Iron deficiency decreases dopamine D_1 and D_2 receptors in rat brain

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Abstract:
Iron deficiency (ID) in early life is known to alter neurological development and functioning, but data regarding specific effects on dopamine biology are lacking. The objective of this study was to determine the extent of functional alterations in dopamine receptors in two dopaminergic tracts in young, growing, iron-deficient rats. Forty male and 40 female weanling Sprague–Dawley rats were fed either an iron-deficient (ID) diet or control (CN) diet for 6 weeks. ID decreased densities of D_1 and D_2 receptors in the caudate–putamen and decreased D_2 receptor densities in the nucleus accumbens. There were no apparent effects of ID on the affinities for the ligands in either receptor in several brain regions. In situ hybridization studies for both dopamine receptors revealed no significant effect of ID on mRNA expression for either receptor. Iron-deficient rats had a significantly higher ED_{50} for raclopride-induced hypolocomotion in male and female rats compared to control rats of each sex. The loss of iron in the striatum due to dietary ID was significantly correlated with the decrease in D_2 receptor density; however, this relationship was not apparent in other brain regions. These experiments thus demonstrate abnormal dopamine receptor density and functioning in several brain regions that are related to brain regional iron loss. Importantly, the impact of ID on dopamine was more pronounced in males than females, demonstrating sex-related different sensitivities to nutrient deprivation.
Keywords: Iron deficiency; Rats; Dopamine receptors; Sex; Brain iron

Article:
1. Introduction
Iron deficiency (ID) is one of the most common nutritional disorders in the world, affecting nearly 5 billion people (ACC/SCN, 1992). Symptoms of ID include lethargy, lack of concentration and decreased cognitive and attentional processes (Lozoff and Brittenham, 1986; Pollit, 1993; Walter, 1993). There is evidence showing that iron does play a role in neurobiological processes (Beard et al., 1993; Rocangliolo et al., 1998; Youdim et al., 1989); however, the neurobiological mechanisms behind iron's role in behavior and cognition remain unknown. Whether ID early in life has persistent neurobehavioral effects is the topic of research for several investigators. For example, one question deals with whether deficits in cognition resulting from ID in infancy can be reversed by an iron-adequate diet later in life.

In the rat, postweaning ID produces a decrease in brain iron content that is reversible with iron repletion (Chen et al., 1995a; Erikson et al., 1997; Pinero et al., 2000). Furthermore, ID-related decreased brain iron content has been linked to many neurological alterations, including hypomyelination (Larkin and Rao, 1990), delayed neuromaturation (Rocangliolo et al., 1998) and altered dopaminergic functioning (Nelson et al., 1997). The effects of ID on dopamine function include decreased D2 receptor density in caudate–putamen (Youdim et al., 1983) and increased extracellular dopamine levels (Beard et al., 1994; Chen et al., 1995b; Nelson et al., 1997). Other researchers have restricted the examination of ID to the undifferentiated striatum; however, we recently observed differential effects of ID between the dorsal striatum (containing the caudate and putamen) and the ventral striatum (containing the nucleus accumbens) (Erikson et al., 2000). A recent study from our laboratory demonstrated a significant decrease in DA transporter density in several brain regions with an associated altered sensitivity to cocaine (Erikson et al., 2000).

Other neurotransmitter systems may be involved as well because iron is known to be involved in the synthesis and catabolism of other monoamines. For example, ID decreases the density of serotonin transporters in mice (Morse et al., 1999) and serotonin levels in rat brain (Youdim et al., 1989). Also, noradrenergic function is altered (Beard et al., 1994; Chen et al., 1995b; Youdim et al., 1989). Behavioral consequences include altered locomotor and exploratory activity (Glover and Jacobs, 1972; Hunt et al., 1994; unpublished observations from our laboratory). It is important to note that the alterations in dopamine function result from decreased brain iron content and not anemia per se (Ashkenazi et al., 1982; Nelson et al., 1997; Youdim et al., 1989) and are reversible with iron therapy if the ID occurs after weaning (Nelson et al., 1997; Youdim et al., 1981).

To date, five dopamine receptors have been identified and subdivided into two groups, D_2-like (D_2, D_3 and D_4) and D_1-like (D_1 and D_5), based on pharmacological and biochemical characteristics (reviewed in Vallone et al., 2000). Previous studies examining alterations in striatal dopamine function related to ID demonstrated that D_2 receptor density was significantly decreased, whereas there was no effect on D_1 receptor density (Youdim et al., 1979, 1989) or on tissue concentrations of dopamine. Our own reports (Beard et al., 1994; Chen et al., 1995b; Nelson et al., 1997) of elevations in extracellular DA in striatum of iron-deficient rats suggest a high likelihood of changes in D_1 and D_2 receptor densities, although there is no clear evidence for changes in D_1 receptor density or affinity in the extant literature. We propose that a careful examination of the impact of ID in several brain regions and on multiple dopamine receptor types is crucial to a more complete understanding of the impact of ID.

Our overall hypothesis was that ID affects dopaminergic brain regions by decreasing both D_2 and D_1 receptor densities in terminal fields of dopaminergic tracts. We also sought to elucidate the relationship between ID-altered dopamine receptor expression and dopamine-related behaviors.

