

Iron Deficiency Alters Dopamine Transporter Functioning in Rat Striatum

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Abstract:

Iron deficiency anemia In early life produces profound changes in both in vivo and In vitro evaluations of dopamine (DA) functioning. This study employed both behavioral and biochemical approaches to examine the biological bases of alterations in striate! DA metabolism seen in Iron-deficient rats. The purpose was to determine whether the DA transporter (DAT) was functionally altered in postweaning iron deficiency. Male and female 21-d-old Sprague-Dewley rats ($n = 40$) were fed either an iron-deficient (ID) diet (3 mg Fe/kg diet) or a control (CN) diet (35 mg Fe/kg diet) for 4 wk before behavioral testing. Motor activity responses to graded doses (3.75-30 mg/kg body) of the DA uptake inhibitor, cocaine, were significantly blunted in iron-deficient rats with a 50% higher half-maximal effective dose (ED_{50}) in both males and females (CN-female, 7.1 ± 0.9 mg/kg; ID-female, 11.2 ± 1.3 mg/kg; CN-male, 12.0 ± 0.7 mg/kg; and ID-male, 17.0 ± 1.8 mg/kg). Radioligand binding assays with ^3H -1-(2- (diphenylmethoxy)-ethyl)-4-(3-phenylpropyl) piperazine (^3H -GBR12935) demonstrated that Iron deficiency did not alter the affinity of the ligand for the DAT but did significantly decrease the density of the transporter by 30% in caudate putamen and 20% In nucleus accumbens. Iron deficiency also significantly decreased ^3H -DA uptake into striatal synaptosomes, but did not affect release of DA with potassium chloride stimulation. These experiments provide supporting evidence that elevated levels of extracellular DA in the striatum of Iron-deficient rats is likely to be the result of decreased DAT functioning and not increased rates of release.

KEY WORDS: iron deficiency anemia, rat brain, dopamine, cocaine, dopamine transporter

Article:

Iron deficiency is one of the most common nutritional disorders in the world, affecting nearly two billion people (W1-10/FAO, 1998). Symptoms of iron deficiency include lethargy, lack of concentration and decreased cognitive and attentional processes (Idjadinata and Pollitt 1993, Lozoff and Brittenham 1986, Pollitt 1993, Walter 1993). There is evidence that iron plays a role in neurobiological processes, although the exact neurobiological mechanism(s) remains unknown. Various studies have proposed changes in neurotransmitter metabolism (Beard et al. 1994, Chen et al. 1995, Youdim et al, 1989), myelin formation (Larkin and Rao 1990) and hippocampal functioning (Rao et al. 1999). Recent studies in both humans and rodents clearly demonstrate that in early life, the brain is quite susceptible to iron deficiency (Erikson et al. 1997, Kwik-Urbe et al. 1999, Pinero et al. 2000, Ran et al. 1999). Although the bulk of the evidence in humans suggests an irreversible alteration in neurodevelopment (Lozoff et al. 1998), the recent human studies of auditorily evoked potential changes are consistent with alterations in both myelination and neurochemistry (Roncagliolo et al. 1998).

In rats, postweaning iron deficiency produces a substantial decrease in brain iron concentration that is reversible with iron repletion (Erikson et al. 1997, Pinero et al. 2000). Furthermore, iron deficiency-related decreased brain iron concentration has been linked to altered dopaminergic functioning (Chen et al. 1995, Nelson et al. 1997, Youdim et al. 1989). The effects of iron deficiency on dopamine (DA)² metabolism include decreased D_2 receptor density in caudate putamen (CP) (Erikson et al, unpublished data, Youdim et al. 1983) and increased extracellular DA in the CP (Beard et al. 1994, Chen et al. 1995, Nelson et al. 1997). Behavioral consequences of Iron deficiency-related alterations in striate DA function include altered locomotor activity (Glover and Jacobs 1972, Hunt et al. 1994, unpublished observations from our laboratory). It is important to note that these alterations in DA metabolism are associated with decreased brain iron concentration and not anemia per se

(Ashkenazi et al. 1982, Nelson et al. 1997, Youdim et al. 1989) and may be reversible with iron therapy (Nelson et al. 1997, Youdim et al. 1981).

Approximately 80% of extracellular DA is recycled into presynaptic neurons through a reuptake Na^+ cotransporter, a 70-kDa protein that is both phosphorylated and glycosylated (Ciliax et al. 1995, Miller et al. 1997). The DA transporter (DAT) is a member of the family of Na^+ , Cl^- -dependent transporters whose regulation includes both chronic and acute regulatory factors (Mash and Staley 1997, Reith et al. 1997). It is important to recall that in vivo microdialysis experiments clearly indicated the possibility of a defect in DA uptake in iron-deficient rats (Nelson et al. 1997).

Our overall hypothesis is that iron deficiency—related increased extracellular DA in the striatum is linked directly to decreased DAT activity. Therefore, we tested this hypothesis from behavioral [half-maximal dose (ED_{50}), cocaine-induced hyperlocomotion], functional (synaptosomal ^3H -DA uptake) and biochemical [^3H -1-(2-(diphenylmethoxy)-ethyl)-4-(3-phenylpropyl) piperazine (^3H -GBR12935) ligand binding] perspectives.

MATERIALS AND METHODS

Animals

Male and female 21-d-old Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were randomly divided into two dietary treatment groups as follows: control (CN; 35 mg Fe/kg diet, $n = 32$ males and females) and iron-deficient (ID; 3 mg Fe/kg diet, $n = 32$ males and females). These diets were prepared as described previously (Pinero et al. 2000). Rats had free access to food and water 24 h/d, and lights were turned off between 1800 and 0600 h. The room temperature was maintained at $25 \pm 1^\circ\text{C}$. After 4 wk of dietary treatment, behavioral testing was conducted. The Pennsylvania State University Animal Care and Use Committee approved all animal procedures.

