Iron supplementation dose for perinatal iron deficiency differentially alters the neurochemistry of the frontal cortex and hippocampus in adult rats

Raghavendra Rao[1,2], Ivan Tkac[3], Erica L. Unger[4], Kathleen Ennis[1], Amy Hurst[5], Timothy Schallert[6], James Connor[7], Barbara Felt[5] and Michael K. Georgieff[1,2,8]

BACKGROUND: Long-term prefrontal cortex (PFC)- and hippocampus-based cognitive deficits are the sequelae of perinatal iron deficiency, despite iron supplementation starting in the newborn period. Whether high-dose iron supplementation prevents these deficits is yet to be determined.

METHODS: Perinatal iron deficiency was induced in rat pups using a low-iron (3 mg/kg diet) diet during gestation until postnatal day (P)8. Iron was supplemented using a standard (40 mg/kg diet) or a 10-fold higher (400 mg/kg diet) iron-containing diet until P21. PFC and hippocampal neurochemistry was determined using *in vivo* ^{1}H nuclear magnetic resonance (NMR) spectroscopy at 9.4 Tesla on P90.

RESULTS: Both standard and 10-fold higher iron supplementation doses corrected anemia and brain iron deficiency by P21. The neurochemical profile of the PFC in both supplementation groups was comparable with the control group. In the hippocampus, standard-dose iron supplementation resulted in lower concentrations of N-acetylaspartate (NAA) and phosphoethanolamine (PE) and higher concentrations of N-acetylaspartylglutamate (NAAG) and glycerophosphocholine + phosphocholine (GPC + PC). High-dose iron supplementation resulted in lower PE and higher GPC + PC concentrations.

CONCLUSION: The iron supplementation dose for perinatal iron deficiency differentially alters the neurochemical profile of the PFC and hippocampus in adults. The neurochemical changes suggest altered glutamatergic neurotransmission, hypomyelination, and abnormal phospholipid metabolism in the formerly iron-deficient (FID) hippocampus.

Iron deficiency during gestation and early lactation (perinatal iron deficiency) is common in humans and is associated with cognitive deficits that persist into adulthood (1,2). Abnormalities in recognition memory, attention, planning ability and inhibitory control, increased anxiety and depression, hesitancy, and wariness in novel situations are the long-term sequelae of early-life iron deficiency (3,4). The nature of the cognitive and behavioral deficits suggests the involvement of the prefrontal cortex (PFC) and hippocampus. Both brain regions rapidly develop during the perinatal period, although hippocampal development precedes that of the PFC (5). Animal studies demonstrate that iron deficiency has profound effects on energy metabolism, myelination, and monoamine (primarily dopamine) metabolism (6–10). Perinatal iron deficiency may independently perturb each of these processes. Moreover, these processes work together in integrated, interacting circuits. Both the independent and synergistic effects probably lead to the complex long-term cognitive and behavioral impairments following early-life iron deficiency.

The persistent cognitive and neurometabolic effects of perinatal iron deficiency may be due to incomplete iron repletion before the completion of the rapid phase of brain development (11,12). Thus, a more rapid correction of brain iron deficiency, for example, by using a higher than the standard dose of iron may prevent or lessen the cognitive deficits. Conversely, high-dose iron supplementation presents the potential for excess iron transport and iron-induced neurotoxicity, particularly when iron supplementation is initiated during the neonatal period when iron transport is avid (13,14). The risk of toxicity from excess iron may be further exacerbated in the setting of perinatal iron deficiency due to the earlier appearance and increased expression of iron transport proteins and receptors in specific brain regions, including the PFC and hippocampus (15).

The effects of different doses of iron supplementation for perinatal iron deficiency on long-term brain metabolism have not been studied. The objective of the current study was to compare the effects of two different doses of postnatal iron supplementation, standard dosing and 10-fold higher dosing, on the neurochemical profiles of the PFC and hippocampus in adult rats that were perinatally iron deficient. These brain regions have substantially different developmental trajectories;

