention with the possibility of development of attention deficits phenotype.

3- These animals could be treated using dopamine-stimulating drugs like methylphenidate.

The rat is an excellent organism for this work as the critical period for full development of the dopamine systems is between 7 and 25 days, whereas the same period in humans is late in the third trimester in utero. We can make infant rats iron deficient by merely out-fostering them to lactating, iron-deficient dams. At weaning (21 days of age) we can furnish an iron-adequate diet and then test the animals for attention function at early-pubescence (PND45). Following this test, the animals can be treated with one of 3 doses of methylphenidate vs. vehicle control. At 60 days of age, the animals are tested again for attention.

**Specific aims include:**

**Specific Aim 1**

The first specific aim is to demonstrate the impact of early iron deficiency during the critical window of dopamine system differentiation (PND4 -PND21) on attention. I examined our hypothesis that rats made iron deficient at postnatal day-4 (PND4) to weaning (PND21) have
attention problems. I used the Attention Set Shift Task (ASST) to measure attentional performance and stimulus control in rats.

Specific Aim 2

The second specific aim is to explore the hypothesis that methylphenidate can ameliorate the above effects induced by early iron deficiency.

Specific Aim 3

The third specific aim is to examine the hypothesis that severe iron deficiency in early life (during the critical period of dopamine system differentiation) impairs dopamine neurobiology. I investigated the effects of iron deficiency on monoamine transporter densities especially dopamine and norepinephrine transporters within specific brain areas that are important in regulating attention.

Conclusions:

These aims are designed to advance our understanding of the effects of early ID on brain dopaminergic systems and putative dopamine-related behavior, especially attention. Using a well established rat model of early ID, I showed the extent to which early ID impairs attention. Next, using radioactive ligand binding techniques I tested the effect of ID on DAT and NET levels in certain brain areas dedicated to attention. Finally, I attempted to remedy the effects of ID using the DA-related drug methylphenidate. Overall, the work performed in this dissertation helps to
define the relationship between early life ID, brain catecholaminergic systems and attention deficits.
Chapter 2

Literature Review

2.1. Iron and Normal Brain Functions

2.1.1. Brain Iron Homeostasis (Synopsis)

A wide range of preclinical studies on iron homeostasis have focused on the regulation of systemic iron uptake and transport (Andrews, 2000a, b). Less is known about brain iron homeostasis. The blood brain barrier (BBB) works as a gatekeeper of iron (Fe) transport into the brain (Bradbury, 1997; Burdo et al., 2003). Iron penetrates the BBB by binding to transferrin (Tf) and forming a Tf-iron complex. Brain Tf is produced by oligodendrocytes and in the choroid plexus (Bartlett et al., 1991) and is found on astrocytes, blood vessels, and cortical, striatal and hippocampal neurons (Connor and Menzies, 1995; Hill et al., 1985; Mash et al., 1990). It is noteworthy that iron deficiency increases brain Tf in developing rats through a yet to be defined mechanism (Piñero et al., 2000). Furthermore, mice that lack Tf show iron overload in liver despite overall anemia (Bernstein, 1987). The Tf-iron complex is then endocytosed into the epithelial cell via TfR (receptor)-mediated endocytosis (Pardridge et al., 1987). Within the epithelial cell, iron is removed from the complex by acidification in the endosome (Connor and Fine, 1987; Yamashiro and Maxfield, 1984). After that, iron is removed from the endosome and transported into the cytosol via divalent metal iron transporter 1 (DMT1) (Burdo et al, 2001; Moos et al., 2000). There is also evidence that non-transferrin bound Fe can penetrate the BBB from the plasma pool (Burdo et al., 2003). It is possible, therefore that there are multiple pathways for brain iron metabolism that operate regionally in the brain. Each of these pathways presents a possible avenue for brain iron regulation and dysregulation.
Iron is unevenly distributed throughout the brain in rats (Beard et al., 1993a; Benkovic and Connor, 1993; Burdo et al., 2001; Connor et al., 1994; Dwork et al., 1990; Focht et al., 1997; Hill and Switzer, 1984; Jiang et al., 2002; Roskams and Connor, 1994), mice (Beard et al., 2005a; Jones et al., 2003; Knutson et al., 2004; Zechel et al., 2006) and humans (Hallgren and Sourander, 1958). Dwork et al. (1990) investigated the effects of intra-peritoneal injection of radio labeled iron on brain iron distribution in young rats. They found that iron initially accumulated in the caudate-putamen and over time moved to the substantia nigra and globus pallidus. These data suggest that there is some sort of intra-cerebral redistribution of brain iron, likely through axonal transport (Mescher and Kiffmeyer, 1992). Brain iron concentration is greatest in each of the brain regions at PND2, and then it declines by PND17 in rats. Furthermore the concentration of iron within the cerebral cortex is 3 times higher at PND2 than at any other age. During adulthood (PND75), iron concentration is reduced in each brain region relative to early life. These findings are supported by the fact that in rats iron uptake in the brain during early postnatal development is rapid with brain iron concentrations at 3 weeks of life that are one half that of the adult rat (Dwork et al., 1990). Later, perhaps as a result of altering brain region requirements for iron at different neurodevelopment stages, iron uptake decreases with age (Beard, 1999; Dwork et al., 1990). Moreover, aged animals are unable to remove free iron due to limited iron binding and this leads to free radical production and possible cell death (Barkai et al., 1991; Connor and Fine, 1986).

Brain iron significantly varies across regions regardless of dietary treatment as shown by Erikson et al. (1997). They fed 21-day-old male Sprague-Dawley rats a low-iron diet for 4 weeks. Compared to other regions, the cortex and hippocampus were the most affected by low-iron diet. Early (PND10- PND21) and later (PND21-PND35) ID significantly decreased iron content in the following brain regions; cortex, deep cerebellum, pons, superficial cerebellum, substantia nigra
and striatum, but not in hippocampus and thalamus as compared to age-matched control rats (Piñero et al., 2000; 2001).

The results from animal studies are reflected in humans. Magnetic resonance imaging (MRI) enables us to quantify the amount of stored iron within the brain of living subjects (Bartzokis et al., 2004; Schneck and Zimmerman, 2004). Within the human brain, oligodendrocytes are rich in iron (Connor et al., 1990; Connor and Menzies, 1996; Le Vine and Macklin, 1990) and iron distribution is affected by age with a specific developmental timeline (Aoki et al., 1989; Connor et al., 1990; Hallgren and Sourander, 1958; Hock et al., 1975).

Furthermore, iron is unequally distributed throughout the adult brain with concentrations from the highest to the lowest as follows: the globus pallidus, red nucleus, substantia nigra, and the putamen, followed by the dentate nucleus, the caudate nucleus, and finally the frontal white matter (Hallgren and Sourander, 1958).

It should be kept in mind that too much brain iron is as harmful as too little brain iron. Decreased brain iron during development results in altered behavior and functioning such as poor cognitive, motor and social-emotional functions (Lozoff et al., 2006), while excess brain iron leads to neurodegenerative diseases (Epstein, 1999). What seems clear is that not all brain regions respond to ID in the same fashion. Hence, we should consider the age of the iron deficient subjects (human or animal), the length of dietary treatment if it is an option as well as the local brain iron regulatory mechanisms.

### 2.1.2. Functional consequences of iron deficiency

Several animal studies have linked deficits in brain iron to reduced energy metabolism (Beard et al., 1993a, b), altered hippocampal functioning and structure (Brunette et al., 2010; Jorgenson et al., 2005; Rao et al., 1999; 2011), changes in brain development (Beard, 2003;
Conner and Fine, 1987), neuropathology (Beard and Connor, 2003; Beard et al., 2003a, b; Unger et al., 2006; 2007), reduced iron metabolism in general (Beard, 2001; Piñero et al., 2000), and altered behavior (Felt et al., 2006a; Lozoff et al., 2006, Mohamed et al., 2011).

In rats, and likely in humans, iron is important for myelin formation (Larkin and Rao, 1990; Ortiz et al., 2004) and brain oligodendrocytes, the cells that form myelin in the central nervous system, contain high levels of iron (Beard et al., 2003a; Connor and Menzies, 1996). Nearly 70% of brain iron is linked to myelin which explains the marked reduction in the white matter in 21-day-old rat pups with persistent ID as compared to controls (de los Monteros et al., 2000). Moreover, prenatal or lactational iron deficiency in Sprague-Dawley dams results in delayed myelination or hypomyelination in outcome pups (Yu et al., 1986). Besides myelin formation, oligodendrocytes help in iron storage and mobilization in the central nervous system of rats (Connor and Menzies, 1996).

The results of the animal studies have clear parallels in humans. Several human studies have highlighted the link between iron deficiency and neuropathology (Epstein, 1999), nutrition (Beard and Connor, 2003), cognition (Akman et al., 2004; Lozoff et al., 1987; McCann and Ames, 2007; Walker et al., 2007) and development (Perez et al., 2005). In humans, oligodendrocytes are central to iron storage and mobilization (Gerber and Connor, 1989), and iron deficiency early in life is associated with impaired behavior and cognition (see for example; Beard and Connor, 2003; Lozoff et al., 2006).

Human studies over three decades have reported the signs of generalized neurological damage associated with altered iron metabolism. Brain iron deficiency has been linked to Restless Leg Syndrome (RLS) (Allen, 2004; Barriere et al., 2005; Connor et al., 2003) and Attention Deficit-Hyperactive Disorder (ADHD) (Cortese et al., 2008; Oner and Oner, 2008). On the other hand, excess brain iron is associated with neurodegenerative disorders including Parkinson’s disease (Bartzokis et al., 1999a; Berg and Hochstrasser, 2006; Dexter et al., 1987; Sofic et al.,
1988), Alzheimer’s disease (Connor et al., 1992; Scherbatykh and Carpenter, 2007), Huntington’s disease (Bartzokis et al., 1999b), and Hallervorden-Spatz syndrome (Koeppen and Dickson, 2001; Swaiman, 2001). There are other neurological sequelae of ID including pediatric stroke, breath holding spells, pseudo-tumor cerebri and cranial nerve palsy (Yager and Hartfield, 2002).

In light of these reports, it is clear that iron deficiency in the brain is associated with widespread alterations in neurological functions that could yield several neurological diseases. Therefore, proper iron regulation is critical for normal development and functioning of the nervous system.

### 2.1.3. Timing of Iron Deficiency

There are three important issues regarding the impact of iron deficiency during early development; timing, severity, and duration. The age of onset of the dietary iron deficiency may have an important impact on how much and where brain iron is lost, and on the possible reversibility with subsequent iron repletion. A critical issue in conducting ID research is when to initiate iron deficiency. Understanding the timing of ID with regard to the brain growth peak in different species must be taken into account in extrapolation of results obtained in one species to any other (Dobbing and Sands, 1979). For a comparison between human brain development and rat brain development, please refer to table 1.

Within this context, in the rat, iron deficiency has been initiated during gestation to early adulthood with greater sensitivity of very young animals to ID (de los Monteros et al., 2000; Lozoff et al., 2006). Interestingly enough, many of the resulting changes in behavior and biochemical abnormalities in the brain persist despite iron therapy (de los Monteros et al., 2000; Lozoff et al., 2006). The human brain appears most vulnerable to the deleterious effects of ID
during times of rapid brain “growth”, including the last trimester in utero and the first 2 years of childhood (McCann and Ames, 2007).

The peak of the human brain growth is between 2 and 3 months after birth, while the peak in the rat is between PND7 and 10 (Dobbing and Sands, 1979). This difference in brain growth timing is not a general rule as within the nervous system, each tract or system has its own growth spurt and vulnerability. For example the myelination process coincides with the brain growth spurt for humans and rats (Davidson and Dobbing, 1966) and early acquisition of iron occurs mainly during the third trimester of pregnancy in humans and post-natally in rats (Piñero et al., 2000). As for dopaminergic system development, Tarazi and colleagues (1999) have described the developmental course of DA receptor systems in rat brain. For D₁ and D₂ receptors in caudate and nucleus accumbens, there is a steady increase in density between PND7 and PND28 followed by a decline (Tarazi et al., 1999; Tarazi and Baldessarini, 2000). Furthermore, the dopamine transporter in the caudate-putamine and nucleus accumbens increases in density from low levels at PND7 to maximal level (about 7 fold increase) at PND60 (Tarazi et al., 1998). Based on the above information and in accordance with the work of Ben-Shachar et al., (1986) and Piñero et al., (2000;2001), to study effect of early ID and capture this critical period, iron deficiency dietary treatment should start at PND4 by out-fostering pups on iron-deficient, lactating dams. On the other hand, animal studies demonstrated that neonatal and post-weaning ID could be remedied quickly with iron repletion (with iron sufficient diet given two to four weeks post-natally) (Beard et al., 1996, 2007; Erikson et al., 1997). Whereas, in utero ID effects are irreversible even after iron repletion (Beard et al., 2003b; Felt and Lozoff, 1996; Kwik-UrIBE et al., 2000).
**Table 1:** Comparison between brain development in rat and human

<table>
<thead>
<tr>
<th></th>
<th>Rat brain</th>
<th>Human brain</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Brain weight</strong></td>
<td>400 gm body weight</td>
<td>Newborn (350-400gm)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adult (1300-1400gm)</td>
</tr>
<tr>
<td><strong>Substantia nigra</strong></td>
<td>poor in iron during the first 60 days of life</td>
<td>poor in iron until the age of 12-15y</td>
</tr>
<tr>
<td><strong>Peak myelination</strong></td>
<td>PND11-12</td>
<td>14-18 month old</td>
</tr>
<tr>
<td><strong>Brain growth peak</strong></td>
<td>PND7-10</td>
<td>2-3 month old</td>
</tr>
<tr>
<td><strong>Early iron acquisition</strong></td>
<td>postnatal</td>
<td>3rd trimestre of pregnancy</td>
</tr>
<tr>
<td><strong>Age matching</strong></td>
<td>rat brain at 10 d old</td>
<td>full term human neonate brain</td>
</tr>
<tr>
<td></td>
<td>brain at weaning (PND21)</td>
<td>late toddler age child</td>
</tr>
<tr>
<td></td>
<td>events during PND7-25</td>
<td>events during 3-16 month postnatal</td>
</tr>
<tr>
<td><strong>Dietary Iron</strong></td>
<td>decrease brain iron within 10 days and replete it within 14 days</td>
<td>chronic ID without severe reduction in iron status or growth</td>
</tr>
</tbody>
</table>

The course of brain development in the rat has the same sequence of events as human brain, however it is more compressed. For references, see for example; Davidson and Dobbing, 1966; Dobbing and Sands, 1979; Donaldson, 1908.
2.2. Neurochemical Effects of Iron Deficiency

2.2.1. Response of Brain to Iron Deficiency

The brain is able to regulate its own iron with respect to tissue iron demands (Erikson et al., 1997; Piñero et al., 2000). ID in young rats lowered brain iron and ferritin concentrations with a compensatory increase in brain iron uptake (Dallman et al., 1975; Dallman and Spirito, 1977; Erikson et al., 1997). Biochemically, iron deficiency alters nucleic acid biosynthesis, mitochondrial functions and catecholamine metabolism (Oski, 1979; Prasad and Prasad, 1991). Furthermore, neonatal brain iron deficiency has been proposed to alter glutathione metabolism thus decreasing cerebral protection from oxidation (Moriarty et al., 1995), as well as, predisposing the neonatal hippocampus to injury from hypoxic-ischemic insult (Rao et al., 1999). Given that the oligodendrocyte is a predominant iron-containing cell in the brain and is also responsible for myelination (Connor and Menzies, 1996), iron deficiency produces immature oligodendrocytes (Connor and Menzies, 1990; Erikson et al., 1997).

2.2.2. Iron Deficiency impairs Dopamine Systems and Related Behaviors

Dopamine (DA) is a catecholamine neurotransmitter in the mammalian brain that is involved in such functions as locomotion, cognition, attention, affect and neuro-endocrine secretion (Dunnett et al., 2005; Nieoullon, 2002). Its effects are mediated by at least five distinct DA receptor subtypes (D_1-D_5) (Missale et al., 1998). Dysregulation of dopaminergic transmission predisposes the organism to a number of disorders including Parkinson’s disease, schizophrenia, attention deficit/hyperkinetic disorder (ADHD), affective disorders and drug use/misuse. Furthermore, many drugs that are used to treat these disorders are believed to work through their effects in central dopamine systems.
Over the past few years a growing body of research shows that there is impairment in dopaminergic functioning in iron deficient animals. For example, several reports using rats showed increased extracellular dopamine concentrations, decreased densities of striatal D1 and D2 receptors and DAT levels, altered behavioral sensitivity to cocaine and decrease cocaine i.v. self-administration (Ashkenazi et al., 1982; Beard et al., 1994; 2003b; 2006b; Ben-Shachar et al., 1986; Burhans et al., 2005; Chen et al., 1995; Erikson et al., 2000, 2001; Jones et al., 2002; Nelson et al., 1997; Youdim et al., 1989; Youdim, 1988; 1990; 2000; Youdim and Yehuda, 2000). It is noteworthy that dopaminergic dysfunction is not the sole neurological effect of early life ID. Altered neurotransmitter levels, myelination, dendritogenesis and neurometabolism have also been observed (Beard, 2003; 2007; Georgieff, 2008; Lozoff and Georgieff, 2006; Youdim, 1988).

Dopamine, like other monoamines, is removed from the synapse by diffusion and about 70% of the extracellular DA is removed from the synaptic space through the dopamine transporter. DA is co-localized with iron in the brain and to date is the most thoroughly studied of all neurotransmitters relative to iron status (Beard et al., 1993a). The role of iron in dopamine neurobiology is the subject of study by several groups including our research team. For example in some very early work in our laboratory, we investigated the effect of iron deficiency on brain monoamine metabolism using in vivo microdialysis techniques. We found that there was a 53% increase in extracellular caudate-putamen DA concentration in 11-week-old iron-deficient rats (Beard et al., 1994). Two themes emerging from this work are: 1) iron deficiency causes a down regulation of dopamine receptors including D2 receptors, (Yehuda and Youdim, 1989; Youdim, 1990), D1 receptors (Erikson et al., 2001) and the dopamine transporter (DAT) (Beard et al., 2006b; Bianco et al., 2008; Erikson et al., 2000; Wiesinger et al., 2007) and 2) iron deficiency early in postnatal development produces deficits in dopamine-related behaviors that are not reversible with later iron repletion in mice (Kwik-Uribe et al., 2000) and rats (Piñero et al., 2001).
Ben-Shachar et al., (1986) showed in rats that in contrast to post-weaning iron deficiency, iron deficiency beginning at PND10 produced persistent decreases in caudate D₂ receptor density with associated diminished behavioral responses to apomorphine that could not be reversed by six weeks of treatment with an iron-adequate diet. More recently, Erikson et al., (2000; 2001) showed that these effects of irreversible impairment extended to the D₁ receptors and to the dopamine transporter. Several reports support the function of D₂ receptors as a DAT modulator in rats (Cass and Gerhardt, 1994; Meiergerd et al., 1993) and mice (Dickenson et al., 1999; Jones et al., 1999). This is in accordance with Unger et al., (2007) as they found that dysregulation of D₂-receptor precedes DAT alterations in early developmental iron-deficient rats. Similarly, D₂ receptor knockout mice exhibit a loss in DAT functioning (Dickenson et al., 1999) with a parallel decrease in D₂ receptor densities (Jones et al., 1999).

