Nutritional Neurosciences

Perinatal Iron Deficiency Alters the Neurochemical Profile of the Developing Rat Hippocampus[1,2]

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ABSTRACT   Cognitive deficits in human infants at risk for gestationally acquired perinatal iron deficiency suggest involvement of the developing hippocampus. To understand the plausible biological explanations for hippocampal injury in perinatal iron deficiency, a neurochemical profile of 16 metabolites in the iron-deficient rat hippocampus was evaluated longitudinally by ^{1}H NMR spectroscopy at 9.4 T. Metabolites were quantified from an 11–24 μL volume centered in the hippocampus in 18 iron-deficient and 16 iron-sufficient rats on postnatal day (PD) 7, PD10, PD14, PD21 and PD28. Perinatal iron deficiency was induced by feeding the pregnant dam an iron-deficient diet from gestational d 3 to PD7. The brain iron concentration of the iron-deficient group was 60% lower on PD7 and 19% lower on PD28 ($P < 0.001$ each). The concentration of 12 of the 16 measured metabolites changed over time between PD7 and PD28 in both groups ($P < 0.001$ each). Compared with the iron-sufficient group, phosphocreatine, glutamate, N-acetylaspartate, aspartate, γ-aminobutyric acid, phosphorylethanolamine and taurine concentrations, and the phosphocreatine/creatine ratio were elevated in the iron-deficient group ($P < 0.02$ each). These neurochemical alterations suggest persistent changes in resting energy status, neurotransmission and myelination in perinatal iron deficiency. An altered neurochemical profile of the developing hippocampus may underlie some of the cognitive deficits observed in human infants with perinatal iron deficiency.   J. Nutr. 133: 3215–3221, 2003.

KEY WORDS: • hippocampus • iron • neurochemical profile • NMR spectroscopy • perinatal iron deficiency

Human infants whose gestations were complicated by maternal iron deficiency, maternal diabetes mellitus, intrauterine growth retardation or prematurity are at risk for perinatal iron deficiency (1–3). Perinatal iron deficiency is associated with long-term cognitive abnormalities (4–6). Iron plays an important role in normal neurodevelopment through enzymes controlling neurotransmitter synthesis (7), cell division (8), neuronal energy metabolism (8,9) and myelination (10,11). Any or all of these mechanisms may be perturbed in states of perinatal iron deficiency.

Electrophysiologic studies in infants at risk for perinatal iron deficiency suggest functional perturbations of the developing hippocampus, an area central to recognition memory (4,5). Hippocampal development begins prenatally and continues postnatally in humans and rats (12–14). In rats, continued neurogenesis, extensive remodeling of pyramidal cell dendrites, peak velocities of myelination and proliferation of synapses result in an exponential increase in the size of the hippocampus during the first four postnatal weeks (12–14). The rapid growth rate is accompanied by upregulation of regional iron transporters and presumably increased iron utilization by the developing hippocampus (15). The accelerated growth rate also increases the vulnerability of the developing hippocampus to various perinatal and postnatal injuries (9,16–19).

A perturbation in the normal development of the hippocampus by perinatal iron deficiency may play a role in cognitive abnormalities in at-risk human infants. Histochemical (9) and behavioral studies (16) in rats have demonstrated selective involvement of the developing hippocampus in perinatal iron deficiency.

Nevertheless, the biochemical mechanisms through which perinatal iron deficiency specifically affects the developing hippocampus have not been comprehensively studied. A longitudinal evaluation of neurochemical changes during hippocampal development may help explain the potential pathways through which perinatal iron deficiency alters hippocampal development and function. Previous studies focused on specific biochemical alterations in models of postna-

tal iron deficiency (8,20). The invasive nature of such biochemical methods precludes longitudinal study designs. In vivo ^{1}H NMR spectroscopy is a sensitive and noninvasive method for simultaneous evaluation of several neurochemicals from distinct regions of the developing brain (21,22). At high magnetic field strengths, such as 9.4 T, neurotransmitter and amino acid concentrations, as well as metabolite markers of energy status, myelination and neuronal integrity, can be reliably measured longitudinally from well-defined brain regions, including the developing hippocampus (21).

