Regional Brain Iron, Ferritin and Transferrin Concentrations during Iron Deficiency and Iron Repletion in Developing Rats

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Abstract:
Iron deficiency in young rats leads to a decrease In brain iron and ferritin concentrations, an increase *in* transferrin (Tf) concentration, and an increased rate of uptake of iron from the plasma pool. We conducted two experiments to determine whether brain iron, Tf and ferritin respond quickly to iron repletion and to determine whether brain regions respond heterogeneously. Weanling male Sprague-Dawley rats were fed an iron-deficient diet (<5 mg/kg Fe) for 2 wk followed by an Iron-adequate diet (REPL group, 35 mg/kg Fe in Experiment 1 and 15 mg/kg Fe in Experiment 2) for 2 or 4 wks, respectively. Age-matched iron-deficient (ID) and control rats composed the other two groups, Fourteen days of repletion with 35 mg/kg Fe dietary treatment were adequate to normalize hematology, brain microsomal and cytosolic Fe and brain ferritin (Experiment 1). Brain transferrin concentrations in REPL rats, however, were significantly above the levels of controls, Regional brain iron decreased heterogeneously due to dietary iron deficiency (Experiment 2), with some regions having a propensity to keep iron (e.g., substantia nigra, pons, and thalamus) and others losing significant amounts of iron (cortex and hippocampus). Ferritin and Tf concentrations also varied significantly across brain regions in ID and control rats. The hippocampus had the most dramatic Tf response to iron deficiency with elevations of approximately 100%, whereas other regions, except striatum, were unaffected. The brain of developing rats thus distributes iron and iron regulatory proteins differently from the brain of adult rats and is quite avid in its reacquisition of iron during iron therapy.
Key Words: iron deficiency anemia, transferrin, ferritin, brain, rats

Article:
Iron deficiency and anemia are major nutritional concerns throughout the world (Baynes and Bothwell 1990). Iron deficiency has been associated with hematological changes (red blood cell deformation), stunted growth, altered thermoregulatory function and decreased cognitive function (Ashkenazi et al. 1982, Brigham and Beard 1996, Lozoff and Brittenham 1986). Iron is essential for proper central nervous system metabolism through its role in the synthesis of neurotransmitters, myelin formation and brain growth (reviewed by Beard et al. 1993). In iron-deficient rats, there is a significant decrease in brain iron, increase in brain transferrin and a slight decrease in brain ferritin concentration (Chen. et al, 1995b). It is unclear whether these changes in iron metabolism are due to altered acquisition or slow turnover rate of brain iron Waltman and Spirito 1977). By examining changes in brain iron and iron regulatory proteins due to iron repletion in rats, we sought to gain a clearer sense of how the brain regulates changes in iron concentration due to iron deficiency.

Iron is heterogeneously distributed in the brain of adults, with the highest concentration being in the basal ganglia, substantia nigra and deep cerebellar nuclei (Hill 1988). It has recently been shown that some of the areas of the brain that are iron rich in adult no brains are not iron rich for the first 60 d of life (Benkovic and Connor 1993). Because the concentration of iron is highest in the brain at birth, decreases through weaning and then begins to increase during critical periods of development, e.g., myelination (Roskams and Connor 1994), it is of great interest to examine the regional responses of the brain to dietary iron deficiency during growth. Developmental iron deficiency clearly alters the functioning of the brain in rats with functional sequelae (Felt and Lozoff 1996).

Iron is stored in the brain primarily as ferritin, whereas transferrin is responsible for transporting iron in cerebral spinal fluid and in plasma (Connor et al. 1992, Octave et al. 1983). Both ferritin and transferrin levels are highest at birth in newborn rats and decline thereafter (Roskams and Connor 1994). Overall, very little is known about the regional response of transferrin and ferritin to iron deficiency and iron repletion during development.

The reversibility of the observed alterations in brain iron metabolism due to iron deficiency has yet to be elucidated (Dallman and Spirit° 1977). Although young iron-deficient rats respond rapidly to iron repletion therapy with respect to hematology and storage of non-heme liver iron, it is not clear if the brain transferrin and ferritin concentrations respond to iron repletion in a rapid fashion. Further, it is unclear if the brain responds heterogeneously with respect to iron repletion, i,e., does an supposedly iron-rich region such as the substantia nigra acquire the same amount of iron, transferrin and ferritin as regions, such as the cortex, that have a lower concentration of iron?

Thus, this study consisted of two experiments in post-weaning rats; the first experiment addressed the effect of iron repletion on brain iron, transferrin and ferritin levels, and the second experiment expanded on this by examining changes in different brain regions due to systemic iron deficiency and then repletion. Our hypothesis was that the brain would respond to iron depletion and to repletion in a heterogeneous fashion. This hypothesis was tested at only one developmental stage, but clearly, other developmental periods require careful examination.

TABLE 1

Hematological variables and liver non-heme iron concentration in iron-deficient rats (ID), control rats (CN) and rats fed iron-deficient diet followed by an iron-adequate diet (REPL) in Experiment 1[1]

	ID	REPL	CN
Dietary Fe, *mg/kg*	3	35	35
n	8	40	8
Hemoglobin, *g/L*	53 ± 5.4[a]	141 ± 10.9[b]	150 ± 8.8[b]
Hematocrit	0.18 ± 0.024[a]	0.41 ± 0.026[b]	0.42 ± 0.023[b]
Plasma Fe, *μmol/L*	1.9 ± 0.7[a]	24.1 ± 2.3[b]	29.5 ± 3.7[b]
TIBC,[2] *μmol/L*	121.9 ± 2.8[b]	65.0 ± 4.0[a]	66.0 ± 1.0[a]
Transferrin saturation, %	1.7 ± 0.3[a]	38.3 ± 3.5[b]	42.5 ± 6.2[b]
Liver iron, *μmol/g*	0.49 ± 0.02[a]	1.48 ± 0.11[b]	1.59 ± 0.20[b]

[1] Values are means ± SEM. Different superscript letters in a row indicate significant difference ($P < 0.05$).
[2] When expressed as (plasma Fe/plasma TIBC) · 100, total iron-binding capacity (TIBC) is equal to the percentage of transferrin saturation.

