Neurobehavioral analysis of developmental iron deficiency in rats

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Abstract:

Iron deficiency (ID) in early life alters the course of behavioral and cognitive development in humans, causing decreased physical activity and responsiveness to the environment. The effects of ID on behavior are similar in rats and hypothesized to be related to ID-related impairments in central dopamine pathways. The objective of this study was to examine the association between brain iron measures of dopamine function, and behavioral measures of activity and reactivity. Male and female weanling rats were fed either an iron deficient diet or control diet for 6 weeks. The iron deficient rats showed significantly decreased activity and increased anxiety-like behaviors. Iron deficient rats also showed significant decrements in brain iron content in the corpus striatum, prefrontal cortex, and midbrain and decreases in dopamine receptors and the transporter in the same areas. Multiple regression analysis showed ventral midbrain iron concentration and dopamine D_1 receptor density to be highly associated with exploration and repeated movements, respectively. In addition, the results showed anxiety-like behaviors to be related to prefrontal cortex dopamine transporter and dopamine D_1 receptor densities. We conclude from these analyses that iron concentration in dopamine containing regions and densities of dopamine receptors and the transporter, are significant predictors of measures of activity and reactivity. These observations also strengthen the argument that the Fe-dopamine link is fundamental to understanding biobehavioral difficulties seen in children with ID anemia.
Keywords: Iron deficiency; Rat; Sex; Dopamine; Behavior; Multiple regression

Article:
1. Introduction

Iron deficiency (ID) is reported to be the most prevalent nutritional problem in the world today with an estimated 5 billion people so afflicted [1]. Among the numerous biological effects of iron, there is considerable evidence that iron is also important for neurological functioning [13,18,24,31]. Such functions include neurotransmitter metabolism [3,4,34], myelin formation [16], and brain energy metabolism [6,15,23,25]. Recent studies of auditory evoked potential changes in iron deficient infants point to possible irreversibly slowed central processing [28,30].

In rats, ID begun after weaning produces a significant decrease in brain iron content that is reversible with subsequent iron repletion [6,23]. Moreover, decreased brain iron content during this period causes altered dopaminergic functioning [3,4,22,34]. These effects include decreased D_1 receptor, D_2 receptor, and dopamine transporter (DAT) densities in the caudate-putamen [7,33], decreased D_2 receptor and DAT densities in the nucleus accumbens [7], and increased extracellular dopamine in the caudate putamen [3,4,22]. It is important to note that these alterations in dopamine function result from decreased brain iron content per se and not anemia [2,22] and may be reversible with iron treatment [22,33].

Behavioral effects of ID include decreased physical activity [10,12,33,34], decreased exploration in a novel environment [31], reduced stereotypic behaviors [12,33,34], and reversed diurnal behavioral activity patterns [33].

Motor activity, exploratory behavior, and stereotypy are considered to be dopamine related behaviors [14,27,29] and are adversely affected by ID anemia [10,12,33,34]. In this study, we also investigated the effect of ID in rats

on performance in the light: dark box test for anxiety-like behavior. We conducted these experiments to test the hypothesis advanced in some human studies of ID-related increases in anxiety and hesitancy, but not yet explored in controlled rodent studies [18,19].

The purpose of this study is twofold. First, we extend our knowledge about the behavioral effects of ID and second we report the results of correlational analysis by multiple regression of brain neurobiology related to ID to specific behavioral outcomes.

2. Methods

2. 1. Animals

Male and female 21-day-old Sprague—Dawley rats (Harlan Sprague—/Dawley, Indianapolis, IN) were randomly divided into two dietary treatment groups: control (35 mg Fe per kg diet) and iron-deficient (3 mg Fe per kg diet). The semi-purified diets were prepared as described previously and their content of iron verified [23]. Rats had free access to food and water 24 h/day, and the lights were turned off between 18:00 and 06:00 h. Room temperature was maintained at $25 \pm °C$. After 4 weeks of dietary treatment, the rats were used for behavioral studies. The Pennsylvania State University Animal Care and Use Committee approved all of the animal procedures.