The objective of the present study was to evaluate the effect of fetal and neonatal iron deficiency on selected metabolite concentrations in the developing hippocampus from postnatal day (PD)[4] 7 to PD28 using high field ^{1}H NMR spectroscopy. We hypothesized that perinatal iron deficiency would alter the developmental trajectories of select metabolites that are dependent on intact iron metabolism. This novel technical approach would allow for sequential in vivo tracking of the metabolite changes as they appear over time.

MATERIALS AND METHODS

Animal preparation. All experiments were approved by the Animal Care and Use Committee of the University of Minnesota. Iron-deficient and iron-sufficient male and female Sprague-Dawley rat pups (Harlan Sprague Dawley, Indianapolis, IN) between the ages of PD7 and PD28 [weight range: 13 g (PD7) to 113 g (PD28)] were used in the study. As in our previous studies (9,19), fetal and neonatal iron deficiency was induced by feeding pregnant dams a low iron diet (Formula TD 80396; Harlan-Teklad, Madison, WI; elemental iron concentration: 3–6 mg/kg) from gestational d 2 to PD7, followed by an iron-supplemented diet (Teklad 4% Mouse/Rat Diet 7001, Harlan-Teklad; elemental iron: 198 mg/kg) until PD28. The compositions of the diets were published previously (16,19). The iron-sufficient dams were fed the iron-supplemented diet throughout the experiment. The litters were culled to 8 pups soon after birth to ensure uniform growth. Rats had free access to food and water and were maintained on a 12-h light:dark cycle at room temperature. A total of 32 pups (8 per group on PD7 and PD28) from 4 litters (2 iron-sufficient and 2 iron-deficient) were utilized for tissue iron assay; 34 (18 iron-deficient and 16 iron-sufficient) pups from 8 litters (5 iron-deficient and 3 iron-sufficient) were studied by ^{1}H NMR spectroscopy on PD7, PD10, PD14, PD21 and PD28. The regional neurochemical profiles during brain development in these iron-sufficient rats were reported previously (21). Six rats in each group were studied longitudinally (i.e., on all 5 postnatal days); the remainder were studied in a cross-sectional manner, resulting in 10–14 spectra in the iron-deficient group and 8–12 spectra in the iron-sufficient group on each of the 5 postnatal days.

Tissue iron assay. Tissue iron concentrations of the brain, liver and heart were assayed by atomic absorption spectroscopy as previously described (19). Values were expressed as μmol elemental iron/g wet tissue weight. Brain water content was determined by weighing the brain before and after drying for 72 h and was represented as a percentage of wet weight.

NMR methods. ^{1}H NMR spectra were obtained from spontaneously breathing rats under inhalation anesthesia (1–2% Isoflurane in an equal mixture of O_2 and N_2O) using a horizontal bore 9.4 T/31 cm magnet (Magnex Scientific, Abingdon, UK) interfaced to a Varian INOVA console (Varian, Palo Alto, CA). The detailed technical aspects of the NMR spectroscopy were described previously (21). Briefly, field homogeneity was optimized using the FASTMAP shimming technique (23,24). Spectroscopic localization was performed by an ultrashort echo time single-voxel stimulated-echo acquisition mode sequence (echo time = 2 ms, mixing time = 20 ms, repetition

time = 5 s) combined with outer volume suppression and variable pulse power and optimized relaxation water suppression (25). Positioning of the volume of interest (VOI) was based on multislice rapid acquisition with relaxation enhancement imaging (echo train length = 8, echo time = 48 ms, field of view = 2 × 2 cm, matrix = 256 × 256, slice thickness = 1 mm). The VOI (11–24 μL) was adjusted to match the postnatal increase in hippocampal size. The study of a single rat did not exceed 60 min.

Quantification of metabolites. In vivo ^{1}H NMR spectra were analyzed using LCModel (26) as in our previous studies (21,27). The signals of macromolecules and the following 18 metabolites were quantified from each spectrum: alanine, aspartate, creatine (Cr), γ-aminobutyric acid (GABA), glucose, glutamate, glutamine, glutathione, glycerophosphorylcholine (GPC), lactate, *myo*-inositol, *N*-acetylaspartate (NAA), *N*-acetylaspartylglutamate, phosphocreatine (PCr), phosphorylcholine (PCho), phosphorylethanolamine, *scyllo*-inositol and taurine, and were represented as μmol/g. *Scyllo*-inositol was not further analyzed because it was detected in only 25% of the processed spectra in both groups. The sum of PCho and GPC (PCho+GPC) was used in the statistical analysis because of a strong cross-correlation between these compounds due to their close spectral similarity. Thus, the spectroscopy analysis resulted in 16 metabolites that could be reliably quantified from the averaged spectra from every rat. The ratios of phosphocreatine to creatine (PCr/Cr) and glutamate/glutamine were also calculated.