METHODS

Rats and dietary treatment. Male Sprague-Dawley rats (21 d of age) were purchased from Harlan Sprague Dawley (Indianapolis, IN). Animal procedures were approved by the Pennsylvania State University Institutional Animal Care and Use Committee. The rats were randomly divided into three groups: REPL (Experiment 1, n = 40; Experiment 2, n = 16), ID (both experiments, n = 8) and CN (both experiments, n = 8). The dietary treatment consisted of nutritionally complete identical diets (AIN-76A, AIN 1977), except for the amount of iron in the low iron diet (3 mg Fe/kg), a moderate repletion diet (15 mg Fe/kg) or an iron-adequate diet (35 mg Fe/kg). As we have done previously, this formulation was modified with the replacement of sucrose by cornstarch; cellulose was omitted from the diets because of its variable contribution of contaminant iron (Borel et al. 1993). The iron-replenished groups (REPL) were fed the low iron diet for 2 wk and verified to be anemic [Hemoglobin (Hb) 69 ± 9,2 g/L and hematocrit (Hct) 0.24 ± 0.005]. These rats were then fed the iron-adequate diet for another 2 wk (Experiment 1, 35 mg Fe/kg) or for 4 wk with a more moderate iron-containing diet (Experiment 2, 15 mg Fe/kg). Age-matched control (CN) groups and iron-deficient (ID) groups were maintained for the entire 4 to 6 wk of the experiments. All rats were housed individually under controlled environmental conditions (0600-1800 h light cycle and 25°C) and were provided free access to food and water. Food intake was measured throughout the second phases of each experiment, i.e., over the last 2 wk in Experiment 1 and over the last 4 wk in Experiment 2, and feed efficiency was calculated by standard methods (Koivistoinen et al. 1968).

Hematology and tissue non-heme iron. Hemoglobin and Hct were measured on d 14, 21, and 28 of the experiment via blood samples acquired by tail prick. Hematocrit was determined by centrifugation of blood collected into heparinized microcapillary tubes. Hemoglobin concentration was measured colorimetrically by the cyanmethemoglobin method (procedure no. 525, Sigma Chemical, St. Louis, MO). Blood were collected at the end of the experiments from the abdominal aorta into heparinized syringes, and aliquots were analyzed for Hb and Hct. The remaining blood was cooled to 4°C and centrifuged with a cold clinical centrifuge for 15 min to separate cells from sera. Plasma was frozen at -20°C prior to analysis for iron and total iron-binding capacity (TIBC).

Non-heme iron and plasma iron and total iron-binding capacity. Liver and spleen non-heme iron were measured by acid hydrolysis using the standard spectrophotometric technique as described in Cook (1980) with ferrozine as the color reagent. Plasma iron concentration and TIBC were measured coulombmetrically (Ferrochem II, ESA Inc., Bedford, MA). External standards were used to calibrate each assay.

Microsomal and cytosolic liver and brain iron. Rats were killed by exsanguination and then decapitation with livers and brains being rapidly removed from the rats and immediately frozen at -70°C. The brains were homogenized 1:5 (wt/v) in ice-cold buffer H, which contained 20 mmol/L HEPES, 250 mmol/L sucrose, 1 mmol/L EDTA, 100 mmol/L leupeptin and 100 mmol/L phenylmethylsulphonyl fluoride (PMSF) at pH 7.2 using an Ultra-Turrax homogenizer (Tekmar, Cincinnati, OH) at high output for 30 s. All steps were performed at 4°C. The homogenates were centrifuged at 1000 x g for 10 min, and the crude supernatant was collected and centrifuged at 21,000 x *g* for 20 min. The resulting supernatant was subjected to centrifugation at 100,000 x g for 60 min, yielding a microsomal pellet and a cytosolic supernatant. Aliquots of these fractions were used for the analysis of protein, ferritin and transferrin concentrations; a 30-µL aliquot from each fraction was diluted 1:50 with 3.12 mmol/L ultra-pure nitric acid and analyzed in triplicate for iron by graphite furnace atomic absorption spectrophotometry (model 5100 AA, Perkin-Elmer, Norwalk, CT) (adapted from Chen et al. 1995b). Standards were prepared by diluting iron standard in the specified nitric acid, with blanks prepared from homogenization reagents. Preliminary studies showed minimal matrix effects with this method of analysis specified by Perkin-Elmer.

Regional brain iron. In Experiment 2, rats were perfused with cold PBS, pH 7.4, after exsanguination. Livers and brains were rapidly removed and brains immediately dissected into eight brain regions: cortex, hippocampus, pons, striatum, substantia nigra, superficial cerebellum, deep cerebellar nuclei and thalamus according to published methods (Focht et al. 1997). Brain regions were diluted 1:20 (wt/v) with HEPES buffer and homogenized with a sonicator (Branson Sonifier 250 at the pulse mode with a microtip; Branson Inc., Danbury, CT). A 30-µL portion of the homogenate was added to an equal volume of ultra-pure nitric acid in a 400-µL polypropylene microfuge tube, digested for 48 h at 50°C, and diluted 1:10 with 3.12 mmol/L nitric acid for iron analysis using the graphite furnace atomic absorption spectrophotometer (adapted from vanGelder 1995 and as noted in Focht et al. 1997). Standards, blanks and standard curves were used as previously specified.

Transferrin and ferritin immunoblat. The immunoblot assay WAS performed according to the immunosorbent technique previously described (Roskams and Connor 1994). The protein concentration loaded into slots was 5 µg/100 µL and measured by micro-Lowry method (procedure no. P5656, Sigma Chemical). The primary antibodies were used at 1:2000 dilution, and the secondary antibody was diluted 1:5000. The ferritin primary antibody was rabbit anti-horse ferritin (Jackson ImmunoResearch, West Grove, PA); the tranferrin primary antibody was rabbit anti-rat (a generous gift of Richard Fine, Boston University), the second antibody was goat anti-rabbit IgG alkaline phosphatase conjugated (catalog no. A-3937, Sigma Chemical). The ferritin and transferrin standards were purchased from Sigma Chemical (catalog F-7005 and T-6013, respectively) and diluted in Tris-buffered saline at 0.25 – 15 ng/50 µL and 1 -15 ng/50 µL, respectively. An internal standard composed of a pool of brain homogenic was analyzed on every membrane to ensure quality control between assays. All measurements were performed with standard conditions using a Eagly-eye densitometer (Stratagene, san Diego, CA). Regression analyses were performed on the acquired densities (standard curves on each batch) and data calculated only if the standard curve correlation coefficients were >0.95.

