Neonatal Iron Deficiency Results in Irreversible Changes in Dopamine Function in Rats

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Abstract:
Iron deficiency in human infants and in young animal models produces changes in neural functioning that may be related to monoamine metabolism. This study employed both behavioral and biochemical approaches in a design using cross-fostering to examine alterations in dopamine (DA) function when iron deficiency occurs during the neonatal period. We measured brain Fe, dopamine transporters (DAT) and dopamine receptor density In rats made iron deficient, or not, from postnatal day (PND) 4 to PND 14 or 21. Some pups were then weaned to an iron-deficient diet and others to the control diet to examine the reversibility of these effects. Behaviors related to dopamine function were measured. Dopamine D2 receptor (D_2R), D,R and iron concentrations were ~70, 80 and 30% of control values, respectively, in the nucleus accumbens and striatum in Iron-deficient rats at PND 14. The DAT density was also reduced to 50% of control density In the nucleus accumbens but was unchanged in the striatum. By PND 21, there was also a significant 50% lowering of DAT, D,R and D_2R densities in the prefrontal cortex (PFC). Iron repletion at PND 21-49 normalized D_1R, D_2R, and DAT levels in the nucleus accumbens, PFC and ventral midbrain but not in the striatum. In summary, neonatal Iron deficiency is associated with changes in DA biology that vary with duration of iron deficiency, and are not completely normalized despite replenishment of iron status. Changes in DA-related behaviors that were persistent after postweaning iron repletion suggest the existence of a critical neonatal developmental period that is expressed by alterations in DA functioning. J. Nut, 133: 1174-1179, 2003.
Key Words: dopamine, dopamine receptors, dopamine transporter, iron deficiency, prefrontal cortex, rats

Article:
Iron is involved in numerous neurological functions. The effects of iron deficiency (ID)[2] on behavior have been widely investigated in humans and in experimental animals. Studies in adults and in children demonstrate that iron deficiency is associated with apathy, irritability, lethargy, lack of concentration, increased anxiety, hypoactivity, and decreased cognition and attention. Despite major methodological differences among early (1,2) and later studies [reviewed in (3-7)1, remarkably similar results are reported. The biological basis of 1D-related cognitive and behavioral alterations is unclear; however, a conceptual model of contributing factors must include environmental as well as biological factors (4,8). The biological variables are related to decreased brain iron, and likely include hypomyelination, impaired dopaminergic function and delayed neuromaturation (8-10). Auditory evoked potential studies in 6-mo-old iron-deficient infants showed increased central conduction times consistent with either hypomyelination or altered monoamine metabolism (10). One way of determining causality is to utilize animal models that focus on the relationship of neurotransmitters in limbic structures to behaviors that appear to be adversely affected by iron deficiency in human infants (4).

The effects of iron on neurological functioning in rodents are related to the brain levels of iron, not to anemia per se (11,12), Induction of iron deficiency in postweaning rats results in decreased densities of dopamine (DA) D2 receptors (D_1R), increased extracellular DA, and blunted DA reuptake in vivo, (12-14), Iron-deficient rats are less active than control rats when placed in a novel environment; they explore less and are more "fearful" than control rats (15,16). These behaviors are dependent in part on dopaminergic function in nigrostriatal and mesocorticolimbic pathways and are the basis for our somewhat selective examination of certain brain regions. The previously cited studies demonstrated a reversible relationship of regional brain iron to DA and to

behaviors, but they did not identify a "critical period" of development, In human infants suffering from iron deficiency, that critical period appears to be during the first 12-18 mo of life.

Rodent studies of neonatal iron deficiency demonstrated developmentally dependent rates of depletion of brain iron with dietary deficiency and a failure to correct abnormal behaviors even though the regional brain iron contents were restored to normal levels within 2 wk of feeding a high iron diet (17). In the current studies, we investigated the relationship of neonatal iron deficiency with dopamine metabolism; furthermore, we sought to determine whether the above-mentioned effects were normalized after dietary iron intervention.

MATERIALS AND METHODS

Design. An out-fostering regimen was implemented to produce iron deficiency postnatally but before weaning in pups, as described by Pinero et al. (17). On postnatal day (PND) 4 of life, we placed iron-sufficient pups with lactating darns that had been fed an iron-deficient diet from midgestation to term. We then conducted an intervention study with experimental groups in which rats were made iron deficient between PND 4 and 14, between PND 4 and 21 or between PND 21 and 49. To examine reversibility of effect, we also included rats made iron deficient between days 4 and 21 and subsequently fed an iron-sufficient diet until d 49 of age. Each experimental group was compared with a control cohort group fed an iron-sufficient diet. All experimental (and control) rats were killed on the day of the regimen as specified.