ED_{50} cocaine experiment and locomotor activity

Locomotor activity was measured using a Digiscan Animal Activity Monitor, model RXYZCM (Omnitech Electronics, Columbus, OH) as described previously (Morse et al. 1999a). The apparatus consisted of a set of four identical 40 x 40 x 30.5 cm Plexiglas boxes with vertical and horizontal infrared sensors. The flooring was an elevated acrylic platform with equally spaced holes (4 X 4 X 1.5 cm in diameter). Data were collected by virtue of rat movements breaking a beam of light between photocells and were logged automatically into the computer and analyzed with the Digiscan software. All testing was begun between 0900 and 1100 h in the light cycle, although the testing boxes themselves were dark. A 2-d behavioral testing protocol was implemented. On d 1, rats from each diet and sex group ($n = 5$) were injected intraperitoneally with saline (1 mL/kg body) and immediately placed in the center of the activity monitor. Locomotion was measured for 30 min in six 5-min intervals or bins of data. The index of locomotor activity analyzed for this report was total distance traveled (cm). On d 2, rats from the previous day were injected with one of four doses of cocaine (3.75, 7.5, 15 or 30 mg/kg body) and locomotor activity measured as on the preceding day. The ordering of the dose was random.

This protocol was repeated until all rats were tested ($n = 5$ for each cocaine dose per group). The ED_{50} for cocaine (the dose necessary to increase total distance by 50%) was calculated using a LOG LOGIT transformation of the data followed by regression analysis.

Tissue. Within 2 d of having completed the behavior testing protocol, the rats were killed by decapitation, livers removed for nonheme iron determination and brains rapidly removed for uptake experiments or ligand binding assays. All rats were killed between 0900 and 1100 h. Blood was collected from the trunk of the rats, hemoglobin and hematocrit measured on fresh blood, and plasma obtained by centrifugation as detailed elsewhere (Pinero et al. 2000). The plasma was frozen at -20°C until it was analyzed for Fe and total iron-binding capacity (TIBC) by established procedures (Chen et al. 1995). Transferrin saturation was calculated as serum $\text{Fe}/\text{TIBC} \times 100$.

Synaptosomal ^3H -DA uptake and release experiments

Striatal synaptosome preparation. Striata were dissected on an ice-cold aluminum block, weighed and homogenized in 20 volumes of ice-cold 0.32 mol/L sucrose, pH 7.4, using a teflon/glass homogenizer (Heidolph, Polyscience, Niles, IL). Homogenates were centrifuged at 2000 x g for 10 min at 4°C. The pellets were discarded and the supernatants were centrifuged at 20,000 X g for 15 min at 4°C. The second pellet was resuspended in ice-cold Krebs-Ringer HEPES (KRH: 118 mmol/L NaCl, 4.7 mmol/L KCl, 1.18 mmol/L MgSO_4 , 1.2 mmol/L KH_2PO_4 , 10 mmol/L HEPES, 5.6 mmol/L glucose, 2.5 mmol/L CaCl_2) pH 7.4, containing 10 $\mu\text{mol/L}$ pargyline (RBI, Natick, MA).

Uptake experiments. Three uptake experiments were conducted using the following striate synaptosomes: 5-min uptake, half-maximal dose of the drug (cocaine) for in vitro effects (IC_{50}), and K_m and V_{\max} assays. Assay tubes contained 200 μL of synaptosomal suspension, 50 μL of KRH and 50 μL of cocaine solution (IC_{50} cocaine experiment, 10^{-5} , 10^{-8} mol/L final concentration). Tubes were preincubated for 10 min at 37°C in a water bath, ^3H -DA (NEN Life Sciences, Boston, MA) added and incubated for 5 min. The K_m and V_{\max} experiments used ^3H -DA concentrations ranging from 5 to 200 nmol/L. All other uptake experiments used 100 nmol/L ^3H -DA. The reaction was terminated by the addition of ice-cold KRH to tubes and rapid filtration through GF/F Whatman glass-fiber filters (Kent, UK) on a Millipore (Bedford, MA) sampling manifold. Filters were washed twice with 3 mL ice-cold KRH and placed in scintillation vials; 5 mL scintillation cocktail (Ecoscint, National Diagnostics, Atlanta, GA) was added. Vials were counted on a Beckman liquid scintillation counter (LS-3801, Beckman Instruments, Irvine, CA) 24 h later. Nonspecific uptake was determined by incubating tubes from each group at 4°C. The protein content was determined using a micro-Lowry procedure (P5656 Sigma Chemical, Natick, MA) modified for use on 96-well plates and with 50 μL of sample. Uptake experiments were repeated three times using synaptosomes from two rats per group with each preparation of synaptosomes; thus a total of six independent observations were made for uptake and release experiments.

Release experiments. Striatal synaptosomes were prepared as above and incubated with 100 nmol/L ^3H -DA for 10 min at 37°C. Synaptosomal suspensions equaling 100 μg protein were loaded into chambers of a Brandel SF-12 (Rockville, MD) superfusion apparatus. KRH buffer (4.7 mmol/L KCl) was perfused through the chambers (1 mL/min) for 5 min to establish baseline, and one of four concentrations of KRH (15, 30, 60 or 120 mmol/L KCl) was perfused for 2 min to elicit ^3H -DA release. The experiments were terminated by perfusing deionized distilled water through the chambers, thereby lysing the synaptosomes and releasing the remaining ^3H -DA. Scintillation vials collected samples at 1-min intervals, scintillation cocktail was added and vials counted 24 h later on a Beckman liquid scintillation counter (LS 3801, Beckman Instruments). Experiments were repeated three times with synaptosomes prepared from two rats per group for each experiment.