Brain histochemistry. Some rats from each group in Experiment 2 were also treated at death for brain histochemistry analysis. Rats were perfused with 10% neural buffered formalin. The brains were removed, immersed in the fixative (10% neural buffered formalin) overnight and then cryoprotected by floating in sucrose (10, 20 and 30$) sequentially. The brains were then stored in 30% glycol until they were sectioned on a freezing microtome (30-µm sections). Cellular iron distribution was determined on individual brain sections using a modified Perls' reaction (Dickinson and Connor 1994). The modification (treatment of the sections with 3.3' diaminobenzidine to enhance the visualization of the cellular iron) has become standard practice for microscopic analysis of rodent brains (Roskams and Connor 1994). The cellular analysis of iron distribution was performed without experimenter knowledge of experimental groups.

Statistical analyses. Potential outliers in the data were identified as extreme values in boxplots within the boxplot program in Minitab (Minitab Corp., State College, PA). Outlier data were identified and excluded from the analyses. This consisted of one or two data points each for iron, transferrin and ferritin. Simple one-way ANOVA was used to test for difference in means in Experiment 1. Analysis of variance with two main effects (brain region and dietary treatment) and an interaction term was used for the analysis in Experiment 2 and was conducted after log transformation of data in some cases. The Type III sums of squares was used to calculate F ratios. Dunnett's procedure was performed to determine whether treatments differed from controls (Steel and Torrie 1980). Tukey's test for multiple pair-wise comparisons was performed to compare specific cell means for effects of dietary treatment within brain regions (Steel and Torrie 1980). Treatment region and interaction effects were considered significant at $P \leq 0.05$. All analyses of variance were conducted with either Minitab software or the Statistical Analysis System (SAS Institute, Cary, NC) using the Pennsylvania State University IBM mainframe computer. The General Linear Models (GLM) approach was used. Regression analysis was performed to evaluate the relationship between brain region and hematologic iron status variables and non-heme liver iron. Data are reported as means ± SEM.

RESULTS

Experiment 1. Hemoglobin, Hct, and non-heme iron levels in liver were significantly lower in ID rats than in CN rats)$P < 0.05$, Table 1). Two weeks of iron repletion were adequate for eradicating anemia in REPL rats. Liver non-heme iron concentrations were also indicative of successful repletion because they were significantly higher in the REPL than in the ID rats ($P < 0.05$) and not significantly different from CN rats (Table 1).

Cytological analysis of brain iron did not reveal clear quantitative differences among groups. It is important to note that at this young age, little iron accumulation has yet occurred. Thus dramatically different iron content regions, as seen in adult rat brains, have not yet been generated. At the cellular level, in all three experimental groups, oligodendrocytes were the predominant iron-containing cell type (Fig. 1). However, there were more cells that resembled immature oligodendrocytes in the white matter of the ID group compared with the CN group. The REPL group contained a mixture of morphologically immature and mature oligodendrocytes. These observations are consistent with hypomyelination during iron deficiency, though this was not measured directly.

Brain and liver cytosolic iron concentrations were significantly lower in ID rats than in CN rats (Fig. 2A, $P < 0.05$ and $P < 0.01$ respectively). Similar results were obtained in the microsomal fractions (data not shown). In the REPL rats, brain cytosolic iron concentration was significantly higher than in the ID rats ($P < 0.05$) and not different from the CN rats. In contrast, liver cytosolic iron concentration in REPL rats was 90% greater than in ID rats *(*$P < 0.01$) but was still 30% lower than in CN rats ($P < 0.05$), indicating incomplete restoration of liver iron stores within the time frame of this experiment (Fig. 2A).