Animals and dietary treatment. Male and female Sprague-Dawley rats (180-200 and 140-160 g, respectively) were purchased from Harlan Sprague Dawley (Indianapolis, IN). The female rats were fed a control diet (35 Fe mg/kg diet) and the males were fed a standard nonpurified diet (Laboratory Rodent Diet, PMI Nutrition International, Brentwood, MO). At age 49 d, the females were mated with a male for 5 d (two females and one male), after which the females were removed and placed in individual home cages. Pregnancy was defined by the appearance of vaginal plugs. The dams were weighed every other day to confirm the pregnancies.

After pregnancy was determined, the dams were fed an iron-deficient diet (3 mg Fe/kg diet) at midgestation or continued to consume the control diet. The first morning that the pups appeared in the cages was considered PND 1. At PND 4, rats from the control dams were randomly assigned to iron-deficient dams or to dams fed the control (CN) diet. To control for the effects of outfostering, per se, a number *of* pups born to control dams were fostered with another control dam until weaning. Some of these pups were then weaned to a low iron diet (3 mg Fe/kg) and others to a control diet (35 mg Fe/kg). No pups utilized in these studies experienced iron deficiency in utero. There were roughly equal numbers of male and female pups in each litter, but only data from males are included. Although we did not measure milk iron and pup milk consumption, previous studies (17,18) demonstrated that this protocol decreases milk iron concentration and that pups consume normal amounts of milk per gram of body weight.

Dietary treatment. The diets were prepared as described elsewhere, (18) and met all nutrient requirements with the exception of iron in the low iron diet. All diets were formulated according to the guidelines of the AIN, with cornstarch as the sole source of carbohydrate (19). By analysis, the diet iron concentrations were 36.7 ± 0.7 mg Fe/kg for the control diet and 3.15 ± 0,5 mg Fe/kg *for the* iron-deficient diet. All rats had free access to food and water 24 hid, and lights were turned off between 1800 and 0600 h. The room temperature was maintained at 25 ± 1°C. The Pennsylvania State University Animal Care and Use Committee approved all animal procedures.

The iron status of the dams was closely monitored by measuring hemoglobin (Hb) and hematocrit (Net) before mating, at midpregnancy, at delivery, at midlactation and at weaning as previously described (17).

Hematological and liver nonheme iron. Hemoglobin was measured colorimetrically by the cyanomethemoglobin method and Hct was determined by centrifugation (9,000 *g,* for 5 min) of blood collected into heparinized microcapillary tubes. Liver nonheme iron was determined using the standard colorimetric technique described by Cook (20) with ferrozine as the color reagent.

Iron repletion. Separate groups of ID rats were weaned to a control diet for 4 wk (IDCN) starting at PND 21. Corresponding age-matched control rats (CNCN) that were iron sufficient at all times were also used. After 4 wk of dietary iron repletion, behavioral measures were obtained before killing and brain iron and dopamine variables were measured.

Locomotor behavior tests. Rats at PND 21 and after 4 wk of iron repletion were subjected to behavior tests (ambulation and stereotypy) via an automated activity monitor according to methods previously described (21,22).

Densities of dopamine receptors and the dopamine transporter (DAT). Brains from freshly killed rats were dissected, membrane fractions prepared and radioligand binding assays performed as described previously (22,23). The ligands used were ^{3}H-SCH23390, ^{125}I-epidepride and ^{3}H-GBR 12935 for D_1R, D_2R and the DAT, respectively. All assays were conducted in random order. Maximum binding values were normalized to protein concentrations as determined by the micro-I.owry assay (P5656 Sigma Chemical, Natick, MA).
Iron analysis. Total iron content of brain region homogenates was determined using atomic absorption spectrophotoinetry (24).

Statistical analysis. Dopamine receptor and transport measures were analyzed by ANOVA. The between-subject factor was diet and the within-subject factor was brain region. The behavioral variables were subjected to ANOVA for a between-subjects variables analysis (diet). Student's t test for samples with unequal variances was used to test effects of dietary treatment on hematological measures and liver nonheme iron. Tukey's Honestly Significant Difference post-hoc test was used to evaluate differences between means with **a set** at $P < 0.05$. Estimated tu^2 were calculated for significant main effects and interaction to show the proportion of variance in the measure attributable to the factor or to interaction (25).