2. Materials and methods

2. 1. Animals

Male and female 21-day-old Sprague–Dawley rats (purchased from Harlan Sprague–Dawley, Indianapolis, IN) were randomly divided into two dietary treatment groups: control (CN; 35 mg Fe/kg diet, n = 20–25 males and females) and iron-deficient (ID; 3 mg Fe/kg diet, n = 20– 25 males and females). The design of the experiments was established in a way as to test the effects of dietary treatment, sex of the subjects and potential interactions of these two independent variables on dopamine function and behavior. The animals were fed iron-sufficient and iron-deficient diets, as previously described (Erikson et al., 1997; Pinero et al., 2000), and had free access to food and water 24 h/day. In the colony room, the lights were turned off between 1800 and 0600 h and the temperature was maintained at 25 ± 1°C. As will be seen in the Results section, growth of iron-deficient animals was significantly slower than that of control, but this was not due to anorexia (Beard et al., 1995). To determine if being “underweight” had a significant impact on our dependent variables of interest, we utilized frozen brains from a nearly identical cohort of animals except for the inclusion of a group of controls who were food-restricted (pair-fed) to match their growth rate to those of the controls.

The behavioral studies were performed between 0900 1200 h or approximately 3–6 h after the onset of the light cycle. Animals continued to have free access to food and water the night before behavior testing. The behavioral testing apparatus was located in a room adjacent to animal holding rooms. The Pennsylvania State University Animal Care and Use Committee approved all of the animal procedures.

2.2. Effect of raclopride on locomotor activity

After 4 weeks of dietary treatment, the animals were tested for the behavioral effects of a potent D_2 receptor antagonist, raclopride. Locomotor activity was measured using a Digiscan Animal Activity Monitor, model RXYZCM (Omnitech Electronics, Columbus, OH). It consisted of a set of four 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). A 2-day behavioral testing protocol was implemented. On Day 1, five rats from each group (diet/sex) were injected with saline (1 ml/kg bw) 15 min prior to testing. Rats were then injected with a dose of cocaine–HCl (Sigma Chemicals, Natick, MA) shown previously to increase their

locomotion by 50% (Larkin and Rao, 1990) and immediately placed in the center of the activity monitor. This dose of cocaine was used to increase locomotor activity of iron-deficient rats to a level sufficient to allow us to determine the impact of raclopride. The ED_{50} cocaine doses used were established in previous studies: 7.1 and 12 mg/kg bw, female and male CN, respectively; 11.2 and 17 mg/kg bw, female and male ID, respectively (Erikson et al., 2000). The animals were left in the activity monitor for 30 min. The index of locomotor activity was total distance traveled over 30 min in centimeters. On Day 2, the rats were injected with one of four doses, 0.1, 0.25, 0.5 or 1.0 mg/kg bw of raclopride tartrate (RBI, Natick, MA), 15 min prior to testing. The rats were then injected with their respective ED_{50} cocaine dose and locomotor activity was measured as on the preceding day. The ED_{50} for raclopride to decrease total distance (by 50%) was calculated using a Log–Logit transformation of the data followed by regression analysis. Other behaviors recorded by the Omnitech activity monitors included frequency nose-pokes in the holeboard (a putative measure of exploration), frequency of repeated movements (related to stereotypy) and time spent in the center of the apparatus (a putative measure of temerity). Upon completion of the behavior testing, the rats were killed by decapitation, livers removed for non-heme iron determination (Erikson et al., 1997) and brains rapidly taken for iron analysis and ligand binding assays. The hematological status of each rat was determined in the fresh blood drained from the trunk by published methods (Chen et al., 1995a; Erikson et al., 1997).

2.3. Dopamine D_1 receptor density determinations

Brains from rats were dissected on an ice-cold aluminum block into four regions: prefrontal cortex (PFC), caudate– putamen (CP), nucleus accumbens (NA) and ventral mid-brain (VMB — consisting of the substantia nigra and the ventral tegmedullary region) using well-defined landmarks (Paxinos and Watson, 1986) and regions frozen at -80°C. Membrane fractions were prepared from frozen tissue and assays conducted using published methods with few modifications (Morse et al., 1999). Membrane fractions were washed several times with isolation buffer prior to their utilization in binding assays. Binding reactions were con-ducted in microtiter plates (CoStar brand, Corning 9017) containing: tissue homogenate (25 µl), Tris buffer (50 mM Tris, 120 mM NaCl), pH 7.4 (25 µl), 10 µl ^{3}H-SCH23390 (8 nM) and 0.1 mM Butaclamol (RBI), final concentration, for non-specific binding. Saturation binding was performed using a nano- to micromolar range of SCH23390. Samples were incubated for 1 h at room temperature, filtered onto GF/ B Whatman filters and the filters were placed in scintillation vials containing 5 ml scintillation cocktail. Samples were counted on Beckman liquid scintillation counter (LS 3801; Beckman Instruments, Irvine, CA) 24 h later. Protein concentrations were determined using the micro-Lowry assay (P5656; Sigma).

2.4. Dopamine D_2-like receptor density determinations

Radioligand binding was performed on membrane fractions prepared identically to the above procedure and according to published protocols (Morse et al., 1999) with the following exceptions: Tris buffer (50 mM Tris, 120 mM NaCl), pH 7.8, was used and 10 µl ^{125}I-epidepride (D_2 antagonist, 4 nM) added to all tubes. As above, 0.1 mM Butaclamol (RBI) was used to determine non-specific binding. We did not differentiate between members of the D_2 "family" by using other more specific blocking agents like 7- hydroxy-*N*,*N*-di(I-propyl)-2-aminotetralin (7-OH-DPAT) in these particular studies. Scatchard plot analyses were per-formed using a 10^6 range of epidipride concentrations prior to the determinations of receptor density.

2.5. Iron analysis

Total iron content of brain region homogenates was determined according to our standard laboratory method using acid digestion and analysis with atomic absorption spectrophotometry (Erikson et al., 1997; Pinero et al., 2000).