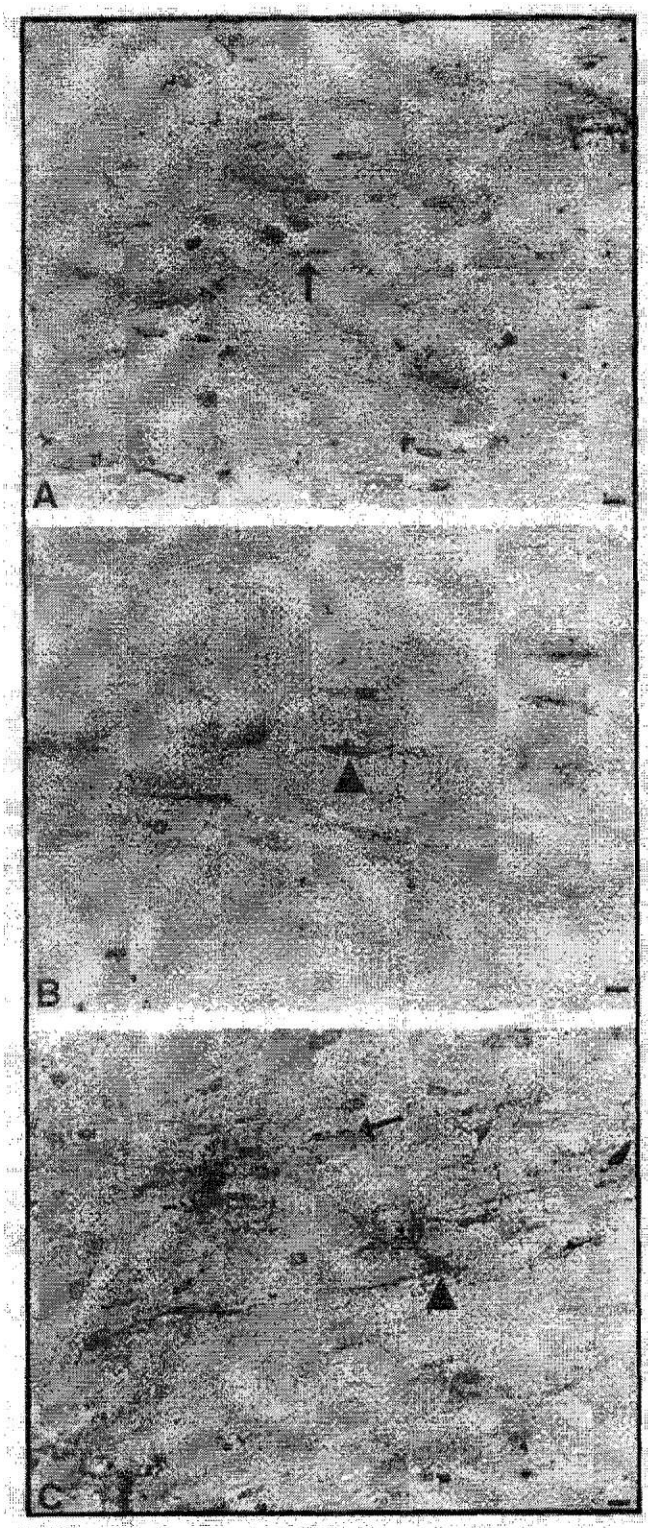

FIGURE 1 Typical histochemical staining patterns of brain cortex of iron-deficient, control and iron-replenished rats. These micrographs are representative of the appearance of the subcortical white matter following a histochemical reaction for iron in the three groups of experimental animal. The cells in the normal rats (panel *A*) are oval-shaped with reaction products mostly confined to one pole of the perikaryal cytoplasm. These cells are typical of morphologically mature oligodendrocytes (arrow). In the iron-deficient group (panel *B*), fewer iron positive cells are visible than normal, and those cells that stain for iron contain a few, beaded processes. These cells are morphologically similar to immature oligodendrocytes (arrowhead). In the iron-replenished group (panel *C*), small, ovoid iron-containing cells with stainable iron exist with iron confined to the soma. However, cells with iron-containing processes are also visible in the same sections, indicating both mature and immature oligodendrocytes. Bar = 10 μm.

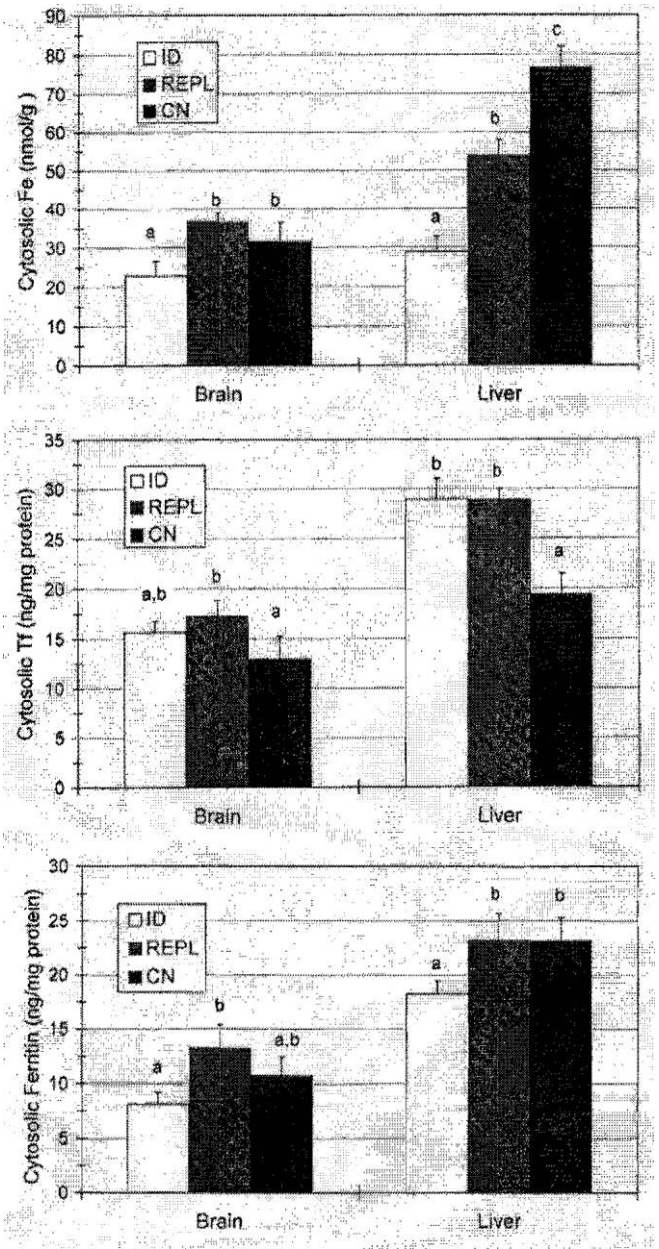

FIGURE 2 Brain and liver cytosolic Fe (*top*), transferrin (Tf) (*middle*) and ferritin (*bottom*) concentrations of iron-deficient rats (ID, n = 8), control rats (CN, n = 8) and rats replenished with a 35 mg/kg iron diet for 2 wk (REPL, n = 40) (Experiment 1). Values are means ± SEM. Different letters indicate significantly different means ($P < 0.05$).

Two weeks of repletion did not normalize brain cytosolic transferrin, despite brain iron concentrations being normal (Fig. 2B). Cytosolic transferrin concentrations in the brains and livers of REPL rats were not different from those of ID rats but were significantly greater than those of CN rats by 30 and 49% respectively ($P <$ 0.05). Liver cytosolic ferritin concentrations were 20% lower in ID rats than in CN and REPL rats ($P < 0.05$, Fig. 2C). Brain cytosolic ferritin was 20% lower in ID rats than in CN rats (Fig. 2C, $P = 0.15$) and 40% lower than in REPL rats ($P < 0.05$). Iron therapy for 2 wk in the REPL rats led to a significant increase in ferritin concentration in both organs ($P < 0.05$), suggesting a prompt upregulation of ferritin during repletion.