Statistical methods. Group means of body and brain weights, brain water content, hematocrit and tissue iron concentrations between the two groups were compared at PD7 (end of iron-deficient dietary period) and PD28 (end of iron supplementation in the study) using two-tailed t tests. Within each group, the differences between PD7 and PD28 were compared using paired t tests. Metabolite concentrations were compared across time and between groups using univariate ANOVA. Univariate analyses were employed instead of a multivariate approach because the focus of the study was to examine group differences in individual metabolites, not differences in the weighted effect of metabolites in combination. Metabolite concentrations of rats studied longitudinally and in a cross-sectional manner were comparable at each time point in both groups. Thus, data from all rats at each time point were assessed together. A statistical analysis software package (SPSS, Version 10.1; SPSS, Chicago, IL) was used for all analyses. Data are presented as means ± SD. A P-value < 0.05 was considered significant.

RESULTS

General. Compared with the iron-sufficient group, the iron-deficient group had higher body and brain weights on PD7 and lower body and brain weights on PD28 (**Table 1**). Brain water content was 87% on PD7 and 80% on PD28 in both groups. The iron-deficient group had a lower hematocrit

TABLE 1

Effect of maternal dietary iron treatment during gestation and early postnatal period on body weight and brain weight of iron-deficient and iron-sufficient Sprague-Dawley rats[1]

Group	Postnatal age	Body weight	Brain weight[2]
	d	*g*	
Iron-sufficient	7	14.2 ± 0.7	0.56 ± 0.03
	28	97.5 ± 11.1	1.14 ± 0.03
Iron-deficient	7	16.6 ± 0.8*	0.61 ± 0.05*
	28	83.6 ± 7.2*	1.05 ± 0.09*

[1] Data are means ± SD, n = 8. Means differed within each group, P < 0.001. * Different from the iron-sufficient group, P < 0.02.

[2] Wet weight of the brain after removal of the cerebellum and brain stem.

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[4] Abbreviations: Cr, creatine; GABA, γ-aminobutyric acid; GPC, glycerophosphorylcholine; NAA, *N*-acetylaspartate; NMDA, *N*-methyl-D-aspartate; PCr, phosphocreatine; PCr/Cr phosphocreatine to creatine ratio; PCho, phosphorylcholine; PD, postnatal day; VOI, volume of interest.

on PD7 and a higher level on PD28 (**Table 2**). In the iron-deficient group, brain iron concentrations were lower on both PD7 and PD28, and liver and heart iron concentrations were lower on PD7.

Hippocampal metabolite concentrations. There were significant changes in the ^{1}H NMR spectra between PD7 and PD28 (**Fig. 1**). The "neurochemical profile" consisted of 16 metabolites that could be reliably quantified from the spectra. The Cramer-Rao lower bounds, which estimate the accuracy of the fitted concentrations, were below 20% for most metabolites at all postnatal ages in both groups. This corresponded to an estimated error of calculated concentration < 0.2 μmol/g. Only a few weakly selected metabolites, such as aspartate and glucose, were quantified with an estimated error ± 0.3 μmol/g.

As in the iron-sufficient group (21), 12 of the 16 metabolites demonstrated significant changes in their concentrations with increasing postnatal age in the iron-deficient group (**Figs. 2–4**) ($P < 0.001$ for each metabolite). Iron deficiency had a significant effect on the concentrations of 7 of these 12 metabolites.