2.6. Analysis of transcription for D_1 and D_2 receptors

Six additional iron-deficient and control rats (three of each dietary treatment), treated as were the others, were killed at PND 63 in order to conduct in situ hybridization histochemistry. Brains were rapidly removed, quickly frozen in isopentane and dry ice and frozen at -80°C until histological preparation (Campbell and Hess, 1999). Twenty-micrometer sagittal sections were cut using a cryostat and thaw-mounted on Superfrost Plus glass slides (Fisher, Pittsburgh, PA). After drying, the slide-mounted sections were stored at -70°C. The assay was

performed in such a way as to always have at least one control brain slice on the slide with an iron-deficient brain slice. Comparisons were always made between slices on the same slide to reduce variability.

The cDNA probe specific to the D1 dopamine receptor in pGEM-3Z was a generous gift from Dr. Marc Caron (Duke University, NC). The clone for the D_2 dopamine receptor was obtained from the IMAGE Consortium (LLNL) cDNA clone ID no. 312056 in pCMV-SPORT. Hybridization assays were conducted as previously described (Campbell and Hess, 1999).

Analysis of mean grain density was performed using the public domain NIH Image program (National Technical Information Service, Springfield, VA). For each region, mean grain density was measured as square pixels remaining after the images were normalized by adjusting the threshold in NIH Image. For single cell quantitation of D_2 dopamine receptor mRNA in the substantia nigra, grain density over individual cells was counted in inverted darkfield views captured at x 20 magnification. At least 96 cells, randomly identified with a random number algorithm, were quantitated per animal for each probe. Slices from the same regions for five of the six animals were examined. To quantitate D_1 dopamine receptor and D2 dopamine mRNA expression in the striatum, images were captured at x 3.0 directly from autoradiographs. The density of at least 64 measurements was assessed per animal for each probe. These densities were then compared between iron-deficient and control slices on the same slide and data expressed as a percentage of control density.

Table 1
Hematological and liver non-heme iron data

Group		Body weight (g)	Hct	Hb (g/l)	Serum Fe (μmol/l)	TIBC (μmol/l)	Tf sat%	Liver Fe (μmol/g tissue)
CN-F	Mean	213.6[a]	0.41[a]	157.3[a]	35.1[a]	85.1[a]	41.5[a]	9.55[a]
	S.E.M.	6.66	0.01	5.14	5.97	7.7	8.9	1.37
ID-F	Mean	180[b]	0.18[b]	65.2[b]	15.8[b]	114[b]	13.6[b]	3.27[b]
	S.E.M.	3.47	0.01	2.88	2.1	10.8	2.6	0.48
CN-M	Mean	323.8[c]	0.43[a]	151.4[a]	23[c]	79.4[a]	27.1[a]	5.40[c]
	S.E.M.	7.45	0.01	2.15	2.7	3.34	3.8	0.57
ID-M	Mean	193.4[d]	0.16[b]	53.3[c]	13.7[b]	130.3	11.1[b]	2.99[b]
	S.E.M.	4.82	0.01	2.24	3.5	18.7	3.7	0.61

Superscript letters signify statistical significance at $P<.05$.
CN-F = control female; ID-F = iron-deficient female; CN-M = control male; ID-M = iron-deficient male.

2.7. Statistical analysis

The data were analyzed using the SAS system for Windows v. 6.12 statistical analysis package (SAS, Cary, NC). Analysis of variance (ANOVA) with repeated-measures factors (brain regions) and between-groups factors (Sex and Diet) was used to test for main effects of and interactions between dietary treatments and sex for ligand binding data. Two-way ANOVA was used to test effects of dietary treatment and sex on hematological parameters, liver non-heme iron and behavioral data. Magnitude of effect for diet and sex and their interactions was evaluated using estimated ω^2 (Hinkle et al., 1998). The Tukey HSD test was used for multiple comparisons. Alpha level for the analyses was set at $P < .05$.

3. Results

3. 1. ED_{50} raclopride experiment

Both males and females fed the iron-deficient diets from weaning were severely iron-deficient and anemic at the time of testing (Table 1). The females were significantly smaller than males, but there was no significant difference in severity of anemia between sexes (Table 1). Under saline treatment, iron-deficient animals evinced less distance traveled than control rats. Total number of repeated movements [$F(1,40)=102.78$, $\omega^2 = 0.523$] and center time [$F(1,40)=16.21$, $\omega^2 = 0.159$] were also significantly affected by ID anemia (Table 2). Increasing doses of raclopride resulted in the expected decreased locomotor activity in both sexes (Fig. 1). Moreover, iron status had a profound affect on the ED50 for raclopride as did the sex of the subjects (Table 2) [$F(1,40) = 16.41$ and $F(1,40)=6.11$]. ID affected females more dramatically than males (ED_{50}=0.70 and 0.41 mg/kg for ID

females and ID males, respectively). There was no effect of sex on the ED_{50} for raclopride in the control rats (0.20 mg/kg for both sexes).

Table 2
Behavioral effects of ID in female and male rats

Group		TD	ED_{50}	#NP[a]	#RM	CT
CN-F	Mean	8414	0.20	16.9	245.7	220.45
	S.E.M.	551.41	0.01	3.26	12.19	43.09
ID-F	Mean	4434	0.70	10.6	190.7	111.75
	S.E.M.	172.84	0.16	1.73	5.73	10.34
CN-M	Mean	7856	0.20	8.55	254.4	233.7
	S.E.M.	286.88	0.01	1.01	3.81	14.99
ID-M	Mean	3394	0.41	7.8	149.55	130.2
	S.E.M.	181.34	0.08	1.35	7.25	24.31

Behavioral data from ED_{50} raclopride experiment. CN-F = control female; ID-F = iron-deficient female; CN-M = control male; ID-M = iron-deficient male. All variables were measured for 30 min. TD = total distance traveled in centimeters; ED_{50} raclopride = dose of raclopride (mg/kg bw) required to decrease TD by 50%; #NP = total number of nosepokes; #RM = total number of repeated movements (stereotypy); CT = total time in center of activity box.