[1]Department of Pediatrics, Neonatology Division, University of Minnesota, Minneapolis, Minnesota; [2]Center for Neurobehavioral Development, University of Minnesota, Minneapolis, Minnesota; [3]Center for Magnetic Resonance Research, University of Minnesota, Minneapolis, Minnesota; [4]Department of Nutritional Sciences, Pennsylvania State University, University Park, Pennsylvania; [5]Department of Pediatrics and Communicable Diseases, University of Michigan, Ann Arbor, Michigan; [6]Department of Psychology, University of Texas-Austin, Austin, Texas; [7]Department of Neurosurgery, Pennsylvania State University, Hershey, Pennsylvania; [8]Institute of Child Development, University of Minnesota, Minneapolis, Minnesota. Correspondence: Raghavendra Rao (raghurao@umn.edu)

Received 20 March 2012; accepted 24 August 2012; advance online publication 5 December 2012. doi:10.1038/pr.2012.143

however, both are affected in perinatal iron deficiency (16–18). We used high-field *in vivo* ^{1}H nuclear magnetic resonance (NMR) spectroscopy to determine the regional neurochemical profiles, consisting of metabolites indexing neuronal and glial integrity, phospholipid and energy metabolism, neurotransmission, osmoregulation, and antioxidants, and related the neurochemical changes to concurrent alterations in the expression of a targeted group of genes.

RESULTS

Effect of Dietary Iron on Milk Iron Concentration

To estimate daily iron intake by the pups, milk samples were collected from dams maintained on different dietary iron concentrations, ranging from 3 to 1,200 mg/kg diet. The milk iron concentration increased steadily with increasing iron concentration in maternal diet up to 400 mg/kg (Table 1). There was no further increase in milk iron concentration with a 1,200 mg/kg iron diet. On the basis of the average daily milk consumption by pups at corresponding ages (19), these data translate to a daily iron delivery of 1 mg/kg body weight during the period of iron deficiency, 2–3 mg/kg body weight during supplementation with standard-dose iron (40 mg/kg diet), and 6 mg/kg body weight during supplementation with high-dose iron (400 mg/kg diet) in maternal diet.

Effect of Iron Supplementation Dose on Hematocrit and Tissue Iron Status in the PFC and Hippocampus

The hematocrit and tissue iron concentration in the PFC and hippocampus were determined on postnatal day (P)21 and P90 in the control group and the two formerly iron-deficient (FID) groups that were supplemented with either standard-dose iron (40 mg/kg diet; FID-40 group) or high-dose iron (400 mg/kg diet; FID-400 group) in maternal diet from P8 to P21 (Figure 1). The hematocrit and tissue iron concentrations in the two FID groups were comparable with the control group on both days (Table 2). However, as compared with the FID-40 group, the hematocrit on P21 was higher and hippocampal iron concentration on P90 was lower in the FID-400 group

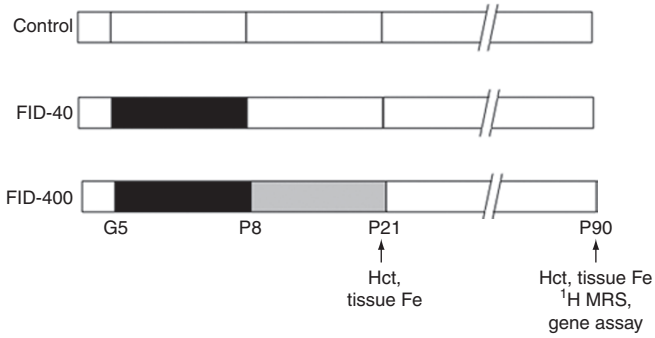

Figure 1. Study design. The control group (control) and the two FID groups were generated by maintaining the dams during the gestation (G) and postnatal (P) periods, and maintaining the pups after weaning on diets containing the different iron concentrations (40 mg/kg diet (white), 3 mg/kg diet (black), and 400 mg/kg diet (gray)). The numbers refer to gestational or postnatal age in days. Fe, iron; FID, formerly iron deficient; Hct, hematocrit; MRS, magnetic resonance spectroscopy.

($P < 0.05$, Table 1). Transferrin receptor 1 (*Tfrc*) mRNA expression in the PFC and hippocampus was determined on P90 to rule out latent tissue iron deficiency. In both FID groups, *Tfrc* expressions in the two brain regions were comparable with those of the control group (Table 2).