The maximum effect of iron deficiency over time was on PCr, glutamate, NAA concentrations and the PCr/Cr ratio, all of which were elevated in the iron-deficient group (all $P < 0.002$) (Fig. 2). Iron deficiency had a moderate effect on aspartate, GABA, phosphorylethanolamine, taurine concentrations (Fig. 3) and the glutamate/glutamine ratio (data not shown) over time ($P < 0.02$ for taurine and $P < 0.005$ for the others). Glutamine, Cr, *myo*-inositol, PCho+GPC (Fig. 4) and alanine (data not shown) concentrations changed with postnatal age but were not affected by iron deficiency.

DISCUSSION

Neurochemical changes accompany structural changes during regional brain development. We demonstrated previously that ^{1}H NMR spectroscopy at 9.4 T can accurately measure changes in the concentrations of 16 metabolites in three specific brain regions in developing rats (21). In the present study, we used this method successfully to demonstrate the effect of perinatal iron deficiency on this neurochemical profile in the developing hippocampus.

In the current study, the severity of brain iron deficiency was slightly greater than that described in human infants with perinatal iron deficiency (2,3). As demonstrated by studies in humans (4,5) and rats (9,16,28), this degree of perinatal iron deficiency affects the structure and function of the developing hippocampus. Similarly, unmet hippocampal iron demands were probably responsible for the neurochemical alterations in
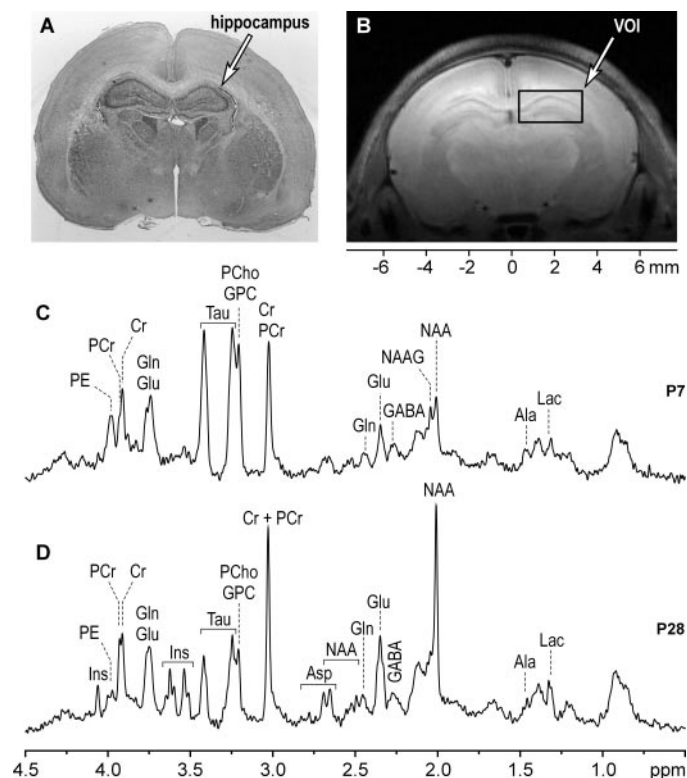

FIGURE 1 Coronal brain section (*A*) and MRI (*B*) on postnatal day (PD) 7, and ^{1}H NMR spectra on PD7 (*C*) and PD28 (*D*) of the iron-deficient rat hippocampus. The brain section (15 μm) was stained for cytochrome *c* oxidase activity. The ^{1}H NMR spectra are from a volume of 13.5 μL (PD7) and 18 μL (PD28) and represent an average of 160 scans each. Abbreviations: Asp, aspartate; Cr, creatine; GABA, γ-aminobutyric acid; Glu, glutamate; GPC, glycerophosphorylcholine; Ins, myo-inositol; Lac, lactate; NAA, *N*-acetylaspartate; NAAG, *N*-acetylaspartylglutamate; PCr, phosphocreatine; PCho, phosphorylcholine; PE, phosphorylethanolamine; VOI, volume of interest.

the present study. Even though the iron-deficient rats were anemic, cerebral hypoxia secondary to anemia is unlikely to have played a major role in these neurochemical changes. Gestational iron deficiency does not result in fetal hypoxia (29). Furthermore, neurochemical alterations due to chronic postnatal hypoxia differ from those observed in the present study (30). Finally, the neurochemical changes persisted in the iron-deficient group after anemia was adequately corrected.