There is strong agreement across animal, non-human primate and human studies, about the fact that iron is an important factor in the development and functioning of the 4 major dopamine (DA) pathways (Lozoff, 2011) namely; mesocortical; mesolimbic, nigrostriatal and tuberohypophyseal (Bjorklund and Dunnett, 2007). Moreover, it is likely that iron deficiency per se and not anemia is responsible for alterations in dopamine systems with subsequent effects on neurological functioning (Youdim et al., 1989). This notion was supported by two observations: first, hemolytic anemia induced by phenylhydrazine did not affect the brain non-heme iron, or behavioral responses to apomorphine (Ashkenazi et al., 1982; Ben-Shachar et al., 1985). Secondly, iron deficiency produces reliable increases in extracellular dopamine with blunted dopamine reuptake, while phenylhydrazine actually causes reliable decreases in extracellular dopamine (Nelson et al., 1997).
**Altered mesocortical pathway and higher cognitive function**

The mesocortical dopamine pathway is related to executive functions, sustained attention, working memory, emotion and motivation (Seamans and Yang, 2004; Volkow et al., 2007; 2009). Early ID induces reductions of dopaminergic activity in rats (Youdim et al., 1983) especially within the prefrontal-striatal circuit (Lozoff, 2011) with poorer control of the higher cognitive functions (Brozoski et al., 1979; Sawaguchi et al., 1990).

Lozoff and colleagues conducted a long-term follow up study in Costa Rica to show the link between early ID and the impairment of the mesocortical functions. They examined a cohort of children at different ages; infancy (Lozoff et al., 1987; 1998), at 5 y (Corapci et al., 2006; Lozoff et al., 1991), 11-14 y (Lozoff et al., 2000) and 19 y (Lukowski et al., 2010). They found that children with chronic severe iron deficiency scored lower on global measures of cognitive, affective, motor and executive functioning, as compared to non-deficient children. These findings are consistent with their longitudinal study in Chile where they reported that formerly iron deficient anemic children had poorer performance in executive functions compared with non-anemic children (Algarin et al., 2004). Similarly, in African American infants, studies found that 9-10-mo-old infants with IDA had poor executive function control compared with those who were iron sufficient (Carter et al., 2010; Lozoff et al., 2008; Shafir et al., 2008; 2009). In the light of these reports, we would suggest that early iron deficiency has long-term effects on higher order cognitive functioning especially executive functions.

**Altered mesolimbic pathway**

The mesolimbic pathway plays a major role in behavioral control, affect and reward (Bressan and Crippa, 2005). It was therefore hypothesized that iron deficiency during early
development may contribute to affective and reward motivation disorders (Dahl and Spears, 2004). For example, iron deficiency in rats beginning at 21 days of age to the age of 57 days old, decreases acquisition of and suppresses responding for cocaine self administration (Jones et al., 2002).

Results from non-human primates and human infants studies point to altered infant social-emotional behavior in early ID. For example; there is evidence that infants of monkey mothers without pre-natal iron supplementation showed increased boldness and impulsivity with increased tenseness and emotionality compared with controls (Golub et al., 2006a, b; 2007; 2009). In human neonates, a negative linear correlation has been shown between cord-blood iron status and emotionality (Wachs et al., 2005). Studies in human infants also support the direct connection of poorer iron status with increased shyness, increased solemnity, and decreased orientation/engagement (Deinard et al., 1986; Lozoff et al., 1985; 1986; 1998; 2003; 2008; Walter et al., 1983).

Altered nigrostriatal pathway

The nigrostriatal pathway is important for movement control and regulation (Dunnett et al., 2005). Dopamine deficits in this pathway may impair the ability to process environmental cues leading to impaired movements, bradykinesia and altered perception (Cooper et al., 1996). Several studies show the behavioral effects of early ID specifically on the dopaminergic system. For instance, Weinberg et al. (1979) used exploratory tasks and active and passive avoidance learning to test rats exposed to ID for the first 28 days of life. These rats showed a persistent deficit in brain non-heme iron in adulthood as well as long term behavioral and physiological effects. Furthermore, Youdim et al. (1981) investigated the possible behavioral effects of an iron free diet for 28 days in rats. These rats exhibited decreased motor activity and reversed circadian
rhythms of thermoregulation. Moreover, the hypermotility and the hypothermic effects of d-amphetamine were significantly reduced in these rats. These effects may be explained by the importance of iron for normal brain dopaminergic function. Comparable results have been described by Felt et al., (2006a) who found that rats with gestational and/or lactational iron deficiency had fewer complete grooming chains than control animals during adulthood. It should be emphasized that behaviors that are dependent on the nigrostriatal pathway remain changed in ID rats despite iron therapy for 4 weeks (Beard et al., 2003b). Taken together these reports highlighted the link between reduced dopamine functions and ID.

_Altered tuberohypophyseal pathway_

The tubero-hypophyseal pathway controls prolactin release (Ben-Jonathan and Hnasko, 2001). There is evidence that induction of ID in PND21 in male rat for 28 days induced a 3 fold increase in serum prolactin levels and a 7 fold increase in liver prolactin-binding sites (Barkey et al., 1985; 1986). Moreover infants with IDA showed wary and hesitant behavior with high serum prolactin level (Felt et al., 2006b; Lozoff et al., 1995) as a result of reduction in dopaminergic activity. Prolactin is not the only hormone so affected. Iron deficiency in infancy has been shown to lower serum cortisol levels (Dimitrijevic et al., 2008).

**2.2.3. Iron Deficiency and Norepinephrine**

Iron deficiency can alter not only the DA neurotransmitter system but it also affects norepinephrine (NE). Several animal studies showed evidence of increased plasma and urine concentrations of norepinephrine in iron-deficient adult rats which normalized after iron therapy (Beard et al., 1990). Furthermore heart NE concentration decreased in ID anemic rats (Borel et
Tyrosine hydroxylase (TH) is the rate limiting enzyme for catecholamine (DA and NE) synthesis, which is an iron containing enzyme (Kumer and Vrana, 1996). Therefore it seems that ID might cause decreased activities of this enzyme (Dunkley et al., 2004) with decreased NE level. However, the effect of ID on TH may depend on age, iron status and brain region sampled (Beard et al., 2003a; Unger et al., 2007; Youdim and Green, 1978; Youdim et al., 1989). NE reuptake into the cell via the NE transporter (NET) may be affected by ID. Indeed, Burhans et al. (2005) reported reduced NET density in ID brain in a number of brain regions. It is interesting to note that cell culture experiments reported a dose-response effect of iron chelators on NET levels and NE uptake (Beard et al., 2006a; Burhans et al., 2005). Furthermore, other rodent studies reported a reduced NE turnover (Tobin and Beard, 1990), while others show elevated NE turnover during IDA (Smith and Beard, 1989). Altogether, these findings show that iron deficiency affects not only central dopaminergic receptors but also affects central NE receptors.

Turning to humans, Voorhess et al. (1975) investigated the neuronal effects of ID among 11 children. They observed increased pretreatment urinary NE excretion that returned to normal after iron therapy. This could be explained by the dependence on iron especially MAO (monoamine oxidase) enzyme which is responsible for inactivation of catecholamines. Comparable results have been described by Webb et al., (1982) as they found a negative correlation between urinary excretions of NE with serum ferritin among children 7-12 years of age.

### 2.2.4. Iron Deficiency and Other neurotransmitters

γ-Aminobutyric acid (GABA) is an inhibitory neurotransmitter that is co-localized with iron in certain brain areas such as the ventral pallidum, globus pallidus and substantia nigra (Li, 1998). It has been demonstrated that GABA metabolism is affected by iron deficiency (Taneja et al., 1986; 1990). For example, gestational exposure of rats to iron deficiency decreased activity of
enzymes related to GABA metabolism (e.g. glutamate decarboxylase; GAD) (Li, 1998) which is irreversible even with iron-enriched diet (Taneja et al., 1990). Likewise serotonin is affected by ID; iron deficiency in rats after weaning results in reversible reduction in brain 5-hydroxytryptamine levels (Shukla et al., 1989). Another study shows that, in rat pups, iron deficiency after weaning leads to decreased activity of aldehyde oxidase, leading to increased brain serotonin (Mackler et al., 1978). Moreover Mackler and Finch (1982) reported elevated plasma concentration of phenylalanine in iron deficient rats with phenylketonuria-like effects.

2.3. Iron Deficiency and Cognitive Impairment

2.3.1. Human Studies of Developmental Iron Deficiency

2.3.1.1. Newborn, Infancy and Early Childhood

The association between iron deficiency and behavioral, cognitive and biochemical alterations is well established, although the specific mechanisms are not yet understood. There is evidence that ID causes neurophysiological and developmental deficits in infants, children, adolescents, and some of these changes may continue into adulthood (Andrews, 2001; Madan et al., 2011). Indeed the most vulnerable groups to the deleterious effects of iron deficiency are infants (6-24 months), children (3-5 years) and even newborn babies. There is an inverse relationship between levels of neonatal serum iron and neonatal negative emotionality as manifested by greater distress and distress-mediated activity (Wachs et al., 2005). Furthermore in newborn infants of diabetic mothers, iron deficiency alters their auditory recognition memory in response to the mother’s voice compared with a stranger’s voice due to the possible ID effects on hippocampal development (Siddappa et al., 2004).
Researchers examined infants at various ages; birth to 15 months (Walter et al., 1989); 9-10 months (Armony-Sivan et al., 2010); 9-12 months (Carter et al., 2010; Oski et al., 1983) and 12-23 months (Lozoff et al., 1987; 2006a) to the deleterious effects of ID. All of these reports agreed about the fact that infants at the age of 6-23 months are vulnerable to ID effects, especially unfolding of fundamental, mental and motor processes. A large body of research shows poorer motor, cognitive and socio-emotional functioning in infants with IDA (Lozoff et al., 1982, 2006a; Lozoff, 1989; Pollitt et al., 1985; Walter et al., 1983). Moreover, infants with IDA do not follow normal neuro-maturational patterns (Algarin et al., 2003; Angulo-Kinzler et al., 2002; Roncagliolo et al., 1998). Mothers of 6-18 month-old infants reported that their IDA infants are more likely to have increased night waking and reduced total sleep duration (Kordas et al., 2008) with altered characteristics of short- and long-term sleep patterns (Peirano et al., 2007; 2010). Given that sleep plays an essential role in early development (Dahl, 1996), we can conclude that early ID likely accounts for poorer cognitive and behavioral development in infants (Peirano et al., 2010). Not surprisingly, therefore, evidence suggests that severe iron deficiency during infancy has been associated with increased fearfulness, unhappiness, hesitancy, fatigue, solemnity and wariness (Lozoff et al., 1985; 1986; 1996; 1998; Lozoff and Black, 2003; Walter et al., 1989; Williams et al., 1999). Furthermore, IDA in infancy was associated with less optimal mother-infant interaction during feeding as compared to non-anemic peers (Armony-Sivan et al., 2010). Moreover in South Africa, Murray-Kolb and Beard, (2009) found that maternal ID negatively impacted mother-child interactions with altered maternal sensitivity and child responsiveness as compared to non-ID children.

Carter et al. (2010) found that 9-month-old infants with IDA showed poor short-term memory. Comparable results have been described in short-term follow up studies. For example Walter et al. (1989) and Walter (1994) examined ID infants at various ages (3, 12.5 and 15 months) using the Bayley scales of infant development. They reported that IDA during infancy
may be associated with irreversible cognitive impairment. The results of these studies support the findings of a publication by Walter (2003) who follows up a cohort of ID infants (6, 12 and 18; and at 4 years) for neurologic maturation using auditory brain stem responses and sleep-wakefulness cycle. Infants at all ages showed slower auditory brain stem responses despite iron therapy beginning at 6 months. Likewise results have been reported by Lozoff and colleagues who conducted a long-term follow up study to investigate the relationship between early iron deficiency and neurodevelopment on a group of Costa Rican children starting from infancy to adolescence. They concluded that severe iron deficiency early in infancy resulted in prolonged irreversible cognitive deficits with poor developmental outcome at 5 years of age (Lozoff et al., 1987; 1991; 1996), at age of 11-14 years (Lozoff et al., 2000), and at age of 19 years (Lukowski et al., 2010).

The above findings are consistent with the seminal work by Lozoff and collaborators (Lozoff et al., 2006a). They have shown that iron deficiency in utero and early infancy may produce long-lasting developmental delays in cognitive, affective and motor function. Some of these effects persist into late adolescence and young adulthood. This is in parallel with large number of controlled clinical trials that have been conducted on the consequences of iron deficiency in the first year of life (Beard, 2007; Lozoff et al., 2006a; Lozoff and Georgieff, 2006). It is interesting to note the linear trends of iron status in 9-month-old infants with gross motor milestones and fine motor skills with small objects (Angulo-Barroso et al., 2011). Therefore, infants with IDA demonstrated sustained decrease in motor activity with worse reaching and grasping patterns compared with non-anemic infants (Shafir et al., 2008; 2009). This reduction in motor activity decreases infants’ opportunities to interact with their physical and social environment ending with changes in experience-expectant developmental processes; re-routing development. These limitations, over time, can contribute to the poorer cognitive, attention, motor and socio-emotional functioning (Angulo-Kinzler et al., 2002; Lozoff et al., 2000),
Likewise in Chile, Lozoff et al. (2003) found that a greater percentage of unsupplemented infants never smiled, never interacted socially and never showed social referencing, compared to infants who received iron supplementation. From preschool follow up studies, it is evident that children at age 5 with chronic severe iron deficiency anemia in infancy show poorer cognitive and motor development (Dommergues et al., 1989; Lozoff et al., 1991; Palti et al., 1983), lower alertness level and negative affect as compared to 5 years old children with good iron status in infancy (Corapci et al., 2006; Tamura et al., 2002). Not surprisingly, therefore, other studies detected a wary/inhibited behavior pattern i.e. less mother-child reciprocity, more worry, less pleasure; in infants with IDA during infancy (Lozoff et al., 1986; 1998) and again at the age of 5 years (Corapci et al., 2006).

It is noteworthy that iron deficient infants show prolonged latencies and reduced amplitudes of waves in Evoked Response Potentials (ERPs); either auditory brain responses (ABRs) or visual evoked responses (VEPs). ERPs are a non-invasive measure of CNS maturation at different levels. Within this context, Roncagliolo et al., (1998) documented iron’s essential role in myelin formation and maintenance. These investigators used ABRs to examine iron-deficient infants at 6 months of age. They demonstrated a slowed nerve conduction velocity with increased stimulus-response latencies of ABRs in iron-deficient infants indicating delayed maturation of their auditory brain stem responses. These effects did not normalize despite 12 months of iron therapy. Finally they concluded that iron deficiency in infants during the critical period for peak myelination (age 6-12 months) results in irreversible hypomyelination. This is in accordance with Algarin et al., (2003) who replicated the previous study at approximately 4 years of age on iron deficient anemic children. They found that iron-deficient children continued to show longer auditory brainstem responses and visual evoked potential latencies. Hence, they concluded that
iron deficiency anemia in infancy had long lasting effects on pathway transmission in both the visual and auditory systems (Algarin et al., 2003) even after treatment with 3-4 mg/kg/day of elemental iron for 12 weeks (Bandhu et al, 2003). This is in contrast with Sarici et al., (2001) who reported a reversal of prolonged N2 latencies in VEP in iron deficient infants after iron supplementation for 12 weeks. Furthermore Burden et al., (2007) compared ERP data in iron deficient infants to iron sufficient ones. They found that the iron sufficient group showed greater response to mother’s face with a good memory at 9 months of age while the deficient group did not show the same result till 12 months indicating a cognitive impairment with iron deficiency anemia.

2.3.1.2. Preschool and School-Age Children

There is strong evidence that IDA delays psychomotor development and impairs cognitive performance of preschool and school-age children in many countries including, Costa Rica (Lozoff et al., 1991), Greece (Metallinos-Katsaras et al., 2004), Guatemala (Pollitt et al., 1983; 1986; Pollitt, 1991), India (Seshadri and Gopaldas, 1989), Palestine (Palti et al., 1985), USA (Hurtado et al., 1999), China (Change et al., 2011) and Indonesia (Soemantri, 1985; 1989). ID during childhood impairs motor, cognitive and socio-emotional functioning that interferes with optimal development (Grantham-McGrego and Ani, 2001; Lozoff, 2007). Furthermore, iron deficiency has been linked to altered sleep patterns in children (Peirano et al., 2009) manifested as tiredness, inattentiveness and irritability (Dahl, 1996). These effects may explain the high prevalence of increased anxiety/depression and social problems among 11-14 years old children who had ID during infancy (Lozoff et al., 2000).

Many longitudinal studies investigated the possible causal relationship between hemoglobin level in early childhood and cognitive development in later childhood (e.g. Hurtado...
et al. 1999; Lozoff et al. 1991; 2000; Wasserman et al. 1992; 1994). Within this context, Palti et al. (1985) demonstrated lower achievement scores among 7-year-old Palestinian children who were formally anemic in infancy as compared with their non-anemic peers. Likewise, American children who had ID during infancy showed mental retardation at 10 years of age (Hurtado et al., 1999). Similarly, preschool-aged Chinese children, 4 year-old, who had chronic IDA in infancy, showed less frustration tolerance, more passive behavior, and delay gratification compared to non-anemic peers (Chang et al., 2011). Furthermore, Shankar et al., (2000) examined the effect of ID on sensory brain function, using brainstem auditory evoked potential responses, in a group of children with an age range from three to 11 years. They reported increased latencies with reduced amplitudes of the waves which mean that there is a significant linear correlation between the severity of anemia and the conduction time indicating some sort of neurophysiological deficits among those anemic children.