2.2. Light/dark box test (n = 5 per diet and sex condition)

2.2. 1. Apparatus

Light/dark box activity was measured using a Digiscan Animal Activity Monitor, model RXYZCM (Omnitech Electronics, Columbus, OH). The apparatus consisted of a set of four 40 x 40 x 30.5 cm Plexiglas^{TM} boxes with vertical and horizontal infrared sensors encased in wooden boxes illuminated with 100-W bulbs. The dark compartment consisted of a darkened acrylic box in the right half of each of the four boxes; the light was oriented to illuminate the light side of the box and to minimize the light entry into the darkened acrylic side of the box. Thus, one half of each box was brightly illuminated and the other half was relatively dark.

2.2.2. Protocol

All animals were tested naïve and all testing was performed between 10:00 and 14:00 h. Testing began with placing the animals in the light side of the box. Data were collected for 15 min in three five-min intervals. Variables measured were:

Time in light and dark.
    Time spent in the lighted or dark side of the box recorded in seconds.

Latency to enter dark side.
    Time in seconds required for the animal to cross from the light side to the dark area of the box.

2.3. Locomotor activity and exploration protocol (n =20 per diet and sex condition)

2.3.1. Apparatus

Locomotor activity was measured using a Digiscan Animal Activity Monitor; model RXYZCM (Omnitech Electronics). The apparatus consisted of a set of four 40 x 40 x 30.5 cm Plexiglas^{TM} boxes with vertical and horizontal infrared sensors. The flooring was an elevated acrylic platform with 16 equally spaced holes of 1.5 cm in diameter.

2.3.2. Protocol

These animals were also part of another study on the effects of ID on cocaine sensitivity. All animals were tested on 2 consecutive days between 10:00 and 14:00 h. On day 1 they received an i.p. injection of saline at a volume of 1.0 ml/kg and on day 2 they received cocaine by the same route. The cocaine data will be reported elsewhere (unpublished data).

2.4. Behavioral data recorded included

Total distance traveled (cm)
    The distance traveled (cm) by the animal in the 30- min period [9].

Number of repeated movements
    Number of same photobeam breaks repeatedly recorded. This corresponds to stereotyped movements.

Center time (seconds)
    Time spent by the animal away from the walls of the box. This measure is complementary to thigmotaxis (wall-seeking) a putative measure of anxiety [29].

Number of nosepokes
    Each time the animal breaks the beam of the vertical sensor, this measure is incremented by one.

Rate of habituation to a novel environment
    This measure was calculated by regressing distance traveled versus time during each 5-min data collection period. The data used were the regression coefficients (slopes).

2.5. Body weights, hematology and liver non-heme iron

Upon completion of the behavior testing, the rats were killed by decapitation, livers removed for non-heme iron determination. Blood was collected from the trunk of the animal, hemoglobin and hematocrit were measured in fresh blood and plasma as detailed elsewhere [23]. The plasma was frozen at -20 °C until it was analyzed for Fe, and total iron binding capacity by established procedures [6,23].

2.6. Neurochemical measures

The methods utilized have been described in detail elsewhere [7] and were used in this study for multiple regression analysis to explore functional relationships among brain iron status, dopamine receptor densities and behaviors related to ID.

2.7. Data analysis

Data were analyzed using the SAS system for Windows v 6.12 statistical analysis package (SAS, Cary, NC). Analysis of variance for a two between-subjects variables experiment was used to test effects of dietary treatment and sex on light/dark activity and locomotor activity data. The magnitude of effect for main effects (diet and sex) and the interaction terms was evaluated using estimated ω^2 [11]. Alpha level for the analyses was set at $P < 0.05$. Stepwise multiple regression was used to assess associations among neurochemical variables (brain iron and dopamine receptor/ transporter densities) and behaviors sensitive to ID.