TABLE 2

Effect of maternal dietary iron treatment during gestation and early postnatal period on hematocrit and tissue iron concentrations of iron-deficient and iron-sufficient Sprague-Dawley rats[1]

			Tissue iron concentration		
Group	Postnatal age	Hematocrit	Brain	Liver	Heart
	d	%	*μmol/g wet weight*
Iron-sufficient	7	32.8 ± 2.9	0.10 ± 0.02	2.99 ± 0.63a	0.45 ± 0.10a
	28	39.3 ± 1.4	0.18 ± 0.01	2.38 ± 1.14a	0.51 ± 0.14a
Iron-deficient	7	17.4 ± 1.5*	0.04 ± 0.01*	0.27 ± 0.06*	0.23 ± 0.02*
	28	44.8 ± 2.1*	0.15 ± 0.01*	2.70 ± 1.15	0.44 ± 0.15

[1] Data are means ± SD, $n = 8$. For each group, means without a common letter differ, $P < 0.005$. * Different from the iron-sufficient group, $P < 0.02$.
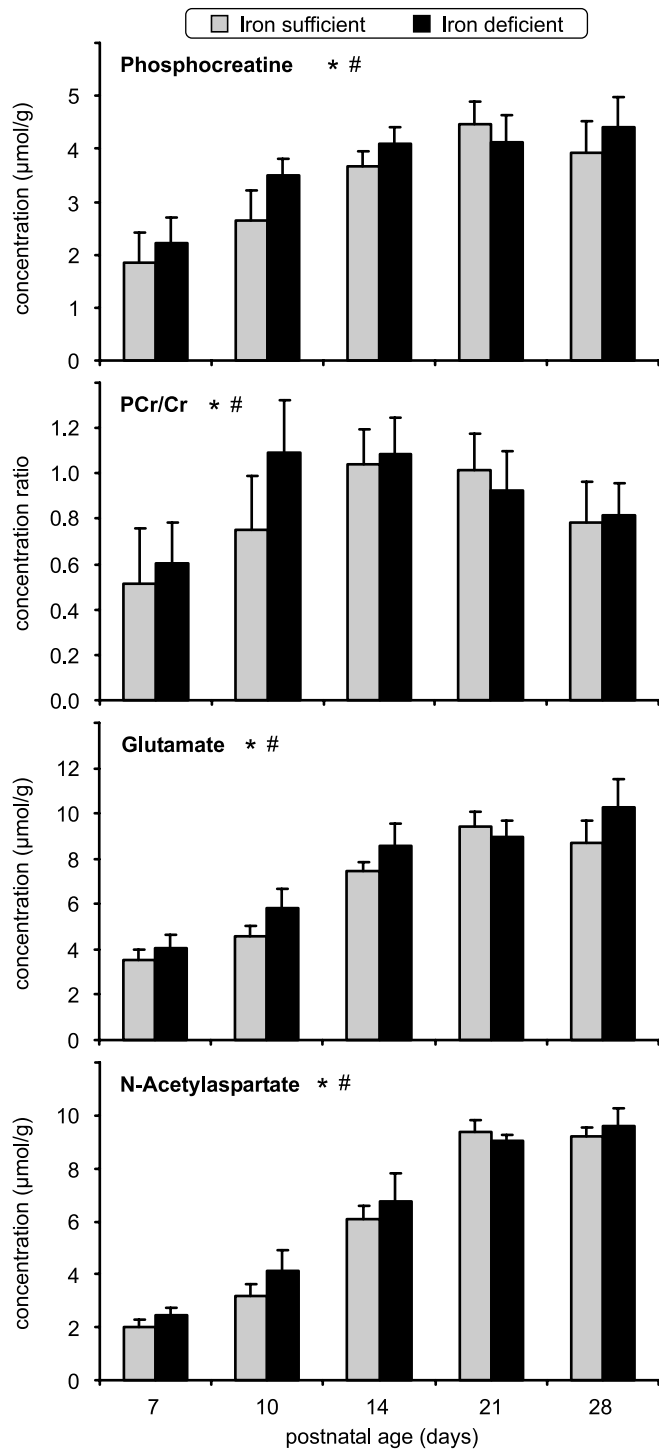
FIGURE 2 Effect of maternal dietary iron treatment during gestation and early postnatal period on phosphocreatine concentration, the phosphocreatine/creatine ratio (PCr/Cr), glutamate and *N*-acetylaspartate concentrations between postnatal days 7 and 28 in iron-deficient and iron-sufficient Sprague-Dawley rats. The iron-sufficient data were published previously (21). Values are means ± SD, n = 8–12 (iron-sufficient) and 10–14 (iron-deficient) at each postnatal age. *Different over time for each group, P < 0.001; #different between groups over time, P < 0.002.