Effect of Iron Supplementation Dose on Neurochemical Profiles of the PFC and Hippocampus

The effects of iron supplementation dose on neurochemical profiles of the brain regions were determined using high-field *in vivo* ^{1}H NMR spectroscopy on P90. ^{1}H NMR spectra from the PFC and hippocampus from a rat in the control group are shown in Figure 2. Hippocampal spectra from two rats, one from each FID group, were excluded due to motion artifacts. A total of 17 metabolites were consistently quantified from the remaining NMR spectra. There were no differences between male and female rats in the neurochemical profiles of the two brain regions. Therefore, spectra from both sexes were combined.

Effect of iron supplementation dose on the regional neurochemical profiles. There were no differences between the

Table 1. Effect of maternal dietary iron on milk iron concentration

Iron concentration in the diet (mg/kg)	Iron concentration in the milk (μg/l)
3	1,397 ± 77
80	3,752 ± 204
400	8,736 ± 224
1,200	7,381 ± 885

Values are mean ± SEM, $n = 4–6$.

Table 2. Effect of the iron supplementation dose for perinatal iron deficiency on hematocrit, tissue iron concentration, and transferrin receptor mRNA expression in the PFC and hippocampus of 21-day-old rats and adult rats

Parameter	Age/region	Control group	FID-40 group	FID-400 group
Hematocrit (%)	P21	39.2 ± 0.6	36.0 ± 1.8	41.7 ± 1.0*
	P90	49.3 ± 0.3	49.3 ± 0.9	47.9 ± 0.7
Tissue iron concentration (μg/g) on P21	PFC	16.6 ± 1.4	16.2 ± 1.5	13.4 ± 0.9
	Hippocampus	15.2 ± 1.4	14.8 ± 1.1	15.3 ± 1.3
Tissue iron concentration (μg/g) on P90	PFC	18.1 ± 1.2	18.7 ± 1.3	16.1 ± 0.7
	Hippocampus	18.0 ± 1.1	21.1 ± 1.2	15.5 ± 1.0*
Tfrc expression on P90	PFC	1.00 ± 0.12	1.17 ± 0.11	1.26 ± 0.10
	Hippocampus	1.00 ± 0.09	1.13 ± 0.07	1.19 ± 0.14

Values are mean ± SEM ($n = 6–8$ per group). Iron concentrations are expressed as μg/g wet tissue weight. Transcript data are normalized to the control group for each brain region.

FID-40 group, formerly iron deficient, supplemented with 40 mg/kg iron diet from postnatal day 8; FID-400 group, formerly iron deficient, supplemented with 400 mg/kg iron diet from postnatal day 8; P, postnatal day; PFC, prefrontal cortex; *Tfrc*, transferrin receptor 1 gene.

*Different from FID-40 group, $P < 0.05$; the rest are not significant.

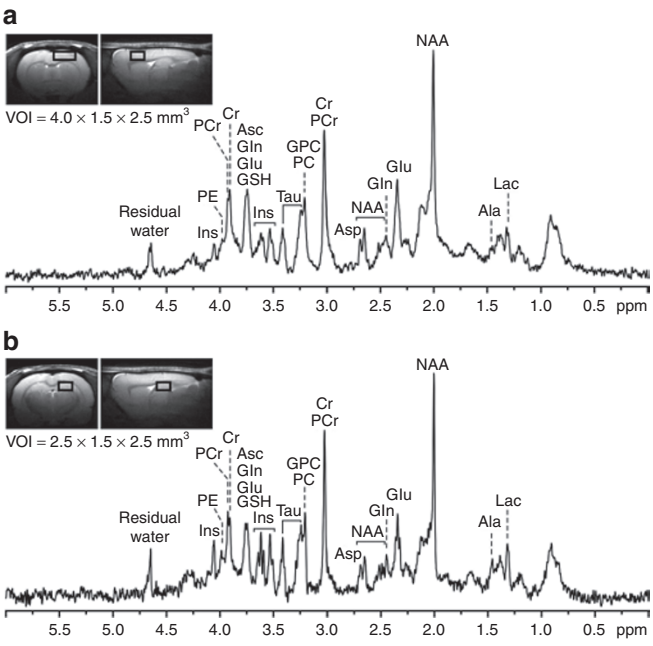

Figure 2. *In vivo* ^{1}H nuclear magnetic resonance spectra from the (**a**) prefrontal cortex and (**b**) hippocampus of a rat at postnatal day 90 in the control group. The magnetic resonance imaging shows the VOI used for acquiring the spectra. Ala, alanine; Asc, ascorbate; Asp, aspartate; Cr, creatine; Glu, glutamate; Gln, glutamine; GSH, glutathione; GPC, glycerophosphocholine; Lac, lactate; Ins, myo-inositol; NAA, N-acetylaspartate; NAAG, N-acetylaspartylglutamate; PC, phosphocholine; PCr, phosphocreatine; PE, phosphoethanolamine; Tau, taurine; VOI, volume of interest.