RESULTS

Biological data. The period of early neonatal ID refers to the dietary iron deficiency n=induced between PND 4 and 14. Complete neonatal iron deficiency refers to iron deficiency from PND 4 to 21. Hermatologic indices demonstrated that rats cross-fostered to iron-deficient dams were iron deficient at PND 14 and 21 and has significantly lower body weights than controls (Table 1). There were age- and region-specific effects of iron deficiency on brain iron concentration. For example, at PND 14, iron-deficient rats had a slightly higher concentration of iron in the prefrontal cortex (PFC) than control rats, but 60-70% lower concentrations in striatum and nucleus accumbens [$F(1,12) = 12.4$, est. $\omega^2 = 0.143$]. Another week of dietary iron deficiency to PND 21 diminished the rapid accumulation of iron in the PFC and ventral midbrain compared with controls [$F(1,12) = 18.6$, est. $\omega^2 = 0.347$] but did not prevent the accumulation of iron in the striatum or nucleus accumbens.

TABLE 1

Hermatological and brain iron concentration in neonatal iron-deficient and control rats[1,2]

Treatment	n	Age	Body Weight	Hct (volume fraction)	Hb	Striatum Fe	Nucleus Accumbens Fe	Prefrontal cortex Fe	Ventral midbrain Fe
		d	g		g/L	---------- µmol/g ----------			
Control	10	14	41 ± 1.4	0.25 ± 0.003	89 ± 3	13.5 ± 1.6	12.4 ± 3.5	1.6 ± 0.1	2.0 ± 0.2
Iron deficient	10	14	30 ± 1.6*	0.14 ± 0.006	45 ± 1*	3.9 ± 0.3*	4.8 ± 0.4*	2.5 ± 0.1*	1.6 ± 0.1
Control	12	21	55 ± 1.9	0.27 ± 0.003	59 ± 3	15.2 ± 0.8	11.1 ± 0.3	15.5 ± 0.3	11.0 ± 1.7
Iron deficient	12	21	43 ± 0.6*	0.13 ± 0.005*	44 ± 2*	12.6 ± 1.9	11.4 ± 1.3	7.0 ± 0.7*	8.0 ± 1.0

[1] Values are means ± SEM. For all variables, diet and age were significant effects)P < 0.001) but there were no interactions.
**** Different from the control of the same age, P < 0.05.***
[2] Hct, hematocrit; Hb, hemoglobin

Binding of D_1, D_2 and DAT ligands. Neonatal iron deficiency affected D_1R density with a significantly lower D_1R density in the striatum at PND 14 [$F(1,12) = 12.3$, $\omega^2 = 0.221$] (Table 2). D_2R density was significantly

lower in striatum at PND 14, [F (1,12) = 10.9, ω^2 = 0.181], but was significantly higher in the PFC [F (1,12) = 8.4, ω^2 = 0.162]. Iron deficiency decreased the density of the DAT in the accumbens and increased its density in the prefrontal cortex [F(1,12) = 12.2, ω^2 = 0.192].

TABLE 2

Brain dopamine receptor and transporter densities in iron-deficient and control rats that experienced Iron deficiency pre- and postweaning[1,2]