Experiment 2. Quantitative regional brain analyses were performed in Experiment 2 using rats with hematologic, growth and iron repletion characteristics similar to those observed in Experiment 1 (Table 2). Body weights were significantly lower in ID rats than in CN rats, and partially recovered in REPL rats ($P <$ 0.001, Table 3). The poor growth in ID rats was not due to anorexia. Their food consumption per gram of body weight was higher than for CN rats ($P < 0.01$, data not shown), but food efficiency was lower than for CN or

REPL rats ($P < 0.05$, Table 3). As in the first experiment, the feeding protocol was adequate to eradicate iron deficiency anemia in the REPL rats at the end of 4 wk of repletion despite a much lower level of dietary iron, 15 mg/kg versus 35 mg Fe/ kg (Table 2).

TABLE 2

Hematological variables and liver non-heme iron concentrations in iron deficient rats (ID), control rats (CN) and rats fed iron-deficient diet followed by an iron-adequate diet (REPL) in Experiment 2[1]

	ID	REPL	CN
Dietary Fe, *mg/kg*	3	15	35
n	8	16	8
Hemoglobin, *g/L*	69 ± 2.2[a]	122 ± 6.1[b]	150 ± 5.2[c]
Hematocrit	0.24 ± 0.01[a]	0.38 ± 0.01[b]	0.42 ± 0.01[b]
Plasma Fe, *μmol/L*	2.6 ± 0.2[a]	10.0 ± 2.1[b]	18.3 ± 1.1[c]
TIBC, *μmol/L*	141.6 ± 11.9[c]	98.9 ± 7.4[b]	67.6 ± 2.3[a]
Transferrin saturation, %	1.9 ± 0.2[a]	11.9 ± 2.8[b]	27.3 ± 1.9[c]
Liver iron, *μmol/g*	0.43 ± 0.02[a]	0.71 ± 0.18[b]	1.86 ± 0.06[c]

[1] Values are means ± SEM. Different superscript letters in a row indicate significant a difference ($P < 0.05$).
[2] When expressed as (plasma Fe/plasma TIBC) · 100, total iron-binding capacity (TIBC) is equal to the percentage of transferrin saturation.

TABLE 3

Growth and food efficiency in iron-deficient rats (ID), control rats (CN) and rats fed iron-deficient diet followed by an iron-adequate diet (REPL) in Experiment 2 over the last 4 wk of the protocol[1]

	ID	REPL	CN
Dietary Fe, *mg/kg*	3	15	35
n	8	16	8
Final body weight, *g*	244.8 ± 5.8[a]	265.4 ± 3.5[a,b]	286.5 ± 6.3[b]
Weight gain, *g/4 wk*	152.2 ± 2.9[a]	181.8 ± 2.4[a,b]	200.4 ± 5.2[b]
Food intake, *g/4 wk*	515.4 ± 11.1[a]	544.1 ± 6.8[a,b]	577.1 ± 13.9[b]
Food efficiency, *g gained/g consumed*	0.302 ± 0.006[a]	0.333 ± 0.003[b]	0.345 ± 0.005[b]

[1] Values are means ± SEM. Different superscript letters in a row indicate significant differences ($P < 0.05$).

Brain regions differed significantly in iron concentrations regardless of dietary treatment groups ($P < 0.0001$, Fig. 3). Dietary treatment also had a significant independent effect on brain region iron concentrations ($P = 0.03$).[4] Interestingly, in the CN rats, hippocampal iron levels were significantly higher than in all other regions, a pattern not observed in our other studies of adult rodent brains (Focht et al. 1997, Roskams and Connor 1994). Hippocampus and cortex were the only two regions with significantly lower levels of iron in response to dietary ID ($P < 0.05$). When regional iron concentration in ID rats was expressed as a percentage of that in CN rats, the hippocampus and cortex were 57 and 64% of CN ($P < 0.05$) compared with ~80 to >100% of CN in all other regions.

Brain transferrin concentration (Fig. 4) also varied significantly across brain regions ($P < 0.0001$), though the variation within just the CN group was marginally significant (P 0.067). Dietary treatment had an independent and significant effect ($P = 0.045$). The interaction of diet and brain region was significant ($P = 0.035$), indicating that not all brain regions responded similarly to dietary treatments. Hippocampal transferrin concentration was higher in ID rats than in CN or REPL rats ($P < 0.05$. Transferrin concentrations in deep cerebellar nuclei, pons, superficial cerebellum and substantia nigra tended to be greater for ID rats than CN rats ($P = 0.13$). Transferrin concentrations did not increase in response to iron deficiency in the other regions. Four weeks of iron repletion led to a normalization of regional transferrin concentrations in REPL rats with the exception of the striatum, in which the striatal transferrin concentration was lower than that observed in CN rats ($P < 0.05$) and similar to the striatal transferrin concentration observed in ID rats.