^3H -GBR 12935 ligand binding

Brains from rats in the behavior protocol were dissected on an ice-cold aluminum block into the following four brain regions: prefrontal cortex (PFC), CP, nucleus accumbens (NA) and ventral midbrain (VMB, composed of both the ventral tegmentum area and the substantia nigra). These regions represent the cell bodies and terminal fields of the nigrostriatal, mesolimbic and mesocortical dopaminergic tracts. Membrane fractions were prepared as follows: brain regions were weighed and ice-cold 0.32 mol/L sucrose, pH 7.4, added to tubes (10 volumes for PFC and VMS, 20 volumes for CP and NA). Brain tissues were homogenized with a teflon/glass homogenizer (Heidolph, Polyscience), and the homogenates were centrifuged at 41,000 X g for 20 min at 4°C. The supernatant was decanted and the pellet resuspended with 300 μL (CP and NA) or 600 μL (PFC and VMB) of 9 g/L saline. This washing procedure was repeated three times. Binding reactions were conducted in microtiter plates (CoStar, Corning, NY) as follows: resuspended membrane pellet (25 μL), sodium phosphate buffer (100 mmol/L Na_2HPO_4 , 120 mmol/L NaCl, 0.2 mmol/L ZnCl_2 , pH 7.5, 25 μL) was added to duplicate tubes, 10 μL ^3H -GBR 12935 added to all tubes, and 10 μL 1-[2-(bis [4-Fluorophenyl]-methoxyethyl)]-4-(3-phenylpropyl)-piperazine (GBR 12909; RBI) added to one tube of each sample to determine nonspecific binding. Incubation reactions had a final volume of 70 μL , and final concentrations of ^3H -GBR 12935 and GBR-12909 were 8 nmol/L and 1 $\mu\text{mol/L}$, respectively. Samples were incubated for 1 h at room temperature, filtered onto GF/B Whatman filter paper, placed in scintillation vials and 5 mL scintillation cocktail added. Samples were counted

on a Beckman liquid scintillation counter (LS 3801, Beckman Instruments) 24 h later. Protein contents were determined using the micro-Lowry assay.

TABLE 1

Body weight and iron status variables of male (M) and female (F) rats fed iron-deficient (ID) or control (CN) diets for 5 wk¹

Group	Body weight	Hematocrit	Hb	Serum Fe	TIBC ²	TSAT	Liver Fe
	g		g/L	μmol/L		%	μmol/g
CN-F	213 ± 6 ^b	0.41 ± .01 ^a	157 ± 5 ^a	35.1 ± 6.0 ^a	85.1 ± 7.7 ^b	41.5 ± 8.9 ^a	9.55 ± 1.37 ^a
ID-F	180 ± 4 ^d	0.18 ± .01 ^b	65 ± 3 ^b	15.8 ± 201 ^c	114.0 ± 10.8 ^a	13.6 ± 2.6 ^b	3.27 ± 0.48 ^c
CN-M	323 ± 7 ^a	0.43 ± .01 ^a	151 ± 2 ^a	23.0 ± 27 ^b	79.4 ± 3.3 ^b	27.1 ± 3.8 ^a	5.40 ± 0.57 ^b
ID-M	193 ± 5 ^c	0.16 ± .01 ^b	52 ± 2 ^c	13.7 ± 3.5 ^c	130.3 ± 18.7 ^a	11.1 ± 3.7 ^b	2.99 ± 0.61 ^c

¹ Values are means ± SEM, *n* = 8. Means in a column not sharing a superscript are significantly different, *P* < 0.05.

² TIBC, total iron binding capacity; TSAT, percentage transferrin saturation.

Iron analysis

Total iron concentration of brain region homogenates from all rats was determined according to our standard laboratory method using acid digestion and analysis with atomic absorption spectrophotometry (Erikson et al. 1997).

Statistical analysis

Data were analyzed using the SAS system for Windows v6.12 statistical analysis package (SAS, Cary, NC). Data were examined for normality of distribution and presence of outliers, ANOVA with repeated-measures factors (brain regions) and between-groups factors was used to test for interactions between dietary treatments and sex for ligand binding data. Two-way ANOVA was used to test effects of dietary treatment and sex on hematologic variables, liver nonheme iron and behavioral data, Tukey's post-hoc test was used to evaluate mean differences, The α -level for the analyses was set at *P* < 0.05. Omega squared (ω^2) calculations were performed to estimate the magnitude of the effect of iron deficiency on certain dependent variables (Myers and Well 1995).

RESULTS

ED₅₀ cocaine experiment. Both male and female rats fed the ID diet were iron deficient and anemic as indicated by the significantly lower hemoglobin, hematocrit, plasma transferrin saturation and liver nonheme iron concentration (Table 1). Iron-deficient rats of both sexes moved significantly less than control rats in the naive testing condition of saline injection (*P* < 0.001, Fig. 1). Increasing doses of cocaine were associated with significant increases in locomotion in all rats, although iron-deficient rats were clearly less responsive than control rats at all doses (*P* < 0.001) and maintained the 50% deficit in movement regardless of the level of blockade of DA uptake. Female rats in both dietary treatments exhibited more motor activity than male rats, and cocaine did not show a differential effect on iron-deficient rats of one sex compared with the other. Analyses of these dose-response curves via LOGIT-LOG transformation and computation of the ED₅₀ were performed to quantify the apparently right-shifted curves. These calculations showed an attenuated response in both male and female ID rats with significant independent effects of iron deficiency and sex on the ED₅₀ dose of cocaine (*F*_{1, 4} = 19.6, *P* = 0.015 and *F*_{1, 4} = 18.2, *P* 0.02, respectively, Table 2).