		D1R			D2R			DAT		
Brain region		14d	21d	49d	14d	21d	49d			
Striatum	Control	0.60 ± 0.04	0.46 ± 0.03	0.50 ± 0.04	1.40 ± 0.72	1.94 ± 0.09	1.75 ± 0.08	19.2 ± 1.4	14.3 ± 0.7	18.8 ± 1.6
	Iron deficient	0.51 ± 0.04*	0.42 ± 0.03	0.39 ± 0.05*	0.87 ± 0.43*	1.73 ± 0.08	1.35 ± 0.06*	19.9 ± 1.4	15.1 ± 1.9	16.9 ± 1.3*
Nucleus	Control	0.49 ± 0.02	0.25 ± 0.02	0.55 ± 0.03	0.58 ± 0.33	0.96 ± 0.02	1.25 ± 0.06	15.3 ± 1.5	14.2 ± 1.5	18.6 ± 0.8
accumbens	Iron deficient	0.36 ± 0.05	0.23 ± 0.02	0.47 ± 0.02*	0.45 ± 0.23	0.91 ± 0.06	0.92 ± 0.05*	7.9 ± 1.9*	11.5 ± 2.3*	13.6 ± 1.4*
Prefrontal	Control	0.17 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.06 ± 0.08	0.06 ± 0.01	0.08 ± 0.01	4.1 ± 0.5	4.9 ± 0.1	3.5 ± 0.2
cortex	Iron deficient	0.16 ± 0.01	0.09 ± 0.01	0.13 ± 0.01	0.11 ± 0.03*	0.05 ± 0.01*	0.19 ± 0.01*	5.7 ± 0.3*	3.6 ± 0.4*	4.6 ± 0.1*
Ventral	Control	0.11 ± 0.01	0.09 ± 0.02	0.08 ± 0.01	0.19 ± 0.10	0.08 ± 0.01	0.12 ± 0.01	5.6 ± 0.4	6.1 ± 0.4	3.5 ± 0.2
Midbrain	Iron deficient	0.09 ± 0.01	0.10 ± 0.01	0.08 ± 0.01	0.20 ± 0.08	0.05 ± 0.01*	0.11 ± 0.01	6.0 ± 0.9	7.4 ± 0.9*	3.2 ± 0.2

[1] Values are means ± SEM, For all variables, diet and age were significant effects (P < 0.001) but there were no interactions.

**** Different from the control of the same age, P < 0.05.***

[2] D_1R dopamine D1 receptor; DAT, dopamine transporter, nmol/mg protein.

The densities of D_1R and D_2R in iron-deficient rats at PND 21 in striatum and in the nucleus accumbens were not different from in controls (Table 2, Fig. 1a,b). In nucleus accumbens and PFC, the DAT density in ID rats was significantly lower than in controls [nucleus accumbens and PFC F (1,22) = 9.6, ω^2 = 0.266, F (1,22) = 7.3, ω^2 = 0.190, respectively].

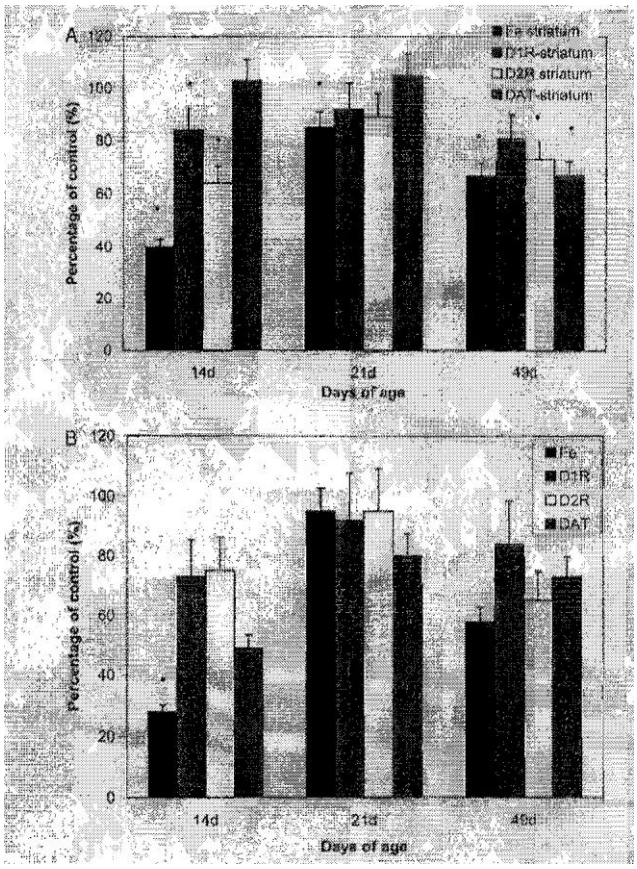


Figure 1. ***Percentage of control dopamine D1 receptor (D_1R), D_2R, dopamine transporter (DAT) densities, and iron concentration in striatum (A) and nucleus accumbens (B) of rats that were iron deficient from postnatal day (PND) 4 to 14 (14 d), from PND 4 to 21 (21 d), and rats fed an iron-deficient diet from PND 21 to 49 (49 d). Values are means ± SEM. See Table 1 for n. * Different from controls.***

The densities of D_1R, D_2R, and DAT were greater in controls than in ID rats in the striatum and accumbens in PND 49, rats {$p < 0.01$ in all cases]. In the PFC, D_2R and DAT densities were greater in the ID rats at PND 49 [$F(1,17) = 7.7$, $\omega^2 = 0.216$].