Evidence suggests that children who had ID in infancy have lower scores in writing, arithmetic, school progress and motor functions as compared with controls (Palti et al., 1985). This is in accordance with Lozoff et al. (2000) who examined a sample of Costa Rican children of 12 years old who had severe chronic IDA during infancy. These children showed more anxiety, depression and social problems as compared with non-anemic group (Lozoff et al., 2000). There is evidence that children with lower cord ferritin concentrations at birth have lower scores in language ability, fine-motor skills and tractability at age of 5 year-old (Tamura et al., 2002). In line with these findings, Halterman et al., (2001) conducted a large follow up study on American children of 6 years old to the adolescent. They found that children with ID with or without anemia are at more risk of having problems with mathematics than normal children of the same age. Ortega et al. (1993) reported better school capability in Spanish 15-16 yr school adolescents with higher hematologic parameters and ferritin concentrations as compared with those with lower hematologic and ferritin parameters. Taken together, these studies came up with the same
conclusion, viz., ID during early childhood leads to impaired motor, cognitive and language development as well as poorer learning and school achievement.

2.3.2. Animal Studies of Developmental Iron Deficiency

A number of reports have highlighted the impairments in cognitive and motor functions in iron deficient rats (Felt and Lozoff, 1996; Ruiz et al., 1984; Yehuda et al., 1986) and mice (Kwik-Uribe et al., 1999; 2000). Moreover, these changes are irreversible even with iron therapy (Felt and Lozoff, 1996; Kwik-Uribe et al., 2000). Behaviorally, rat pups with ID shows delayed forelimb placing reflex (Beard et al., 2006b). Additionally, rats with gestational and lactational ID show disrupted grooming sequences during adulthood (Felt et al., 2006a) with more hesitancy and less exploration in the novel environment (Beard et al., 2002; Felt et al., 2006a; Felt and Lozoff, 1996; Piñero et al., 2001). There is evidence that iron deficiency in rats causes sudden sensori-neural hearing loss due to decreased iron-dependent enzyme activity in the cochlea and changes in the stericelia function (Sun et al., 1992).

It has been shown that prenatal ID infant rhesus monkeys showed reduced spontaneous motor activity compared to iron sufficient controls (Golub et al., 2007). Furthermore, juvenile monkeys with IDA during infancy show lower dopamine levels in cerebrospinal fluid compared to controls (Coe et al., 2009). Hence it is crucial to maintain adequate iron during gestation and lactation for sake of normal brain development and myelination (Kwik-Uribe et al., 2000).

Beard and co-workers explored the underlying mechanism(s) of the developmental effects of early iron deficiency in rats (Beard et al., 2006b; Felt et al., 2006a; Piñero et al., 2001). They found that these effects are mediated through changes in dopamine neurobiology in the nigrostriatal and meso-cortical limbic system (Beard, 2007; Beard et al., 2003b). Another possible explanation for the long term effects of ID is based on the functional isolation framework theory
derived from rat models (Levitsky and Barnes, 1972). Perhaps altered myelin formation is a third mechanism that explains the long-term sequela of early ID. For instance, in rats, iron is important for myelin formation (Larkin and Rao, 1990; Ortiz et al., 2004). Nearly 70% of brain iron is linked to myelin which explains the marked reduction in the white matter in 21 day-old rat pups with persistent ID as compared to controls (de los Monteros et al., 2000). Further, prenatal or lactational iron deficiency in Sprague-Dawley dams results in delayed myelination or hypomyelination in outcome pups (Yu et al., 1986).

2.3.3. Treatment of ID-Induced Attention Deficits

The attention system of the human brain is carried out by a network of anatomical areas (Mesulam, 1981; Rizzolatti et al., 1985). In other terms, the attention system in the human brain is divided into subsystems that perform interrelated functions. These subsystems have the following major functions that account for attention (Posner and Boies, 1971; Posner, 19990): a) orientation to sensory events; b) signal detection for conscious processing, and c) maintaining an alert state. For more details about the human brain networks underlying attention, see Figure 1.

There is mounting evidence tying ID in children to attention deficit-hyperkinetic disorder (ADHD) (Cortese et al., 2008; Oner et al., 2007; Oner and Oner, 2008). Recent reports suggest an association between low serum ferritin status and behavioral problems reported in ADHD subjects (Oner and Oner, 2008) but not cognitive performance (Oner et al., 2008). Furthermore, Lukowski et al., (2010) reported that ID during early infancy produces persistent impairments on cognitive tasks including attention set-shifting. Similarly, Foley et al. (1986) reported possible deleterious effects of mild ID on spatial performance in a sample of university undergraduate students. In line with these findings, Kretsch et al. (1998) reported a positive connection between hemoglobin and transferrin saturation and sustained attention in obese dieting women.
Early oral iron supplementation in human infants with a low birth weight shows a beneficial effect on long term neurocognitive and psychomotor development later in life (5 years) (Steinmacher et al., 2007). Moreover, iron supplementation during early infancy from 1 to 6 months of age improves Bayley mental and psychomotor developmental indexes (Friel et al., 2003). In accordance with these findings Moffatt et al. (1994) reported the protective effect of iron supplementation in preventing infant developmental delays and abnormal behavior. There is evidence that iron supplementation enhances social interaction and affective responsivity among 12 months Chilean infants (Lozoff et al., 2003). This is in accordance with Idjradinata and Pollitt (1993) who reported complete reversal of mental and psychomotor functions in 12-18-month-old Indonesian infants after 4 months of iron therapy as shown from performance in the Bayley scales.

A number of studies investigated the effect of iron supplementation on mental and motor development in children. Some studies show moderate improvement of mental development score in children older than 7 years, nonetheless, iron supplementation fails to correct the deleterious effects of IDA on mental and/or motor development in children less than 27 months (Sachdev et al., 2005). Moreover Seshadri and Gopaldas (1989) reported that iron therapy for 8 months improved certain scores on cognitive tests in Indian school children aged 5-8 years. In line with these findings Agaoglu et al., (2007) studied the effect of iron therapy for 4-6 months on iron deficient Turkish children aged 6-12 year as compared to controls. They found lower IQ scores among the pretreatment IDA children compared to controls. However, after treatment, the IQ scores were significantly higher in the anemic group. Bruner and colleagues (1996) showed that iron supplementation for 8 weeks improved verbal learning and memory in non-anemic adolescent ID girls. Furthermore, Murray-Kolb and Beard (2007) conducted a blinded, placebo-controlled intervention study on ID women during their reproductive years (18-35 y) receiving iron supplementation. They found that iron supplementation for 16 weeks, remedied memory, and
learning and attention deficits. This is in agreement with Groner et al. (1986) who concluded a beneficial effect of iron therapy on psychomotor test-score performance especially attention among pregnant women aged 14-24 years.

Iron supplementation may correct attention problems in ADHD children as indicated by increased serum ferritin and decreased Connor’s Parent and Teacher Rating Scale (Konofal et al., 2005; 2008). In fact for some ADHD children, iron supplementation may diminish some of the symptoms; nonetheless, it is quite likely that many of these children are treated with dopaminergic agents such as methylphenidate (Ritalin®), amphetamine or a cocktail of amphetamine salts (Adderall®) (Mehta et al., 2004; Olfson et al., 2003; Robison et al., 1999; Safer and Krager, 1988; Swanson and Volkow, 2002). Pharmacological studies in humans reported that dopaminergic agonists like methylphenidate (MePh) facilitate cognitive performance (Kimberg and D’Esposito, 2003; Luciana et al., 1998). This is why we selected Meph to treat attention problems induced by infantile iron deficiency in rats.

**Conclusions**

The present work lends credence to the hypothesis that iron plays a fundamental role in health and disease. Based on the aforementioned literatures in human and animals, it is clear that, brain iron deficiency is associated with widespread alterations in neurological functions. There is strong evidence that ID is associated with poor development in infants, and lower scores on cognitive function tests and poor educational achievement in children without any catch-up in cognitive performance despite iron therapy (Lozoff et al., 2006b). Additionally, ID impairs cognitive abilities in adolescent girls (Bruner et al., 1996) and adults (Tucker et al., 1982; 1984). Because of the multiple biochemical pathways that utilize iron, we are in need to fully understand
the complexity of iron regulation within the human body through conduction of intervention studies and basic research.

**Attention System**

**Alerting**
- Posterior area (NE)
- Thalamus (NE)
- Frontal area (DA, NE)
- Reticular activating system (NE)
- Locus ceruleus (NE)

**Orienting**
- Frontal eye field (NE, DA)
- Superior parietal lobe (DA, NE)
- Tempoparietal junction (NE)
- Pulvinar (NE)
- Superior colliculus (NE)

**Executive**
- Prefrontal cortex (DA, NE)
- Anterior cingulate gyrus (DA, NE)
- Nucleus accumbens and striatum (DA)

**Figure 1:** Attention system of the human brain with the proposed mediating neurotransmitters. DA; dopamine mediated; NE; Norepinephrine mediated (Posner and Rothbart, 2009).
Published Paper

Methylphenidate Improves Cognitive Deficits Produced by Infantile Iron Deficiency in Rats

Modified from: Mohamed WM, Unger EL, Kambhampati SK, Jones BC. Methylphenidate improves cognitive deficits produced by infantile iron deficiency in rats. *Behav Brain Res.* 2011; 216:146-52
3.1. Abstract

In humans, iron deficiency early in life produces persistent, impaired cognition. Dietary iron replacement does not ameliorate these problems and to date, no attempt to treat these individuals pharmacologically has been reported. The aim of this work was to test the hypothesis that rats made iron deficient in early infancy exhibit cognitive deficits similar to those seen in humans at adolescence. A second aim was to investigate whether the deficit could be treated pharmacologically. Sprague–Dawley rats were made iron deficient (ID) starting at postnatal day 4 by being placed with iron-deficient dams (vs. control). At weaning, all pups were placed on an iron-sufficient diet for the remainder of the study. At 45 days of age, the animals were tested for attention set shifting. After testing, the animals were assigned to one of three methylphenidate (MePh) dose groups, 1, 5 or 10 mg/kg, p.o., vs. vehicle control and treated daily for 15 days prior to a second round of attention set shift testing and continued throughout testing. The results showed that ID rats performed more poorly than controls overall on attentional set-shift testing. MePh improved ID rats’ performance and lower doses were more effective than higher doses. This is the first demonstration that MePh can improve cognitive deficits produced by early ID in animals. These findings may open the possibility of pharmacotherapy to treat the persistent cognitive difficulties in children who were severely iron deficient in early infancy.

Key words: Infancy, Iron deficiency, Methylphenidate, Attention
3.2. Introduction

Iron deficiency (ID) is reported to be the most common single nutrient disorder worldwide. It is a problem in developing as well as in industrialized countries. An estimated 40% of preschool children in developed countries and about 80% in developing countries are anemic at 1 year of age (WHO, 2006). Worldwide, an estimated 4 billion people are afflicted (Stoltzfus, 2001; WHO, 2001); accordingly, micronutrient malnutrition (including iron deficiency) was identified as one of the priority areas for WHO action (WHO, 2004). According to Looker and colleagues (Looker et al., 1997), the prevalence of ID among full-term toddlers aged 1–2 years in the United States is 9%. The clinical consequences of ID in the first year of life are well known and widely reviewed (Beard, 2007; Grantham-McGregor and Ani, 2001; Lozoff and Georgieff, 2006; Lozoff et al., 2006). The common finding is that ID during infancy is consistently associated with altered cognitive, affective and motor deficits, including attentional difficulties. What is especially notable is that these difficulties persist into adolescence and beyond, in humans and in rats, despite dietary iron replacement (Beard et al., 2003b; Lozoff and Georgieff, 2006; Lozoff et al., 2006; Piñero et al., 2001). In a recent article, Lukowski et al. showed that early iron deficiency in early infancy produced persistent effects on cognitive tasks including attention set-shifting in young human adults (Lukowski et al., 2010).

There is also evidence to suggest that ID in childhood may be one factor contributing to attention-deficit/hyperactivity disorder (ADHD) (Grantham-McGregor and Ani, 2001; Konofal et al., 2004; Oner et al., 2007; Oner and Oner, 2008). Symptoms include inattention, impulsivity and hyperactivity (Faraone et al., 2003), and in 60% of cases, the symptoms of ADHD persist into adulthood (Kessler et al., 2005). Attention-deficit/hyperactivity disorder has been estimated by some to affect 8–12% of school-aged children worldwide and 3–5% of US adults; although, lower estimates (5% or less) are probably more realistic (APA, 1994; Dopheide and Pliszka,
2009). There are many hypotheses about the etiology of ADHD, one of the most prominent being that of central dopamine (DA) dysfunction (Nieoullon, 2002). Iron deficiency impairs DA neurotransmission (Beard et al., 2006b; Unger et al., 2007), and may prove to be irreversible in rats when occurring in infancy, especially from postnatal day (PND) 4 to 21 (Beard et al., 2003b). Although the preponderance of research reports lean toward central DA, there is also evidence from the research literature that norepinephrine may be involved in the pathogenesis of ADHD (e.g., Kuczenski and Segal, 2002). As the relationship of iron to DA neurobiology has been studied, two themes have emerged: (1) ID causes a down regulation of DA receptors and the DA transporter (Beard et al., 2006b; Bianco et al., 2008) and (2) ID early in development produces deficits in DA-related neurobiology, effects that are not reversible with later iron repletion (Beard et al., 2003b; Piñero et al., 2001). The results of these studies in early (pre-weaning) ID rats parallel findings observed in human studies; i.e. that the neurobehavioral effects persist long after replenishment and probably into adulthood (Beard, 2007; Lozoff and Georgieff, 2006; Lozoff et al., 2006). ID also alters central noradrenergic (NE) functioning. Iron deficiency decreases the NE transporters level in PC12 cells (derived from adrenal medulla) and in rat locus coeruleus (Beard et al., 2006a). Anderson and colleagues (Anderson et al., 2009) reported ID to reduce NE transporter protein in globus pallidus and hippocampus in rats and McEchron and colleagues (McEchron et al., 2010) recently reported peri-natal ID to impair NE synaptic transmission in hippocampus of rats. While it is clear that central DA and NE systems are altered by ID, the timing of the deficiency is not as well documented for NE as it is for DA.

There are three important issues regarding the impact of ID anemia during the early developmental period, viz.; timing, severity, and duration of ID. The age of onset of the dietary ID is probably the most important of the three because of the possible long-term effects even with subsequent iron repletion. In this study, we focused on timing in order to capture the putative critical period for dopamine receptor differentiation. The peak for human brain growth includes
the last trimester prenatally through the first 2 years of childhood (Kolb and Whishaw, 2001). In the rat, the peak is between postnatal day (PND) 7 and 10 (Dobbing and Sands, 1979). As for the development of DA systems, Tarazi et al. (Tarazi et al., 1999) elucidated the developmental course of DA receptor systems in rat brain. For D1 and D2 receptors in caudate and nucleus accumbens, there is a steady increase in density between PND7 and PND28 followed by a decline (Tarazi et al., 1999; Tarazi and Baldessarini, 2000). In humans, a similar decline in D1 receptor density in caudate–putamen from childhood into old age was reported by Montague and colleagues (Montague et al., 1999). Furthermore, the DA transporter (DAT) in caudate–putamen and in nucleus accumbens increases in density from low levels at PND7 to maximal level (about a seven-fold increase) at PND60 (Tarazi et al 1998). Thus in order to test the hypothesis that early ID impairs attention, we decided to make our rats iron deficient beginning at PND4, put them on an iron-adequate diet at weaning and test their attentional abilities in adolescence. Finally, because ID affects central DA and NE neurobiology, we hypothesized that attentional deficits produced by ID might be treated effectively by catecholamine-related drugs, in our case, methylphenidate (MePh), an agent that blocks pre-synaptic DA and NE reuptake.

3.3. Methods and materials

3.3.1. Subjects and dietary treatment

We purchased male and female Sprague–Dawley rat breeding stocks from Harlan Sprague–Dawley (Indianapolis, IN). Male breeders were fed rodent chow (Purina Mills Lab Diet 5001) containing 270 parts per million (ppm) Fe while female breeders were divided into 2 groups; one was fed a diet containing 80 ppm Fe (control, CN), and the other was fed a diet containing 4 ppm Fe (iron deficient, ID). These diets were prepared in our laboratory and
followed the American Institute of Nutrition (AIN-93G) diets which met all nutritional requirements with the exception of the iron content for the low iron diet (Piñero et al., 2000; Reeves et al., 1993). Both groups were given distilled water. At 200–220 g body weight, females were placed with males (1 male and 2 females/cage) for 5 days or until a vaginal plug was observed. Pregnant dams were then housed singly and checked daily for delivery. All dams were weighed weekly. The first day pups appeared was considered to be postnatal day 0 (PND0). At PND4 all pups were out-fostered; pups from control dams were out-fostered to other control dams or to iron-deficient dams. All pups weaned at PND21 to Purina Rodent Diet (5001), containing 270 ppm iron, \textit{ad libitum} until the experiment was terminated. All pups were pair-housed in clear plastic shoebox cages measuring 20cm×42.5cm with stainless steel lids in the University Vivarium. The rooms were temperature and humidity-controlled at 22±1 °C with an automatic 12/12 h light/dark cycles (light 0600–1800 h). All experimental animals were weighed weekly and monitored closely for health. All experimental protocols adhered to National Institutes of Health Animal Care Guidelines (1985) and were approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

### 3.3.2. Attentional Set-Shift Task (ASST) protocol

#### 3.3.2.1. Principle and validity of the task

The attentional set-shifting task and its apparatus are modified version of those described by Birrell and Brown (2000); and Lapiz and Morilak (2006). This task is based on two-choice discrimination which is similar to the intra–extra-dimensional shift tests for children introduced by Kendler et al. (1972). Stimulus dimension is defined as arrays of stimuli appropriate to specific sensory modalities or sub-modalities. For example for visual stimuli, color might
constitute one dimension while shape constitutes another (Birrell and Brown, 2000). Ideally, the stimulus dimensions should be species-appropriate in order to facilitate rapid acquisition of discrimination tasks (Birrell and Brown, 2000). Attentional Set-Shift Task includes testing rats on a series of increasingly intra- and extra-dimensional discriminations. Typically, animals are trained to discriminate between two stimuli that vary within a dimension—for example, black vs. white object. On subsequent test sessions, an extra-dimensional, but irrelevant quality is introduced (e.g., shape). Next, the animal is trained to respond to the formerly non-rewarded object (compound discrimination reversal or shift). The fourth task is to have the animal attend to the formerly irrelevant dimension for the rewarded cue and fifth, to acquire cue reversal on the changed dimension. Thus ASST is related to rats’ ability to focus and re-focus attention on familiar and novel arrays (Pantelis et al., 1999).