Uptake experiments. The 5-min uptake of DA and the *V*_{max} were significantly lower in striate synaptosomes of male and female iron-deficient rats compared with control rats (Table 2). Analysis of DA uptake at 30 and 60 s showed no effect of iron deficiency, hut uptakes at 90 and 120 s were 84 and 77% of control synaptosomal uptake (*P* < 0.05, data not shown). There was no effect of iron deficiency on the apparent *K*_m for uptake of DA (Table 2). The IC₅₀ experiments determined the effect of iron deficiency on the ability of cocaine to inhibit the synaptosomal uptake of DA (Table 2). Synaptosomes from the striatum from both iron-deficient male and female rats required ~6 μmol/L cocaine to inhibit 50% of the DA uptake compared with ~3 μmol/L cocaine in synaptosomes from control rats (*P* < 0.001). Because the synaptosomes from iron-deficient rats took up only

~50% as much DA as control synaptosomes to begin with, this amounts to a more than fourfold higher amount of cocaine necessary to achieve the same absolute suppression of DA uptake.

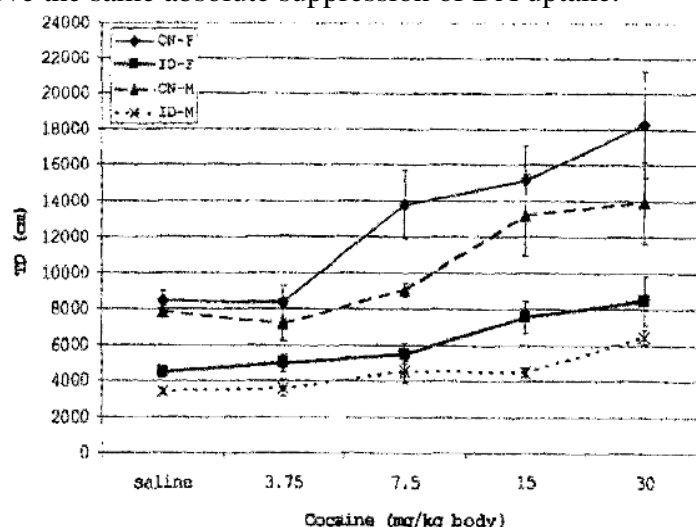


FIGURE 1 Total distance traveled (TD) measured using a Digiscan activity monitor for control female (CN-F), iron-deficient female (ID-F), control male (CN-M) and iron-deficient male (ID-M) rats. Values are means \pm SEM, $n = 5$, for saline, 3.75, 7.5, 15 and 30 mg/kg body cocaine doses. Iron deficiency significantly decreased TD in both males and females compared with control rats ($P < 0.001$) at all doses of cocaine and at the baseline condition of saline injection.

Release experiments. Iron deficiency did not significantly affect KCl-evoked ^3H -DA release from striate synaptosomes compared with synaptosomes from control rats when doses of KCl from 15 to 120 mmol/L were tested (data not shown). That is, there were similar percentages of the intrasynaptosomal pool of DA released in iron-deficient and control synaptosomes at each concentration of KCl. For example, at 120 mmol/L KCl stimulation, there was 68% release of DA above baseline in control synaptosomes and a 72% release above baseline in iron-deficient synaptosomes.

^3H -GBR 12935 ligand binding. Radioligand binding assays were performed to determine the affinity and density of DAT in isolated membranes for a highly specific ligand. Scatchard analyses were performed utilizing GBR 12935 concentrations that ranged from 1 pmol/L to 1 $\mu\text{mol/L}$ and revealed a significant effect of iron deficiency on B_{max} (8.2 ± 0.8 vs. 12.9 ± 1.1 pmol/mg of membrane protein, $P < 0.01$) but not K_d (8.1 ± 0.55 vs. 8.3 ± 0.69 nmol/L, $P = 0.88$) of the DAT for its ligand.

TABLE 2

IC₅₀ ^3H -dopamine uptake into synaptosomes and ED₅₀ dose of cocaine in male (M) and female (F) rats fed control (CN) and iron-deficient (ID) diets after 5 wk of dietary treatment¹

Group	ED ₅₀ cocaine ²	5-min uptake ³	V _{max} ²	K _m ³	IC ₅₀ cocaine ³
	mg/kg body	nmol/mg protein	nmol/(mg protein · 5 min)	nmol/L	$\mu\text{mol/L}$
CN-F	7.1 \pm 0.9 ^c	217.3 \pm 8.4 ^a	82.7 \pm 1.3 ^a	17.87 \pm 2.08 ^b	3.2 \pm 1.3 ^b
ID-F	11.2 \pm 1.3 ^b	128.5 \pm 15.3 ^c	57.9 \pm 8.0 ^b	16.67 \pm 0.95 ^b	6.3 \pm 1.7 ^a
CN-M	12.0 \pm 0.7 ^b	173.6 \pm 18.5 ^b	53.3 \pm 7.2 ^b	21.05 \pm 1.72 ^a	2.8 \pm 0.6 ^b
ID-M	17.0 \pm 1.8 ^a	70.6 \pm 5 ^d	44.1 \pm 1.9 ^c	21.79 \pm 1.08 ^a	5.9 \pm 2.2 ^a

¹ IC₅₀, half-maximal dose for in vitro effects; ED₅₀, half-maximal dose of drug.

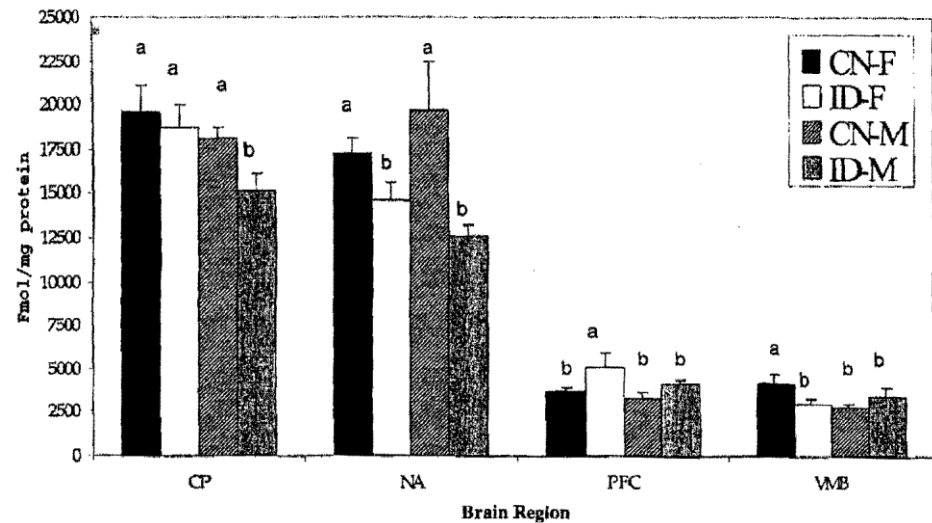
² Refers to data collected during the determination of the ED₅₀ for cocaine dose-response experiments and was calculated for LOGIT-LOG transformation followed by regression analysis. Values are means \pm SEM, $n = 5$ for in vivo studies. Means in a column not sharing a superscript are significantly different from one another ($P < 0.05$).