TABLE 3

Dopamine receptor (D_1R, D_2R) and transporter (DAT) densities in neonatal iron-deficient rats that were made iron replete postweaning (IDCN) and control rats (CNCN)

		D_1R			D_2R			DAT		
	n	Striatum accumbens	Nucleus accumbens	Prefrontal cortex	Ventral midbrain	Striatum	Nucleus accumbens	Prefrontal cortex	Ventral midbrain	Striatum	Nucleus accumbens	Prefrontal cortex	Ventral midbrain
		fmol/mg protein								*nmol/mg protein*			
IDCN	6	268 ± 19	355 ± 32	119 ± 15	85 ± 11	3393 ± 233	2806 ± 462	83 ± 7	84 ± 4	8.81 ± 0.25	17.09 ± 0.81	3.56 ± 0.30	3.42 ± 0.31
CNCN	16	403 ± 19*	401 ± 20	95 ± 9	81 ± 6	1716 ± 126*	1847 ± 164*	68 ± 4	75 ± 8	18.10 ± 0.15*	19.74 ± 0.86	3.31 ± 0.33	2.75 ± 0.22

† Values are means ± SEM. *Different from IDCN, $P < 0.05$.

Postweaning iron supplementation. Iron repletion of neonatal iron-deficient rat pups commenced at weaning (IDCN) and resulted in significant improvements in hematologic measures, and D_1R, D_2R, and DAT in brain regions (Table 3). The rats that were iron deficient from PND 4 to 21 and then made replete (IDCN) had a body weight of 191 ± 10 g, Hct of 0.39 ± 0.02 and liver iron of 2.54 ± 0.38 μmol/g. The rats that were iron sufficient throughout PND 4-49 (CNCN) had a body weight of 212 ± 13 g, Hct of 0.43 ± 0.02 and liver iron of 1.93 ± 0.36 μmol/g tissue. D_1R density was significantly lower in striatum than control levels (CNCN) [F = 1,20; ω^2 = 0.184] even though these rats were no longer anemic and body iron stores has been restored (Fig.2). The D_2R density measurements, however presented the greatest surprise. They were 98 and 52% greater than in controls in the striatum and the nucleus accumbens, respectively [F = 1,28; ω^2 = 0.194]. The DAT density remained lower in the striatum of iron-replete rats [F = 1,22; ω^2 = 0.191] but other regions did not differ.

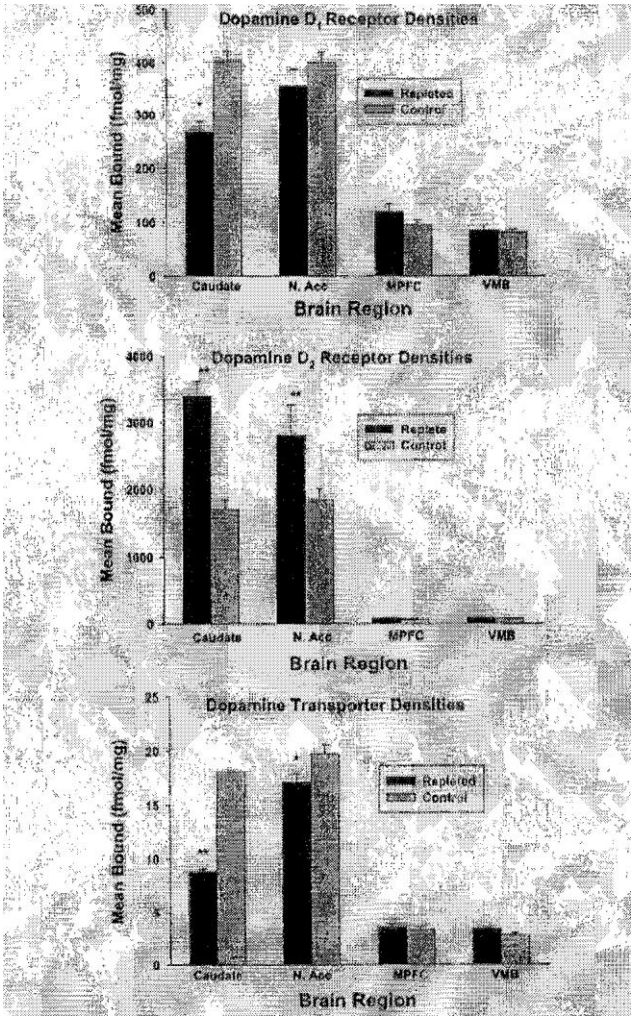
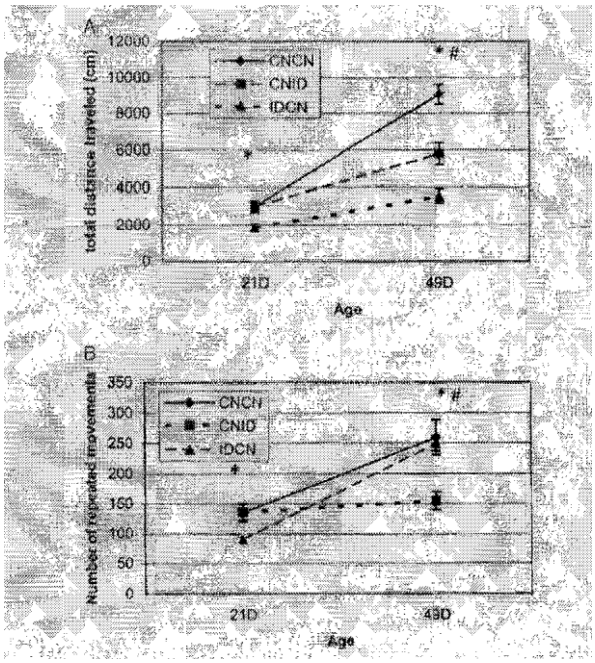