3.3.2.2. Apparatus

The test apparatus consisted of a Plexiglas® cage measuring 59cm×35cm×32 cm. One third of the box was divided into two sections by removable Plexiglas panels, each containing a small ceramic bowl. Wood et al., (1999) showed that rats can be trained to dig in small ceramic bowls filled with sawdust to retrieve food reward. A removable divider separated these sections containing each bowl from the rest of the box so that access to both bowls could be controlled between trials. A smaller divider was used to block access to either one of the two bowls (Birrell and Brown, 2000). The purpose of the dividers was to enable the experimenter to clean the apparatus and change the bowls between trials. Moreover, this separation enabled the experimenter to remove the rat following an incorrect trial. We used PETCO Eggshell Small Animal Ceramic Bowls as digging bowls (PETCO, USA). The bowls had an internal diameter of 10cm and an internal depth of 5 cm. The stimulus dimensions used were texture of the digging
medium and scent. For texture, two kinds of gravel were used to fill the bowls, coarse and fine as can be purchased for fish tanks. For the other dimension, scented liquids as can be obtained in the spice section of a grocery store were used. A few drops were applied to the inner rim of the bowls using a cotton swab at the beginning of each testing session (Birrell and Brown, 2000). The bowls were baited with \(~8\) loops of Honey Nut Cheerios (General Mills, Minneapolis, MN, USA). The bowls were filled with different digging media to mask the food reward visually. Baiting the bowls with 8 loops allowed for continuous testing without re-baiting between trials. It is important to note that although the correct bowl had multiple cheerios loops, the rat was permitted to consume only one per each correct choice.

3.3.2.3. General procedure

All behavioral testing took place in a special procedure room adjacent to the housing area. For each rat, the procedure entailed three phases; acclimation (1 day), training to dig in the media (1 day) and testing which was conducted on three consecutive days following this order: day-1 simple discrimination (SD), day-2 compound discrimination (adding the irrelevant dimension), compound discrimination reversal as described above (CD, CD-R) and day-3 extra-dimensional shift, extra-dimensional reversal (EDS, ED-R). All animals were transported to and from the apparatus in their home cages. The attentional set-shift apparatus was cleaned with soap and water between animals, and at the end of the day. A different bowl was used for each combination of digging medium and scent and only one scent was applied to a given bowl. During all stages of training and testing, both bowls contained equal amounts of crushed Cheerios to prevent the rats from using cereal scent to locate the reward.
3.3.2.4. Acclimation phase

As described by Birrell and Brown (2000), Fox et al., (2003), and Lapiz and Morilak (2006), acclimation was done by placing 2 digging bowls filled with different media combinations and baited with the reward in animals’ home-cages to familiarize the rats with testing materials. The rat was placed in the testing apparatus and given 2 consecutive trials with unlimited free access for 15 min to 2 baited bowls with different media. Both bowls were re-baited as necessary and this phase ended when the rat acquired reliable reward retrieval.

3.3.2.5. Training phase

Following acclimation, rats were trained to dig into the media to obtain the reward. Afterwards, rats were trained on 2 simple discriminations (SD); medium (Cat litter vs. Kaytee bedding) then odor (Mint vs. Strawberry). Placement of the bowl containing the reward on each trial was randomized according to an adapted pseudorandom Gellerman schedule (Gellerman, 1933). Rats were allowed to explore both bowls for the first 4 trials (exploratory trials, 90 s each) regardless of which bowl was dug in first to make an association between food reward and digging. Training trials were initiated by opening the movable divider to give the rat a chance to access the 2 bowls. If the rat started to dig in un-baited bowl, an error was recorded and trial ended. The rats were trained to reach a criterion of six consecutive correct trials out of thirty on each discrimination task. Most rats finished this phase successively during a single session. When a rat failed to reach criterion (after total of 30 trials), it was returned to its home-cage and a second training session was conducted on the next day. There was a maximum of three attempts to train each rat and all rats passed this phase successfully. The discriminanda used in training
(cat litter and Kaytee bedding; mint and strawberry) were not used during subsequent formal testing phase.

3.3.2.6. Attention set-shift testing (Phase 1 testing)

We attempt to mimic as closely as possible the attention problem in humans by selecting rats during specific age periods (refer to Table 6). In general, rats have a brief accelerated childhood compared to humans. For instance, during infancy rats develop rapidly and reach sexual maturity (puberty) at age of 6 weeks (Adams and Boice, 1983) and reach social maturity at about 5 months of age (Adams and Boice, 1983). Humans, on the other hand, develop slowly reaching puberty at 12.5 years and become socially mature at the age of 18 years. During adulthood, each rat month is roughly equal to 2.5 human years (Ruth, 1935). Indeed, there is no concrete agreement among researchers about the exact definition of adolescent rats. However to some extent researchers who used rats of different ages and related their ages to that of humans determine the following age intervals for rats: juvenile (PND21-30), peri-adolescent (PND31-39), adolescent (PND40-50), and young adults (PND60-75) (e.g. Rezvoni and Levin, 2004; Spear and Brake, 1983; Vorhees et al., 2005).

At 40 days of age, we started the first phase of attention set-shifting testing. We tested 92 rats; 47 males and 45 females; 48 control and 44 iron deficient. The day before testing, the target animals were food-deprived from 1500 until 0900 (the day of testing). We weighed all animals weekly without any reported weight loss or any significant difference between both sexes in terms of total body weight (see figures 2, 3, 4). Testing began at 0900. Prior to testing, each rat was placed in the testing apparatus for 1min. A trial was initiated by raising the divider to give the rat access to the two digging bowls, one of which was baited. An error was recorded when the rat dug in the un-baited bowl (McLean et al., 2008). During the testing phase, half of the rats
started with odor as relevant dimension (Tables 2 and 3) and the other half started with digging medium as the relevant dimension. Testing continued until a rat reached criterion of six consecutive correct trials or reached 30 trials without meeting criterion (Birrell and Brown, 2000). Initially, rats performed a SD between two bowls that differed along only one of the two perceptual dimensions (medium or odor). Upon reaching criterion or finishing the 30 trials without reaching criterion, testing progressed to the compound discrimination (CD) task, where the correct and incorrect exemplars of the relevant dimension remained the same as in the SD, but the second (irrelevant) dimension had been introduced. Compound discrimination was followed by a compound discrimination reversal (CD-R), in which the discriminanda and the dimensions were unchanged from the CD, but the previously correct exemplar now incorrect and vice versa. The CD-R was then followed by an extra-dimensional shift (EDS) stage of the task. Rats were now required to attend to the previously irrelevant dimension for rewarded cues. The test sequence ended with extra-dimensional reversal discrimination (ED-R) (Table 2 and 3).
Figure 2: Total body weight in CN and ID animals. The data presented are combined data of both sexes and presented as mean. CN=control; ID=iron deficient.
**Figure 3:** Total body weight in CN animals. The data presented as mean. M: male; F: female.

**Figure 4:** Total body weight in ID animals. The data presented as mean. M: male; F: female.
Table 2: Testing protocol and examples of stimulus pairs used in ASST and the progression through the stages of the ASST (50% of animals start with odor).

<table>
<thead>
<tr>
<th>Task</th>
<th>Dimensions</th>
<th>Stimulus combinations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Relevant</td>
<td>Irrelevant</td>
</tr>
<tr>
<td>SD</td>
<td>Odor</td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>Odor</td>
<td>Medium</td>
</tr>
<tr>
<td>CD-R</td>
<td>Odor</td>
<td>Medium</td>
</tr>
<tr>
<td>EDS</td>
<td>Medium</td>
<td>Odor</td>
</tr>
<tr>
<td>ED-R</td>
<td>Medium</td>
<td>Odor</td>
</tr>
</tbody>
</table>

Half of the rats started with odor as a relevant discriminative stimulus dimension with shifting to the digging medium in the EDS. The remaining rats started with digging medium as a relevant discriminative stimulus dimension with shifting to odor in the EDS, to make a counterbalance between dimensions. SD: simple discrimination; CD: compound discrimination; CD-R: compound discrimination reversal; EDS: extra-dimensional shift; ED-R: extra-dimensional reversal.
### Table 3: Testing protocol and examples of stimulus pairs used in ASST and the progression through the stages of the ASST (50% of animals start with medium).

<table>
<thead>
<tr>
<th>Task</th>
<th>Dimensions</th>
<th>Stimulus combinations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Relevant</td>
<td>Irrelevant</td>
</tr>
<tr>
<td>SD</td>
<td>Medium</td>
<td>M1 (coarse crystal gravel)</td>
</tr>
<tr>
<td>CD</td>
<td>Medium</td>
<td>Odor</td>
</tr>
<tr>
<td>CD-R</td>
<td>Medium</td>
<td>Odor</td>
</tr>
<tr>
<td>ED-S</td>
<td>Odor</td>
<td>Medium</td>
</tr>
<tr>
<td>ED-R</td>
<td>Odor</td>
<td>Medium</td>
</tr>
</tbody>
</table>

Half of the rats started with digging medium as a relevant discriminative stimulus dimension with shifting to the odor in the EDS. The remaining rats started with odor as a relevant discriminative stimulus dimension with shifting to digging medium in the EDS, to make a counterbalance between dimensions. SD: simple discrimination; CD: compound discrimination; CD-R: compound discrimination reversal; EDS: extra-dimensional shift; ED-R: extra-dimensional reversal.
3.3.2.7. Methylphenidate treatment and Phase 2 testing

At the end of Phase 1 testing, all 92 animals were divided into 4 treatment groups and administered one of three doses of MePh or control vehicle daily for 15 days (see below). All test animals were distributed across treatment groups from 13 litters and all pups from these litters were divided into two groups for out-fostering to ID or control dams. After this time, the animals were continued on their dosing regimen and tested a second time on attention set shifting as described above. The numbers of animals by sex (m/f) allocated to each dose group by dietary condition were:

Table 4: Numbers of animals by sex (m/f) allocated to each dose group by dietary condition:

<table>
<thead>
<tr>
<th>Animals</th>
<th>1mg/kg</th>
<th>5mg/kg</th>
<th>10 mg/kg</th>
<th>Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7/6</td>
<td>5/7</td>
<td>8/3</td>
<td>5/7</td>
</tr>
<tr>
<td>ID</td>
<td>8/4</td>
<td>7/6</td>
<td>6/3</td>
<td>4/6</td>
</tr>
</tbody>
</table>

Racemic MePh as the hydrochloride salt was obtained courtesy of the National Institute on Drug Abuse Drug Supply Program. The drug was dissolved in Hawaiian Punch® and administered p.o. by serological pipette. Before dosing, animals were subjected to water deprivation during the day (from 0700–1500) then dosing was conducted 2 h prior to lights out. The doses were 1, 5 and 10 mg/kg. After dosing, the animals had access to Purina diet and water, *ad libitum* during the dark-phase.

In humans, methylphenidate is effective in treatment of ADHD in a dose of 0.25-1.0 mg/kg orally which results in 8-40 ng/ml peak plasma level of methylphenidate (Swanson et al., 1999; Swanson and Volkow, 2002). While in rats, Aoyama et al (1990) suggested that oral MePh doses between 0.5 mg/kg and 3.5 mg/kg induce peak plasma MePh concentrations within the typical clinical range (8-40 ng/ml). In our treatment protocol, we administered MePh once daily
for consecutive 15 days to avoid any behavioral sensitization (Gaytan et al., 2000; Kalivas et al., 1993; McDougall et al., 1999) or tolerance (Ellison and Eison, 1983) to its locomotor and stereotypic effects with intermittent dosing. Although the half life of MePh in rats is much shorter (1-2 hrs) (Melega et al., 1995) than in humans (3-6 hrs) (Anggard et al., 1973), there is an evidence that rats are capable of exhibiting sensitization for MePh treatment even after an extended abstinence period (McDougall et al., 1999). It is evident that in adult rats, chronic treatment with psychostimulants produces behavioral sensitization that can be expressed months after the final drug exposure (Castro et al., 1985; Paulson et al., 1991; Robinson and Becker, 1986). Others (e.g. Izquierdo et al., 2010) gave rats 3-5 days rest (no behavioral testing, in homecage) after MePh treatment before any food restriction or behavioral testing. It is important, therefore to clarify that even though we started behavioral testing after finishing the 15 days treatment, it is possible to see trace effects of MePh.

We did not use intra-peritoneal or subcutaneous administration of methylphenidate to avoid stress of injection and to minimize animals’ manipulation as stress has deleterious effects on behavioral flexibility (Holmes and Wellman, 2008; Iquierdo et al., 2006) especially with the reported elevated level of corticosterone 1-72 h after methamphetamine treatment (Herring et al., 2008). Also, MePh injection produces much higher blood and brain MePh levels (Gerasimov et al, 2000) which might be relevant to drug abuse but not to treatment of attention problems (Gaytan et al, 1997; McDougall et al, 1999; Sproson et al, 2001). Such higher doses produce hyperactivity which might compromise the overall performance (Amini et al, 2004; Wultz et al, 1990). Furthermore, Gerasimov et al., (2000) concluded that the response of male Sprague-Dawley rats to oral methylphenidate (5mg/kg) is effectively equivalent to a lower intra-peritoneal dose of methylphenidate (2mg/kg i.p.). Our selections for oral route is in accordance with Kuzcenski and Segal (2002) who reported that oral administration of a low MePh dose produced plasma levels in rats similar to those in ADHD children with decreasing locomotor activity in rats.
as observed in humans. We used Hawaiian Punch® as a vehicle for oral MePh as it is tasty to rats compared to distilled water as a vehicle (Bethancourt et al., 2009). Moreover, we were sure that the assigned MePh dose dissolved completely in the punch compared to injection of MePh into oyster crackers as a vehicle (Harvey et al., 2011; Wooters et al., 2011). The latter vehicle has some limitations like food restriction prior to cracker feeding, rats need to be trained to consume oyster crackers and the need to be sure that rats get the entire cracker, ensuring administration of the correct dose.

It is evident that, repeated dose administration of MePh in rats attenuate presynaptic striatal dopamine function (Sproson et al, 2001) and blunt certain forms of learning by interfering with the plasticity that would otherwise occurs (Kolb et al., 2003). we believe that our treatment protocol (MePh daily for consecutive 15 days) worked well especially with the fact that, the effect of MePh on behavior varies from one rat strain to another (Amini et al., 2004) and the reported impairment of attention set-shifting after a sensitizing regimen of amphetamine i.e. a 5-week escalating-dose regimen (Featherstone et al., 2008; Fletcher et al., 2005). Indeed, our treatment protocol mimics the drug-free periods (drug holidays) with clinical treatment in humans (Committee on Children with Disabilities, 1996). Finally it is important to note that in our studies, the focus was primarily on the pharmacodynamic rather the pharmacokinetic aspect of MePh. One main concern, which should be considered in future studies, is whether ID alters MePh distribution and clearance.

3.3.3. Hematology and brain iron

All animals were tested for hemoglobin (Hb), hematocrit (Hct), prefrontal cortex, and ventral midbrain iron according to well-established procedures in our laboratory (Erikson et al., 1997; Piñero et al., 2001). The dams had 30µl blood sampled for measuring Hb and Hct by tail
nick before and after parturition. Rat pups were also evaluated for Hb and Hct by 30 µl blood sampled by tail nick at weaning, after completing phase-1 testing and at sacrifice. At the end of the experiment, all animals were sacrificed by decapitation after CO₂ suffocation and tissues harvested. Brain iron determinations were made using atomic absorption spectroscopy (Erikson et al., 1997; Piñero et al., 2001). Because of our earlier work showing dopamine receptor alterations by iron deficiency (Beard et al., 2003b), we chose the medial prefrontal cortex and ventral midbrain for iron analyses. We would expect that the iron concentration in these areas would be the same for ID and control animals, but future work should show ID-related persistent deficiency in dopamine receptors and the transporter.

3.3.4. Time schedule for our studies

Due to the nature of our studies, integrated approaches were implemented. Specific aims have been studied simultaneously to complete successfully all studies in a reasonable time. Table 5 and 6 show the experimental design with illustration of time frame for each key step during the experimental period.

**Table 5:** Time line of each experimental phase for the breeders:

<table>
<thead>
<tr>
<th></th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start age</td>
<td>50 days old</td>
</tr>
<tr>
<td>Habituation</td>
<td>3 days</td>
</tr>
<tr>
<td>Dietary treatment</td>
<td>4 weeks</td>
</tr>
<tr>
<td>Mating</td>
<td>5 days</td>
</tr>
</tbody>
</table>
3.3.5. Data analysis

The number of trials to criterion was subjected to analysis of variance (ANOVA) for a two between-subjects variable (diet, drug dose) and one within-subjects variable (pre/post-drug treatment) experiment. Initial analysis showed that there were no sex-related differences, so the data for males and females were combined. For those animals that did not reach criterion in 30 trials, they were assigned a score of 30, the maximum number of trials permitted. All post hoc pairwise comparisons were made using the Tukey HSD test.

Table 6: Time line of each experimental phase for the pups:

<table>
<thead>
<tr>
<th>PND</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth</td>
<td>0</td>
</tr>
<tr>
<td>Out-fostering</td>
<td>4</td>
</tr>
<tr>
<td>Weaning</td>
<td>21</td>
</tr>
<tr>
<td>ASST-1</td>
<td>45</td>
</tr>
<tr>
<td>MePh treatment</td>
<td>50</td>
</tr>
<tr>
<td>ASST-2</td>
<td>65</td>
</tr>
<tr>
<td>Sacrifice</td>
<td>75</td>
</tr>
</tbody>
</table>
3.4. Results

3.4.1. Number of animals failing to meet criterion during phase-1 testing

Table 7 presents numbers of animals failing to meet criterion (six consecutive correct choices) during pre-drug baseline testing. The total numbers of animals were 48 and 44 for control and ID, respectively, tested on each of the five tasks. As can be seen, far greater numbers early ID animals failed to meet criterion in all phases of testing than seen among controls, $x^2 = 13.61$, $p < 0.01$. Regardless of success or failure to meet criterion on one task, all animals were tested on all tasks successively. The table presents the number of control (out of 48) and ID (out of 44) that failed to meet criterion on each task.