³ Refers to in vitro experiments from three separate assay cohorts with synaptosomes from two rats in each treatment group in each assay cohort ($n = 6$ total observations for each treatment and sex group).

Because there was no effect of iron deficiency on apparent affinity, a concentration of 16 nmol/L ^3H -GBR 12935, twice the apparent K_d , was used to examine B_{max} in four brain regions (Fig. 2). The effect of iron

deficiency on ^3H -GBR 12935 binding in all four brain regions examined was significant ($P = 0.0021$, $\omega^2 = 0.21$) as was the expected variation among brain regions ($P < 0.0001$), with $\text{CP} > \text{NA} >> \text{PFC} > \text{VMB}$ (Fig. 2). In addition, iron deficiency did not affect all regions in both sexes similarly because there was a significant interaction of dietary treatment and brain region with sex of the rats ($P < 0.001$). Iron-deficient males had significantly lower B_{max} in striatum (CP, $P = 0.035$), whereas both sexes were similarly and significantly affected by iron deficiency in the NA ($P = 0.002$). The terminal field of the mesocortical track, the PFC, actually had a significant 18% higher DAT density in female iron-deficient rats ($P = 0.046$) compared with control females. The VMB is comprised of the ventral tegmentum area and the substantia nigra, and was sensitive to effects of iron deficiency only in females ($P = 0.032$).

FIGURE 2 ^3H -1-(2-(diphenyl-methoxy)-ethyl)-4-(3-phenylpropyl) piperazine (^3H -GBR12935) radioligand binding in membrane fractions prepared from four brain regions of iron-deficient male (ID-M) and female (ID-F) and control male (CN-M) and female (CN-F) rats. Values are means \pm SEM, $n = 5$. The brain regions examined were caudate putamen (CP), nucleus accumbens (NA), prefrontal cortex (PFC) and ventral midbrain (VMB). There was an overall main effect of iron deficiency decreasing ^3H -GBR12935 binding across regions ($P = 0.002$). There was also a significant variation in ligand binding from region to region ($P = 0.0001$) ($\text{CP} > \text{NA} >> \text{PFC} > \text{VMB}$) and significant interaction of region \times treatment \times sex ($P = 0.02$). Bars within a region not sharing a letter are significantly different, $P < 0.05$.



Brain region iron concentrations. Iron deficiency for 4 wk caused a significant decline in iron concentration in nearly all four brain regions examined (Fig. 3; $F_{1, 16} = 33.7$, $P < 0.0001$). The greatest drop in brain iron concentration was in the cell bodies of the VMB ($F_{3, 64} = 13.8$, $P = 0.001$) (estimated $\omega^2 = 0.59$) (Fig. 3), although significantly lower brain iron concentrations were also observed in CP and PFC ($F_{3, 64} = 12.2$, $P < 0.001$ and $F_{3, 64} = 11.9$, $P < 0.001$, respectively). It is important to note that the iron concentration was not affected in the NA in female rats with this dietary protocol, whereas the iron concentration in this region in iron-deficient male rats was significantly ($P < 0.05$) lower than in controls.

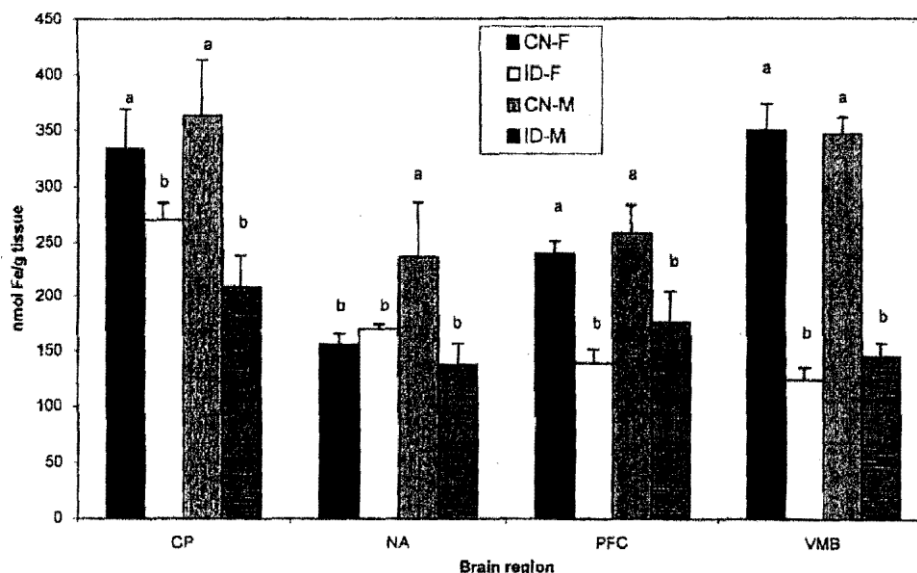


FIGURE 3 Total iron concentration in brain region membrane fractions prepared from four brain regions of iron-deficient male (ID-M) and female (ID-F) and control male (CN-M) and female (CN-F) rats. Values are means \pm SEM, $n = 5$. The brain regions examined were caudate putamen (CP), nucleus accumbens (NA), prefrontal cortex (PFC) and ventral midbrain (VMB). There was a significantly lower iron concentration across brain regions from rats fed the low iron diets ($P = 0.0001$) as well as a significant difference in iron concentrations among regions ($P = 0.0001$). There was no main effect of sex on brain region iron concentration. Bars within a region not sharing a letter are significantly different, $P < 0.05$.