[a] ID had profound effects on all variables measured except #NP. There was a significant effect of sex on #NP [$F(1,40) = 7.46$, $\omega^2 = 0.073$].

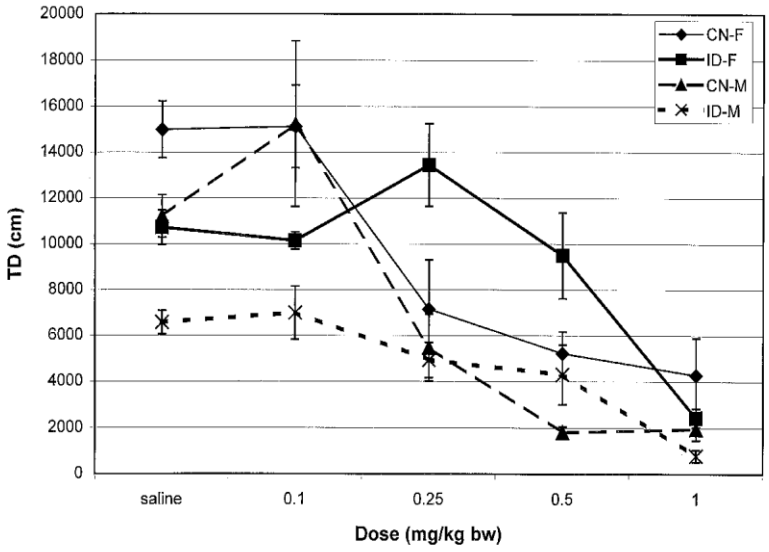

Fig. 1. Total distance traveled (TD) measured using a Digiscan activity monitor for control female (CN-F), iron-deficient female (ID-F), control male (CN-M) and iron-deficient male (ID-M) rats. Data are mean ± S.E.M. for saline and 0.1, 0.25, 0.5 and 1.0 mg/kg bw raclopride doses. ID significantly decreased TD in both males and females ($P < .001$).

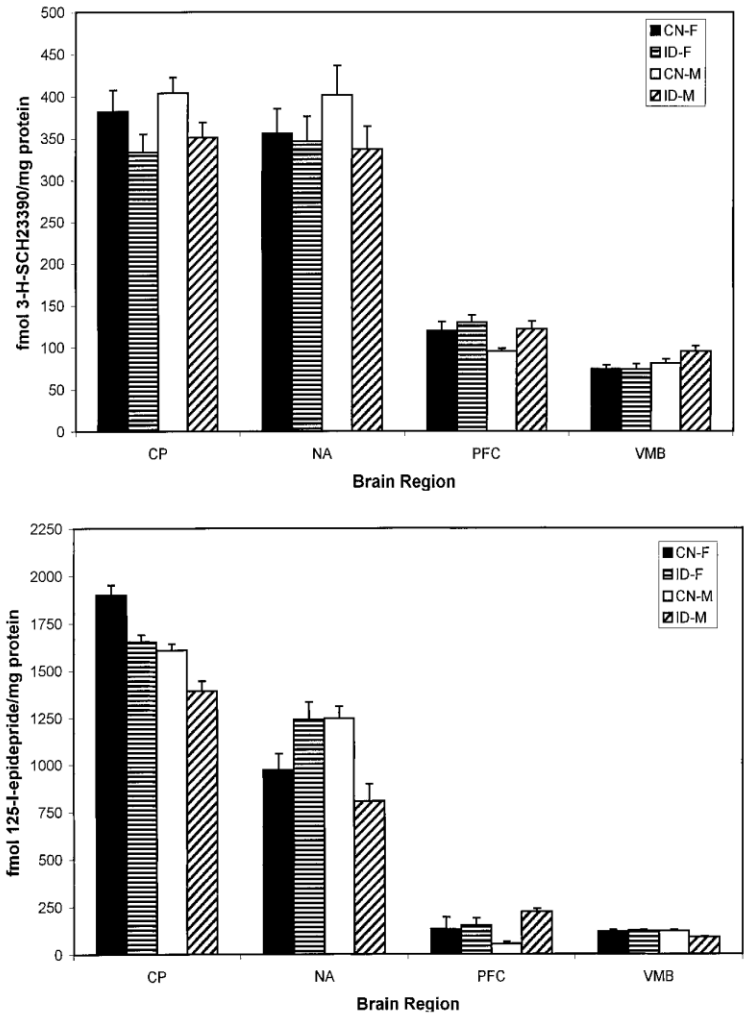

Fig. 2. (a) ^{3}H-SCH23390 radioligand binding in membrane fractions prepared from four brain regions of control female (CN-F), iron-deficient female (ID-F), control male (CN-M) and iron-deficient male (ID-M). The brain regions examined were caudate–putamen (CP), nucleus accumbens (NA), prefrontal cortex (PFC) and ventral midbrain (VMB). ID significantly decreased D_1 receptor density in caudate–putmen [$F(1,16)=5.8$, $P<.05$] and increased D_1 receptor density in PFC [$F(1,16)=4.7$, $P<.05$] across sexes. (b) ^{125}I-epidepride radioligand binding in membrane fractions prepared from four brain regions of control female (CN-F), iron-deficient female (ID-F), control male (CN-M) and iron-deficient male (ID-M). The brain regions examined were caudate–putamen (CP), nucleus accumbens (NA), prefrontal cortex (PFC) and ventral midbrain (VMB). ID had an overall main effect on decreasing D_2 receptor density across all brain regions [$F(1,32)=5.3$, $P<.05$]; there was a main effect of sex [females with higher density, $F(1,32)=13.4$, $P<.01$] and a significant interaction of group by sex across brain regions [D_2 density decreased most severely in ID-M, $F(1,16)=4.9$, $P<.05$].