Table 7: Numbers of animals failing to meet criterion in pre-drug baseline attention set shift testing.

<table>
<thead>
<tr>
<th>Animals</th>
<th>SD</th>
<th>CD</th>
<th>CD-R</th>
<th>EDS</th>
<th>ED-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>11</td>
<td>15</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>ID</td>
<td>37</td>
<td>38</td>
<td>36</td>
<td>28</td>
<td>33</td>
</tr>
</tbody>
</table>
3.4.2. Effect of methylphenidate on attention set shifting in early iron-deficient rats

3.4.2.1. Simple discrimination

Analysis of variance revealed significant main effects of diet and treatment ($F_{1,84} = 148.05$, $p < 0.001$; $F_{3,84} = 3.23$, $p < 0.03$) and a significant interaction between diet and treatment ($F_{3,84} = 2.73$, $p < 0.05$). There was also a significant main effect of pre/post drug treatment ($F_{1,84} = 57.31$, $p < 0.001$) and significant pre/post by diet and pre/post by dose interactions ($F_{1,84} = 46.24$, $p < 0.001$; $F_{3,84} = 8.54$, $p < 0.001$). As shown in Figs. 5 and 6, MePh produced no significant effect on performance in control animals at any dose, but showed a non-significant tendency to interfere with performance at 10 mg/kg. By contrast, in ID animals, MePh improved performance at each dose ($p < 0.01$ all). Vehicle control post testing revealed no significant improvement in simple discrimination for either control or ID animals.
Figure 5: Effect of Methylphenidate on simple discrimination in control animals. The data presented are mean ± S.E.M. of mean trial to criterion, pre-drug treatment vs. post-drug treatment. Pairwise comparisons are for pre- and post drug treatment.
Figure 6: Effect of Methylphenidate on simple discrimination in ID animals. The data presented are mean ± S.E.M. of mean trial to criterion, pre-drug treatment vs. post-drug treatment. Pairwise comparisons are for pre- and post drug treatment.
3.4.2.2. Compound discrimination

Analysis of variance revealed significant main effects of diet and treatment ($F_{1,84} = 45.06$, $p < 0.001$; $F_{3,84} = 5.99$, $p < 0.002$) and a non-significant interaction between diet and treatment ($F_{3,84} < 1$). There was also a significant main effect of pre–post-drug treatment ($F_{1,84} = 33.45$, $p < 0.001$) and a non-significant pre/post by diet interaction. ($F_{1,84} = 9.88$, $p < 0.001$). As shown in Figs. 7 and 8, MePh produced no significant effect on performance in control animals at any dose, but showed a no significant tendency to interfere with performance at 10 mg/kg. In ID animals, MePh improved performance significantly at 1, 5 and 10 mg/kg dose ($p < 0.01$, $p < 0.01$, $p < 0.05$). Vehicle control post testing revealed no significant improvement in compound discrimination.
Figure 7: Effect of Methylphenidate on compound discrimination in control animals. The data presented are mean ± S.E.M. of mean trial to criterion, pre-drug treatment vs. post-drug treatment. Pairwise comparisons are for pre- and post drug treatment.
Figure 8: Effect of Methylphenidate on compound discrimination in ID animals. The data presented are mean ± S.E.M. of mean trial to criterion, pre-drug treatment vs. post-drug treatment. Pairwise comparisons are for pre- and post drug treatment.
3.4.2.3. Compound discrimination reversal

We observed significant main effects of diet but not treatment ($F_{1,84} = 16.26, p < 0.001$; $F_{3,84} = 1.48, p > 0.05$). There was no significant interaction between diet and treatment ($F_{3,84} < 1$). There was a significant main effect of pre–post-drug treatment ($F_{1,84} = 23.77, p < 0.001$) and a significant pre/post by diet and pre/post by dose interactions ($F_{1,84} = 4.73, p < 0.05$; $F_{3,84} = 3.02, p < 0.05$). As shown in Figs. 9 and 10, MePh produced no significant effect on performance in control animals at any dose. In ID animals, MePh significantly improved performance at 1 and 5mg/kg dose ($p < 0.01, p < 0.05$). Moreover, the 10 mg/kg dose and vehicle control revealed no significant improvement in compound discrimination reversal.
Figure 9: Effect of Methylphenidate on compound discrimination reversal in control animals. The data presented are mean ± S.E.M. of mean trial to criterion, pre-drug treatment vs. post-drug treatment. Pairwise comparisons are for pre- and post drug treatment.
**Figure 10:** Effect of Methylphenidate on compound discrimination reversal in ID animals. The data presented are mean ± S.E.M. of mean trial to criterion, pre-drug treatment vs. post-drug treatment. Pairwise comparisons are for pre- and post drug treatment.
3.4.2.4. Extra-dimensional shift

There were no main effects of diet or treatment on this behavior (F\(_1, 84\) = 3.25, p > 0.05; F\(_3, 84\) = 1.30, p > 0.05). There was a no significant interaction between diet and treatment (F\(_3, 84\) < 1). There were significant effects of pre/post by diet interaction (F\(_1, 84\) = 6.68, p < 0.05) and a non-significant pre/post by dose interaction (F\(_3, 84\) = 1.06, p > 0.05). There was a significant main effect of pre/post-drug treatment (F\(_1, 84\) = 7.48, p < 0.001). As shown in Figs. 11 and 12, MePh produced no significant effect on performance in control animals at any dose. MePh improved performance at 10 mg/kg (p < 0.05) in ID animals but not at 1 or 5 mg/kg. As with the other tasks, there was no effect of vehicle control testing.
Figure 11: Effect of Methylphenidate on extra-dimensional shift in control animals. The data presented are mean ± S.E.M. of mean trial to criterion, pre-drug treatment vs. post-drug treatment. Pairwise comparisons are for pre- and post drug treatment.
**Extradimensional Shift**

**Early Iron Deficient Animals**

Methylphenidate (mg/kg)

Mean Trials to Criterion

0

10

20

30

Pretreatment

Posttreatment

1 5 10 Vehicle

* p<0.05

**p<0.01

**Figure 12:** Effect of Methylphenidate on extra-dimensional shift in ID animals. The data presented are mean ± S.E.M. of mean trial to criterion, pre-drug treatment vs. post-drug treatment. Pairwise comparisons are for pre- and post drug treatment.
3.4.2.5. Extra-dimensional shift reversal

Analysis of variance revealed a significant main effect of diet but no significant effect of treatment ($F_{1, 84} = 5.22, p < 0.05; F_{3, 84} < 1$). There was a significant main effect of pre/post-drug treatment ($F_{1, 84} = 7.87, p < 0.001$) and a significant pre/post by diet interaction ($F_{1, 84} = 10.13, p < 0.001$). As shown in Figs. 13 and 14, MePh produced no significant effect on performance in control animals at any dose. In ID animals, MePh significantly improved animals’ performance at 5mg/kg ($p < 0.05$). Furthermore, there was no significant effect of the 10 mg/kg dose or vehicle control post testing on extra-dimensional shift reversal.
**Figure 13:** Effect of Methylphenidate on extra-dimensional reversal in control animals. The data presented are mean ± S.E.M. of mean trial to criterion, pre-drug treatment vs. post-drug treatment. Pairwise comparisons are for pre- and post drug treatment.
**Extradimensional Shift Reversal**
**Early Iron Deficient Animals**

<table>
<thead>
<tr>
<th>Methylphenidate (mg/kg)</th>
<th>Pretreatment</th>
<th>Posttreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p<0.05
** p<0.01

**Figure 14:** Effect of Methylphenidate on extra-dimensional reversal in ID animals. The data presented are mean ± S.E.M. of mean trial to criterion, pre-drug treatment vs. post-drug treatment. Pairwise comparisons are for pre- and post drug treatment.
3.4.2.6. Biological data

These data show that the ID animals had lower Hct at weaning than controls and being placed on an iron-adequate diet immediately after weaning normalized both Hb and Hct at Phase-I testing. The brain iron values for the ID animals were not significantly different from controls at sacrifice.

Table 8. Hematological and brain iron concentrations in iron-deficient and control rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Stage</th>
<th>Age</th>
<th>N</th>
<th>Hb</th>
<th>Hct</th>
<th>PFC Iron</th>
<th>VMB Iron</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Days</td>
<td></td>
<td>g/L</td>
<td>Volume %</td>
<td>µmol/g</td>
<td></td>
</tr>
<tr>
<td>CN</td>
<td>Weaning</td>
<td>21</td>
<td>53</td>
<td>8.584±0.217</td>
<td>41.930±1.439</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Phase-1</td>
<td>45</td>
<td>30</td>
<td>10.317±0.418</td>
<td>43.903±1.314</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Sacrifice</td>
<td>75</td>
<td>53</td>
<td>15.786±0.306</td>
<td>-</td>
<td>37.377±1.905</td>
<td>18.521±0.677</td>
</tr>
<tr>
<td>ID</td>
<td>Weaning</td>
<td>21</td>
<td>53</td>
<td>6.540±0.229*</td>
<td>30.058±1.618**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Phase-1</td>
<td>45</td>
<td>39</td>
<td>9.965±0.536</td>
<td>47.538±1.231*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Sacrifice</td>
<td>75</td>
<td>49</td>
<td>15.694±0.650</td>
<td>-</td>
<td>41.130±1.929</td>
<td>17.627±0.812</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ±S.E.M.

Abbreviations: Hct, Hematocrit; Hb, Hemoglobin; PFC, Prefrontal cortex; VMB, Ventral midbrain.

For Hb; * p<0.05 compared with CN group at weaning.
For Hct; ** p<0.001, compared with CN group at weaning, * p<0.05 compared with CN group at start of Phase 1 testing.
3.5. Discussion

To our knowledge, the current report is the first to show that MePh can effectively treat cognitive-task performance deficits in rats produced by ID in early life as measured by attentional set shifting. Ideally, ASST required rats to shift attention between different testing cues (odor, texture). Thus the optimal task performance required working memory (to remember which bowl was baited and which was un-baited), response inhibition (avoiding the un-baited bowl) and the ability to sustain and shift attention, i.e., to learn to attend to a previously unrewarded stimulus.

Overall, we showed that ID rats performed more poorly than controls in all aspects of ASST testing. This was a trace effect of having been iron deficient early in infancy; however, the behavioral difficulties persisted even though the blood and brain iron parameters had normalized. As expected, MePh did not improve the performance in control animals; however, MePh did so significantly in ID animals and more dramatically in the simpler tasks. Generally, lower doses produced better performance than higher in ID animals. Although not significant across tasks 10 mg/kg of MePh tended to impair the performance in control rats and based on informal observations, this high dose tended to make the animals hyperactive and exhibited behaviors incompatible with obtaining reward.

The exact mechanism underlying ID-related deficiency in ASST performance is unknown at this time; however, based on our previous work, and based on the fact that MePh was able to facilitate performance, we believe that catecholamine functioning in the meso-cortico-limbic system and hippocampus are reasonable places to start. Previously, we showed that early ID produces persistent down regulation of DA receptors including the DA transporter (Beard et al., 2006b; Bianco et al., 2008) in the limbic and striatal areas but we did not study the effect of ID in
frontal cortex DA systems. We have shown similar effects of ID on central NE function, but not to the extent that we have studied DA. Methylphenidate inhibits reuptake of both catecholamines.

Regionally, MePh increases extracellular concentrations of NE in the hippocampus and dopamine in the nucleus accumbens (Kuczenski and Segal, 2002). In non-human primates, frontal cortex lesions have been shown to impair reversal learning, intra-dimensional and extra-dimensional set shifting (Birrell and Brown, 2000; Dias et al., 1996a; b). This is in accordance with other studies, using rats that reported frontal cortex injury-related impaired shifting between response rules (Joel et al., 1990; Ragozzino et al., 1999a; b), impaired reversal learning to discriminate stimuli (Bussey et al., 1997) and delayed response tasks (Delatour et al., 1999). Furthermore, similar impairment is seen in patients with frontal resections (Owen et al., 1991), marmosets with lateral prefrontal damage (Dias et al., 1996a; b) and rats with mPFC lesions (Birrell JM, Brown, 2000). The large differences MePh efficacy that we observed between ID and control animals at all doses supports our hypothesis that at least to some extent, catecholamine neurobiology is involved in ID-based impaired cognition. When we treated our ID animals with MePh, the results indicated that lower, rather than larger doses of MePh were more effective. This result may be consistent with impaired DA, or perhaps, NE function, but at this time we cannot state exactly in which of the major catecholamine pathways may be involved. Our results, in terms of enhancement of ID rats’ performance after MePh administration, are consistent with MePh therapeutic effect in humans with ADHD (Biederman, 2005). We chose oral administration to avoid the stress of injection as a possible confounding factor (Holmes and Wellman, 2008).

Our ID protocol providing an iron depleted diet from PND4–PND21 only. As described above, this period captures the critical period for the differentiation of at least one of the central catecholamine systems that we believe is critical to understanding the persistence of the effects of ID during this developmental epoch (Beard et al., 2003b). In this respect, the rodent model fairly parallels the persistence of ID effects in humans as reported by Lozoff and Georgieff, (2006) and
Lozoff et al., (2006). After having been placed on an iron-adequate diet, the animals’ brain and systemic iron-related parameters normalized. Their performance on ASST did not, however, showing us that like early ID in children, the cognitive deficits persisted well into adolescence and young adulthood.

This is the first study that shows early iron deficiency deficits in ASST performance and as such these preliminary results require some caution in interpretation. What we do not know is the extent to which the impaired performance resulted from sensory/motor or motivational difficulties resulting from early iron deficiency. In a previous study, we measured the effect of post-weaning iron deficiency on cocaine intravenous self-administration and found iron-related delays in acquisition and maintenance of responding for cocaine (Jones et al., 2002). When we tested the animals for responding for oral sucrose solution, however, we found no differences between the iron deficient and control groups. These data should be viewed with caution; however as the timing of the iron deficiency did not include pre-weaning ID. In relation to satiety as a confounding factor for poor performance of ID rats, probably not, as each animal was allowed to consume just one cheerio following making a correct choice and the test was conducted on three successive days.

**Possible Caveats and rationales**

For time and severity of iron deficiency, we examined only iron deficient rats during the critical period of dopamine system differentiation (PND4-21). We did not examine iron deficiency effects following the period of rapid dopamine system differentiation (post-weaning). Growth of ID animals was slower than that of controls (Figure 2) until the 7th week of age, but this is not due to anorexia (Beard et al., 1995). We matched the growth curve of our animals (Figure 3, 4) with the standard growth curve obtained from Harlan Laboratories (Hsd: Sprague
Dawely® SD® (Growth Curve) to exclude possible impact of underweight rats and/or sex on our variable of interest. It could be argued that comparing growth curves is not an accurate method for this as others (e.g. Izquierdo et al., 2010) used food restriction techniques by feeding rats 15g of food each day to maintain a target weight of 85% normal weight. We believe that applying this technique on our ID rats is too risky especially with reporting some fatalities of unknown causes among ID rats throughout the experiment.

Many neurological disorders show sex differences in their incidence, including ADHD (Quinn, 2005). Furthermore, brain structures and functions differ between male and female (Cahill, 2006) especially dopamine function (Andersen et al., 1997; Wong et al., 1988). Additionally, there is evidence that sex and genetics are important cofactors in assessing the impact of ID on the developing mouse brain (Morese et al., 1999b). At the beginning of our work, we considered sex in our model to include both male and female. However, initial statistical analysis of our data shows no sex-differences between male and female rats in terms of total trial to criterion so we combined our male and female data. It was better if we used only male Sprague-Dawley rats to avoid sex (male’s dopaminergic system may respond differently to ID than does female’s dopaminergic system in rats) and phase-of-cycle effects (when estrogen concentrations are highest; proestrus and estrus). For example, rats show elevated sensitivity to locomotor activating effects of cocaine (Sell et al., 2000) and increased intravenous self-administration of cocaine (Hecht et al., 1999) during the cycle when estrogen concentrations are highest. Also, estrogen modifies the expression of D2 receptors (DiPaolo et al., 1988), as well as DAT sensitivity to cocaine (Post et al., 1987).

In relation to selection of specific time frame to our studies, it should be noted that, our studies are developmental studies so we have designed the sequence of tests with specific ages so as to try to minimize sequence confounds, avoid any possible interaction between the testing sequence and the dietary treatment, and to catch specific developmental time windows (see Table
1) With reference to using Sprague-Dawley rats in our study, the majority or ID research has been conducted in Sprague-Dawley rats, and our study serves to build on the current published literature.

We selected MePh in our pharmacological study as the prevalence of its use in USA to treat ADHD increased dramatically between 1990 and 1995 for both preschool (Zito et al., 2000) and school-age (Robison et al., 1999) children. Additionally, there is an evidence for improving set-shifting in ADHD children using MePh (Mehta et al., 2004). Further, there is some evidence that relates ADHD treatment by stimulants to substance (primarily tobacco) abuse (Lambert and Hartsough, 1998). Therefore understanding the interaction between developmental iron deficiency and treatment with dopaminergic agents is a critical issue in its own right.

Conclusions

In summary, these data support our hypothesis that in rats, early ID impairs their performance on an attention-related task, an effect that persists into adolescence. Furthermore, MePh “rescued” the phenotype, especially on the easier tasks and at the lower of the tested doses. To our knowledge, our findings are the first of their kind and may lead eventually to the successful treatment of persistent cognitive dysfunction characteristic of children and youths who suffered from ID early in infancy.
Acknowledgments

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Chapter 4

Effects of early iron deficiency on catecholaminergic transporters in rat brain

4.1. Introduction

Iron deficiency (ID) is the most common single nutrient disorder worldwide (ACC/SCN, 1992; DeMaeyer and Adiels-Tegman, 1985) and is associated with increased risk of delayed mental and motor development (Lozoff et al., 1991, 2000). Iron plays a vital role within the nervous system and is necessary for proper myelination, optimal metabolic activity and acts as a co-enzyme for monoamine neurotransmitter synthesis (Beard et al., 1993a, b; 1994). Furthermore, a number of reports have highlighted the importance of iron for normal neurotransmitter synthesis and regulation in rats (Beard, 2003; Beard et al., 2003b, 2006a; Burhans et al., 2005, 2006; Erikson et al., 2000; 2001) and mice (Salvatore et al., 2005).

