In Vivo Dopamine Metabolism Is Altered in Iron-Deficient Anemic Rats

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Abstract:
Previous studies of dopamine metabolism in iron-deficient rats demonstrated an elevation in extra-neuronal levels of dopamine and a depression in the number of dopamine D2 receptors; however, the importance of anemia per se and the reversibility of these observations are not completely resolved. The purpose of this study was to determine if in vivo reuptake of caudate dopamine is altered by iron deficiency anemia, if it is reversible with iron therapy, and if anemia per se produced the same effects on dopamine metabolism. Male Sprague-Dawley rats (21-d old) were fed an iron-deficient diet (4 mg Fe/kg diet) and then iron repleted (5 mg iron dextran), or were fed an iron adequate diet (35 mg Fe/kg diet) and then given phenylhydrazine to induce hemolytic anemia. In vivo microdialysis was performed in steady-state conditions both before and after iron or no therapy and was followed by an intraperitoneal injection of a dopamine reuptake blocker (cocaine-HCl 30 mg/kg). Thirty percent higher extracellular dopamine levels in the caudate-putamen were observed in iron-deficient rats compared with control rats, but no differences were observed in tissue levels. Hemolytic anemic and iron-repleted rats had normal extracellular dopamine levels. The response to dopamine reuptake blockade was significantly attenuated in iron-deficient rats compared with control, iron-repleted, or hemolytic anemic rats. These experiments provide evidence that iron deficiency blunts the dopamine reuptake mechanism, that this is a reversible process in postweaning rats, and that anemia per se does not cause the increased extracellular dopamine levels.

Key Words: iron deficiency anemia, rat brain, dopamine, cocaine, microdialysis

Article:
Iron deficiency is one of the most common nutritional disorders in the world, affecting ~15% of the world's population with likely functional consequences in many of those individuals (Baynes and Bothwell 1990). An important question relating to the effects of iron deficiency in early life on brain development, neural functioning and behavioral development remains unanswered (Beard 1996, Felt and Lozoff 1996, Lozoff 1990). Little is known about the biological consequences of iron deficiency in early life on brain functioning, or even if brain iron deficits exist in humans. It is quite clear from animal studies that dietary iron deficiency can quickly change brain iron content and have behavioral consequences, regardless of the timing of this iron deprivation (Chen et al. 1995b, Felt and Lozoff 1996, Yehuda and Youdim 1989). Importantly, the staging of iron deficiency relative to brain development has received comparatively little attention. Most studies have been conducted in postweaning rats, demonstrating reversibility of alterations with long-term iron refeeding (Yehuda 1990, Yehuda and Youdim 1989, Youdim 1990). In contrast, iron deficiency before postnatal day (PND) 21 is associated with irreversible changes in brain iron content and in behavior (Dallman and Spirito 1977, Felt and Lozoff 1996). Differentiation between iron deficiency anemia and anemia per se is also lacking and is critical to our understanding of causality. Phenylhydrazine (PHZ) causes a hemolytic anemia when injected repeatedly in rats but is without an effect on dopamine (DA) D2 receptor density or on striatum iron content (Ashkenazi et al. 1982, Youdim et al. 1989).

There is a strong correlation between increased peripheral sympathetic nervous system activity and severity of iron deficiency, with a resulting increase in circulating norepinephrine (NE), tissue NE turnover, and appearance of NE in urine (Beard 1987). Indeed, the first publications on developmental delays in iron-deficient infants showed increased excretion of NE before the administration of iron therapy (Oski al, 1983). Although
there is some colocalization of brain iron and catecholaminergic neurons in adult rodent brain (Hill and Switzer 1984), relationships between changes in brain iron concentration and changes in local brain catecholamine metabolism are lacking or have been unexplored. The exception to this is the elevation in dopamine and a depression in the density of dopamine D2 receptors in the striatum of iron-deficient rats (Beard et al. 1993, Chen et al. 1995a, Youdim et al. 1989). In vitro measurements fail to consistently reveal alterations in the concentration of monoamines or in the activity of the iron-dependent enzymes tyrosine hydroxylase or tryptophan hydroxylase (Yehuda and Youdim 1989), although clear behavioral consequences have been observed in both the preweaning and postweaning iron deficiency models (Felt and Lozoff 1996, Youdim 1990). Behavioral responses returned to normal quickly after iron repletion therapy (Youdim et al. 1979 and 1981) as do dopamine D2 receptor Bmax levels (Ashkenazi et al. 1982, Ben-Shachar et al. 1986). In vivo measurements of dopamine metabolism, however, have not yet shown reversibility with iron therapy, nor has the possibility of altered catabolism of dopamine been carefully examined.