3.2. ^{3}H-SCH23390 and ^{125}I-epidepride ligand binding

There was no effect of ID on the affinity of either ligand (K_d) to D_1 - or D_2-like receptors in the caudate–putamen as determined by Scatchard plot analyses (D_2 K_d = 0. 162 ± 0.02 nM for control caudate, K_d = 0.164 ± 0.022 nM for iron-deficient caudate; SCH23390 D_1 K_d=1.52±0.38 nM for control, K_d = 1.30±0.21 for iron-deficient caudate) We performed D_2 K_d determinations in pooled samples from control animals using epidipride in prefrontal cortex (0.98±0.028 nM), nucleus accumbens (1.26 ± 0.019 nM) and ventral mid-brain (1.65 ± 0.011 nM), but did not have sufficient material to verify that there were no significant effects of ID on D_2-like receptor affinity in those regions. D_1 K_d in ventral midbrain was also unaffected by brain ID (K_d = 2.20 nM in control compared to K_d = 1.89 nM in ID).

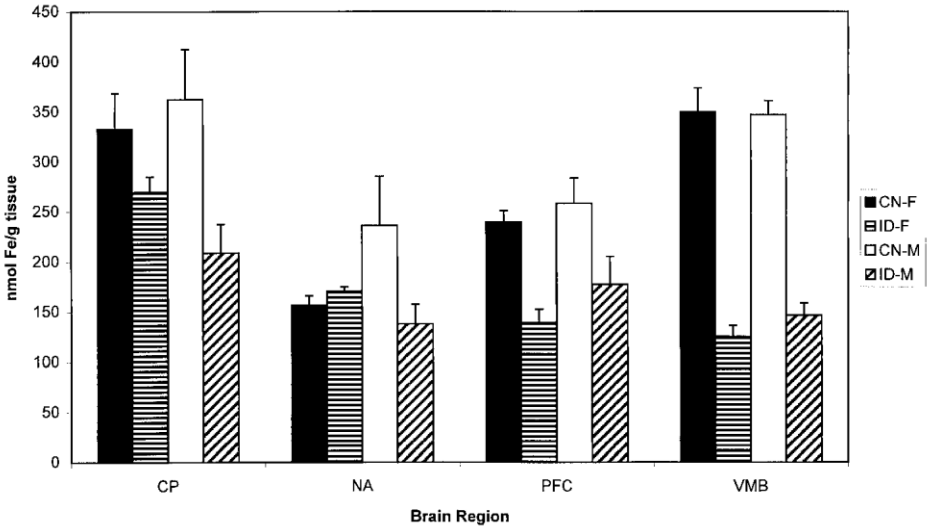

Fig. 3. Total iron content in tissue homogenates prepared from four brain regions of control female (CN-F), iron-deficient female (ID-F), control male (CN-M) and iron-deficient male (ID-M). The brain regions examined were caudate–putamen (CP), nucleus accumbens (NA), prefrontal cortex (PFC) and ventral midbrain (VMB). ID significantly decreased iron content across brain regions [$F(1,32)=33.7$, $P<.001$].

For both ligands, there were variations in receptor densities by brain region [F(3,64) = 327.2, P <.00 1; F(3,64) = 213.7, P<.001 (D_1 and D_2, respectively)] (Fig. 2). There was also a main effect of dietary treatment [F(1,32)=5.3, P ≤ .05 (estimated ω^2 =0.191)] with ID decreasing D_2 receptor density (Fig. 2). ID caused a significant decrease in both D_1 and D_2/D_3 densities in CP [F(1,16)=5.74, P<.05 (estimated ω^2=0.048; F(1,16)=4.55, P < .05 (estimated ω^2 = 0. 110)], respectively, and a significant increase in D_1 and D_2 densities in prefrontal cortex [F(1,16) = 4.7, P ≤ .05 (estimated ω^2 = 0.039, 0.0328); F(1,16)=5.44, P ≤ .05 (estimated ω^2 = 0.157)], respectively The nucleus accumbens and ventral midbrain were not significantly affected. Females had a significantly higher overall D_2 receptor density [F(1,32)=13.4, P ≤ .01 (estimated ω^2 = 0.546)] (Fig. 2b), but not D_1 density, compared to male rats. A significant interaction between diet and sex resulted in iron-deficient males, but not females [F(1,16)=7.6, P < .05 (estimated ω^2 = 0.286)], evincing decreased D_2-like receptor density. There was no such interaction for D_1 receptor density. Controlling for body weight did not remove the significant effects of either iron status or sex of the subjects.

Because the growth of the iron-deficient rats was significantly less than that of controls, we retrospectively examined D_1 and D_2 receptor density in brains of pair-fed control rats, ad libitum-fed control rats and iron-deficient rats that had been raised similarly to the rats examined in the current report. In this much smaller cohort of rats (n = 5-6 per treatment group, males only), we observed no significant differences in caudate–putamen D_2 receptor densities of pair-fed controls compared to ad libitum controls (1540±123 fmol/mg pair-fed controls compared to 1374 ± 89 fmol/mg in ad libitum controls), with both control groups' densities substantially higher than in iron-deficient caudate–putamen (1060±107 fmol/mg). Insufficient samples and inappropriate storage procedures precluded D_1 or D_2 analyses in other brain regions.