Figure 2. ***Dopamine D1 receptor (D_1R) densities (upper panel), D_2R density (middle panel) and dopamine transporter (DAT) density (lower panel) in brains of iron-replete rats and control rats. N. Acc., nucleus accumbens; MPFC, medial prefrontal cortex; VMB, ventral midbrain. Values are means ± SEM, n = 8-16. * Different from controls, P < 0.01; ** different from controls, P < 0.001.***

Behavioral testing. Iron-deficient rats at PND 21 showed significantly decreased levels of spontaneous movement and exploration of a novel environment than age-matched controls [F (1,10) = 7.7, ω^2 = 0.245]. Iron repletion for 4 wk did not completely correct this behavioral effect because a significant difference in locomotion remained at PND 49 [F (1,10) = 11.2, ω^2 = 0.493] (Fig. 3a). Stereotypy (number of repeated movements) was also significantly lower in iron-deficient neonatal rats [F (1,8) = 4.6, ω^2 = 0.177]; however, postweaning iron repletion reversed this effect (Fig. 3b).



Figures 3. ***Total distance traveled by male rats of variable iron status in an open field chamber for a 20-min period (A) and number of repeated movements (B) at both 21 and 49 d of age. The groups of rats are controls (CNCN), neonatal iron-deficient rats made iron replete (IDCN) and postweaning iron-deficient rats (CNID). Values are means ± SEM. See Table 2 for n. * Different from other groups, P < 0.001; ** different from CNCN, P < 0.001.***

Discussion

The current experiments answered three important questions: 1) Does deficiency during the neonatal period change DA function? 2) Is there an effect of the duration of iron deficiency during the neonatal period on DA? 3) Is there a "complete normalization" of DA metabolism after iron repletion? We found that neonatal rat pups subjected to iron deficiency for only 10 or 17 d (PND 14 or 21) developed changes in DA function and behavior that were not corrected despite normalization of hematology and iron stores. This was suspected on the basis of our earlier studies that showed that brief periods of neonatal iron deficiency were sufficient to significantly alter brain iron concentrations (17). In their study, 2 wk of iron therapy did normalize the iron content of most regions of the brain, but some behaviors were resistant to complete normalization within that short time period (17, 21). The current report provides some possible biologic explanations for those observations because we were able to demonstrate that DA metabolism is also not completely normalized after a longer 4-wk repletion period.