We were interested in several related questions regarding dopamine metabolism in young growing iron-deficient rats. 1) Is the elevation in extracellular dopamine readily reversible with iron therapy? 2) What is the role of tissue iron deficiency versus anemia per se on this elevation in DA? 3) Could the elevation in extracellular dopamine be caused by decreased reuptake of the neurotransmitter because the in vitro data did not suggest any alteration in synthesis? We employed the method of in vivo microdialysis to examine these questions in postweaning male rats and utilized pharmacologic blockade of dopamine reuptake to evaluate the hypothesis that clearance of dopamine from the extracellular space was altered in iron deficiency.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN), 21-d old, were randomly divided into two dietary treatment groups: control (CN; 35 mg Fe/kg diet) and iron-deficient (ID; 4 mg Fe/kg diet). These diets were prepared as described in Borel et al. (1991). Rats had free access to food and water 24 h/d, and the lights were turned off between 1100 and 2300 h. The temperature was maintained at 25 ± 1°C. After 4 wk of dietary treatment, the rats were prepared for surgery. All animal procedures were approved by The Pennsylvania State University Animal Care and Use Committee.

Surgery. On the morning of the surgery day, each rat was anesthetized with an intramuscular injection of a combination of ketamine HCl and xylazine (0.75 and 0.38 mL/kg, respectively); stereotaxic placement of a CMA/12 microdialysis guide was perforated as previously described (Beard et al. 1994). The guide was located as follows: 0.4-mm anterior, 3.0-mm lateral and 4.0-mm vertical from bregma, thus located in the middle of the caudate-putamen (Beard et al. 1994). Location of the probe was verified at death.

Microdialysis. Four days after surgery, and after all rats were again gaining weight, rats were prepared for microdialysis. Each rat was placed in a Plexiglas cage on the fourth evening after surgery. The cage was devoid of food, but rats had access to water and the temperature was maintained at 25 ± 1°C. A microdialysis probe (CM A/12, BioAnalytical Systems, West Lafayette, IN) was inserted into the guide with the dialysis exchange membrane extending 2 mm into the brain tissue. The probe was connected to a high precision syringe pump (CMA/100, BioAnalytical Systems). Sterile synthetic cerebral spinal fluid (CSF, 128 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L CaCl2, 2 mmol/L MgCl2, pH 7.3) was pumped at 0.9 μL/min, and the fluid was allowed to perfuse overnight with the lights off. At ~0900 h the following morning, the perfusion rate was increased to 1.5 μL/min, and collections began 1 h afterwards. Previous studies demonstrated that this protocol is sufficient to provide steady-state neurotransmitter levels in the dialysate (Chen et al. 1995a). A total of 13 15-min samples of dialysate were collected. Samples were collected in microdialysis tubes, with 3 μL of 100 μmol/L acetic acid used as a preservative. The first two collections were base-line data. The rats were then injected intraperitoneally with 100 μL of sterile saline, and three more collections were taken. The rats were then injected intraperitoneally with cocaine with a dose sufficient to elicit a maximal response (35 mg/kg body weight in 100 μL saline) (Nicolaysen et al. 1988), and the remaining collections were taken. Other studies demonstrate that cocaine appears in the brain in rats within several minutes (Morse et al. 1995) and is then rapidly oxidized and cleared.
**Treatment.** The design of the protocol is outlined in Figure 1. After the final microdialysis collection of the first part of the experiment, the rats were disconnected from the microdialysis apparatus and returned to their cages for 7 d. During this intervening week, the rats were subjected to one of the following four treatments:

1. Rats continued to consume the control diet for several days, and then were killed for brain region analysis (CN group).
2. Rats continued consuming the iron-deficient diet for several days, and then were killed for brain region analysis (ID group).
3. Rats that were previously fed the control diet were injected with phenylhydrazine (50 mg/kg) every other day (Ashkenazi et al. 1982) and fed the control diet (PHZ group).
4. Rats that were previously fed the iron-deficient diet were injected with iron dextran (5 mg, intraperitoneal) (Beard et al., 1990) and fed the control diet (REPL group).

After 7 d, microdialysis was performed again using the previously described procedure. Four rats from the iron-deficient group and one from the control group did not complete the experiment because of their removal of the probe and damage during the course of data collection. At the termination of the experiment, rats were killed by decapitation, the brain and liver were removed and weighed, and the position of the probe was verified. One hemisphere of the brain was used in a portion of rats for the determination of tissue levels of neurotransmitters. Four brain regions were analyzed for DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-hydroxytryptamine, 5-HT), 3,4-dihydroxyphenylalanine (L-DOPA), norepinephrine (NE) and 5-hydroxyindole-3-acetic acid (5-HIAA) content in order to encompass the dopaminergic pathways that were sampled with the microdialysis experiments. These regions were the medial prefrontal cortex, the nucleus accumbens, the caudate-putamen and the ventral midbrain. The other hemisphere was used for verification of the probe's location by sectioning and visual inspection. The hematologic status of each rat was determined on the fresh blood drained from the trunk by standard methods, and plasma was stored at -20°C until analysis for Fe and total iron-binding capacity (Chen et al., 1995b).

**Analysis.** Microbore reverse-phase HPLC was used to analyze concentrations of monoamines and their metabolites in the dialysate as previously described (Beard et al. 1994) and in tissue (Morse, A., Beard, J. and Jones, B., unpublished results). The compounds analyzed included the catecholamines DA, DOPAC, HVA, L-DOPA, NE, 5-HIAA and 5-HT. Conditions were optimized in this case for the determination of dopamine and
its metabolites; hence, inconsistent data on 5-HIAA and 5-HT were obtained and are not reported. Concentrations were determined by comparison of peak heights with known concentrations of standards analyzed with each set of samples (correlation coefficient >97% for all standard curves). Determinations of tissue neurotransmitters were performed on microdissections of brain sections as defined in an atlas for rats (Paxinos and Watson 1986). These dissections were performed immediately at death, and brain sections frozen in dry ice and then stored at -80°C. Brain regions were thawed slightly, homogenized with a Teflon pestle in 50 mmol/L HClO₄ (5:1, v/wt) at 4°C, 250 pg of dihydroxy benzylamine (DHBA) added as an internal standard, and then analyzed by HPLC. All analyses were performed at electrode potentials of +800 mV relative to the reference Ag-AgCl electrode. The minimum detectable concentration was ~3 μg/L for all of the monoamines and metabolites. Microdialysate and plasma were also analyzed for amino acid concentration by HPLC (Sizemore et al. 1995) to test for possible alterations in substrate availability.

Statistical analysis. Data were corrected for day-to-day variation in column conditions by analyzing an external DHBA standard each day and then correcting the sample values accordingly. All data were subjected to a box-plot analysis in Minitab (State College, PA) to determine the presence of outliers; tests for normality also were performed before ANOVA. ANOVA was used for statistical analysis of hematologic and dialysis data when data from all four groups were compared; the Type III sums of squares was used to calculate F ratios. When group variances were unequal, data were log-transformed before analysis. Tukey's test for multiple pairwise comparisons was performed to compare specific cell means for treatment effects (Hinkle et al 1988). Significance was assumed at P ≤ 0.05.