3.3. Brain region iron content

Dietary ID caused a significant decrease in brain iron content [F(1,32)=33.7, P ≤ .01 (estimated ω^2 = 0.578)] with significant effects in all four brain regions examined in this study. There was a much greater drop in brain iron concentration in ventral midbrain (60% decrease) than in prefrontal cortex (37% decrease). In the caudate–putamen, ID decreased iron concentration by approximately 30%, and in the nucleus accumbens, the decrease was 20% (Fig. 3). Iron concentrations in the caudate–putamen and nucleus accumbens in females were less affected by diet than that seen in the males.

3.4. Correlational analysis

In an attempt to determine the degree to which dopamine receptor densities are related to regional iron concentrations, we performed a correlational analysis between iron concentration and receptor density (Fig. 4a). In the CP, prefrontal cortex and nucleus accumbens, D_2-like receptor densities showed a significant correlation with iron ($r = .91$, $P < .001$; $r = .42$, $r = .44$, $P < .05$, respectively), No such correlation was found between iron concentration and D1 receptor density.

3.5. In situ hybridization histochemistry

Quantitative in situ hybridization studies focused on the substantia nigra and caudate–putamen and revealed no significant effects of ID on mRNA levels for dopamine D_1 or D_2 receptors when individual cells within each of these brain regions were examined. Brains (n = 3) from iron-deficient rats had 102%, 97% and 101% of control mean grain density when the cDNA probe for the D_1 receptor was used and 96 randomly chosen cells in each region were counted. The densities for D_2 receptor mRNA were 101 %, 100% and 96% of control densities in the nigra.

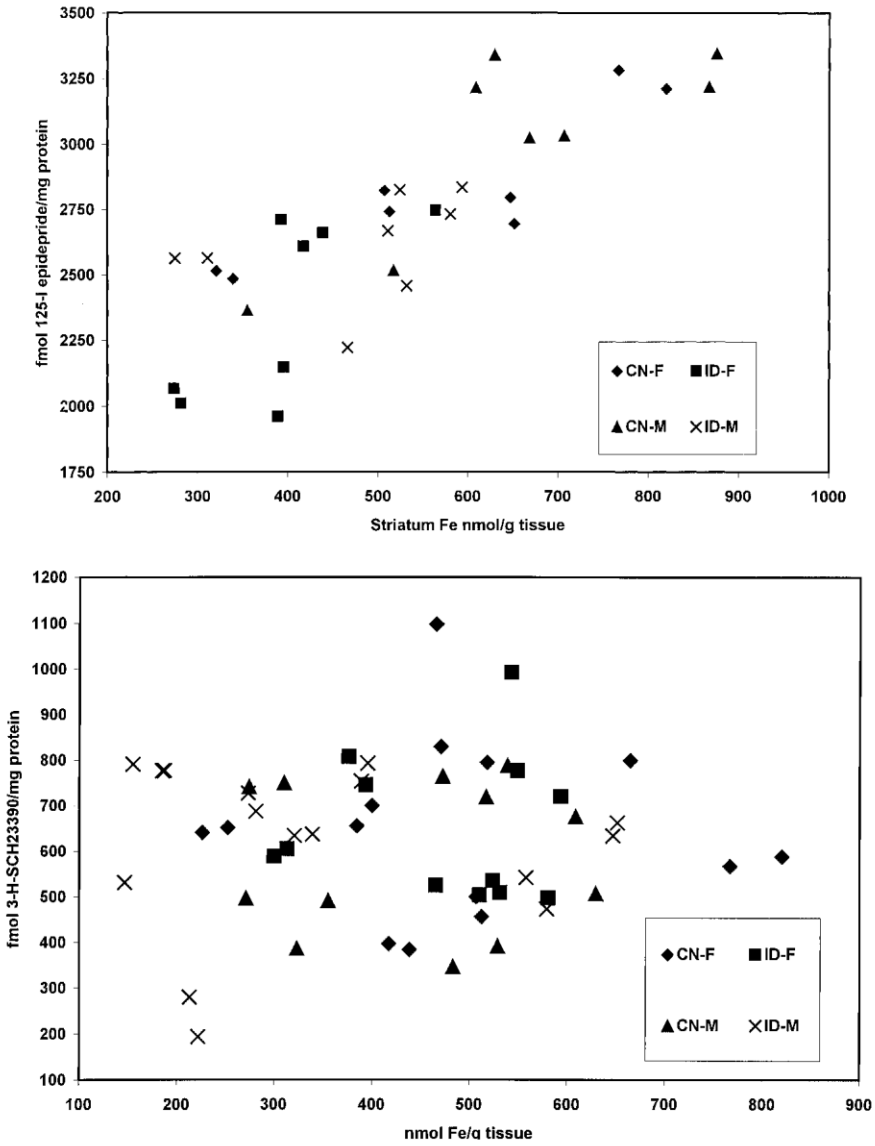

Fig. 4. Correlational analysis of brain region iron concentration vs. dopamine receptor concentration. (a). Striatum (CP+NA) iron concentration correlates significantly with striatal D_2 receptor density (r=.91, P<.01), whereas (b) striatal iron does not correlate with D_1 receptor density (r=.12).