Table 1
Hematological and liver non-heme iron data

Group		Body weight (g)	Hb (g/l)	Serum Fe (µmol/l)	TIBC (µmol/l)	Tf sat%	Liver Fe (µmol/g) tissue
CN-F	Mean	213.6[a]	157.3[a]	35.1[a]	85.1[a]	41.5[a]	9.55[a]
	S.E.M.	6.66	5.14	5.97	7.7	8.9	1.37
ID-F	Mean	180[b]	65.2[b]	15.8[b]	114[b]	13.6[b]	3.27[b]
	S.E.M.	3.47	2.88	2.1	10.8	2.6	0.48
CN-M	Mean	323.8[c]	151.4[a]	23[c]	79.4[a]	27.1[a]	5.40[c]
	S.E.M.	7.45	2.15	2.7	3.34	3.8	0.57
ID-M	Mean	193.4[d]	53.3[c]	13.7[b]	130.3[b]	11.1[b]	2.99[b]
	S.E.M.	4.82	2.24	3.5	18.7	3.7	0.61

Superscript letters signify statistical significance $P < 0.05$. TIBC, total iron binding capacity, Tf sat%, percent of transferrin saturation. CN-F, control female, ID-F, iron deficient female, CN-M, control male, ID-M, iron deficient male.

Table 2

Brain iron, DA receptor, and DA transporter densities in four brain regions of adult Sprague–Dawley rats

	Cp-Fe (nmol/g)	NA-Fe (nmol/g)	VMB-Fe (nmol/g)	PFC-Fe (nmol/g)	CP-D$_2$R (fmol/mg)	NA-D$_2$R (fmol/mg)	VMB-D$_2$R (fmol/mg)	PFC-D$_2$R (fmol/mg)	CP-D$_1$R fmol/mg protein	NA-D$_1$R fmol/mg protein	VMB-D$_1$R fmol/mg protein	PFC-D$_1$R fmol/mg protein	CP-DAT (fmol/mg)	NA-DAT (fmol/mg)	VMB-DAT (fmol/mg)	PFC-DAT (fmol/mg)
ID	212±26*	223±87	133±32**	152±91**	1392±134&**	1029±138&	109±12*	164±28*	279±35	279±21	74±9	108±12	15221±1572*	13872±855	2410±77&*	2785±134
CN[a]	328±46	188±21	362±44	248±76	1676±260	1085±52	132±9	91±25	337±24	302±25	66±5	90±3	20344±2339	17359±1753	2744±185	2711±109

Table of mean±S.E.M. *, significantly different from control rats, $P < 0.075$; **, significantly different from control rats, $P < 0.001$; &, significant sex × treatment interaction, $P < 0.01$.

[a] ID, iron deficient rats ($n = 16$, equal numbers of males and females), CN, control rats ($n = 16$, equal numbers of males and female rats).
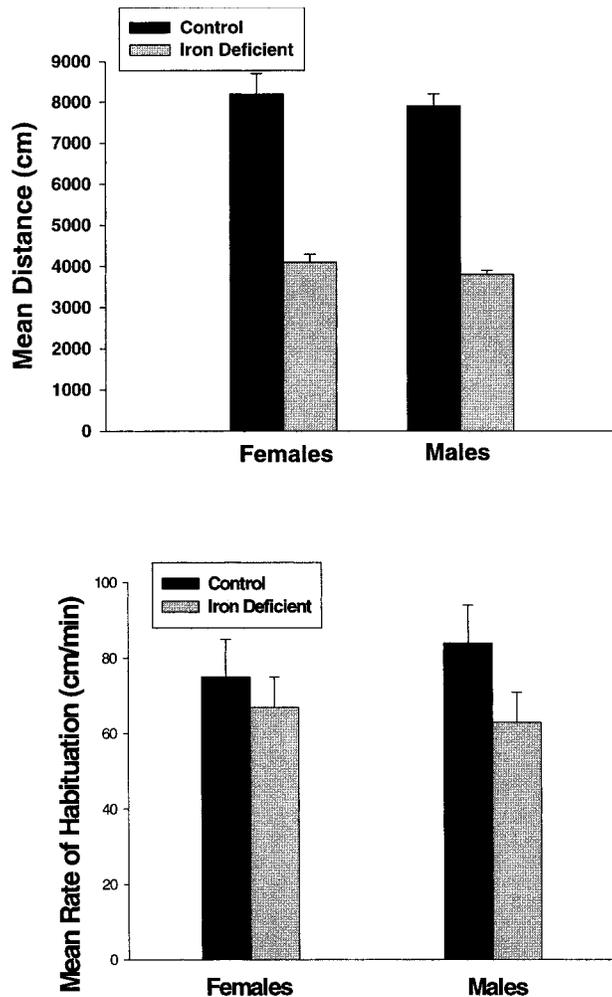
Fig. 1. (Top panel) ID caused a significant decrease in ambulation ($P < 0.001$). (Bottom panel) ID significantly ($P < 0.05$) attenuated the rate of habituation in locomotor activity to a novel environment.