Consequently early life (during infancy) ID is believed to be linked to impaired cognition, altered thermoregulation, neurodegenerative diseases (Beard and Connor, 2003; Beard et al., 2003a; Tran et al., 2008; Unger et al., 2007) including Parkinson’s disease (Powers et al., 2003) and restless leg syndrome (Earley et al., 2000), as well as impaired physical growth (Gambling et al., 2004; Shahbazi et al., 2009). The particular effects on behavior including cognition and affect may not be totally normalized by iron supplementation (Beard and Connor, 2003; Felt et al., 2006a; Lozoff et al., 2006; Shafir et al., 2008).

Investigators have performed many neurobehavioral analyses on iron deficient rats. For example, Beard et al., (2002) reported that ID rats showed more anxiety-like behavior, reduced exploration and a slower habituation rate in a new environment as compared to controls. These behavioral changes accompany changes in dopamine metabolism within the ID rat brain (Beard et
al., 1994). Furthermore, Beard and colleagues (2003b) found that neonatal iron deficiency downregulates nigrostriatal and mesolimbic dopamine receptors (Beard et al., 2003b). This is in accordance with other scientific reports that illustrate the effects of early ID on central dopamine receptor and DA transporter (DAT) densities in rats (Chen et al., 1995; Erikson et al., 2000; 2001; Nelson et al., 1997; Youdim and Green, 1978; Youdim et al., 1980) and in mice (Kwik-Uribe et al., 2000; Morse et al., 1999b). Too much iron can also be a problem, as other researchers showed that in rats dietary iron overload was associated with neurobehavioral dysfunction including decreased motor activity, reduced habituation of exploratory behavior and attenuated acoustic startle response (Sobotka et al., 1996).

Evidence from positron emission tomography (PET) studies in humans show that striatal DA neurotransmission is crucial for performance in tasks that require inhibitory control such as card sorting (Monchi et al., 2006). Similar observations in animals showed that striatal DA transmission is essential for any flexible shifting of response (O’Neill and Brown, 2007; Haluk and Floresco, 2009). Additionally, it has been reported that the fronto-striatal system is critical for executive control (Dalley et al., 2008; Robbins, 2005). From the neurochemical point of view, it is evident that catecholamines (DA, NE) play a critical role in modulating the prefrontal cognitive function (Arnsten, 1998). Further, DA depletion in marmosets impairs their ability to maintain attention to one of the perceptual dimension (Crofts et al., 2001). Despite the fact that the DA transporter shares many amino acid fragment sequences with other monoamine transporters (norepinephrine, serotonin), the effects of ID have focused almost exclusively on the DAT. Therefore, in this study, we investigated the possible effects of infantile ID on catecholaminergic membrane transporter densities in the brain, especially the dopamine transporter (DAT) and the norepinephrine transporter (NET). We examined DA and NE transporters densities within the rat brain at different age periods viz; 21, 45 and 75 days old using radioactive ligand binding.
4.2. Methods and materials

4.2.1. Subjects and dietary treatment

We purchased male and female Sprague–Dawley rat breeding stocks from Harlan Sprague–Dawley (Indianapolis, IN). Male breeders were fed rodent chow (Purina Mills Lab Diet 5001) containing 270 parts per million (ppm) Fe while female breeders were divided into 2 groups; one was fed a diet containing 80 ppm Fe (control, CN), and the other was fed a diet containing 4 ppm Fe (iron deficient, ID). We purchased Teklad custom diet pellets from Harlan Laboratories which included either TD.09588 iron adjusted diet (80ppm) as a the CN diet, or TD.80396 iron deficient diet (4ppm) as an ID diet. Both groups were given distilled water. At 200–220 g body weight, females were placed with males (1 male and 2 females/cage) for 5 days or until a vaginal plug was observed. Pregnant dams were then housed singly and checked daily for delivery. All dams were weighed weekly. The first day pups appeared was considered to be postnatal day 0 (PND0). At PND4, all pups were out-fostered; pups from control dams were out-fostered to other control dams or to iron-deficient dams, and pups from ID dams were euthanized. All pups were weaned at PND21 to Purina Rodent Diet (5001), containing 270 ppm iron, ad libitum until the time of sacrifice. All pups were pair-housed in clear plastic shoebox cages measuring 20cm×42.5cm with stainless steel lids in the University Vivarium. The rooms were temperature and humidity-controlled at 22±1 °C with an automatic 12/12 h light/dark cycles (light 0600–1800 h). All experimental animals were weighed weekly and monitored closely for health. All experimental protocols adhered to National Institutes of Health Animal Care Guidelines (1985) and were approved by the Pennsylvania State University Institutional Animal Care and Use Committee.
4.2.2. Brain dissection and sectioning

We followed the same procedure as described in Burhans et al., (2005; 2006). Animals were sacrificed by CO₂ followed by decapitation. We sacrificed the animals at 21, 45 and 75 days of age. The numbers of animals by sex (m/f) allocated to each age group by dietary condition are shown in Tables 9 and 10. There were uneven numbers of males and females for each experiment due to the limited availability of samples.

**Table 9:** Total number of animals by sex and diet assigned for DAT ligand binding:

<table>
<thead>
<tr>
<th></th>
<th>21d</th>
<th>45d</th>
<th>75d</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>-</td>
<td>8</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>3</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td><strong>ID</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>-</td>
<td>7</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Female</td>
<td>7</td>
<td>6</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>15</strong></td>
<td><strong>24</strong></td>
<td><strong>11</strong></td>
<td><strong>50</strong></td>
</tr>
</tbody>
</table>

*13 cases are deleted from statistical analyzer due to missing data: 2 cases from 21-day-old rats, 8 cases from 45-day-old rats, and 3 cases from 75-day-old rats.

**Table 10:** Total number of animals by sex and diet assigned for NET ligand binding:

<table>
<thead>
<tr>
<th></th>
<th>21d</th>
<th>45d</th>
<th>75d</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>-</td>
<td>4</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td><strong>ID</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>-</td>
<td>5</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>14</strong></td>
<td><strong>17</strong></td>
<td><strong>10</strong></td>
<td><strong>41</strong></td>
</tr>
</tbody>
</table>
The brains were rapidly removed from the skull and then divided mid-sagittally on ice. The brains were divided into 2 hemispheres however we used only the right hemisphere and kept the left one in reserve. The right hemisphere was immediately frozen in isopentane cooled by dry ice and stored at -80 °C until tissue sectioning. All brains were sectioned within 4-5 months of harvest. We cut serial sagittal sections, 20µm thick, starting at the midline at -19 °C using a Leica CM1950 cryostat (Leica Microsystems GmnH, Germany). The sections were placed on gelatin-coated slides with 2 sections per slide. These sections included individual brain regions specific for DAT; striatum, nucleus accumbens (NA), substantia nigra (SN), and olfactory tubercle (OT); and others specific for NET; frontal cortex (FC), dentate gyrus (DG), and locus coeruleus (LC). These brain regions were identified according to the mouse brain atlas by Swanson (1998). We prepared gelatin coated slides by immersion in 0.95% gelatin (VWR International, West Chester, PA) and 0.0014% chromium (III) potassium sulfate (Alfa Aesar, War Hill, MA) dissolved in double distilled H₂O. After that the slides were dried at room temperature over night, placed in sealed plastic bags, closed tightly and stored for 4 months at refrigerator until ligand binding.

4.2.3. DA transporter ligand binding

DA transporter ligand binding was performed as reported by Burhans et al., (2005) and Andrews et al., (1996). [¹²⁵I]-RTI-55 was purchased from Perkin Elmer (Boston, MA). The slides were incubated in a solution of [¹²⁵I]-RTI-55 (1098.7 µCi/ml, 2200 Ci/mmol) and protease inhibitor cocktail (PIC) diluted in a phosphate buffer (50mM NaH₂PO₄; 50mM NaHPO₄; pH 7.4) 1:10 for 90 min at 4°C. The solution also contained 10 µM fluoxetine hydrochloride (Eli Lilly Company, Indianapolis, IN) to block serotonin transporter binding. Non-specific binding was done in the presence of GBR 12935 (1 µM) and fluoxetine hydrochloride (10 µM). After the incubation period, the slides were washed 3 times in ice-cold fresh phosphate buffer for 5 min
each. Immediately following the final wash, the slides were quickly dipped once in ice-cold
double distilled H₂O to desalt the tissue and dried by a steady flow of air overnight at room
temperature. DAT slides and an autoradiographic [¹²⁵I] Microscale (Amersham Biosciences,
Picataway, NJ) were exposed to Kodak BioMax MR-1 film (Amersham Biosciences) at 4°C for
24 hours.

4.2.4. Norepinephrine transporter ligand binding

NET transporter ligand binding procedures were modified from those reported by Tejani-
Butt, (1992). Nisoxetine HCl [N-Methyl-³H] was purchased from Perkin Elmer (Boston, MA).
The slides were incubated in fresh ice-cold Tris buffer containing 1nM [³H]-Nisoxetine
hydrochloride (82 Ci/mmol) for 3 hours at 4°C. Non-specific binding was determined in the
presence of 1 µM desipramine (Sigma, USA). The slides were washed immediately after
incubation using fresh ice-cold Tris buffer (NaCl 300mM; KCl 5mM; andTris 50mn4 in dd H₂O)
3 times for 5 min each, quickly dipped once in ice-cold double distilled H₂O to desalt the tissue
then dried by a steady flow of air at room temperature overnight. Slides and a [³H] microscale
(Amersham Biosciences, Picataway, NJ) were exposed Kodak BioMax MR-1 film (Amersham
Biosciences, Picataway, NJ) at 4°C for 10 weeks.

4.2.5. Quantification of transporter ligand binding

We followed the procedures as described in Burhans et al., (2005). Ligand binding slides
were quantified using NIH Image (Bethesda, MD). The standard curve was based on the level of
radioactivity of the microscale on the day the film was developed. Individual brain regions were
outlined and the average amount of the bound radio-ligand was measured by NIH Image using
the standard curve and the Rodbard prediction equation that assumes curvilinear relationships of optical density and radio-ligand binding. For each individual rat, the amount of transporter was obtained by calculating the average of the specific binding sections (2 sections per slide) and subtracting the average of non-specific binding sections (2 sections per slide). Data were then averaged across treatment groups. The original data were expressed as nanoCuries, however the final binding values were reported in femtomes (fmol) of bound radioligand (refer to the appendix section for data conversion). It is important to note that not all sections were used to determine receptor binding because of folding, tears, etc., which explains the inconsistency of the animal numbers through various age groups. Furthermore, we repeated the ligand binding for some animals, which reduced the number of available slides for the subsequent binding experiment. This explains why the 21-day-old group includes only females in the following analyses.

4.2.6. Data analysis

Experimental data were expressed as fmol of bound radioligand. The values represent mean ± SEM. The total numbers of examined animals were 14, 17, and 10 representing 21 d, 45 d, and 75 days old rats respectively. The distributions of data were examined for outliers and for normal distribution (>3 SD from the mean), but no points needed to be removed for DAT and NET data. The transporter densities were subjected to multivariate analysis of variance (MANOVA) for two between-subject variables (diet, age), and multiple dependent variables for DAT (STR, NA, OT, SN) and NET (FC, DG, LC). Statistical significance was determined at α = 0.05. Tukey’s HSD post-hoc analyses were used when appropriate. All data were analyzed using SYSTAT 12 (SYSTAT Software, Inc., USA).
4.3. Results

4.3.1. DA transporters

MANOVA reveals a significant age effect on DEAT levels in the nucleus accumbens (NA), olfactory tubercle (OT), and substantia nigra (SN) \([F_{(2,31)}=7.54, \ p<0.05; \ F_{(2,31)}=23.22, \ p<0.05; \ F_{(2,31)}=12.32, \ p<0.05]\) respectively but not in the striatum. Specifically, 21 day old rats had greater DAT levels compared to 45 day old rats in the NA, OT and SN (\(p<0.05\) for all regions) as well as in the OT compared to 75 day old rats (\(p<0.05\)). There was no main effect for diet and no diet-age interactions (see Tables 11, 12, 13 Figures 15, 16, 17, 18).

Table 11: DAT ligand binding \(^{125}\text{I}-\text{RTI-55}\) in four brain regions of Sprague-Dawley rats at the age of 21 days

<table>
<thead>
<tr>
<th>Group</th>
<th>Striatum</th>
<th>Nucleus accumbens</th>
<th>Olfactory tubercle</th>
<th>Substantia nigra</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>13.59±1.87</td>
<td>10.54±1.04*</td>
<td>7.10±0.76*</td>
<td>6.17±1.37*</td>
</tr>
<tr>
<td></td>
<td>(n=8)</td>
<td>(n=8)</td>
<td>(n=8)</td>
<td>(n=7)</td>
</tr>
<tr>
<td>ID</td>
<td>17.34±4.05</td>
<td>14.14±3.14*</td>
<td>7.46±0.85*</td>
<td>4.16±0.95*</td>
</tr>
<tr>
<td></td>
<td>(n=7)</td>
<td>(n=7)</td>
<td>(n=7)</td>
<td>(n=6)</td>
</tr>
</tbody>
</table>

*Significant difference from 45 day old rats, \(p<0.05\).

CN: control, ID: iron deficient. Table of mean ± SEM.

Concentrations in fmol RTI-55.
**Table 12:** DAT ligand binding (\(^{125}\text{I}-\text{RTI-55}\)) in four brain regions of Sprague-Dawley rats at the age of 45 days

<table>
<thead>
<tr>
<th>Group</th>
<th>Striatum</th>
<th>Nucleus accumbens</th>
<th>Olfactory tubercle</th>
<th>Substantia nigra</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>8.99±1.84 (n=11)</td>
<td>6.36±1.12 (n=10)</td>
<td>2.83±0.67 (n=9)</td>
<td>1.8±0.47 (n=7)</td>
</tr>
<tr>
<td>ID</td>
<td>7.98±1.27 (n=13)</td>
<td>6.17±0.68 (n=13)</td>
<td>2.70±0.37 (n=13)</td>
<td>1.48±0.28 (n=9)</td>
</tr>
</tbody>
</table>

CN: control, ID: iron deficient. Table of mean ± SEM.

Concentrations in fmol RTI-55.

**Table 13:** DAT ligand binding (\(^{125}\text{I}-\text{RTI-55}\)) in four brain regions of Sprague-Dawley rats at the age of 75 days

<table>
<thead>
<tr>
<th>Group</th>
<th>Striatum</th>
<th>Nucleus accumbens</th>
<th>Olfactory tubercle</th>
<th>Substantia nigra</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>12.59±0.74 (n=5)</td>
<td>9.14±0.64 (n=5)</td>
<td>5.05±0.37* (n=5)</td>
<td>2.86±0.34 (n=4)</td>
</tr>
<tr>
<td>ID</td>
<td>13.98±0.56 (n=6)</td>
<td>10.68±0.45 (n=6)</td>
<td>5.04±0.31* (n=6)</td>
<td>3.51±0.60 (n=4)</td>
</tr>
</tbody>
</table>

*Significant difference from 21 day old rats, p<0.05.

CN: control, ID: iron deficient. Table of mean ± SEM.

Concentrations in fmol RTI-55.
**Figure 15:** DAT ligand binding ($^{125}$I-RTI-55) in striatum of Sprague-Dawley rats at different ages. CN: control; ID: iron deficient
Figure 16: DAT ligand binding ($^{125}$I-RTI-55) in nucleus accumbens of Sprague-Dawley rats at different ages. *Significant difference from 45 day old rats, p<0.05. CN: control; ID: iron deficient
**Figure 17:** DAT ligand binding (\(^{125}\text{I}\)-RTI-55) in olfactory tubercle of Sprague-Dawley rats at different ages.*Significant difference from 45 day old rats, p<0.05. †Significant difference from 21 day old rats, p<0.05. CN: control; ID: iron deficient.
Figure 18: DAT ligand binding (\(^{125}\)I-RTI-55) in substantia nigra of Sprague-Dawley rats at different ages. *Significant difference from 45 day old rats, p<0.05. CN: control; ID: iron deficient
4.3.2. Norpinephrine transporter

MANOVA revealed a significant age difference on NET levels in the dentate gyrus [F(2, 35) = 4.00, p<0.05] but not in the frontal cortex or the locus coeruleus. Specifically, NET levels were increased among 45 day old rats compared to 75 day old rats (p<0.05). There was no main effect for diet and no diet-age interaction on any of the dependent variables (see Tables 14, 15, 16; Figures 19, 20, 21).

**Table 14:** NET ligand binding ([H]-nisoxetine) in three brain regions of Sprague-Dawley rats at the age of 21 days

<table>
<thead>
<tr>
<th>Group</th>
<th>Frontal cortex</th>
<th>Dentate gyrus</th>
<th>Locus coeruleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>40.16±4.46 (n=8)</td>
<td>68.1±11.72 (n=8)</td>
<td>61.2±6.91 (n=8)</td>
</tr>
<tr>
<td>ID</td>
<td>53.31±4.61 (n=6)</td>
<td>78.52±7.08 (n=6)</td>
<td>60.91±5.19 (n=6)</td>
</tr>
</tbody>
</table>

CN: control, ID: iron deficient. Table of mean ± SEM.

Concentrations in fmol [H]-Nisoxetine.
**Table 15:** NET ligand binding ($^3$H-nisoxetine) in three brain regions of Sprague-Dawley rats at the age of 45 days

<table>
<thead>
<tr>
<th>Group</th>
<th>Frontal cortex</th>
<th>Dentate gyrus</th>
<th>Locus coeruleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>46.27±4.69 (n=8)</td>
<td>72.61±13.59* (n=8)</td>
<td>60.01±10.17 (n=8)</td>
</tr>
<tr>
<td>ID</td>
<td>57.48±10.82 (n=9)</td>
<td>91.90±10.94* (n=9)</td>
<td>61.22±5.45 (n=9)</td>
</tr>
</tbody>
</table>

*Significant difference from 75 day old rats, p<0.05.

CN: control, ID: iron deficient. Table of mean ± SEM.


**Table 16:** NET ligand binding ($^3$H-nisoxetine) in three brain regions of Sprague-Dawley rats at the age of 75 days

<table>
<thead>
<tr>
<th>Group</th>
<th>Frontal cortex</th>
<th>Dentate gyrus</th>
<th>Locus coeruleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>31.10±5.71 (n=5)</td>
<td>43.14±11.35 (n=5)</td>
<td>45.25±4.56 (n=5)</td>
</tr>
<tr>
<td>ID</td>
<td>52.34±13.23 (n=5)</td>
<td>51.39±9.82 (n=5)</td>
<td>73.65±12.26 (n=5)</td>
</tr>
</tbody>
</table>

CN: control, ID: iron deficient. Table of mean ± SEM.