roles (8,31,32). The temporal evolution of the neurochemical profile of the iron-deficient hippocampus paralleled that of iron-sufficient rats, yet the two profiles were not identical.
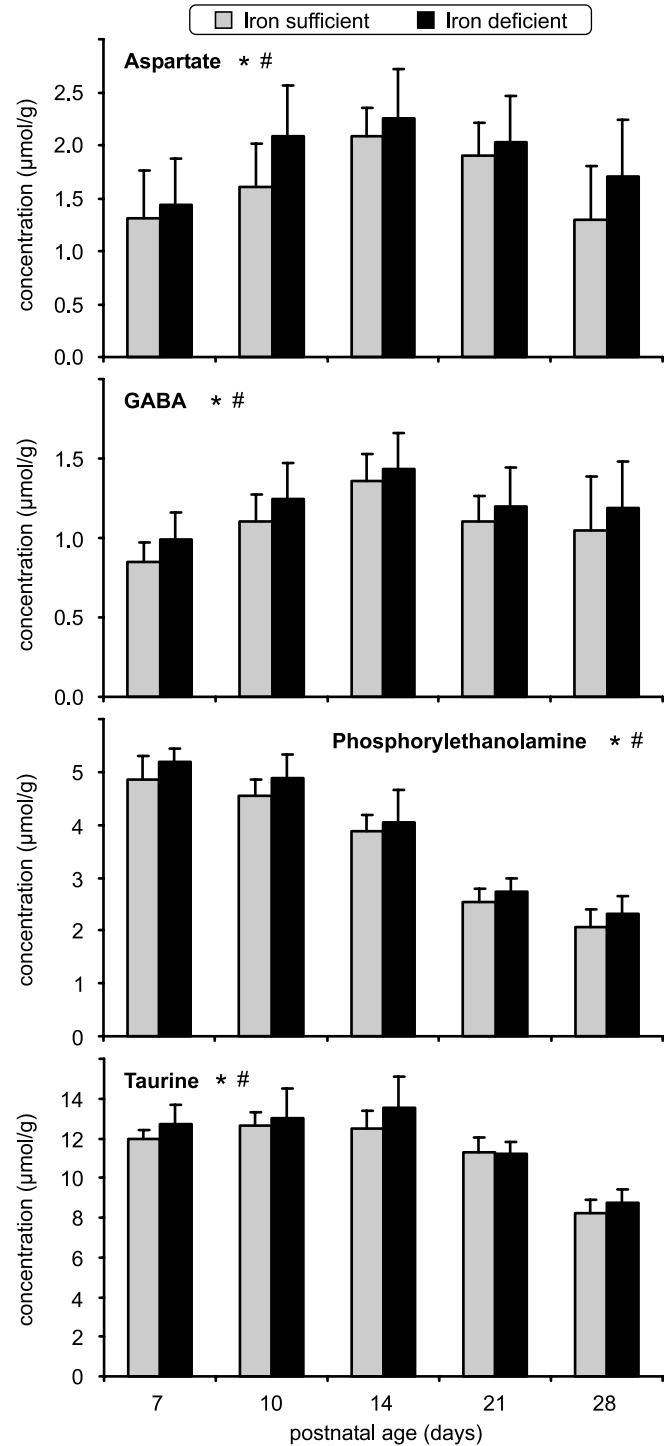

FIGURE 3 Effect of maternal dietary iron treatment during gestation and early postnatal period on aspartate, γ-aminobutyric acid (GABA), phosphorylethanolamine and taurine concentrations between postnatal days 7 and 28 in iron-deficient and iron-sufficient Sprague-Dawley rats. The iron-sufficient data were published previously (21). Values are means ± SD, n = 8–12 (iron-sufficient) and 10–14 (iron-deficient) at each postnatal age. *Different over time for each group, P < 0.001; #different between groups over time, P < 0.02 for taurine, and P < 0.005 for the others.