In an attempt to examine the relationship of systemic iron status to brain iron status, we correlated liver nonheme iron concentration with regional brain iron concentration and observed a significant correlation of

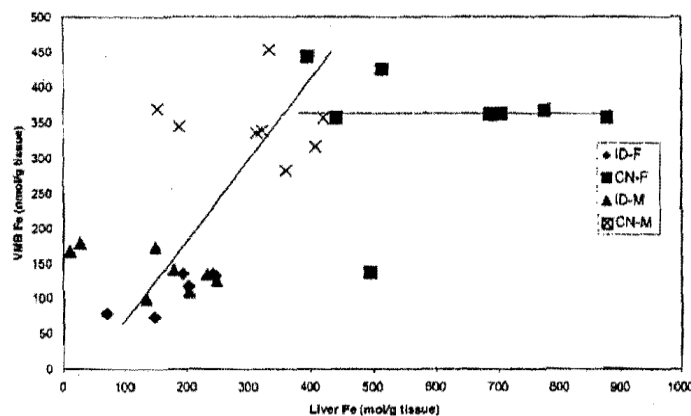
liver Fe with VMB Fe (Fig. 4; $r = 0.462$, $P < 0.0002$) when there was some depletion of liver iron (e.g., the liver iron concentration was $<400 \mu\text{mol/g}$). There was no relationship between these two variables in control rats; thus the nature of the curve relating regional brain iron to liver iron stores remains somewhat speculative. There was no relationship between liver iron and regional brain iron concentration in other brain regions. Within the brain, we observed significant correlations of VMB (substantia nigra and ventral tegmentum) cell body iron with regional iron concentrations in the terminal fields of these Dopaminergic tracts in striatum ($r = 0.41$, $P = 0.022$) and PFC ($r = 0.45$, $P = 0.015$).

DISCUSSION

These experiments demonstrate that iron deficiency alters both in vivo and in vitro functioning of the DAT in rat striatum. These alterations due to iron deficiency were brain region specific (occurring in striatum not PFC) and affected males more dramatically than females. This work thus extends the previous observations that iron deficiency is associated with a decreased D_2 receptor density (Ashkenazi et al, 1982, Youdim et al. 1983) and increased extracellular DA (Beard et al. 1994, Nelson et al. 1997).

The current observations of attenuated in vivo and in vitro responses to cocaine also provide evidence that increased extracellular DA in iron deficiency is due primarily to altered clearance (Nelson et al. 1997). Synaptosomal release data from the current experiment are not consistent with an increased release of DA that is large enough to account for the observations made in vivo (Fig. 3); nor is it likely that the elevated DA is the result of increased DA synthesis because tyrosine hydroxylase activity was modestly decreased, not increased (unpublished observations). The most obvious explanation for this increased extracellular DA is that iron deficiency affects DA reuptake into the presynaptic membrane by altering DAT functioning because this is the route of removal of $> 70\%$ of the extracellular DA from the synaptic space. Although there is no known direct role of iron for synthesis and/or regulation of the DAT, there are possible avenues of effect that involve gene regulation, post-translational modification, second messenger phosphorylation reactions or perhaps direct interactions with the ligand receptor site.

One of the key regulators of the DAT is the activation of the D_2 autoreceptor on the presynaptic membrane (Dickinson et al. 1999, Meiergerd et al. 1993). Increased agonist binding to this receptor increases the DAT activity (Meiergerd et al. 1993). Conversely, in D_2 receptor—deficient mice, in vivo voltammetry demonstrated that exogenous DA clearance was attenuated, suggesting altered DA uptake (Dickinson et al. 1999). Both of the aforementioned studies also demonstrated that the effect of the D_2 receptor occupancy on extracellular DA levels was not due to its effect on DA release. Thus, the down regulation of the D_2 receptor observed numerous times [see review of Youdim et al. (1989)] in the striatum of iron-deficient rats might also play an important mechanistic role in the decreased functioning of the transporter.



A second novel finding in the current studies is the observation that the dopaminergic system of female rats responds differently to iron deficiency than does the dopaminergic system of male rats. Others have demonstrated that female rats are more sensitive to cocaine than male rats and have a greater DAT density in striatum' (Morissette and DiPaulo 1993, Post et al. 1987). The current studies replicate the increased sensitivity (IC_{50}) of females compared with males because female rats required significantly less cocaine than males to elicit a 50% increase in locomotor activity. We showed previously that sex and strain of highly inbred strains of mice are strong determinants of the biologic responses to iron deficiency in early life (Morse et al. 1999a and 1999b). Although it appears that both male and female rats have similar alterations in brain iron concentration during postweaning iron deficiency in this and previous studies (Pinero et al. 2000), there are seemingly more subtle interactive effects exerted on the dopaminergic system than were previously appreciated.

Alterations in D_2 receptor functioning in iron-deficient rats has been alluded to a number of times as being causally related to abnormal locomotion (Youdim et al. 1989), poor home nesting behavior (Felt and Lozoff 1995), reversal of the dark; light activity cycle (Ben-Schachar et al. 1986) and perhaps altered proprioception. The current study used a more pharmacologic approach to demonstrate that indeed, profound alterations in behavior persist despite increasing extracellular DA with large amounts of cocaine. The ED_{50} cocaine experiment supports the concept that much higher concentrations of extracellular DA are required to have the same activation of locomotion as in control rats. Our previous microdialysis experiments with cocaine demonstrated that ID rats have higher than normal DA concentrations, both before and after cocaine administration (Nelson et al. 1997). The ability of cocaine to increase motor behavior demonstrates that at some functional level, all of the intracellular machinery and fundamental neural connections are working in the iron-deficient anemic rodent. The iron-deficient rats, however, never reached the same level of activity as the control rats, despite high doses of cocaine. This could clearly be due to fewer D_1 and D_2 receptors in several parts of the brain. In studies related to the current report, we indeed found decreased DA receptor densities in many of the same regions (unpublished results). Although the mechanistic relationship of local iron deficiency to DAT and receptor functioning remains unknown, the current report demonstrates convincingly that DAT functioning is altered in iron deficiency.