control group and the two FID groups in any of the 17 metabolites in the PFC (**Figure 3a**). In contrast, the two iron supplementation doses had differential effects on hippocampal neurochemistry (**Figure 3b**). As compared with the control group, there was a 6% decrease in N-acetylaspartate (NAA), a 37% increase in N-acetylaspartylglutamate (NAAG), a 15% decrease in phosphoethanolamine (PE), and a 17% increase in glycerophosphocholine + phosphocholine (GPC + PC) concentrations in the FID-40 group ($P < 0.05$, **Figure 3b**). In the FID-400 group, NAA and NAAG concentrations were comparable with the control group, but there was a 15% decrease in PE and a 27% increase in GPC + PC concentrations ($P < 0.05$, **Figure 3b**).

Effect of Iron Supplementation Dose on Gene Expression in the PFC and Hippocampus

The mRNA expression of a targeted group of genes was determined on P90 to relate neurochemistry results to specific neurodevelopmental processes. Similar to neurochemistry results, myelin basic protein (Mbp), profilin 1 (Pfn1), and calcium/calmodulin-dependent protein kinase II α (Camk2a) mRNA expressions in PFC in both FID groups were comparable with those in the control group (**Table 3**). In the hippocampus, Mbp and Pfn1 expressions were increased in both FID groups (control group <FID-40 group <FID-400 group, $P < 0.05$). Camk2a was increased only in the FID-400 group ($P < 0.05$; **Table 3**).