2.8. Results
2.8. 1. General biological effects of iron deficiency

As expected, and as illustrated in Table 1, dietary ID caused significant anemia, lowered body weight, and decreased liver iron stores in rats of both sexes. In addition, it can be seen that dietary ID was associated with a significant reduction in brain iron content in three out of four brain regions examined with only the nucleus accumbens not being affected by ID (Table 2). The densities of D_2 and D_1 dopamine receptors varied significantly by region examined; however, not all regions were equally affected by poor iron status. We observed significant, but modestly lower D_2 receptor densities in caudate putamen and ventral midbrain. The D_1 receptor densities were not significantly altered by iron status. The density of the DA transporter was modestly reduced in several brain regions, namely the caudate putamen and the ventral mid-brain. Significant sex-by-treatment interactions were noted for several variables though no consistent interaction pattern was observed.

2.8.2. Locomotion, exploration and anxiety-like behaviors

2.8.2.1. Ambulation. As presented in Fig. 1, top panel, iron deficient rats evinced significantly decreased loco-motion than did controls ($F_{1,76} = 158.6$, $P < 0.001$, estimated $\omega^2 = 0.65$).

2.8.2.2. Habituation. As illustrated in the bottom panel of Fig. 1, the rate of habituation in locomotor activity to the novel environment of the activity boxes was significantly slowed in iron deficient rats ($F_{1,76} = 6.74$, $P < 0.05$, estimated $\omega^2 = 0.08$). Adjustment for baseline levels of activity as a covariate did not significantly alter the relationship of habituation to iron status.

2.8.2.3. Nosepokes. ID resulted in a non-significant trend toward a decrease in this exploratory behavior as shown in Fig. 2, top panel ($F_{1,76} = 3.02$, $P < 0.10$). However, there was a significant sex effect with females producing more nosepokes than did males ($F_{1,76} = 7.56$, $P < 0.01$, $\omega^2 = 0.07$).

2.8.2.4. Repeated movements. Fig. 2, bottom panel shows that ID reduced repetitive movements in males and in females ($F_{1,76} = 102.78$, $P < 0.001$, estimated $\omega^2 = 0.52$). A significant treatment by sex interaction showed males to be more affected by ID in this measure than females ($F_{1,76} = 10.00$, $P < 0.01$, estimated $\omega^2 = 0.05$).

2.8.2.5. Center time. Iron deficient animals spent significantly less time in the center of the activity monitor than did the controls (Fig. 3) ($F_{1,76} = 16.20$, $P < 0.01$, estimated $\omega^2 = 0.16$).
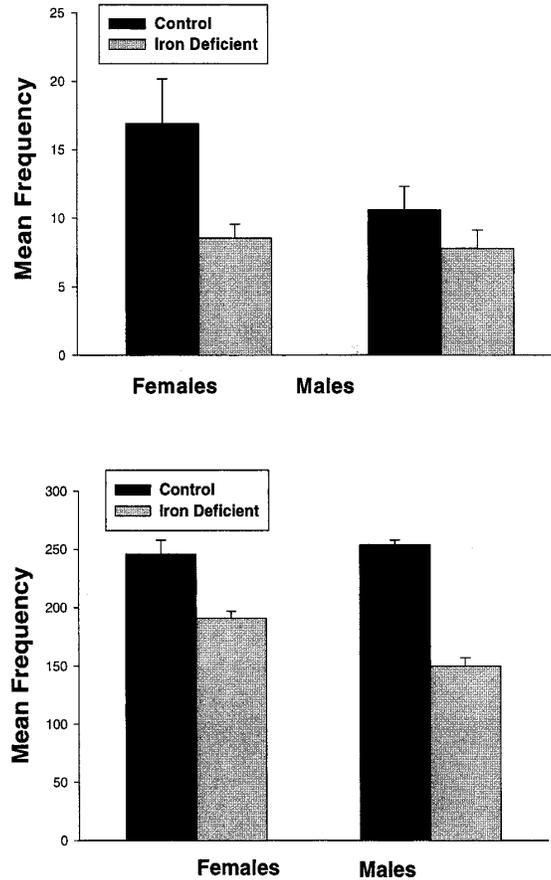