The final set of novel observations is derived from the examination of four brain regions, which represent three dopaminergic tracts (nigrostriatal, mesolimbic and mesocortical) rather than only one as had been studied previously (Youdim et al. 1989). Because striatum is the brain region with the richest innervation of dopaminergic tracts, it has been the region of choice to examine iron deficiency's involvement in DA metabolism. The current experiments showed that although the effect of iron deficiency on the DAT is fairly robust, it is not universal in all parts of dopaminergic tracts. Ventral midbrain, a point of origin of the three dopaminergic tracts studied, was relatively unaffected with respect to a drop in iron concentration. The PFC had substantially less iron in the iron-deficient rats compared with controls, but had greater DAT activity. The CP was very sensitive to iron depletion, and as a terminal field for the mesolimbic tract, had significant changes in DAT activity, but only in male rats. The present report is the first to demonstrate a lower iron concentration in the NA as a function of dietary iron deficiency. This region also showed significantly lower DAT activity due to iron deficiency. The sensitivity of the CP to iron deficiency was expected on the basis of our previous data, but because we had not measured accumbens iron concentrations in previous studies, we did not know whether iron concentrations there would be affected. However, in our efforts to explore the "robustness" of the effect of iron deficiency on brain DA metabolism, we had to include several dopaminergic tracts. The role of these regions in exploratory behavior is partially what determined the behavior tests that were administered. That is, lesions in the caudate and the NA can decrease motor activity. Spatial learning, avoidance tasks and anxiety protocols would be more sensitive to alterations in other brain regions such as the PFC and hippocampus and will be employed in future protocols. We do not yet have the cellular detail to determine whether iron deficiency is more important to functioning of cell bodies or is exerting a more important role in the terminal fields of these dopaminergic tracts. However, the data from this experiment suggest that even very modest limitations in iron supply to the cell body may have an important effect on dopaminergic functioning.

In conclusion, we believe that important new findings resulted from these experiments. Strong and consistent in vivo and in vitro data demonstrate that the DAT is only ~50% as sensitive in iron-deficient rats as control rats. Moreover, studies in three dopaminergic tracts revealed dissimilar effects of iron deficiency, thus demonstrating regional specificity of effect. That is, iron deficiency caused alterations in the nigrostriatal tract and had the opposite effect in the mesocortical tract. Finally, sex of the subject has a strong influence on the effect of iron deficiency in vitro. Behavioral studies support the concept that alterations in DA metabolism play a role in the behavioral outcomes of iron deficiency. Although a mechanism is still unclear, lowered DAT functioning and lowered DA D2 receptor density are consistent with a coupling of iron-related events. We hope to probe this question in subsequent studies.

Notes:

² Abbreviations used: CN-F, control female rats; CN-M, control male rats; CP, caudate putamen; DA, dopamine; DAT, dopamine transporters; ED₅₀, half-maximal dose of drug; G8R 12935, 1-(2-(diphenylmethoxy)-ethyl)-4-(3-phenylpropyl) piperazine; GBR 12909, 1-[2-(bis[4-fluorophenyl]-methoxyethyl)-4-[3-phenylpropyl]-piperazine; IC₅₀, half-maximal dose of drug for in vitro effects; ID-F, iron-deficient female rats; ID-M, iron-deficient male rats; KRH, Krebs-Ringer HEPES; NA, nucleus accumbens; PFC, prefrontal cortex; TIBC, total iron-binding capacity; VMB, ventral midbrain.

LITERATURE CITED

- Ashkenazi, R., Ben-Shachar, D. & Youdim, M.B.H. (1952) Nutritional iron and dopamine binding sites In the rat brain. *Pharmacol. Biochem. Behav.* 17 (suppl. 1): 43-47.
- Beard, J. L., Chen, O., Connor, J. R. & Jones, B. C. (1994) Altered monoamine metabolism In caudate putamen of iron-deficient rats, *Pharmacol. Biochem. Behav.* 48: 821-824.
- Beard, J. L., Connor, J. R. & Jones, B. C. (1993) Iron in the brain. *Nutr. Rev.* 51: 157-170.
- Ben-Shachar, D., Ashkenazi, R. & Youdim, M.B.H. (1986) Long term consequences of early Iron deficiency on dopaminergic transmission. *J. Dev. Neurosci.* 4: 81-88.
- Chen, O., Connor, J. R. & Beard J. L. (1995) Brain iron, transferrin and ferritin concentrations are altered in developing iron-deficient rats. *J. Nutr.* 125: 1529-1535.
- Ciriak, B. J., Hellman, C., Demchyshyn, L. L., Pristupa, Z. B., Ince, E., Hersch, S. M., Niznik, H. B. & Levey, A. I. (1995) The dopamine transporter. Immunohistochemical characterization and localization in brain. *J. Neurosci.* 15: 1714-1723.
- Dickinson, S. D., Sabeti, J., Larson, G. A., Giardina, K., Rubinstein, M., Kelly, M. A., Grandy, D. K., Low, M. J., Gerhardt, G. A. & Zahniser, N. R. (1999) Dopamine D receptor-deficient mice exhibit decreased dopamine transporter function but no changes in dopamine release In dorsal striatum. *J. Neurochem.* 72: 148-156.
- Erikson, K. M., Pinero, D. J., Connor, J. R. & Beard, J. L. (1997) Regional brain Iron, ferritin and transferrin concentrations during Iron deficiency and iron repletion In developing rats. *J. Nutr.* 127: 2030-2038.
- Glover, J. & Jacobs, A. (1972) Activity pattern of iron-deficient rats, *Br. Med. J.* 2: 627-628
- Hunt, J. F., Zito, C. A., Erjavec, J. & Johnson, L. (1994) Severe or marginal iron deficiency affects spontaneous physical activity in rats. *Am. J. Clin. Nutr.* 59: 413-418.
- Idjrandinata, P. & Pollitt, E. (1993) Reversal of developmental delays In Iron-deficient anemic infants treated with iron. *Lancet* 341: 1-4.
- Kwik-Urbe, C. L., Golub, M. S. & Keen, C. L. (1999) Behavioral consequences of marginal Iron deficiency during development in a murine model. *Neurotoxicol. Teratol.* 21: 661-672.
- Larkin, E. C. & Rao, G. A. (1990) Importance of fetal and neonatal iron: adequacy for normal development of central nervous system. In: *Brain, Behavior and Iron in the Infant Diet* (Dabbing, J., ed.), pp. 43-63. Springer-Verlag, London, UK.
- Lozoff, B. & Brittenham, G. M. (1986) Behavioral aspects of iron deficiency. *Prog. Hematol.* 14: 23-53.
- Lozoff, B., Klein, N. K., Nelson, E. C., McClish, D. K., Manuel, M. & Chacon, M. E. (1998) Behavior of Infants with Iron deficiency anemia. *Child Dev.* 69: 24-36.
- Mash, D. C. & Staley, J. K. (1997) The dopamine transporter in human brain. In: *Neurotransmitter Transporters: Structure, Function and Regulation* (Reith, M.E.A., ed.), pp. 315-343, Humana Press, Totowa, N.J.