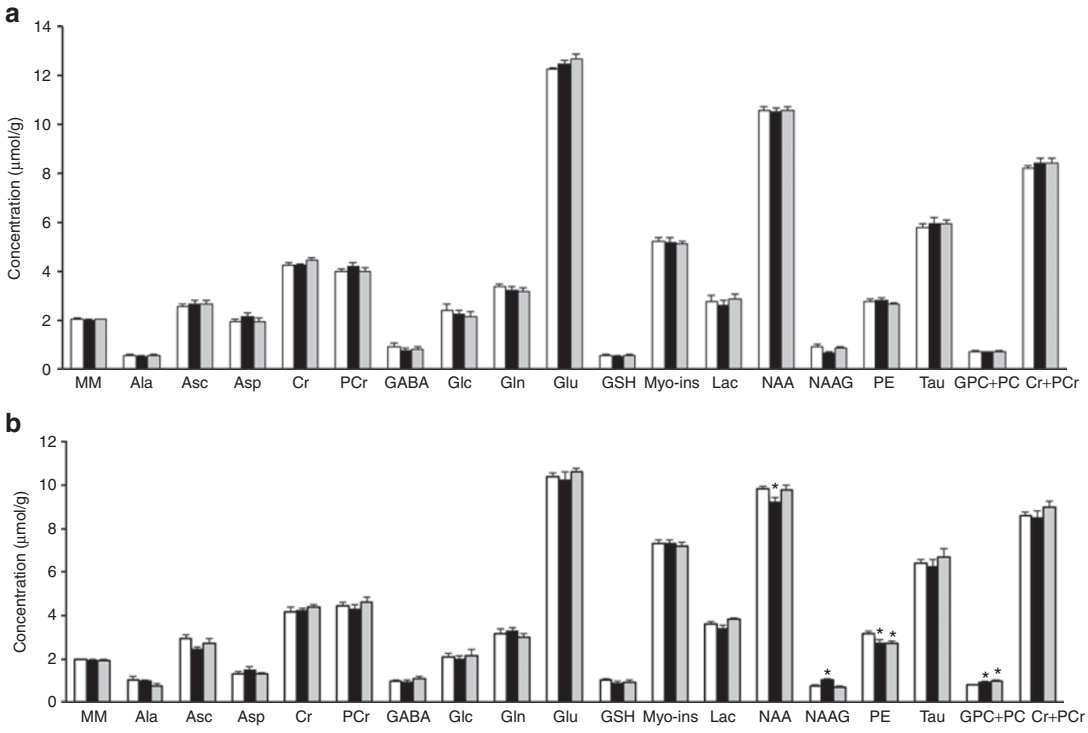

Figure 3. The neurochemical profiles of the (**a**) prefrontal cortex and (**b**) hippocampus in the control (white), FID-40 (black), and FID-400 (gray) groups. Values are mean ± SEM, $n = 7–8$; *$P < 0.05$ vs. the control group. Ala, alanine; Asc, ascorbate; Asp, aspartate; Cr, creatine; FID-40, formerly iron deficient, supplemented with 40 mg/kg iron diet from postnatal day 8; FID-400, formerly iron deficient, supplemented with 400 mg/kg iron diet from postnatal day 8; GABA, γ-aminobutyric acid; Glc, glucose; Gln, glutamine; Glu, glutamate; GSH, glutathione; GPC, glycerophosphocholine; Lac, lactate; Ins, myo-inositol; MM, macromolecules; NAA, N-acetylaspartate; NAAG, N-acetylaspartylglutamate; PC, phosphocholine; PCr, phosphocreatine; PE, phosphoethanolamine; Tau, taurine.

Table 3. Effect of the iron supplementation dose for perinatal iron deficiency on mRNA expression of a target group of genes in the PFC and hippocampus in adult rats

	PFC			Hippocampus		
Gene	Control group	FID-40 group	FID-400 group	Control group	FID-40 group	FID-400 group
Mbp	1.00 ± 0.10	1.08 ± 0.05	1.36 ± 0.20	1.00 ± 0.10	1.22 ± 0.06[a]	1.75 ± 0.19*,**
Camk2a	1.00 ± 0.24	1.03 ± 0.50	1.39 ± 0.29	1.00 ± 0.10	1.19 ± 0.08	1.32 ± 0.05*
Pfn1	1.00 ± 0.11	1.08 ± 0.07	1.25 ± 0.20	1.00 ± 0.08	1.28 ± 0.05*	1.54 ± 0.03*,**

Values are mean ± SEM normalized to the control group ($n = 6–8$ per group). There was a group effect for all genes in the hippocampus, $P < 0.04$ (ANOVA), but not in the PFC.

Camk2a, calcium/calmodulin-dependent protein kinase II α; FID-40 group, formerly iron deficient, supplemented with 40 mg/kg iron diet from postnatal day 8; FID-400 group, formerly iron deficient, supplemented with 400 mg/kg iron diet from postnatal day 8; *Mbp*, myelin basic protein; PFC, prefrontal cortex; *Pfn1*, profilin 1.

*Different from the control group, $P < 0.05$; **different from the FID-40 group, $P < 0.05$ (Bonferroni-adjusted unpaired *t* tests).

DISCUSSION

This study demonstrates that the iron supplementation dose for perinatal iron deficiency has a region-specific long-term effect on the neurochemical profile and gene expression in the PFC and hippocampus of adult rats. Both doses of iron supplementation, beginning in the neonatal period, corrected anemia and normalized tissue iron concentrations in both brain regions. Whereas the neurochemical profile of the PFC in both FID groups was comparable with that in the control group, the hippocampal neurochemical profile was abnormal in adulthood. These region-specific neurochemical effects, which index glutamatergic neurotransmission, myelination, and phospholipid metabolism, may have a role in the hippocampus-mediated cognitive deficits that persist in adulthood following early-life iron deficiency. Furthermore, these region-specific changes may cause an imbalance in the inputs and outputs of these two circuits on other critical brain systems (e.g., ventral tegmentum) that are necessary for normal behavioral function (2,20).

The concentration of 10 of the 17 metabolites differed by 10–40% in the PFC and hippocampus in the control group. Similar interregional metabolite concentration differences were also present in the two FID groups, with few exceptions. Such large, disparate differences in individual metabolites suggest that the data reflect region-specific effects and rule out technical factors, such as partial volume effect for the results. These data probably stem from the differences in metabolic demands and processes inherent to each brain region (5) and suggest that the effect of perinatal iron deficiency on a brain region depends on its developmental stage and metabolic demand at the time of iron deficiency.