RESULTS
Rats were iron deficient and anemic after 4 wk of consuming the low iron diet and had severely depressed hemoglobin, hematocrit, transferrin saturation and liver nonheme iron (Table 1). Iron therapy with iron dextran, 5 mg intraperitoneally, and a control iron diet resulted in normalization of these hematologic indices of iron status within 7 d. In addition, hemolytic anemia induced by the injection of 50 mg/kg PHZ resulted in anemic rats with a hemoglobin concentration that was <100 g/L. There was also a significantly greater accumulation of iron in the liver and a higher circulating plasma Fe in these hemolytic anemic rats (PHZ group) compared with controls.

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<td>Hematologic variables and liver iron concentration of control (CN), iron-deficient (ID), iron-repleted (REPL) and hemolytic anemia (PHZ) rats used in microdialysis and in vitro determinations of effects of iron deficiency!</td>
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Values are means ± SEM. b,c Significant differences (P < 0.05) within a row are indicated by different letters.

Base-line extracellular neurotransmitter concentrations were dramatically affected by the different treatments with the caudate dopamine, i.e., nearly 67% higher in the iron-deficient anemic rats than in control rats (Fig. 2A). Although DOPAC was unaffected by iron deficiency (Fig. 2B), the other primary catabolite, HVA, was significantly higher in the iron-deficient anemic rats compared with control rats (Fig. 2C). In contrast, hemolytic anemia was associated with significantly lower extracellular DA, DOPAC and HVA than controls. Control rats and iron-repleted rats did not differ in base-line concentrations of DA, DOPAC and HVA. The administration of intraperitoneal saline at 30 min elicited a prompt response that was most pronounced in the ID anemic rats with a significant rise in both concentration and area-under-the-curve (AUC). These peaks occurred ~15-30 min after injection (45-60 min on the axis) and were all significantly smaller in magnitude in all other groups than in the ID anemic rats. The injection of cocaine-HCl in a dose sufficient to block the uptake of DA back into
presynaptic neurons resulted in a similar size response (both peak height and AUC) as the saline injection in ID rats. The response of control rats to cocaine injection was much more pronounced than that of iron-deficient rats. The net AUC for control rats was 32-fold greater than that of iron-deficient rats (16 vs. 0.5 cm², Fig. 2). All other treatment groups, however, had much larger dopamine responses to cocaine than to saline injection. In addition, the response was delayed by 15 min compared with control rats. Iron-repleted rats did not differ from controls in their dopamine response to cocaine. Phenylhydrazine anemic rats showed a temporal profile similar to that of iron-deficient anemics but with a much larger effect of cocaine than saline. The intra- and extracellular production of HVA within the paradigms demonstrated higher concentrations of HVA in iron-deficient anemic rats and generally lower levels in hemolytic anemic rats compared with control rats (Fig. 2C). There was no difference between iron-repleted and control rats. In contrast, DOPAC was lower in hemolytic anemia rats, dropping to concentrations less than 30% of normal with a blunted response to cocaine injection (Fig. 2B).

Because one explanation of the changes in dopamine metabolism in iron-deficient anemic rats could be the altered availability of substrate amino acids for synthesis, we measured the steady-state concentrations of certain amino acids in the dialysate (Fig. 3). There was no effect of iron deficiency on the levels of amino acids in brain extracellular fluid or plasma (data not shown).
Our final effort to explore neurotransmitter metabolism in this experiment was the measurement of neurotransmitters and metabolites in postmortem brain tissue of iron-deficient, control and iron-replete rats (Table 2). As expected, there was a >10-fold variation in dopamine concentration and metabolite concentration across the regions studied, i.e., caudate-putamen, medial prefrontal cortex nucleus accumbens and ventral midbrain, with caudate-putamen and nucleus accumbens having the highest concentrations. Iron deficiency anemia was associated with an elevation in dopamine in the nucleus accumbens but not in the other brain regions, although there was a clear tendency \( (P = 0.08) \) for an elevation to occur in most regions examined. It is interesting to note that in caudate-putamen, the region in which microdialysis had shown a significant in vivo effect of iron deficiency on extracellular DA, there was no significant effect on tissue concentration of DA, DOPAC or HVA.