Fig. 2. (Top panel) Female rats significantly produced more nose-pokes than male rats ($P < 0.01$), but ID had no effect. (Bottom panel) ID significantly reduces the number of repetitive movements in both male and female rats ($P < 0.001$), however this effect affected male rats more than female rats ($P < 0.01$).
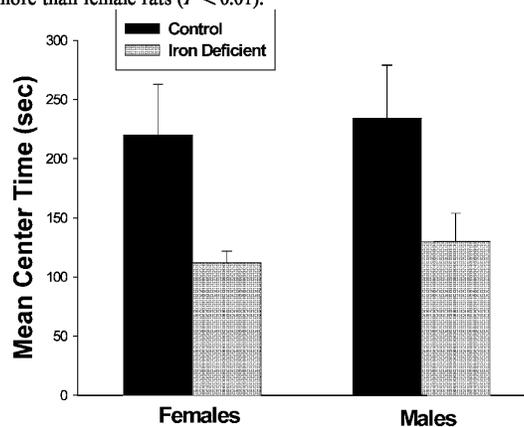

Fig. 3. Iron deficient rats spent significantly less time in the center of the box than did control rats ($P < 0.01$).

2.8.3. Light—/dark box

ID did not have a significant effect on time spent in the light compartment (Fig. 4, upper panel); however, it did significantly affect the rapidity at which the rats moved into the dark compartment (lower panel) ($F_{1,19} = 5.21$, $P < 0.05$, estimated $\omega^2 = 0.24$). Iron deficient male rats moved into the darkened area about four times more quickly than did the controls demonstrating a particularly strong effect of iron status on latency to enter the dark.
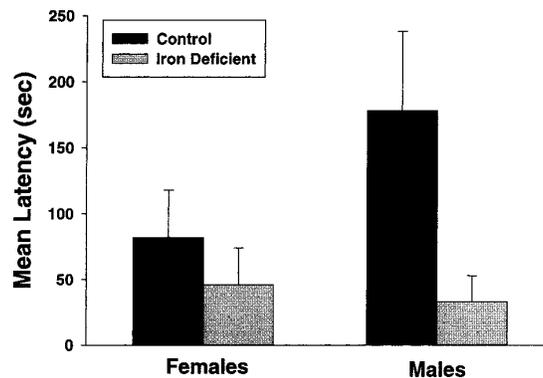

Fig. 4. (Top panel) ID had no effect on time spent in the light compartment, however iron deficient rats moved into the darkened area (latency) more quickly than controls ($P < 0.05$) (Bottom panel).

2.8.4. Neurochemical indices

Iron-related neurobiological measures included brain iron content, dopamine D_1 and D_2 receptors and the transporter in medial prefrontal cortex, nucleus accumbens, and caudate-putamen and ventral midbrain. The data for these measures in response to ID are summarized in Table 2.

2.8.4. 1. Multiple regression analysis. Stepwise multiple regression (forward stepping with criterion of alpha= 0.05) of distance traveled revealed three significant predictor variables with a combined R^2 of 0.65 (Table 2). The most salient of the predictors was the concentration of iron in the ventral midbrain, accounting for 52% of the variance, followed by the density of dopamine D_1 receptors in the ventral midbrain and density of D_1 receptors in the caudate-putamen. A similar pattern was observed for repeated movements, but with the third factor being density of D_1 receptors in the medial prefrontal cortex for a total of 65% of the variance explained. Rate of habituation to a novel environment was most influenced by the concentration of iron in the medial prefrontal cortex with a R^2 of 0.285, followed by iron concentration in the nucleus accumbens and iron concentration in the ventral mid-brain for a total of 49% of the variance explained. The frequency of nosepokes was influenced by iron concentration in the medial prefrontal cortex only, $R^2 = 0.10$. Center time was influenced by the density of D_2 receptors in the caudate-putamen for 25% of explained variance and iron concentration in the nucleus accumbens for another 24% for a total of 49% of variance explained.