The neurochemical profile of the PFC in both FID groups was comparable with that of the control group. On the basis of the previously demonstrated impaired energy metabolism in this region during perinatal iron deficiency (16) and persistent PFC-mediated behavioral deficits in iron-deficient human infants at adulthood (1), we expected neurochemical changes in the PFC. One possible explanation for our results is that both iron supplementation doses corrected tissue iron deficiency in the PFC by P21, i.e., before the onset of its peak developmental period (5) and may have protected the region from the deleterious effects of perinatal iron deficiency. Consistent with this possibility, PFC-mediated behaviors are preserved in adult rats that were perinatally iron deficient and that received iron supplementation from P8 (17).

Unlike the PFC, hippocampal neurochemistry was abnormal in both FID groups, despite the normalization of tissue iron concentration by P21. Latent or new-onset hippocampal iron deficiency is unlikely to explain these results, because tissue iron concentration and *Tfrc* expression in this region were normal at P90. Although the magnitude of the neurochemical changes (6–37%) in the FID hippocampus may appear modest, it is comparable with those reported during the period of iron deficiency and following iron supplementation in a slightly more severe perinatal iron deficiency model (10,11) and is associated with cognitive deficits in humans (21,22). Unlike the PFC, the period of brain iron deficiency and anemia in our model coincides with the period of peak hippocampal development (5) and may have perturbed its development. The iron supplementation doses also appear to have a more variable effect. A two to threefold higher daily iron intake was ineffective in achieving normal hippocampal neurochemistry. Collectively, these data suggest that the hippocampus is more sensitive to perinatal iron deficiency and may not benefit from iron repletion paradigms starting in the newborn period. It is not known whether starting iron supplementation earlier than P8 or continuing it beyond P90 would have normalized the hippocampal neurochemical profile. A previous study demonstrated that iron supplementation from P4 normalizes monoamine metabolism and behavior in a slightly less severe model of perinatal iron deficiency (12).

The abnormal aspects of hippocampal neurochemistry are informative in terms of the processes they index. Decreased NAA and increased NAAG in the context of iron deficiency suggest altered glutamatergic turnover (23). Our previous studies have demonstrated suppressed glutamate–glutamine cycling and *N*-methyl-D-aspartate receptor expression in the hippocampus during perinatal iron deficiency (10,24). NAAG modulates *N*-methyl-D-aspartate receptor function and suppresses long-term potentiation in the hippocampus (25), a finding consistent with reduced long-term potentiation at adulthood following perinatal iron deficiency (26). The NAA and NAAG changes in the FID-40 hippocampus are potentially correctable with higher doses of iron supplementation, given that these changes were absent in the FID-400 hippocampus. *Camk2a* upregulation, which is necessary for the induction of long-term potentiation (27) in the FID-400 group, but not in the FID-40 group, supports this interpretation.

In addition to its direct effect on hippocampus-specific cognitive function, altered glutamatergic neurotransmission may

indirectly influence the overall memory system in perinatal iron deficiency. The interconnections between the hippocampus, PFC, and ventral tegmentum, known as the ventral tegmental area loop, are regulated by the glutamatergic, γ-aminobutyric acid–ergic, and dopaminergic neurotransmitter systems. The loop is important for long-term memory and cognitive flexibility (20). The ventral tegmentum integrates glutamatergic inputs from the hippocampus and PFC and subsequently sends output to these structures via dopaminergic neurons, in turn modifying their activity. Of note, early-life iron deficiency profoundly alters dopaminergic metabolism in all three of these structures in adulthood (28). Combined with the altered glutamatergic changes demonstrated in our study, these neurotransmitter changes probably cause long-term impairments in cognitive performance and behavior (3,7,28).

Altered PE and GPC + PC, which index phospholipid metabolism in the hippocampus, have been previously demonstrated during the period of perinatal iron deficiency (10). Similar results in both FID groups in the current study suggest that perinatal iron deficiency irreversibly alters phospholipid metabolism in the hippocampus and that this effect is not amenable to correction with iron supplementation even at higher doses. Decreased PE suggests hypomyelination or demyelination (9,29). A corresponding increase in *Mbp* probably represents a compensatory response toward remyelination, similar to other demyelinating neuropathologies (30,31). Although the magnitude of PE decrease was similar in the two FID groups, a dose–response effect was present in the magnitude of *Mbp* expression (control group < FID-40 group < FID-400 group), suggesting the potential for greater compensatory remyelination with a higher dose of iron supplementation.