Meiergerd, S. M., Patterson, T. A. & Schenk, J. O. (1993) D₂ receptors may modulate the function of the striate transporter for dopamine: kinetic evidence from studies in vitro and in vivo. *J. Neurochem*, 61: 764-767.

Miller, J. W., Kievan, D. T., Domin, B. A. & Fremeau, A., Jr. (1997) Cloned sodium- (and chloride-) dependent high-affinity transporters for GABA, glycine, praline, betaine, taurine and creatine, In: *Neurotransmitter Transporters: Structure, Function and Regulation* (Reith, M.E.A., ed.), pp. 101-149. Humana Press, Totowa, NJ.

Morrisette, M. & DiPaolo, T. (1993) Sex and estrous cycle variations of rat striate dopamine uptake sites. *Neuroendocrinology* 58: 16-22.

Morse, A. C., Beard, J. L., Azar, M. R. & Jones, B. C. (1999a) Sex end genetics are important cofactors in assessing the impact of iron deficiency on the developing mouse brain. *Nutr, Neuro*. 2: 323-335.

Morse, A. C., Beard, L. & Jones, B. C. (1999b) A genetic developmental model of iron deficiency: biological aspects. *Proc. Soc. Exp. Biol. Med.* 220: 147-152.

Myers, J. L. & Well, A. D. (1995) *Research Design and Statistical Analysis*. Lawrence Earlbaum, Mahwah, NJ.

Nelson, C., Erikson, K., Pinero, D. J. & Beard, J. L. (1997) In vivo dopamine metabolism Is altered in Iron-deficient anemic rats. *J. Nutr*, 127: 2282-2288.

Pinero, D. J., Li, N. Q., Connor, J. R. & Beard, J. L. (2000) Variations in dietary iron alter brain iron metabolism In developing rats. *J. Nutr*. 130: 254-263.

Pollit, E. (1993) Iron deficiency and cognitive function. *Annu. Rev. Nutr*, 13: 521-635

Post, R. M., Weiss, S.R.B., Pert, A. & Uhde, T. W, (1987) Chronic cocaine administration: sensitization and kindling effects. In: *Cocaine: Clinical and Biobehavioral Aspects* (Fisher, S., Raskin, A. & Uhlenhut, E. H., eds.), pp. 108-173, Oxford University Press, New York, NY.

Rao, S. G., de Ungria, M., Sullivan, D., Wu, P., Wobken, J. D., Nelson, C. A., Georgieff, M. K. (1999) Perinatal Iron deficiency increased the vulnerability of rat hippocampus to hypoxic ischemic Insult. *J, Nutr*. 129: 199-206.

Reith, M.E.A., Xu, C. & Chen, N. H. (1997) Pharmacology and regulation of the neuronal dopamine transporter, *Eur. J. Pharmacol*, 324: 1-10.

Rocagliolo M., Garrido, M., Walter, T., Peirano, P. & Lozoff, B. (1998) Evidence of altered central nervous system development In infants with iron deficiency anemia at 6 mo: delayed maturation of auditory brainstem responses, *Am. J. Chn, Nutr*, 68: 683-690,

Walter, T. (1993) Impact of Iron deficiency on cognition in infancy and childhood. *Eur. J. CHI., Nutr*, 47: 307-316.

Youdim, M.B.H., Ben-Shachar, D., Ashkenazi, R. & Yehuda, S. (1983) Brain iron and dopamine receptor function. In: *CNS Receptors from Molecular Pharmacology to Behavior* (Mandel, P. & DeFeudls, F.V., eds.), pp. 309-322. Raven Press, New York, NY.

Youdim, M.B.H., Ben-Shachar, D. & Yehuda, S. (1989) Putative biological mechanisms of the effect of Iron deficiency on brain biochemistry and behavior. *Am. J. Clin, Nutr*. 50; 807-617.

Youdim, M.B.H., Yehuda, S. & Ben-Uriah, Y. (1981) Iron deficiency induced circadian rhythm reversal of dopaminergic mediated behaviors and thermoregulation in rats. *Eur. J Pharmacol*, 74; 295-301.