Table 3

Stepwise regression models relating activity variables to brain dopamine and iron values

Dependent variable	Step	Predictor variable	Parameter estimate	Cumulative R^2	P
Total distance	1	VMB-Fe	15.14	0.515	< 0.01
	2	VMB-Fe; VMB-D_1	13.99 and −27.08	0.599	< 0.01
	3	VMB-Fe; VMB-D_1; CP-D_1	13.99 and −24.34 and −8.603	0.650	< 0.01
Repeated movements	1	VMB-Fe	0.30	0.506	< 0.01
	2	VMB-Fe; VMB-D_1	0.28 and −0.47	0.568	< 0.01
	3	VMB-Fe; VMB-D_1; PFC-D_1	0.30 and −0.54 and 0.51	0.615	< 0.01
Nosepokes	1	PFC Fe	0.88	0.10	< 0.02
Center time	1	CP-D_2	0.20	0.247	< 0.05
	2	NA-Fe	−0.76	0.488	< 0.02
Habituation	1	PFC-Fe	−0.16	0.285	< 0.01
	2	PFC-Fe; NA-Fe	−0.17 and 0.064	0.396	< 0.01
	3	PFC-Fe; NA-Fe; VMB-Fe	−0.12 and 0.073 and −0.0790	0.490	< 0.01

Stepwise regression analysis performed with SAS-STEPWISE procedure. Abbreviations used in the table include: CP, caudate putamen; PFC, prefrontal cortex; NA, nucleus accumbens; VMB, ventral midbrain comprised of both the ventral tegmentum and the substantia nigra; DAT, dopamine transporter; D_1, dopamine D_1 receptor; D_2, dopamine D_2 receptor. Fe, iron concentration in that brain region. The parameter estimates are included in the order of the predictor variables entered into the models.

The concentration of dopamine transporters in the medial prefrontal cortex explained 23% of the variance of time spent in the lighted part of the light—dark box, followed by D_1 receptor concentrations in the medial prefrontal cortex and caudate-putamen, respectively, for a total R^2 of 0.45 (Tables 3 and 4). Analysis of latency to enter the dark revealed two factors, D_2 receptor density in the nucleus accumbens with a R^2 of 0.396 and dopamine transporter density in the nucleus accumbens for a total variance explained estimate of 43%.

Table 4
Stepwise regression models relating the light:dark box measures to brain dopamine variables

Dependent variable	Step	Predictor variable(s)	Parameter estimates	Cumulative R^2	P
Time in the light	1	PFC-DAT	0.3573	0.2306	< 0.06
	2	PFC-DAT; PFC-D_1	0.6472 and −0.0824	0.3801	< 0.05
	3	PFC-DAT; PFC-D_1; CP-D_1	0.7855 and −0.0755 and 0.3635	0.4549	< 0.06
Latency	1	NA-D_2	0.3486	0.3962	< 0.01
	2	NA-D_2; NA-DAT	0.2582 and 0.0279	0.4337	< 0.03

Stepwise regression analysis performed with SAS-STEPWISE procedure. Abbreviations used in the table include: PFC, prefrontal cortex; NA, nucleus accumbens; VMB, ventral midbrain; DAT, dopamine transporter; D_1, dopamine D_1 receptor; D_2, dopamine D_2 receptor. The parameter estimates are included in the order of the predictor variables entered into the model. Brain iron values were not available for this analysis.

3. Discussion

The experiments presented in this report confirm our earlier findings showing ID's profound effect on exploratory and locomotor behaviors in rats [23] and extends our knowledge to ID being associated with fear-like behaviors. Our previous studies also showed age-dependent effects of ID on behavior that were not entirely reversed with iron therapy [23,8].