Increased GPC + PC also indicates altered phospholipid metabolism due to previous iron deficiency in the two FID groups. Increased GPC and/or PC could be responsible for the increased GPC + PC, because the close spectral similarities of the two compounds preclude their differentiation using *in vivo* NMR spectroscopy. However, increased *Pfn1* expression, which regulates neurite formation (32), suggests increased PC more than GPC but probably caused increased GPC + PC in both FID groups. In this regard, we have previously demonstrated increased, but abnormal-appearing neurites in the FID hippocampus (24,33). Our recent study in FID monkeys also demonstrates persistent changes in cerebrospinal fluid protein profiles that are indicative of abnormal neurite formation (unpublished data). Unlike PE, a dose–response effect was present for GPC + PC and *Pfn1* expression (control group < FID-40 group < FID-400 group). However, greater PC concentration may not portend a beneficial effect with higher doses of iron. Indeed, increased PC and neurite formation is a hallmark of neurodegenerative disorders such as early-stage Alzheimer's disease (34). In this context, we have previously demonstrated premature upregulation of Alzheimer's disease–related genes in the hippocampus during perinatal iron deficiency (35). Considering the potential role of iron dyshomeostasis in Alzheimer's disease (36), we posit that increased GPC + PC may indicate a greater risk of Alzheimer's disease in the FID-400 group. Future studies are necessary to test this hypothesis.

In summary, our study supports the presence of long-term region-specific adverse effects of perinatal iron deficiency on the developing brain despite iron supplementation. The dietary protocols resulted in a daily iron intake of 2–6 mg/kg body weight and rapidly corrected anemia and brain iron deficiency but were unable to achieve neurochemical normalcy in the hippocampus at adulthood. These data may have relevance to iron-deficient human infants and toddlers. The iron supplementation dose in the current study falls within the range used for treating these children (37). Although the data may not be directly translatable to humans, the inability to achieve neurochemical normalcy even with high doses of iron suggests the importance of additional research on treatment strategies for ensuring normal neurodevelopment in these infants.

METHODS

Animals and Dietary Treatment

All procedures conformed to the guidelines of the National Institutes of Health and were approved by the institutional animal care and use committees at the University of Michigan and the University of Minnesota. Male and female Sprague-Dawley rats were used. The overall study design is shown in **Figure 1**. Custom research diets were purchased (Harlan Laboratories, Madison, WI). Pregnant dams were maintained on a low-iron diet (TD80396; iron concentration, 3 mg/kg diet) from gestational day 5 to P7 to induce anemia and brain iron deficiency in the pups. On P8, pups were treated by cross-fostering to dams maintained on diets containing one of two iron concentrations, 40 mg/kg diet (TD89300) or 400 mg/kg diet (TD02545), until weaning on P21 to create the FID-40 group and the FID-400 group, respectively. Pups born to dams maintained on the 40 mg/kg iron–containing diet throughout gestation and postnatal periods formed the always iron-sufficient control group (control group). All groups were maintained on the 40 mg iron/kg diet from P21. Each group consisted of pups from at least two litters.

Determination of Milk and Tissue Iron Concentration

Collection of milk. Milk samples were collected on P9, P12, and P15 from a separate group of dams maintained on a diet containing one of the following iron concentrations: 3, 80, 400 and 1,200 mg/kg. Under 3% isoflurane anesthesia, the abdomen was cleaned with nanopure water followed by ethanol. Milk was collected by manual expression after injecting oxytocin (20 U/ml solution; 300 µl, s.c.) to stimulate milk production. The volume of collected milk was recorded, and samples were stored at −20 °C. The entire procedure took <20 min.

Collection of brain tissue. Brain tissue was collected from a separate group of rats on P21 and P90 after *in situ* perfusion with phosphate-buffered saline. The PFC and the hippocampus were dissected on ice and kept frozen at −80 °C until analysis.