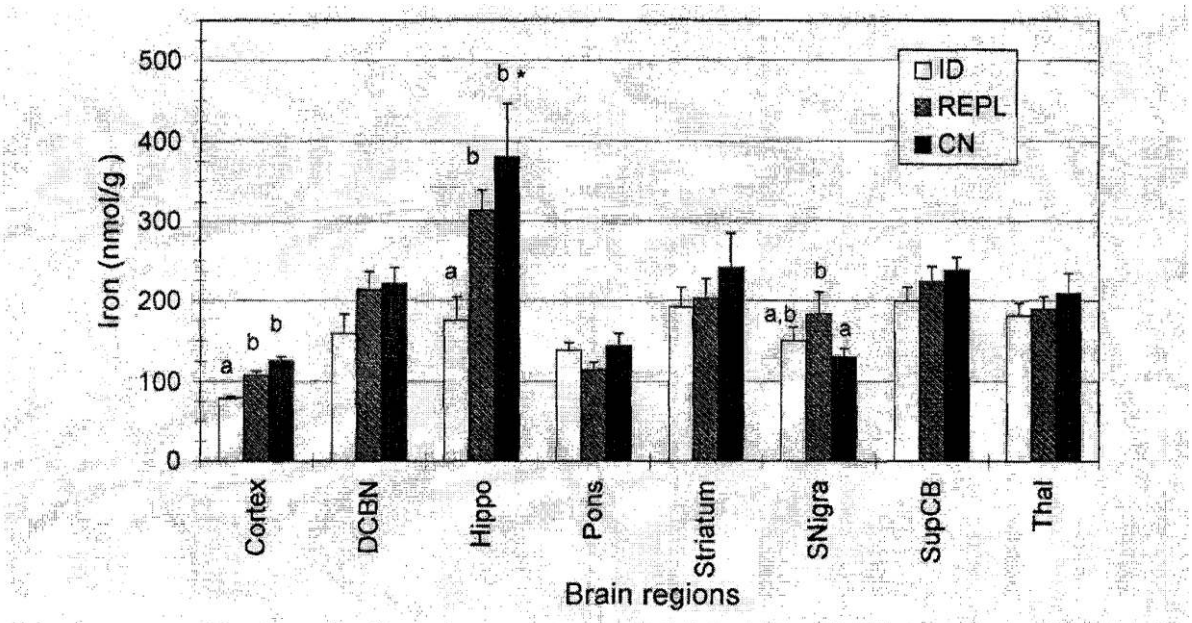

FIGURE 3 Regional brain iron concentrations of iron-deficient (ID, $n = 8$), control (CN, $n = 8$) and iron-replenished rats (REPL, $n = 16$) measured in tissue homogenates (Experiment 2). Values are means ± SEM. Brain regions examined were cerebral cortex (cortex), deep cerebellar nuclei (DCBN), hippocampus (Hippo), pons, striatum, substantia nigra (SNigra), superficial cerebellum (SupCB) and thalamus (Thal). The distribution of iron was significantly different across regions ($P < 0.01$) within all three treatment groups. Iron deficiency had an overall main effect of lowering brain iron ($P < 0.01$). Means with different letters within a brain region differed significantly ($P < 0.05$). Control rats had significantly higher concentrations ($P < 0.01$) of iron in hippocampus than any other brain region as noted by an asterisk.

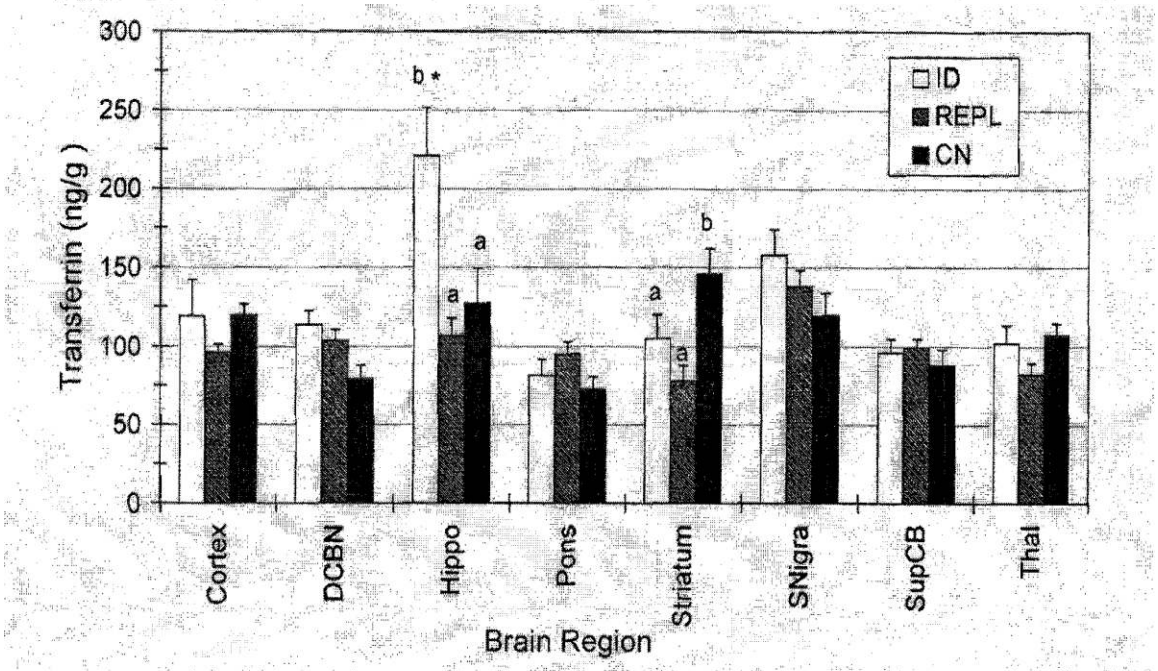

FIGURE 4 Regional brain transferrin concentrations for iron-deficient (ID, $n = 8$), control (CN, $n = 8$), and iron-replenished rats (REPL, $n = 16$) measured in tissue homogenates. Brain regions examined were cerebral cortex (cortex), deep cerebellar nuclei (DCBN), hippocampus (Hippo), pons, striatum, substantia nigra (SNigra), superficial cerebellum (SupCB) and thalamus (Thal). The distribution of transferrin was significantly different across regions ($P < 0.01$) in the ID and REPL treatment groups and approached significance in the CN treatment group ($P = 0.067$). Means with different letters within a brain region differed significantly ($P < 0.05$). Iron deficiency had an overall main effect of increasing brain transferrin ($P < 0.05$), though hippocampus was the only region in which transferrin was significantly higher ($P < 0.01$) than in any other brain region, as noted by an asterisk.

Ferritin concentrations also varied significantly across the eight brain regions examined *(P* 0.004, Fig. 5). Dietary iron deficiency significantly affected ferritin concentrations *(P* = 0.05), but no particular brain region appeared more susceptible than other brain regions.

The relationship of Hb concentration, an index of systemic iron deficiency, to regional brain iron concentration was investigated. Hemoglobin concentration and substantia nigra iron concentration were not significantly

correlated (Fig. 6, bottom panel), whereas there was a strong positive and significant relationship between Hb and cerebral cortex iron concentrations (r = 0,883, $P < 0.001$, Fig. 6, top panel). Similarly, the relationship between liver iron, another index of systemic iron status, and substantia nigra iron was not significant, whereas there was again a strong positive curvilinear relationship between liver iron and cortex iron (data not shown).