**DISCUSSION**

Several new and important observations are reported in these experiments: 1) extracellular dopamine in the caudate-putamen returned to normal with the normalization of iron status; 2) elevations in dopamine or other neurotransmitters are not due to anemia per se; and 3) dopaminergic transporters may be altered in iron deficiency. This report extends our previous observations (Beard et al. 1994, Chen et al. 1995a) of elevations of extracellular dopamine and metabolites by demonstrating reversibility with iron therapy in a short period of time and suggests further that it is removal of dopamine from the interneuronal cleft that is responsible for this elevation. Availability of substrate amino acids is not altered in this brain region, thus eliminating this possibility for the explanation of altered neurotransmitter concentrations.

One research group (see review of Youdim et al. 1989) established that dopamine is perturbed by iron deficiency in the rat animal model. They observed a decrease in \( D_2 \) receptor density in the caudate-putamen that was irreversible if iron deficiency is begun in early preweaning or intrauterine life. These investigators also note that certain behaviors, such as poor responses to adverse stimuli (foot shock), decreased temperature regulation after apomorphine and a reversal of the diurnal cycle, are likely related to this alteration in dopamine metabolism. Iron deficiency postweaning did not affect tyrosine hydroxylase or tryptophan hydroxylase, both iron-containing enzymes, nor were there effects on serotonin, adrenergic or gabaminergic receptor populations (Youdim et al. 1989). Thus, for many years, the focus and attention in this area of work resided on the \( D_2 \) receptor in one brain region despite a lack of critical studies by other research groups into other aspects of
Recent studies provide some additional information. Iron heterogeneously distributed in the brain (Hill 1988), and this distribution is not achieved until early adulthood (Roskams and Connor 1994). Furthermore, we have demonstrated that iron deficiency does not affect all brain regions equally (Erikson et al. 1997). Dietary iron restriction reduces brain iron concentration within 14-21 d (Chen et al. 1995b, Erikson et al. 1997), despite the fact that iron reportedly turns over slowly in the central nervous system (Dallman and Spirito 1977). Specifically pertinent to the current report is the observation that caudate-putamen has a 30% loss of iron concentration in iron deficiency, whereas other regions such as the substantia nigra are unaffected (Ben-Shachar et al. 1986, Erikson et al. 1997).

The results of this in vivo study are consistent with previous results from this laboratory that demonstrated elevation in extracellular dopamine (Beard et al, 1994, Chen et al. 1995a). Because extracellular dopamine in the synaptic cleft can undergo rapid catabolism to HVA, can be removed by the dopamine transporter or can bind to pre- and postsynaptic dopamine receptors, we felt it necessary to explore the quantitatively most important route of disappearance—the dopamine transporter (Cilax et al. 1995, Povlock et al. 1996). A decrease or inhibition in the activity of the dopamine transporter produces increased extracellular concentrations of dopamine and HVA and might explain the elevated dopamine in the extracellular space. Roughly 80% of interstitial dopamine is recycled through a Na+/Cl−-dependent membrane dopamine transporter after its release from presynaptic neurons (Cilax et al. 1995, Nicolaysen and Justice 1988, Povlock et al. 1996). The synaptic concentration of dopamine and the amount of dopamine available for receptor stimulation are thus largely regulated by the dopamine transporter activity. The administration of cocaine, a potent dopamine transporter antagonist (Giros and Caron 1993, Nicolaysen et al. 1988), had no real effect on the dopamine concentrations of
the iron-deficient rat beyond what was seen with a placebo saline injection. We know that there is a very rapid appearance of cocaine and its metabolites in the brain after peripheral injection (Morse et al. 1995). Injection of cocaine caused an increase in the levels of dopamine and its metabolites in the first post-cocaine collection in control rats. In iron-deficient rats, the increase was not seen until 30 min post-cocaine injection, indicating a delay in the onset effect of the blockade. This delay could be interpreted as an indication of a decreased appearance of cocaine in the brain of iron-deficient rats, a decreased binding of cocaine to these transporters and/or a decreased number of functioning dopamine transporters in the striatum of iron-deficient rats. Experiments with mice, however, demonstrate no effect of iron deficiency on the rate of appearance of cocaine or its metabolites in brain after an intraperitoneal injection (Morse, A., Beard, J. and Jones, B., unpublished results). Thus we can tentatively conclude that dopamine clearance by this mechanism is altered in iron deficiency. Iron therapy rapidly normalized these metabolite patterns as well as the recovery of a normal hematologic status, demonstrating a clear iron responsive process. Direct measurements of the amount of dopamine transporter and its functioning are necessary before a clear role for iron is firmly established.