Determination of iron concentration. Milk and the brain regions were thawed on ice and wet digested using published protocols (38). The iron concentration was determined using atomic absorption spectrometry (AAnalyst 600, Perkin-Elmer, Norwalk, CT). Standards were prepared by diluting iron standards (PE#N9300126; Perkin-Elmer) in 0.2% ultrapure nitric acid. Blanks were prepared by digesting and diluting reagents to control for possible contamination. All standard curves exceeded $r > 0.99$.

In vivo ^{1}H NMR Spectroscopy

In vivo ^{1}H NMR spectroscopy was performed on P90 in spontaneously breathing rats under inhalational anesthesia (isoflurane, 3% for induction and 1–2% for maintenance in a 1:1 mixture of O_2 and N_2O). Measurements were performed using a horizontal bore 9.4 Tesla/31 cm magnet (Varian/Magnex Scientific; Oxford, UK) interfaced to Varian INOVA/Direct Drive consoles (Varian, Palo Alto, CA) using previously published protocols (2,10). The volume of interest was ~15 µl ($4.0 \times 1.5 \times 2.5\,mm^3$) for the PFC and 9 µl ($2.5 \times 1.5 \times 2.5\,mm^3$) for

the hippocampus and was based on multislice fast spin-echo magnetic resonance imaging (echo train length = 8, echo spacing = 12 ms, echo time = 48 ms, field of view = 3 cm × 3 cm, matrix = 256 × 256, slice thickness = 1 mm). The order of data acquisition from the two regions was randomly chosen in each rat. The study of a single rat did not exceed 100 min.

Quantification of neurochemicals. *In vivo* ^{1}H NMR spectra were analyzed with the spectrum of fast-relaxing macromolecules included in the basis set as previously described (2,10). Unsuppressed water signal was used as internal reference assuming 80% brain water content. Experiments with poor signal-to-noise ratio (Cramer Rao lower bounds >30%) were excluded from the analysis. The following metabolites were included in the final analysis: alanine, ascorbate, aspartate, creatine, phosphocreatine, γ-aminobutyric acid, glucose, glutamate, glutamine, glutathione, lactate, *myo*-inositol, NAA, NAAG, PE, and taurine. In addition, the sum of GPC and PC was determined, given that it was not possible to differentiate the two metabolites due to their close spectral similarity. Thus, the neurochemical profile consisted of 17 metabolites.

Quantitative RT-PCR

Rats were killed after NMR spectroscopy using an overdose of pentobarbital (120 mg/kg i.p.). The brain was removed, and the PFC and hippocampi were dissected out, flash frozen, and stored at −80 °C. The mRNA expression was measured using published methods (39) and ready-made primer/probes (TaqMan Gene Expression Assays; Life Technologies, Carlsbad, CA; **Supplementary Table S1** online). Samples were assayed in duplicate and normalized against ribosomal protein S18.

Statistical Analysis

The effects of iron supplementation dose on regional neurochemistry and mRNA expression were determined using ANOVA and Bonferroni-corrected *t* tests. Data are presented as mean ± SEM. Significance was set at $P < 0.05$.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/pr

ACKNOWLEDGMENTS

The authors acknowledge the technical assistance of Liam Callahan, Katie Thibert, and Kim-Thao Le.

STATEMENT OF FINANCIAL SUPPORT

This work was supported by a grant (P01 HD039386, principal investigator: B. Lozoff) from the National Institutes of Health. The Center for Magnetic Resonance Research is supported by a National Center for Research Resource grant (P41 RR008079), a Neuroscience Center Core Blueprint Award (P30 NS057091), and the W.M. Keck Foundation. The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Disclosure: This study was presented as abstracts during the annual meetings of the Pediatric Academic Societies (R.R., I.T., K. Czerniak, A.H., B. F., and M.K.G.; Long-term effects of perinatal iron deficiency and its treatment on the neurochemical profile of the hippocampus and frontal cortex in adult rats) on 2 May 2011, Denver, CO, and the International Society of Magnetic Resonance in Medicine (I.T., K. Czerniak, A.H., B.F., M.K.G., and R.R.; Long-term dosage effects in iron repletion treatment on neurochemical profiles and gene expression in rat model of neonatal iron deficiency) on 8 May 2012, Melbourne, Australia.

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