Figure 19: NET ligand binding (³H-nisoxetine) in frontal cortex of Sprague-Dawley rats at different ages. CN=control; ID= iron deficient
Figure 20: NET ligand binding ($^3$H-nisoxetine) in dentate gyrus of Sprague-Dawley rats at different ages. *Significant difference from 75 day old rats, p<0.05. CN=control; ID= iron deficient
Figure 21: NET ligand binding (³H-nisoxetine) in locus coeruleus of Sprague-Dawley rats at different ages. CN=control; ID= iron deficient
4.4. Discussion

The current experiments yielded several interesting findings regarding dietary iron deficiency and brain functioning across the early life span. The first finding is that there is a significant age effect on DAT levels in the nucleus accumbens (NA), olfactory tubercle (OT), and substantia nigra (SN) but not in the striatum. Specifically, 21-day-old rats had greater DAT levels compared to 45-day-old rats in the NA, OT, and SN as well as in the OT compared to 75-day-old rats. The second finding is that there is a significant age difference on NET levels in the dentate gyrus but not in the frontal cortex or the locus coeruleus. Specifically, NET levels were increased among 45-day-old rats compared to 75-day-old rats. The final observation is that there was no main effect for diet and no diet-age interactions on DAT and NET levels.

Our results are in agreement with Burhans et al., (2005) who found that dietary treatment did not significantly affect NET ligand binding in both male and female rats. In contrast, our results are contradicted with those of Burhans et al., (2005; 2006) who reported the dietary effect of ID on DAT levels with the greatest concentration of DAT level in NA > OT > SN. Consistent with Burhans et al., (2005), our results show that the greatest concentration of DAT level in NA>OT>SN, the lowest DAT concentration was observed in the substantia nigra and the least NET concentration was reported in the frontal cortex. Given that frontal cortex is an important part of the attentional system (see Figure 1) and the substantia nigra considered a part of nigrostriatal pathway that controls movement, we can at least partially explain the ID-related deficiency in ASST performance by catecholamine deficiency in these two regions. It is possible that early ID affect the dopaminergic/noradrenergic balance in the fronto-striatal system involved in critical aspects of executive control (Dalley et al., 2008; Robbins, 2005).

Our results are consistent with the postnatal developmental pattern of DAT throughout different age groups. For instance, within the same dietary group e.g. CN or ID, our DAT results
showed a trend for DAT levels to be high at 21 days of age, after that, DAT levels declined at the age of 45 day old and finally elevated at the age of 75 day old. Such a pattern is nearly similar to the postnatal development of dopamine D1 receptors that increase in their level in rat striatum to a maximal level at PND35-40, followed by significant elimination of excessive receptors (pruning) to stable levels sustained into adulthood (Gelbard et al., 1989; Giorgi et al., 1987). This supports our time selection for induction of ID during the critical window of dopamine system differentiation (PND4-21) as the age of onset of the dietary iron deficiency may have an important impact on how much and where brain iron is lost, and on the possible reversibility with subsequent iron repletion. We did not observe the same pattern in NET levels, which explained by the presence of another developmental time window for NET that differs from DAT. Another explanation for this discrepancy is that NET might compensate to some extent for the reduction in DAT levels (Shukla et al., 1989).

Beyond the desire to replicate previous findings, we sought to relate our findings to the impaired performance of ID rats in ASST. Contrary to our expectations to find a significant dietary effect on DAT and/or NET levels within several examined brain regions, our data revealed no significant dietary effect on DAT and NET levels. However, we reported a significant age effect on DAT and NET levels. As we reported before (Mohamed et al., 2011) ID rats performed poorly in ASST as compared to CN at 45 day old with performance improvement at 65 day old age after MePh treatment. Although, there is a restoration of systemic iron dependent proteins like hemoglobin (Hb) and hematocrit (Hct), the impact of ID on the central nervous system is likely irreversible in this model. This explained by the fact that the effects of ID in utero or during lactation (i.e. preweaning) appear to be irreversible in terms of DA metabolism in rats (Beard et al., 2003b) as well as in mice (Kwik-Uribe et al., 2000). Furthermore, it is apparent that cognitive impairment may not attributed to a single neurotransmitter, but rather, alterations and interactions of several systems in different brain regions. Additionally, we could not explain
the poor performance of ID rats based on catecholaminergic levels in specific brain area per se; nonetheless, several brain areas are responsible for this poor performance. For instance, we did not find any significant dietary effect on striatal DAT levels. Despite that, there is evidence that variations in baseline striatal DA synthesis capacity alter individual human performance in reversal learning (Cools et al., 2009).

Possible Caveats and rationales

Needless to say, our study has important methodological limitations. First, we did not have enough biological brain samples to represent an equal number of males and females, therefore; we did not consider animal sex as a variable. Given that females are largely refractory to the effects of iron deficiency on DA receptors (Erikson et al., 2000; 2001), and male rats showed a greater effect of ID on DAT levels than did the female rats (Burhans et al., 2006) combining data from both male and female animals may affect our final conclusions. Second, we did not measure 5-HT transporter levels which might show some sort of compensation to the reduction of DAT or NET levels (Shukla et al., 1989) and also we were unable to measure DAT in PFC because of low density levels. Lastly, our 75 days old rats received methylphenidate (Meph) treatment for 15 days which might affect the results by inducing up regulation of DA and NE within the brain through inhibition of their reuptake (Kuczenski and Segal, 2002). Additionally, Meph blocks the DA and the NE transporter molecules; however it improves ID rat performance in ASST. Such improvements may be attributed to the possible reductions in regional cerebral blood flow in some of the fronto-parietal circuit with enhancement of the efficiency of information processing (Mehta et al., 2000). Also it is possible that MePh, via its actions on catecholamines, boosts signal-to-noise in PET (Mehta et al., 2000). There is evidence
that striatum is the most sensitive area in the brain to the DA-depleting effects of MePh (Eisch et al., 1992), which might explain the non-significant effect of diet on striatum at age of 75 days.

It is worth stressing once more that comparing our ligand binding data with that of Burhans et al., (2005) should be taken cautiously as they used 21 days old Sprague-Dawley rats and sacrificed males and females after 8 or 5 weeks of dietary treatment respectively. Additionally they made rats iron deficient post-weaning while in our model rats are ID at PND4 with out-fostering to ID dams (i.e. lactational).

**Conclusions**

In summary, early ID in rats alters many monoaminergic-mediated behaviors, including learning, spatial memory, and other complex tasks. Such changes might be irreversible despite the fact that there is a restoration of peripheral and/or central iron. Moreover, we examined only DAT and NET levels; however, other neurotransmitter systems may also be affected by early ID, and these systems need further attention in subsequent studies. It could be argued that levels of monoamine transporters are far less powerful predictors of the alterations in attention and animal performance in ASST. Future studies measuring monoamine transporter activities may highlight the effects of brain iron deficiency on various neural pathways with further defining the functional ramifications.
Chapter 5

Conclusions and Future Directions

General Conclusions

The high prevalence of ID, especially in developing countries, warrants urgent attention from clinicians, researchers and policy makers. ID impairs cognitive functions especially memory, learning and attention. Accordingly a full understanding of the complexity of iron regulation throughout the human body has a tremendous broad implication in our mental health. The studies of this dissertation were designed to examine the relationship between early (PND4-21) iron deficiency in rats and its neurobehavioral sequelae. We have demonstrated several novel findings regarding the neurobehavioral effects of early ID using a rat animal model as well as extending our knowledge regarding the underlying mechanism(s).

Our first aim explored the impact of early ID, during the critical window of DA system differentiation (PND4-21) on attention. Using attention set shift testing (ASST) apparatus, we found that early ID impairs the performance of ID rats on attention-related tasks, an effect that persists into adolescence. This effect is obvious from comparing the total number of ID animals failed to meet criterion in pre-drug baseline ASST to that of non-ID animals. These results are summed in table 17 and figure 22. Overall, we showed that ID rats performed more poorly than controls in all aspects of ASST testing with larger number of errors, longer response time and more total number of trials to criterion as compared to non-ID rats. This was a trace effect of having been iron deficient early in infancy; however, the behavioral difficulties persisted even though the blood and brain iron parameters were normalized. These results support our hypothesis that early iron deficiency has persistent effects on attentional processes. Thus, this
experiment is in strong agreement with suggestions from other research groups that ID in childhood may be one factor contributing to ADHD (Grantham-McGregor and Ani, 2001; Konofal et al., 2004; Oner et al., 2007; Oner and Oner, 2008).

**Table 17:** Numbers of animals failing to meet criterion in pre-drug baseline attention set shift testing.

<table>
<thead>
<tr>
<th>Animals</th>
<th>SD</th>
<th>CD</th>
<th>CD-R</th>
<th>EDS</th>
<th>ED-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>11</td>
<td>15</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>ID</td>
<td>37</td>
<td>38</td>
<td>36</td>
<td>28</td>
<td>33</td>
</tr>
</tbody>
</table>

**Figure 22:** Attention Set Shifting baseline performance between ID and CN animals. CN: control; ID: iron deficient
The second aim focused on the ability of methylphenidate (MePh) to ameliorate these effects induced by early iron deficiency. Because ID affects central DA (Beard et al., 2006; Bianco et al., 2008) and NE (Anderson et al., 2009; McEchron et al., 2010) neurobiology, we hypothesized that attentional deficits produced by ID might be treated effectively by catecholamine-related drugs, in our case, MePh, an agent that blocks presynaptic DA and NE reuptake. We observed that MePh did not improve the performance significantly in control animals; however, MePh did so significantly in ID animals and more dramatically in the simpler tasks. Generally, lower doses produced better performance than higher in ID animals, i.e. there was a tendency in this group for 1 mg/kg to be more effective in improving performance compared to 5 and 10 mg/kg doses. Although not significant across tasks, 10mg/kg of MePh tended to impair the performance in control rats and based on informal observations. This high dose tended to make the animals hyperactive and exhibited behaviors incompatible with obtaining reward. The impaired performance with 10mg/kg fits well with findings of Izquierdo et al., (2010) who reported an impairment of reversal-specific learning in rats after a binge (4×2mg/kg) regimen of methamphetamine using ASST. The large differences in MePh effect that we observed between ID and CN animals at all doses supports our hypothesis that at least to some extent, catecholamine neurobiology is involved in ID-based impaired cognition. MePh increases extracellular concentration of NE in the hippocampus and dopamine in the nucleus accumbens (Kuczenski and Segal, 2002). Our results in terms of enhancement of ID rats’ performance after MePh administration are consistent with MePh therapeutic effect in humans with ADHD (Biederman, 2005). Our results may be consistent with impaired DA, or perhaps, NE function, therefore, we designed the third aim to probe which of the major catecholamine pathways might be involved. To our knowledge, this is the first report to show that MePh can effectively treat cognitive-task performance deficits in rats produced by ID in early life as measured by attentional set shifting.
The third aim focused on exploring the underlying mechanism(s) responsible for impaired attention among ID rats. We examined different brain areas dedicated to attention for DAT and NET levels using ligand-binding technique. Results from these experiments reveal a significant age effect on DAT levels in the nucleus accumbens (NA), olfactory tubercle (OT), and substantia nigra (SN) but not in the striatum. Specifically, 21-day-old rats had greater DAT levels compared to 45-day-old rats in the NA, OT, and SN as well as in the OT compared to 75-day-old rats. Additionally, there is a significant age difference on NET levels in the dentate gyrus but not in the frontal cortex or the locus coeruleus. Specifically, NET levels were increased among 45-day-old rats compared to 75-day-old rats. However, there is no main effect for diet and no diet-age interactions on DAT and NET levels. These results are summed in Tables 18 and 19. These experiments demonstrated an important aspect regarding dietary iron deficiency and brain functioning: there is a significant age effect on DAT levels in certain brain regions. Our results are consistent with the postnatal developmental pattern of DAT throughout different age groups. This in turn supports our hypothesis for selection of a specific time window to start ID (PND4-21). There was no main effect for diet on DAT and NET levels throughout the examined brain regions. The later findings do not support our earlier hypothesis that deficits in DAT and NET levels are responsible for poor performance of ID rats in ASST. However, the reason for this may be due to using the ligand binding technique, which measures only the surface proteins. These experiments extend our knowledge to include NET as a potential place for ID effects within a specific developmental time window.
Table 18: DAT ligand binding ($^{125}$I-RTI-55) in four brain regions of Sprague-Dawley rats at the age of 21 days

<table>
<thead>
<tr>
<th>Group</th>
<th>Striatum</th>
<th>Nucleus accumbens</th>
<th>Olfactory tubercle</th>
<th>Substantia nigra</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>13.59±1.87</td>
<td>10.54±1.04*</td>
<td>7.10±0.76*</td>
<td>6.17±1.37*</td>
</tr>
<tr>
<td></td>
<td>(n=8)</td>
<td>(n=8)</td>
<td>(n=8)</td>
<td>(n=7)</td>
</tr>
<tr>
<td>ID</td>
<td>17.34±4.05</td>
<td>14.14±3.14*</td>
<td>7.46±0.85*</td>
<td>4.16±0.95*</td>
</tr>
<tr>
<td></td>
<td>(n=7)</td>
<td>(n=7)</td>
<td>(n=7)</td>
<td>(n=6)</td>
</tr>
</tbody>
</table>

*Significant difference from 45 day old rats, p<0.05.

CN: control, ID: iron deficient.

Table of mean ± SEM. Concentrations in fmol RTI-55.

Table 19: NET ligand binding ($^3$H-nisoxetine) in three brain regions of Sprague-Dawley rats at the age of 45 days

<table>
<thead>
<tr>
<th>Group</th>
<th>Frontal cortex</th>
<th>Dentate gyrus</th>
<th>Locus coeruleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>46.27±4.69</td>
<td>72.61±13.59*</td>
<td>60.01±10.17</td>
</tr>
<tr>
<td></td>
<td>(n=8)</td>
<td>(n=8)</td>
<td>(n=8)</td>
</tr>
<tr>
<td>ID</td>
<td>57.48±10.82</td>
<td>91.90±10.94*</td>
<td>61.22±5.45</td>
</tr>
<tr>
<td></td>
<td>(n=9)</td>
<td>(n=9)</td>
<td>(n=9)</td>
</tr>
</tbody>
</table>

*Significant difference from 75 day old rats, p<0.05.

CN: control, ID: iron deficient. Table of mean ± SEM.

Concentrations in fmol $[^3$H]-Nisoxetine.
While the novel contributions we made to ID by describing its effects on attention in an animal model are of value in their own right, ultimately the importance of our findings is in the context of iron-dopamine-attention connection. Our work shows the causal link between early ID and attention impairment accompanied with changes in DAT and NET levels in certain brain areas. Therefore our data are in agreement with others (e.g. Volkow et al., 2009) who support the hypothesis of an impairment of the DA pathway(s) in ADHD. Given that current treatment approaches for ADHD in children focus primarily on the dopaminergic system, these data allude to a potential new treatment strategy avenue. Consequently, iron supplementation may be used as a first line of treatment (or at least to be an adjuvant to stimulants to reduce their dose and/or their treatment period) for children with ADHD as iron therapy may be well tolerated and effective as compared to stimulants.

Our data helps in understanding of the interaction between developmental ID (considering its impact on dopamine pathways and related behavior) and treatment of children with dopaminergic agent. More relevant to our work is the matter of whether early nutritional status i.e. micronutrients especially iron can be a contributing factor to ADHD symptoms, dopaminergic drug efficacy (methylphenidate) or later drug abuse. From a public health perspective, such knowledge could be instrumental in drug abuse prevention programs, in drug therapy protocol development, or at least in reduction of drug abuse relapse. Because iron regulation in rats and humans is very similar, we expect that our findings could be extrapolated to human especially school age children.
Future Directions

In the last few decades, considerable progress has been made in order to understand the relationship of early iron deficiency to brain and behavior. All studies so far in humans and monkeys’ infants, and rats’ pups have focused on the differential effects of pre- vs. postnatal iron deficiency and their reversibility. It is clear from what we and others have shown that iron plays a crucial role in neurobiological processes with unclear neurobiological mechanism(s). The main work of this dissertation has shown the relationship between iron and attention with possible implications on DA and attention.

The association between central dopamine systems and drug abuse is well established. Because ID affects DA, it might also affect how animals respond to drugs. Erikson et al., (2000) showed attenuation of in-vivo and in vitro responses of ID rats to cocaine self-administration as compared to controls. Jones et al., (2002) examined the effects of severe ID on cocaine self-administration, the ID animals showed a retarded acquisition of i.v. cocaine self-administration, lowered break-point for progressive-ratio reinforcement schedules and altered dose-response responding. Additionally, there is some evidence for a link between childhood ADHD and subsequent substance use disorder among adolescents and adults (Wilson and Levin, 2005) including cocaine (Schubiner et al., 2000) and nicotine (Ohlmier et al., 2007). Therefore our future project in collaboration with Hershey Medical Center aims to examine the possible effects of early iron deficiency on cocaine distribution and clearance using an ID rat model as well as to examine whether the effect of iron deficiency is specific to cocaine rather than other abused drugs. It is important to note that in such study, the focus should include the pharmacodynamic as well as the pharmacokinetic aspect of drug self-administration.
We hope to probe some questions and extend our hypotheses in subsequent studies. For example, an area which warrants investigation is translation of our MePh treatment protocol into novel treatments for kids with attention deficits problems. We believe that we are still far from this as we are in need for further pharmacological studies on the effect of early ID on MePh profile including: dose response curve, toxicological, pharmacokinetic (absorption, distribution, metabolism) and pharmacodynamic ($t_{1/2}$, mode of action) studies. Also, there are many factors that should be taken in the account in such studies including; sex, ID duration, changes in its severity and its timing especially during developmental period.