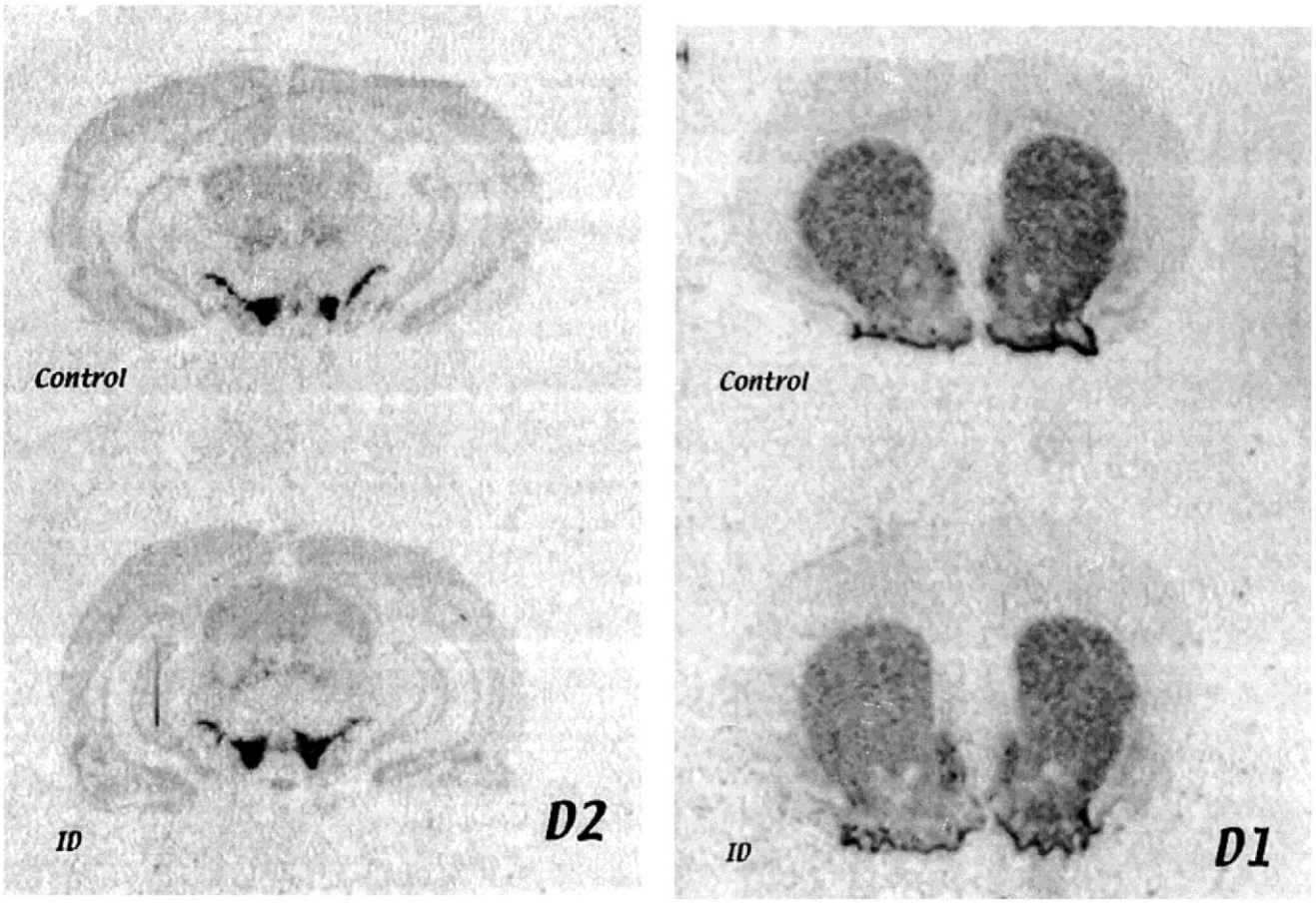

Fig. 5. In situ hybridization studies were conducted in coronal sections with highly specific cDNA. There was no discernible effect of ID on mRNA levels for either the D_2 (a) or the D_1 receptor (b).

Representative autoradiographs from coronal sections are shown in Fig. 5.

4. Discussion

These experiments demonstrate that ID alters both in vivo and in vitro functioning of dopamine receptors in the rat brain. These alterations were brain-region-specific, and affected males more dramatically than females. This work thus extends the previous observations that ID anemia causes a decreased D_2 receptor density in caudate–putamen (Youdim et al., 1979, 1983, 1989). Our approach of examining terminal fields of two different dopaminergic path-ways extends the conceptual framework regarding the interaction between local ID and brain dopamine functioning, since we now demonstrate that downregulation of the D_2-like receptor is not universal in all terminal fields of dopaminergic tracts. In addition, we demonstrate that the affinity of these receptors in the caudate–putamen for highly specific ligands is not altered by ID. The current report is in agreement with the previous studies that used less specific ligands to demonstrate a 15–25% drop in D_2- like receptor density in male Sprague–Dawley rats (Ben-Shachar et al., 1986; Youdim et al., 1983, 1989). We extend those observations to now demonstrate a downregulation in the nucleus accumbens and ventral midbrain in males. Our studies suffer a somewhat similar limitation as the earlier studies — non-specificity of the ligands used. In this case, epidipride binds to D_2 and D_3 receptors, both of which are in high density in the nucleus accumbens and the caudate– putamen. Since there are many fewer D_3 receptors in the cortex, this complication does not limit our interpretation of the data from that region where D_2 density was actually higher in iron-deficient males than controls. This finding is not in concert with the published literature that suggested a universal negative effect of ID on DA function (Ben-Shachar et al., 1986; Youdim et al., 1979, 1983, 1989). Finally, the less robust findings in the midbrain area may well be a result of the lack of precision of definition and the large mass of tissue analyzed — a result that likely decreased the signal-to-noise ratio. The second important new observation regarding ID and D_2-like receptors was the importance of sex on the receptor density