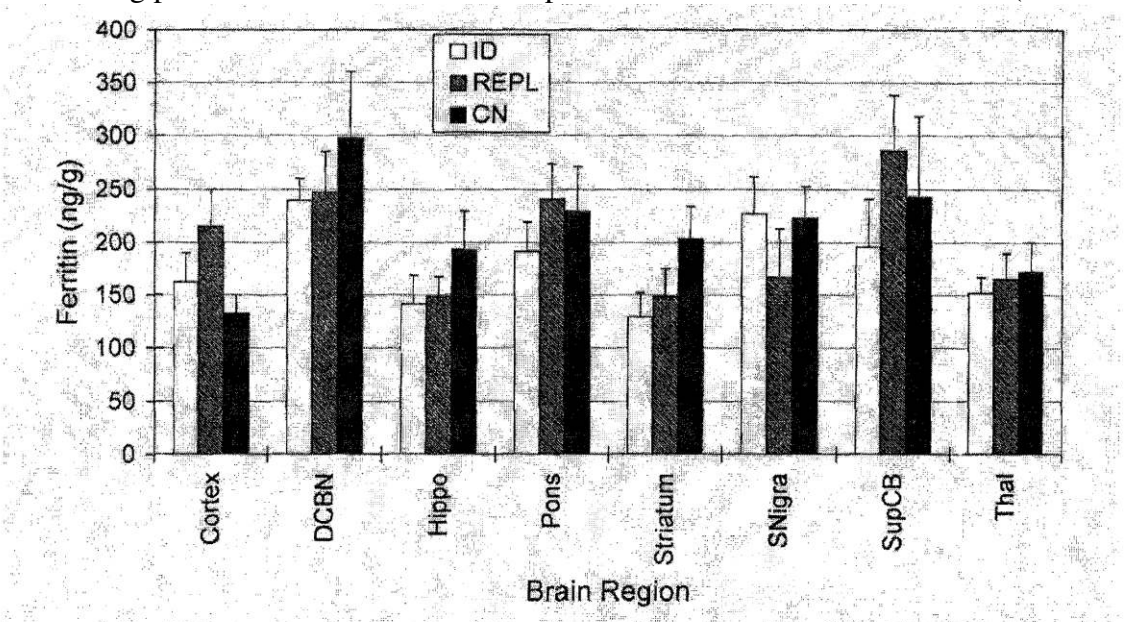

FIGURE 5 Regional brain ferritin concentrations for iron-deficient (ID, $n = 8$), control (CN, $n = 8$) and iron-replenished rats (IR, $n = 16$) determined in cytosolic fractions. Brain regions examined were cerebral cortex (cortex), deep cerebellar nuclei (DCBN), hippocampus (Hippo), pons, striatum, substantia nigra (SNigra), superficial cerebellum (SupCB) and thalamus (Thal). The distribution of ferritin was significantly different across regions ($P < 0.01$) in all three treatment groups. Iron deficiency had an overal main effect of decreasing brain ferritin concentration ($P < 0.05$).

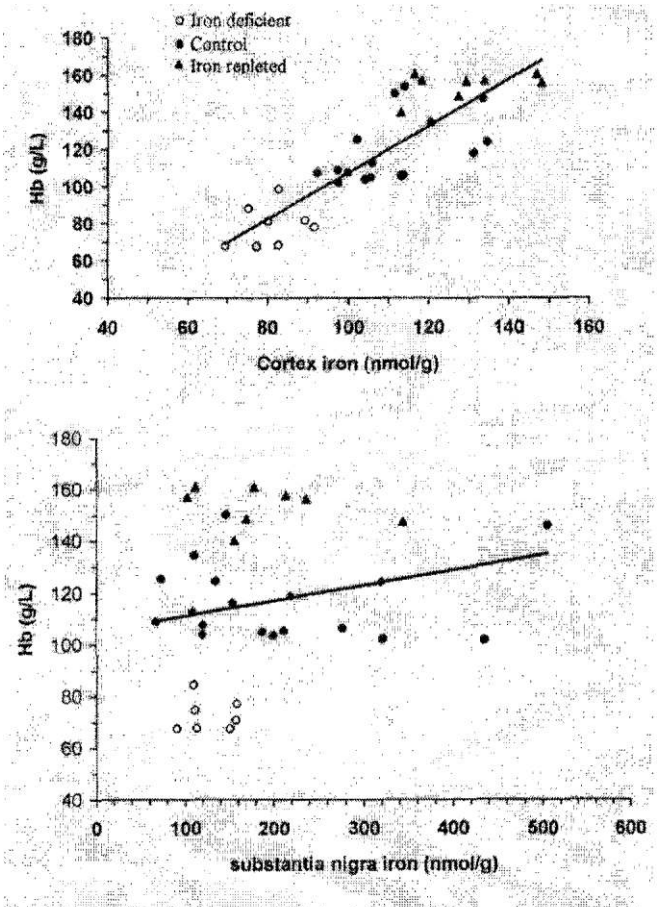

FIGURE 6 Scatter plots of blood hemoglobin concentration (Hb) vs. brain iron concentration in cerebral cortex (*top panel*) and in substantia nigra (*bottom panel*) in iron-deficient anemic, control and iron-replenished rats. The univariate correlation was significant ($r = 0.883$, $P < 0.001$) in cortex but not in the substantia nigra.

DISCUSSION

These experiments demonstrate several new aspects regarding iron metabolism in the brain of post-weaning rats. 1) Two weeks of iron repletion are adequate for correcting the overall iron concentration of the brain after a short duration of iron deficiency. However, adaptive processes such as changes in transferrin levels are still ongoing at this time (Experiment 1). 2) Iron and iron regulatory proteins, transferrin and ferritin, are distributed heterogeneously in the young rat brain and respond to iron repletion in a heterogeneous fashion (Experiment 2). In other words, brain regions have the apparent capacity to regulate their iron concentrations in response to local needs when faced with an alteration in systemic iron delivery. Although the mechanisms of this local regulation are not yet clear, this is the first demonstration that not all regions of the brain are altered equally by systemic iron deficiency.