ID decreased the time spent in the center of the activity monitor and decreased the latency for movement from the lighted area of the light—dark box to the darkened area. Thus, our observations that iron deficient rats showed more anxiety-like behaviors than did control rats support anecdotal observations reported in human studies [18,19]. Moreover, in this study we observed that the behaviors that we observed are sensitive to brain iron status and dopamine function.

Iron concentration and D_1 receptor density in the ventral midbrain are important predictors of measures of spontaneous activity (locomotion, repeated movements, whereas prefrontal cortical iron concentration predicts nosepokes and rate of habituation. Additionally, iron concentration in the nucleus accumbens predicts the time spent in the center and rate of habituation, behaviors which other authors might collect under the rubric, reactivity [25]. Significant decrements in brain iron concentration were observed in caudate, VMB, and prefrontal cortex and are in agreement with our earlier studies of changes in brain iron concentration with dietary ID [6,7,23]. The regression analyses identify which of the four brain regions examined is most highly related to locomotion and reactivity behaviors. For the anxiety-like behaviors it appears that VMB and prefrontal cortex iron concentrations are critical. This is the first opportunity afforded to analyze the animal experiments on ID in this manner, but does not preclude the possibility, or likelihood, that other neurotransmitters also play significant roles in these behaviors. In addition, it is feasible that metabolism of other micronutrients, and macronutrients, are altered by primary ID, which raises the possibility that some of our observations reflect relationships mediated by these other factors and not directly by iron.

The time spent in the light compartment has been shown to be sensitive to dopaminergic regulation [5,32]. In our analysis, we demonstrated prefrontal cortex DA transporter concentration and D_1 receptor concentration to be highly significant predictors of this measure. Previously, and in this study, we showed that iron deficient rats evinced a greater than 50% drop in iron concentration in the ventral midbrain, but only minimal changes in the densities of the dopamine transporter and D_1 receptor [6,7]. Nevertheless, small changes in densities of the dopamine transporter and D_1 receptor have a significant impact on the amount of time that the rats spend in the light compartment. The negative regression coefficient for D_1 in the prefrontal cortex is consistent with our observations that ID rats tend to have higher concentrations of D_1 in this region [6]. The prefrontal cortex, thus appears to play a major role in an animal's tolerance of an aversive environment.

The latency to move from the light to the darkened compartment presents a different picture. In this mea-sure of anxiety-like behavior, the nucleus accumbens was the sole player with significant effects of densities of the D_2 receptor and the dopamine transporter.

In conclusion, in this study, we extend our knowledge about the behavioral effects of ID during development to anxiety-like behaviors. Moreover, through the analytical technique of multiple regression, we have demonstrated the impact of regional iron and dopamine receptor/transporter status on activity and reactivity related behaviors. The final question, then is whether our research has any relevance to the observation made in iron deficient children. The rather striking parallels between hesitancy and anxiety-like behavior in iron deficient animals and reports of similar behaviors in iron deficient humans leads us to assert the heuristic value of the rat as model organism. Iron deficient children show attentional and information processing problems [18,19,24] and are often described anecdotally as more fearful. Thus, the cognitive difficulties in children so afflicted may originate from multiple behavioral difficulties, including timidity and inattention to the environment. In the 'real world' life is far more complex than the more controlled environment of our rodent studies. This animal model is not an exact developmental analog to the human studies of anxiety though in both species there were periods of ID during weaning and subsequent development. Nonetheless, we are now able to tie similar kinds of behavioral difficulties to brain iron status and dopaminergic functioning' steps leading to the generation of hypotheses concerning the biobehavioral-mechanisms underlying ID, including the effect of ID on other neurotransmitter systems. For example, in our own research, we have shown that ID affects serotonergic function [20,21] and others have demonstrated that ID influences gamma-amino butyric acid functions [17]. ID can be shown to produce numerous changes in the development of neurological functioning that may be morphologic as well as biochemical [26]. Multiple regression analysis and other, multivariate analyses can be valuable aids to help untangle the complexity of early nutritional deficiencies and point the way to mechanisms. In the future we will perhaps be able to account for the potential influence of secondary effects of iron status on energy metabolism, micro and macro-nutrient metabolism, and other potential biological alterations that could further clarify the role of nutrient deficiencies in brain functioning and behavior.

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