In another line of research, we need to find the possible avenues of ID effects on synthesis and/or regulation of DAT that may involve, for example, compensatory changes in other monoamine metabolism, manipulation of DAT turnover and trafficking through synaptic PKC, gene regulation, modification of second messenger, and/or direct ligand receptor interaction. Also we are in need to account for the possible secondary effects of iron status on energy metabolism and micro-and macro-nutrients metabolism with a possible role in brain functioning and behavior. Moreover, we are in a desperate need to find the possible deleterious effects of ID during different critical windows of different organs and tissues to ameliorate many ID effects with proper nutritional intervention programs. Finally, we hope to address the similarities and differences between rat fetus and human infants in terms of the rate of neurogenesis, the amount of iron demands and the compensatory mechanisms to ID during early development to make our model more applicable to human condition.
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Appendix: Methods and protocols

A.1. Vertebrate Animals (Rats) handling

For the whole study, rats were used as the animal model. To achieve iron deficiency forty-five day old Sprague-Dawley rat breeding stocks were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and housed 2/cage. Upon arrival male breeders were fed rodent chow (Purina Mills Lab Diet 5001) containing 270 parts per million (ppm) Fe while female breeders were divided into 2 groups; one was fed a diet containing 80 ppm Fe (control, CN), and the other was fed a diet containing 4 ppm Fe (iron deficient, ID). At 200–220 g body weight, females were placed with males (1 male and 2 females/cage) for 5 days or until a vaginal plug was observed. Pregnant dams were then housed singly and checked daily for delivery. All dams were weighed weekly. All animals were maintained in a temperature (23 ± 2°C) and humidity (40%) controlled room on a 12:12 hr light/dark cycle (lights on at 0600).

All husbandry and testing were conducted in accordance with the recommended principles of laboratory animal care by National Research Council (2003). All protocols were reviewed and approved by the Penn State Institutional Animal Care and Use Committee prior to start of work. The Pennsylvania State University has an efficient, central animal resource facility, AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) approved, fully staffed with veterinarians and technicians. The animal use began at PND4 and continued throughout testing beginning at 50 days of age. All animals were examined daily and weighed weekly to monitor the health. Prior to behavioral testing, all animals were individually housed in transparent shoebox cages, acclimated to the colony room and handled daily by laboratory staff for one week.
A.2. Diets:

Male breeders were fed rodent chow (Purina Mills Lab Diet 5001) containing 270 ppm Fe while female breeders were divided into 2 groups; one was fed a diet containing 80 ppm Fe (CN), and the other was fed a diet containing 4 ppm Fe (ID). For the MePh experiment, we prepared the diet in our laboratory including special ingredients. This diet was prepared in our laboratory and followed the American Institute of Nutrition (AIN-93G) diets which met all nutritional requirements with the exception of the iron content for the low iron diet. For the rest of the experiment, we purchased Teklad diet pelts from Harlan laboratories either; TD.09588 (iron adjusted diet, 80 ppm) or TD.80396 (iron deficient diet, 4 ppm). All pups were weaned at PND21 to Purina Rodent Diet (5001), containing 270 ppm iron, ad libitum until the experiment was terminated. Distilled water was available from Nalgene cylinders fitted with silicon stoppers and stainless steel drinking tubes.
A.3. Hematocrit

2. Seal tube using crito-seal.
3. Spin the blood sample in a micro-capillary centrifuge for 5 minutes.
4. Measure the height of the red blood cell line.
5. Measure the total height of the sample.
6. Divide the height of red blood cells by the total height of the sample. Multiply by 100 this is % hematocrit.
A.4. Hemoglobin

1. Add 2.5 mL of Drabkin’s solution to 10 mL vials.

2. Collect 10 μL of blood through tail prick into a capillary tube and dissolve in Drabkin’s solution.

3. Read standards and samples at 540 nm on spectrophotometer.

**Standards (Stan Bio)**

To prepare standards add amount of cyanmethemoglobin standard to Drabkin’s reagent

<table>
<thead>
<tr>
<th>Standard</th>
<th>Standard amount (mL)</th>
<th>Drabkin’s reagent (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>E</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

Standards are stable for at least 6 months at 2-8 °C.

**Drabkin’s reagent (Sigma)**

Reconstitute 1 vial Drabkin’s reagent in 1 L of nano-pure H2O. Add 0.5 mL 30% Brj 35 solution (included in kit). Mix well and filter insoluble particles.
A.5. Gelatin coating slides (slide subbing procedure, porcine gel)

Supplies:

Glass slides 1x3, frosted, 1 mm thick, 6 to 8 boxes

Pig gel (Sigma Type A, G-2500) 3 gram

Chromium potassium sulfate (Fisher C337-500) 0.3 gram

D2O, 600 ml

80% EtOH to fill tub

Slide racks, specially used for subbing (10) and handle

Staining dish, specially used for subbing

Tray with pad for stacking racks

Plastic tubs

Glass funnel and filter paper

Detergent

Methods:

1-Place glass slides in racks. Save boxes for later use. Fill one plastic tub with warm water and soap, place racks with slides in tub and let soak for one hour.

2-Drain tub and rinse slides with running tap water until all suds are gone. Remove racked slides and place in another tub with 80% EtOH, allow to soak for 1 hour. Make sure all slides are submerged.

3-Clean other tub and fill with DZO. Transfer slides from the EtOH to the D2O tub and rinse in running D2O for several complete rinses. Allow to soak while gelatin is being made. EtOH will prevent gelatin from sticking to the glass slide.
4-Warm 150 ml of D2O to 50o C on a hot plate; add 3 g of gelatin while stirring. When mixed, add the remaining 450 ml of D2O and the chromium potassium sulfate (0.3 g). Mix, then, filtered directly into the staining dish.

5- Remove slide rack from D2O, drain excess water off rack, then place rack into staining dish with subbing solution. Let slides sit for 5 seconds, then remove from dish.

6-Drain rack and separate any slides that may have stuck together. Place rack on lab soaker to dry, angle slide racks to allow excess liquid to flow off slides and rack. May place in convection oven to dry at 34oC or let dry at room temperature.

7-When thoroughly dried, remove from racks and place back in original boxes and label with date and initials. Place in freezer for storage.
A.6. Brain sectioning steps by Cryostat

1-Take off frozen brain sample out off the freezer and put it for 30 min in the cryostat to take its temp which is about -19°C. Adjust for 20 µm section.

2-Stick the brain to the probe in the cryostat in an oblique direction (45 degree) with the olfactory area upwards and the occipital (cerebellum) downwards

3-We are interested to measure Dopamine transporter and D2 receptors, so, 10 slides per rats should be sufficient, with 2 sections per slide. That is 110 slides. These slides should be gelatin coated. This means that sections will be between 0.9 - 2.9 are most important. Dopamine protein levels are hard to measure in PFC using ligand binding. VMB, nucleus accumbens and striatum will need to be included. All of these regions can be analyzed on the same section (1.9-2.4 mm from midline). Usually we start at the level where anterior commissure starts to appear and will continue until its disappearance so that we included substantia nigra.

4-After slicing the section try to straighten it using the brush and then stick to the slide. Try one section at a time. You should paste both section to one side of the slide adjacent to one edge. Try to mark the slide that contains anterior commissure most clearly as it will be a landmark slide.

5-After finishing all slides …. let them to dry for 30 mn using slide warmer then put the opened slid rack inside the dissecator for 15 mn to remove any humidity then put the rack closed in a zopic bag with adding a tube (pierced from the top) filled with a material to remove any humidity….store the bag at -80 C. Remember to leave 2 empty slots between every animal slides set (set of 10).
A.7. Dopamine Transporter (DAT) ligand binding

**DAY 1**

A) Thaw appropriate amount of Protease Inhibitor Cocktail (PIC) buffer in refrigerator overnight.

B) Fill bottles (rinse them out first) with ddH2O, store in fridge overnight.

C) Make fresh 0.4M PO4 buffer

Combine buffers, pH 7.4

Dilute 0.5M PO₄ to 0.050 M PO₄ buffer solution

Need approximately 2.5L 0.050M PBS for dipping one set of slides,

Each additional set of slides will require approximately 1.5L PBS more.

Store PBS in 4°C overnight, must be cold for ligand binding.

D) Make sure you have adequate and proper radioactive waste disposal containers for next day.

This includes solid and liquid waste.

**DAY 2**

1. Dry slides by fan on low.

2. Determine total volume required in each beakers so that every section will be completely immersed in ligand solution. Ex. 120ml

3. Make fresh GBRI2935 (10mM) stock solution (0.00487g)

4. Make fresh fluoxetine (10mM) stock solution (0.00345g)

5. Make PIC/BSA solution: 50mg bovine albumin (stored in 4°C) to 50ml cold PIC solution. Stir solution slowly to avoid bubbles.

To determine volume of PIC/BSA solution you will need: will make a 1:10 PIC/BSA:
PO₄ mixture in each beaker for DAT and NSB. Aliquot solution into disposable plastic beaker! Keep solution cold on ice

Ex. If need 100 ml total volume, will add 10 ml PIC/BSA solution to 90ml PBS.
10 ml PIC/BSA for each DAT and NSB = 30ml + 3 ml excess =33 ml PIC/BSA

6. Add ligand (RTI-55) (stored in -4°C) to PIC/BSA solution under acid hood! Cover with foil, label with radioactive tape, swirl solution gently to mix (6.21 µl)

\[
\left(\frac{1098.7 \text{ mci}}{1000}\right) \left(\frac{1000}{707}\right) \left(\frac{1 \text{ mL}}{10^3 \mu L}\right) \left(\frac{1 \text{ Ci}}{10^3 \text{ mCi}}\right) \left(\frac{3.7 \times 10^{10}}{\text{Cis min}}\right) \left(\frac{0.7 \text{ cpm}}{\text{dpm}}\right) = 2414964.3
\]

\[
(2414964.3 \times \mu \text{L}) = \left(\frac{150.000}{100}\right)(10 \times 10^3) = 6.21 \mu \text{L}
\]

7. Count sample in scintillation counter. Add 100ul ligand PIC/BSA solution to 5 mL Ecoscint A scintillation fluid. Counts should be around 150,000 cpm. With instrumental efficiency of 97%

8. On ice, add appropriate volume 0.050M PBS to DAT, NSB (non specific binding) disposable beakers.

9. To each beaker add PIC/BSA ligand solution.

10. To DAT beaker add fluoxetine (10uM) to block SERT.

11. To NSB beaker add fluoxetine (l0uM) to block SERT. Use same volumes as in steps 8 & 9.

12. Pour ligand solution into appropriate Tissue Tek slide boxes. Make sure boxes are labeled correctly and have radioactive caution tape. Cover boxes with lids, put in 4°C fridge for 30 minutes.

13. Inoculate slides in ligand solution for 90 minutes in 4°C refrigerator.

14. Wash slides in cold 0.05M PBS for 5 minutes, 3 times.

15. Radiacwash first three PBS slide boxes two times, rinse well with dd H₂O.

16. Dip slides in cold dd H₂O to desalt (in the three radiacwashed boxes).
17. Dry slides by fan on low overnight.

18. Repeat steps #13-17 for each additional set of slides.

**Film Exposure and Data Analysis**

Slides will be exposed to Kodak Bio Max MR-1 film at 4°C for 24 hours

**Calculations:**

- Specific activity for $^{125}$I-RTI-55 = 2200 Ci/mmol
- Concentration of $^{125}$I-RTI-55 = 1098.7 µCi/ml
- Half life = 60 days
- Volume of $^{125}$I-RTI-55 used in ligand binding = 6.21 µl
- Conversion of data from nanoCuri into fmol:

  Specific activity = 2200 Ci/mmol

  1 Ci = $2.2 \times 10^{12}$ cpm = 1 m mol

  = $2.2 \times 10^9$ = 1 umol

  = $2.2 \times 10^6$ = 1 nmol

  = $2.2 \times 10^3$ = 1 pmol

  = $2.2 \times 10$ = 1 fmol

  1 Ci = $2.2 \times 10$ = 1 fmol

  $2.2/1 = 10/X$ ...... so $X = 10/2.2 = 4.54 \times 10^{-12}$ mol = $4.54 \times 10^{-1}$ fmol = 0.45 fmol

Therefore, I multiplied data by 0.45 to express data in fmol
A.8. Norepinephrine Transporter (NET) ligand binding

DAY 1

A) Fill bottles (rinse them out first) with ddH₂O, store in fridge overnight. Amount depends on how many sets of slides will be inoculated on following day. Approx. 1 L for one set of slides.

B) Make fresh Tris buffer: NaCl 300mM; KCl 5mM; and Tris 50mM in ddH₂O.
   
   If making 2.0 L (enough for dipping one set of slides = 3 full racks)
   Add to ddH₂O (leave room for pH corrections and then fill to 2.0L after pH is complete):
   12.11 g Trizma base; 35.1g NaCl; and 0.75g KCl
   PH buffer to 7.4

   Need approximately 2.0 L Tris buffer for dipping one set of slides, each additional set of slides will require approximately 1.5L PBS more.

   Pour Tris buffer into 500ml bottles and store in 4°C overnight; must be cold for ligand binding.

C) Make sure you have adequate and proper radioactive waste containers for next day.

   This includes solid and liquid containers.

DAY 2

1. Dry slides by fan on low.

2. Determine total volume required in each beaker so that every section will be completely immersed in ligand solution. Ex. 100ml

3. Make fresh Desipramine (10mM) stock solution, 3.028 mg

4. On ice, add appropriate volume buffer (usually 120ml) to NET and NSB Tissue Tek slide boxes.
5. To NSB beaker add 1µM Desipramine from fresh Desipramine stock solution to block NET binding. Desipramine dilution – 1µM, 12µl Desiperamine into NSB beaker of 120ml Tris buffer

\[ M_1V_1 = M_2V_2 \]

\[ (10 \times 10^3 \mu M) \times \mu L = (1\mu M)(120 \times 10^3 \mu L) \]

\[ \times \mu L = \left( \frac{(1\mu M)(120 \times 10^3 \mu L)}{10 \times 10^3 \mu M} \right) \]

= 12 µL Desipramine into NSB beaker of 120mLs Tris buffer

6. Add 24 µl of [³H]-Nisoxetine HCl ligand in acid hood to buffer solution in Tissue Tek slide boxes. Make sure boxes are labeled correctly (NET and NSB) and have radioactive caution tape. Cover boxes with lids, put in 4°C fridge for 30 minutes. *If you have time, this would be a good thing to do to ensure ligand is distributed equally throughout solution.

\[
\left( \frac{1 \text{ mCi}}{\text{mL}} \right) = \left( \frac{\text{mmol}}{82\text{Ci}} \right) = 12.2 \mu M
\]

\[ (12.2 \mu M) \times \mu L = (3nM)(100\mu L) \]

\[ \times = 24 \mu L \text{ into 100ml of buffer} \]

7. Inoculate slides in ligand/buffer solution for 4 hours in 4°C fridge.

8. Wash slides in cold Tris buffer for 5 minutes x 3 times.

9. Radiacwash first three buffer "rinse" slide boxes two times and rinse well with dd H₂O.

10. Dip slides in cold ddH₂O to desalt (in the three radiacwashed boxes).

11. Dry slides by fan on low overnight.

12. Repeats steps# 9 - 13 for each additional set of slides.

**Film Exposure and Data Analysis**

Slides will be exposed to Kodak Bio Max MR-1 film at 4°C for 4 weeks.
**Calculations:**

- Specific activity for [³H]-Nisoxetine HCl = 82 Ci/mmol
- Concentration of [³H]-Nisoxetine HCl = 1 mCi/ml
- Volume of [³H]-Nisoxetine HCl used in ligand binding, 24 µl into 100 ml of buffer
- Conversion of data from nanoCuri into fmol:

I followed the same steps as in DAT. However due to different specific activity = 82 Ci/mmol, I multiplied data by 12 to reach to fmol.
### A.9. Statistical Output

**DAT AMANOVA**

<table>
<thead>
<tr>
<th>Effect</th>
<th>Value</th>
<th>F</th>
<th>Hypothesis df</th>
<th>Error df</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>Pillai's Trace</td>
<td>.915</td>
<td>75.531&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.000</td>
<td>28.000</td>
</tr>
<tr>
<td></td>
<td>Wilks' Lambda</td>
<td>.085</td>
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a. Exact statistic
b. The statistic is an upper bound on F that yields a lower bound on the significance level.
c. Design: Intercept + DIET + AGE + DIET * AGE
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a. R Squared = .212 (Adjusted R Squared = .085)
b. R Squared = .380 (Adjusted R Squared = .280)
c. R Squared = .607 (Adjusted R Squared = .543)
d. R Squared = .484 (Adjusted R Squared = .400)
### NET MANOVA

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**a.** Exact statistic  
**b.** The statistic is an upper bound on F that yields a lower bound on the significance level.  
**c.** Design: Intercept + DIET + AGE + DIET * AGE
## Tests of Between-Subjects Effects

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a. R Squared = .129 (Adjusted R Squared = .005)
b. R Squared = .228 (Adjusted R Squared = .118)
c. R Squared = .114 (Adjusted R Squared = -.013)
CURRICULUM VITAE

Wael M.Y. Mohamed

Education and Training:

Academic

2006 - Present  Ph.D. Candidate, Interdisciplinary Graduate Programs (Neuroscience)  The Pennsylvania State University, USA

2003 - 2006  M.Sc., Clinical Pharmacology  EGYPT

1996  M.D.  EGYPT

Clinical Training

1998 - 1999  General Practitioner  Health Ministry, EGYPT

1996 - 1998  Medicine Intern (H.O.)  Menoufiya University Hospital, EGYPT

Neurosurgery Practicum

Awards/Fellowships:

2010-2011  Graduate Assistantship  – Penn State Hershey Medical Center, College of Medicine

2006-2010  PhD. Graduate Fellowship  - Egyptian Ministry of Higher Education

September-2006  Student Travel Award  - Safety Pharmacology Society (SPS) 6th Annual Meeting, San Diego, California, USA

Grants and Funding:

Penn State CYFC (Children, Youth and Families Consortium) 423-15 1001 CYFC. Early Iron Deficiency and Attentional Deficits: From Basic Science to Treatment. October 2008 to October 2010 (Research Associate). Principal Investigator: Dr. Byron C Jones.

Membership in Professional Organizations:

2010-present  National Academy of Neuropsychology (NAN)

2009-present  American Neuropsychiatric Association (ANPA)

2008-present  The Society for Neuroscience (SfN)

2006-present  American Society for Pharmacology and Experimental Therapeutics (ASPET)

Selected peer-reviewed Publications (in chronological order):


Mohamed WM, Hamida SB, de Vasconcelos AP, Cassel JC, Jones BC. Interactions between3,4-Methylenedioxymethamphetamine and Ethanol in Humans and Rodents. Neuropsychobiology. 2009; 60(3-4):188-94.