The in vitro data suggest that other brain regions may also be adversely affected by iron status, although none were examined by the in vivo method. These data contrast with Youdim's experiments, which showed no significant effect in vitro of iron deficiency on dopamine, serotonin norepinephrine concentrations in brain regions (Youdim et al, 1989). Peripheral sympathetic nervous system activity is altered by iron deficiency, with increased concentrations of norepinephrine in plasma and urine and decreased concentrations in tissue (see review of Brigham and Beard 1996), an observation that could also be explained by a decreased reuptake of that catecholamine (Kanner and Schulinder 1987).

Fourteen days of dietary iron therapy are sufficient to restore brain iron concentration to normal (Chen et al. 1995b), and this study also shows rapid normalization of neurochemical alterations. These results support previous studies showing that rat behavioral responses return to normal after 7 d of iron repletion therapy (Youdim et al, 1979 and 1981) and that $D_2$ receptor $B_{max}$ levels are restored rapidly by iron therapy in postweaning rats (Ashkenazi et al. 1982, Ben-Shachar et al. 1986). Iron therapy probably will not correct the alterations in brain neurochemistry suspected in preweaning iron deficiency, which have irreversible consequences (Felt and Lozoff 1996), although this was not tested in the current study. Because it is speculation at this time to surmise that the most important "critical period" is the time of active myelinogenesis, PND 8–14 in rats, and the first year of postnatal life in humans, continued active investigations using the developmental perspective must be conducted (Dobbing 1990).

Previous studies from our laboratory (Beard et al.1994, Chen et al. 1995a) did not resolve whether anemia per se was, responsible for the elevation of CSF dopamine in the striatum, because the dietary model of severe iron deficiency anemia cannot itself distinguish and separate tissue iron deficiency effects from oxygen transport effects. Several protocols are available, such as exchange transfusion or rapid repletion, to differentiate these effects (Beard et al. 1990). In this study, we used hemolytic anemia, induced with phenylhydrazine, an alternate form of anemia that should not have direct effects on brain iron metabolism (Ashkenazi et al. 1982). Extracellular levels of dopamine and its metabolites, DOPAC and HVA, in hemolytic anemic rats were below control values, demonstrating that anemia per se is not responsible for the elevated extracellular dopamine levels found in ID anemic rats and that oxygen transport to brain is not a likely cause for the alterations.

In conclusion, we demonstrated in this experiment that iron deficiency is associated with a significant elevation of dopamine in young rats and that anemia itself is not the cause. This functional pool of dopamine is normalized within 7 d of iron therapy. Additional evidence suggests that proper functioning of the dopamine transporter is faulty in iron deficiency. These animal studies thus provide some further biologic support for a causal relationship of iron status to cognitive functioning via the dopaminergic system.

Notes:

4 Abbreviations used: AUC, area under the curve; CN, control; CSF, cerebral spinal fluid; DA, dopamine; OHBA, dihydroxybenzylamine acid; DOPAC, dlhydroxyphenyl acetic acid; L-DOPA, 3,4-
Dihydroxyphenylalanine; 5-HIAA, 5-hydroxyindole-3-acetic acid; 5-HT, serotonin (5-hydroxytryptamine); HVA, homovanillic acid; ID, iron deficient; NE, norepinephrine; PHZ, phenylhydrazine; PND, postnatal day; REPL, iron repleted.

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