response to dietary ID. Females did not respond to ID in the same manner as males, especially in the nucleus accumbens. Estrogen clearly modifies the expression of D_2 receptors (DiPaolo et al., 1988; Levesque and DiPaolo, 1990), as well as the sensitivity of the DA transporter to cocaine (Post et al., 1987). The basis of the significant interaction between iron status and sex of the subject is unknown at this time, as there is little evidence that ID alters estrogen metabolism. However, the fact that iron concentrations in the nucleus accumbens did not fall with dietary ID, in contrast to male rats, suggests to us that regional iron metabolism is important. We do not differentiate presynaptic autoreceptors from postsynaptic receptors with the approach taken in the current study. These autoreceptors are known to interact with the DA transporter in the regulation of clearance of DA from the intersynaptic space (Meiergerd et al., 1993; Vallone et al., 2000). We recently reported that ID causes a significant drop in DA transporter density in the striatum and accumbens of young rats (Erikson et al., 2000). It is certainly possible that these two observations are related; i.e., a decreased D_2 autoreceptor functioning leads to a decreased DA transporter functioning with a resultant significant elevation in striatal DA content (Meiergerd et al., 1993). Indeed, the D_2-like receptor density is significantly correlated with DA transporter density ($r = .668$, $P < .001$).

In addition to the effects of ID anemia on D_2 receptor density, we also observed alterations in D_1 receptor density. The D_1 receptor density was lower in caudate–putamen, but not nucleus accumbens. This, of course, is in contrast to lower D_1 density in ID males in both brain regions. One previous study (Youdim et al., 1979) reported no effect of ID on D_1 receptor density in the caudate–putamen in male rats, but the ligand used in that older study lacked the specificity of currently available ligands and regional iron concentrations were not measured. It is clear from our in situ hybridization studies that D_1 and D_2 mRNA expression was not altered by ID, suggesting that ID is not having an effect on gene transcription events. The inconsistent effect of ID on regional dopamine receptor density is likely related to the regional variation in brain iron sensitivity to dietary ID (Erikson et al., 1997; Pinero et al., 2000). In addition, there are receptor subtypes that are not differentiated by the ligands used in the current study. For example, in prefrontal cortex, the ratio of D_4 to $D_{2/3}$ receptors is much greater than in the striatum (Tarazi et al., 1997). Therefore, it is plausible that ID alters the D_4 to $D_{2/3}$ ratio, leading to increased ^{125}I-epidepride binding in the PFC in male rats.

The pharmacological impact of ID is manifest in our observation of an attenuated response to raclopride in iron-deficient anemic rats of both sexes. Decreased motor activity has been observed previously in iron-deficient male rats (Glover and Jacobs, 1972; Hunt et al., 1994; Youdim et al., 1981, 1989) and is confirmed in saline-treated rats in this study. We cannot differentiate between mesolimbic and nigrostriatal contribution to locomotion in this research, as both pathways were affected by poor iron status. Previous studies document a highly significant drop in DA transporter density and functioning in caudate of iron-deficient rats (Erikson et al., 2000). In addition, we know that there is an excess of DA in the extracellular space in this brain region (Beard et al., 1994; Chen et al., 1995b; Nelson et al., 1997). Alterations in extracellular DA in other brain regions might be expected, but is speculation at this time. Although the impact of ID on DA-related intracellular signaling and neurotransmission efficiency is unknown, we believe that these are the next avenues for study. One of the key activities of the D_2 receptor on the presynaptic membrane is the feedback regulation of the dopamine transporter (DAT) (Dickinson et al., 1999; Meiergerd et al., 1993). Increased agonist binding to this receptor increases DAT activity and increases the rate of removal of DA from the synaptic space (Dickinson et al., 1999; Meiergerd et al., 1993). Downregulation of the D_2 receptor in the striatum of iron-deficient rats may thus lead to a dysfunction of the DAT. From our raclopride experiments, it appears possible that the functional connection between the D_2 and the DAT could also be desensitized, implying that it takes significantly more DA to stimulate the DAT. Exactly how the downregulation of the two dopamine receptors is related to the downregulation of the dopamine transporter is the basis of ongoing investigations in our laboratory.

Our observations of sex differences in DA receptor density in rats made iron-deficient agree with our previous studies in mice (Morse et al., 1999). Estrogen secretion is related to the density of dopamine receptors (DiPaolo et al., 1988; Levesque and DiPaolo, 1990; Andersen et al., 1990), as well as the sensitivity of the DA transporter to cocaine (Post et al., 1987). The current study clearly notes that the sensitivity of the brain to ID is also sex-dependent (Morse et al., 1999; Pinero et al., 2000). Thus, brain regions that are not sensitive to loss of iron (e.g.,

nucleus accumbens) in females do not evince diminished D_2 receptor densities. Correlational analysis verified this strong association of caudate iron and caudate receptor density. This relationship did not exist for the D_1 receptor and suggests additional avenues of effect of iron of receptor metabolism.

It is clear from the careful examination of the growth data in Table 1 that iron-deficient rats grew less well than the control rats, and it is reasonable to question whether or not "undernutrition" could be related to the changes in dopamine function as opposed to ID. We, and others, have used pair-fed control animals in studies of metabolism in mineral deficiencies and find that slower growth caused by the mineral deficiency does not necessarily impact brain catecholamine or peripheral catecholamine metabolism (Beard et al., 1995; Halas et al., 1982). Our brief exploration of dopamine receptor density in frozen samples in the current report supports this conclusion. Perinatal undernutrition has been shown to alter both dopamine receptor function and behavioral responses to drugs (Keller et al., 1990; Shen et al., 1995), but similar effects by mild postweaning under nutrition are lacking.

5. Conclusion

These studies demonstrate strong effects of postweaning ID on both D_1 and D_2 receptor density and functioning (D_2). Sex of the subject was a key determinant of the receptor density variations, but not of the functional outcomes: ID universally decreased locomotor sensitivity to attenuation by raclopride. Future studies will focus on potential mechanisms by which local iron in the brain alters functioning of these DA receptors.

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