The acquisition of iron by different regions is quite different in the first neonatal period studied compared with the iron accumulation in the second neonatal period. The absolute concentrations of iron in the four regions examined show that striatum and accumbens accumulated much more iron in the first 10-14 d of the neonatal period than in the next 7 d, whereas the PFC and ventral midbrain displayed a "burst" of iron accumulation between PND 14 and 21. Thus, the effect of iron deficiency on brain iron concentrations is very dependent on the "timing" of the dietary deprivation. For instance, the striatum is sensitive to dietary iron depletion from PND 4 to 14, but an additional 7 d of dietary iron restriction did not reduce iron concentrations to levels below those at PND 14. In contrast, the nucleus accumbens is "insensitive" to dietary iron restriction during the first 10 d of iron deficiency but then "falls behind" very substantially in iron acquisition over the next week of growth when that region is very actively accumulating iron. The observation that high rates of iron accumulation occur in different brain regions at different ages of postnatal life was noted previously (17). The biological basis of this relative iron accumulation is not understood, but is likely mediated by changes in the cellular iron uptake proteins, divalent metal transporter protein and transferring receptor, as well as changes in iron requirements with regional brain growth and development (21, 26, 27). Iron requirements for myelin

production and oligodendrocyte metabolism would be expected to be high during the development of white matter regions, but other biological processes that require iron remain to be explored (10, 17).

A key finding of this study is that the regional densities of DAT and D_2R closely and positively follow the changes in iron concentration across the different ages studied. We previously observed a strong correlation between regional iron concentration and aspects of DA metabolism in postweaning iron-deficient rats (13,22,28). Multivariate regression analyses demonstrated that combinations of both iron concentrations and DA variables explained between 50 and 67% of the variation in exploratory behaviors and in latency in light-dark box behaviors in those studies. The argument that only DA variables and only certain brain regions are affected exclusively by iron deficiency is not likely to be valid (28); those studies documented only associations between particular dopaminergic pathways and behaviors known to be affected by dysfunction in those brain regions and pathways.

Dopaminergic neurons express transporters and receptors at different times in development and also experience a substantial amount of "pruning" of neural circuits with postnatal aging (29-31). D_2R density in some rat brain regions doubles from PND 7 to 14, increases by another 20% from PND 14 to 21 and by another 20-50% by PND 28 (31). The density of the DAT increases steadily throughout neonatal development to a maximal level by adulthood. In contrast, the density of D_1R in primary dopaminergic regions is not age dependent during the neonatal period (28,30). Our demonstration that the densities of dopamine receptors rise and fall with iron status may indicate that iron has a role in DA neuron-specific apoptosis as well as specific effects on the expression of these proteins. This idea has been explored in cell culture in which acute iron chelation in both PC12 cells and neuroblastoma cells showed dose-response relationships between the expression of the DAT and amount of cellular iron deficiency (32).

The final key observation from the current study was that 4 wk of iron repletion resulted in incomplete normalization of DAT and receptor densities and some improvement, but not complete normalization, in all behaviors that were examined. Thus, our previous hypothesis that longer periods of iron repletion in rats would normalize behaviors is only partially supported (33,34). There is thus some support for the notion that even longer periods of iron therapy would result in complete normalization of the behaviors related to exploration of the environment. The biological explanation of the need for this longer period of iron therapy must hinge on events other than simply the iron content of a brain region because iron concentration is normalized in most brain regions within 14 d of dietary treatment (21, 24). It is tempting to argue that the basis of the alterations in some behaviors that are resistant to normalization are related to irreversible changes in dopamine transporter and receptor levels in certain brain regions. We, and others, have already established that iron-deficient rats have altered motor activity, exploratory behavior and stereotypy (14-16, 21). They also show more anxiety in both open field and light box-dark box behavior paradigms. Regression analysis showed these behaviors to be highly related to dopaminergic function in the nigrostriatal and mesocorticolimbic pathways (11,35) as well as to iron concentrations in ventral midbrain and striatum (34). As noted elsewhere, a number of other possible biological explanations remain viable at this time and are being explored (35).

In conclusion, these studies of neonatal iron deficiency and postweaning iron repletion provide important new evidence regarding the relationship of brain iron deficiency to behavior in several ways: 1) dopaminergic tracts are affected by iron deficiency during the neonatal period; 2) the early neonatal period has a different "susceptibility" than the second half of the neonatal period; 3) iron therapy for 4 wk normalizes DA transporter and receptor densities in the dopaminergic tracts, with the exception of D_1R and DAT densities in striatum; and 4), behaviors known to be dependent on functioning of the striatal dopaminergic neurons remain despite iron therapy. Whether human infants with iron deficiency anemia also demonstrate this dopaminergic sensitivity to regional brain iron concentration must be determined. We contend, however, that these rodent studies strongly support such a hypothesis.

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