Normalization of brain iron, in terms of overall concentrations in either microsomal or cytosolic fractions, extends our previous observations that rat brain iron can be reduced rapidly with post-weaning iron deficiency (Chen et al. 1995b). Repletion studies also demonstrate that the rat brain is fully capable of regaining iron in a rapid fashion, despite early observations that brain turnover (or more exactly, brain iron loss from the body) was a very slow process (Dallman and Spirito 1977). Others have shown an increase in iron uptake into the rat brain during iron deficiency, with transcytosis of iron and transferrin across the blood brain barrier (Crowe and Morgan 1992). The brain obtains iron via regulation of the transferrin receptor on the surface of endothelial cells on the brain microvasculature (Kalaria et al. 1992). The increase in uptake of iron observed in iron deficiency is not reflective of overall changes in blood brain barrier permeability and is highly selective for iron and transferrin. Although speculative at this time, it is reasonable to assume that upregulation of transferrin receptors is partially responsible for this alteration in uptake, because they also have a heterogeneous distribution (Hill et al. 1985, J, R. Connor, unpublished observation).

The regional specificity for brain iron acquisition is of great interest, because the distribution of iron in the developing brain is not the same as that in the adult brain (Connor 1994, Focht et al. 1997, Hill 1988, Roskams and Connor 1994). In a recent quantitative study using brains of 10-wk-old Fisher 344 rats, the striatum, hippocampus, thalamus and frontal cortex had equivalent iron concentrations of approximately 820 nmol/g, followed by brain stem, medial cortex, pons and cerebellum with 625-715 nmol/g (Focht et al. 1997). This is about double the amount measured here, where iron intake was much more highly regulated. Although there is a rough similarity in the distribution of iron, the absolute differences are more pronounced. In the same study, older (24-mo-old) rats had further iron accumulations to at least 890 nmol/g. Systematic evaluations of strain differences in brain iron distribution in rats have not been conducted. The substantia nigra, an iron-rich region in the adult rat brain as measured by histochemical methods (Hill 1988), is not particularly high in iron concentration in young rats. This region did, however, exhibit a dramatic increase in iron concentration during iron repletion, suggesting that iron was somehow "targeted" to go to the substantia nigra. It is interesting to recall that substantia nigra, globus pallidus and caudate putamen accumulate iron in elderly humans, particularly those with Alzheimer's disease and Parkinson's disease (Connor 1992, Good et al. 1992, Loeffler et al. 1995). Direct determinations of the regional responses of the transferrin receptor in future studies will test the possibility that it is upregulation of the transferrin receptor that is responsible for this targeted uptake of iron.

Iron is important for normal dopamine functioning in regions connected to the substantia nigra (Beard et al. 1994, Chen et al. 1995a, Youdim et al. 1989). The substantia nigra is rich in both iron and dopaminergic neurons (Bjorklund and Lindvall 1984, Hill 1988). The nigrostriatal dopaminergic pathway in the striatum has been found to be sensitive to iron deficiency with both decreases in dopamine D_2 receptors and increases in extracellular dopamine observed (Ashkenazi et al, 1982, Chen et al. 1995a, Youdim et al. 1989). Recent studies from our laboratory demonstrate that extracellular dopamine concentrations in the striatum return to normal within this same time frame for restoration of ventral mid-brain iron content (Nelson, C., Erikson, K. and Beard, J. L., unpublished observations). In addition, radioligand binding studies demonstrate alterations in dopamine transporters in striatal pathways in iron deficiency (Morse, A., Jones, B., and Beard, J. L., unpublished observations).

The predominant cell type containing iron in the young brain is the oligodendrocyte, with rapid accumulation of iron in these cells at the onset of myelination (Connor 1994). Cytological examination in the current study revealed "immature" appearing oligodendrocytes as a result of iron deficiency, which do not immediately revert to normal appearance within several weeks of iron therapy. These cells actively express transferrin receptor during development; when their function is compromised, the receptor expression drops dramatically (Roskams and Connor 1992). In addition, transferrin mRNA production and oligodendrocyte maturation are tightly coupled processes, suggesting that iron availability to these cells has a direct influence on their functioning (Bartlett et al. 1991).

Brain ferritin concentrations paralleled those of iron concentrations in some cases (e.g., no effect of iron deficiency in the substantia nigra), but in many brain regions there was a poor relationship between the two. This cannot be explained with our current data, although it is possible that the H and L chain isomers of ferritin are responding differently as the brain is richer in the H than the L isomer (Arosio et al. 1991). Current studies with more refined antibodies for specific chains of ferritin will provide an answer to whether specific HI ratio changes occur in response to development and iron status.

This study also indicated that brain, and certain brain regions, respond to iron deficiency and iron repletion much more aggressively than was expected based on previous data on brain iron turnover (Dalimn and Spirit() 1977). One earlier study noted that caudate putamen iron concentration could be restored within several months of iron therapy in the post-weaning rats (Ben-Shachar et al. 1986). In contrast, pre-weaning or lactational exposure to low iron leads to irreversible changes in brain iron even after repletion for long periods of time (Dallman and Spirito 1977, Felt and Lozoff 1996, Youdim et al. 1989). Because peak myelination occurs around postnatal d 10 in rats, it is reasonable to assume this is a critical period, during which oligodendrocyte requirements for iron are quite high. Our current protocol did not address this question but the observed changes in oligodendrocyte appearance in iron deficiency after postnatal d 21 demonstrate a continued sensitivity to iron status well beyond the early and peak portions of the myelination period.

The higher transferrin concentration in the brain of REPL rats compared CN with rats indicates a biological adaptation that is still not complete after 2-4 wk of iron repletion. The source of this transferrin is not clear because transferrin can be derived from the choroid plexus, oligodendrocytes, as well as transcytosed across the blood brain barrier (Connor and Benkovic 1992, Crowe and Morgan 1992). The regional variation suggests, however, the possibility that transferrin plays a role in some sort of distribution system that likely requires the expression of transferrin and transferrin receptor.

In summary, these studies indicate there is a heterogeneous distribution and response of brain iron and iron regulatory proteins due to iron deficiency and iron repletion, respectively. Further investigations using early developmental periods as elegantly defined by Felt and Lozoff (1996) will help us to identify the critical periods of development during which "reversible" damage becomes "irreversible" damage.

Notes:

[3] Abbreviations used: CN, control rats: HO, hemoglobin, Hct, nematocrit; ID, iron-deficient rats; TIBC, total iron-binding capacity; REPL, iron-replenished rats.

[4] Because these measurements on regional responses were all performed on crude homogenate fractions, and not cytosolic and microsomal fractions as in Experiment 1, the absolute concentrations are different from those in the preceding section.

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