The deleterious effects of iron deficiency have been postulated to be mediated through multiple biochemical pathways in which iron and iron-containing enzymes play important
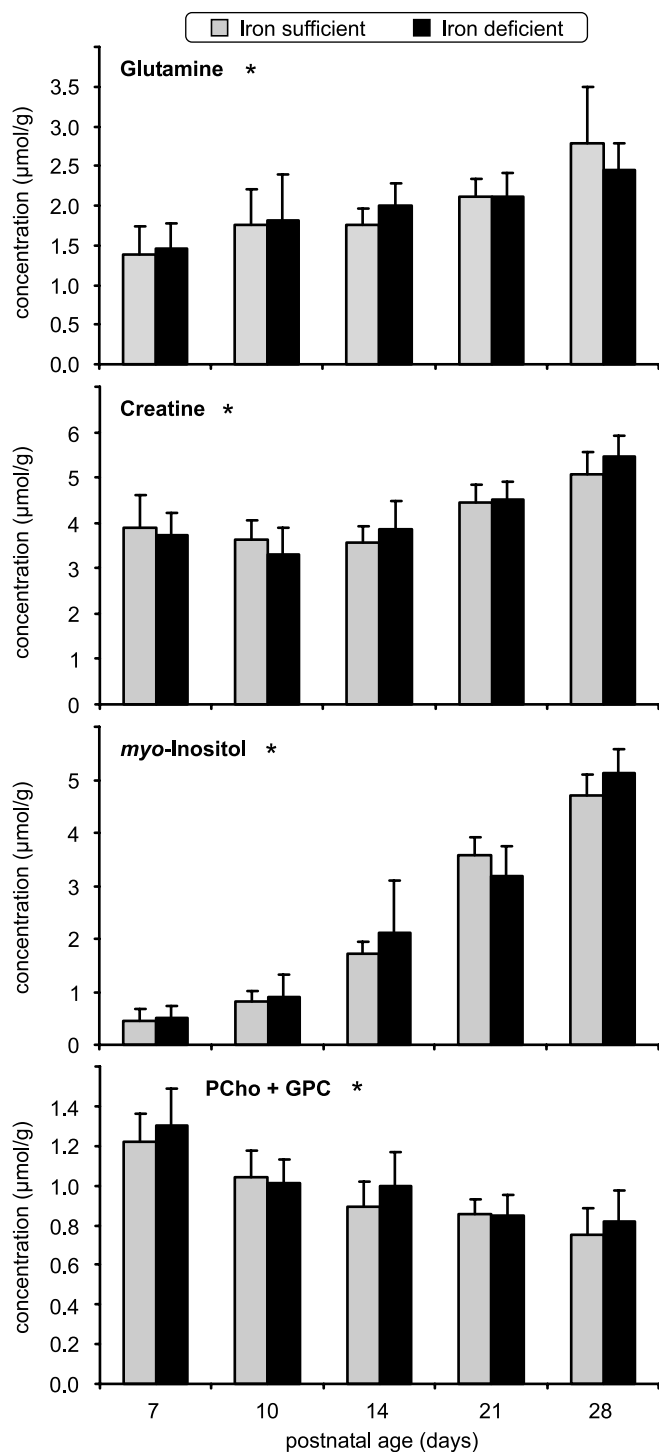
FIGURE 4 Effect of maternal dietary iron treatment during gestation and early postnatal period on glutamine, creatine, myo-inositol and combined phosphorylcholine and glycerophosphorylcholine (PCho+GPC) concentrations between postnatal days 7 and 28 in iron-deficient and iron-sufficient Sprague-Dawley rats. The iron-sufficient data were published previously (21). Values are means $\pm$ SD, $n = $ 8–12 (iron-sufficient) and 10–14 (iron-deficient) at each postnatal age. *Different over time for each group, $P < 0.001$.

Elevations in the concentrations of multiple metabolites in the iron-deficient group could suggest changes in tissue architecture, slightly different quantification conditions or temporal shifts in hippocampal development in perinatal iron defi-

ciency. However, such scalar reasons for the concentration elevations in the present study can be ruled out because the magnitudes of elevation were not uniform across all metabolites and the concentrations of some metabolites with strong postnatal developmental changes, such as myo-inositol, did not differ between the groups. This implies that although the overall progression of hippocampal development may not be altered by perinatal iron deficiency in a major way, substantial deviations occur in its neurochemical profile during this process.

Perinatal iron deficiency resulted in elevated PCr and PCr/Cr ratio, i.e., a higher phosphorylation potential (Fig. 2). These findings most likely indicate altered energy metabolism. PCr is a readily releasable cytosolic store of high energy phosphate for ATP synthesis with excess ATP being converted to PCr for energy storage (33,34). Because iron deficiency adversely affects oxidative production of ATP (8,9), a decrease in PCr concentration would have been expected in perinatal iron deficiency. However, unlike acute injuries, chronic adverse conditions result in complete recovery of PCr concentrations, even when ATP concentrations are restored only partially (35,36). The elevated PCr/Cr ratio in the setting of chronic iron deficiency may therefore reflect a compensatory mechanism to sustain ATP production during periods of increased energy demand. However, this protective effect is likely to be transient because the perinatally iron-deficient hippocampus is highly vulnerable to superimposed acute hypoxic-ischemic injury (19). Finally, because the measurement of PCr by NMR spectroscopy is not neuron specific, the elevated PCr levels may also reflect altered oligodendrocyte energy function in perinatal iron deficiency. In demyelinating disorders, PCr levels are elevated (37).

Altered neuronal energy metabolism may also be responsible for the elevated glutamate concentrations in the iron-deficient group (Fig. 2). The majority of glutamate measured by ^{1}H NMR spectroscopy was localized to the neuronal compartment (38). Thus, the elevated glutamate concentrations suggest either increased synthesis or relatively decreased release of glutamate from the neurons. Glutamate plays an important role in neurodevelopment through its stimulatory effect on N-methyl-D-aspartate (NMDA) receptors (39–41). Glutamate-glutamine cycling between neuron and glia has been accepted as the major mechanism for maintaining glutamatergic neurotransmission (42–44). Glutamatergic neurotransmission is a highly energy-dependent process that accounts for >80% of the total energy expenditure in the brain (45). We postulate that glutamatergic neurotransmission is decreased in perinatal iron deficiency as a result of inefficient energy metabolism and is responsible for the elevated intracellular glutamate levels. The elevated glutamate/glutamine ratio in the present study, the reduced expression of NR2b subunit of the NMDA receptors in the perinatally iron-deficient hippocampus (28) and the reduced glutamate binding to synaptic membranes in iron deficiency states (46) support this postulation. Our working hypothesis is that decreased glutamatergic neurotransmission during development is responsible for the abnormal dendritic arborization recently demonstrated in the hippocampus in perinatal iron deficiency (28).

Aspartate and GABA levels were also elevated in the iron-deficient rats (Fig. 3). Like glutamate, aspartate and GABA were postulated to stimulate dendritic arborization and synaptic plasticity in the developing brain (39,41,47,48). However, beyond PD7, the GABAergic neurotransmission switches from excitatory to inhibitory in the hippocampus (47). Thus, the increased GABA levels may suggest an increased inhibitory drive for reducing the overall neurotrans-

mission rates and brain activity in an energy-limited environment. Both decreased glutamatergic stimulation of NMDA receptors and increased GABAergic inhibitory neurotransmission are associated with impairments in hippocampally based memory tasks in rats (49). We speculate that such a mechanism may be responsible for the abnormalities in hippocampus-specific cognitive tasks in rats (16) and the electrophysiologic tests of recognition memory in human infants with perinatal iron deficiency (4,5).

The mean NAA concentration was higher in the iron-deficient rats (Fig. 2). NAA, synthesized and stored primarily in the neurons, plays a major role in myelin synthesis in the developing brain (50–53). Deacetylation of NAA for myelin synthesis requires optimal functioning of the oligodendrocytes (54). Oligodendrocytes have one of the highest metabolic rates and require considerable amounts of iron for myelin synthesis and oxidative phosphorylation (11). Although the neuronal number and distribution are not affected by gestational iron deficiency (55), the production, and presumably function of oligodendrocytes is decreased (56). Early iron deficiency results in reduced total myelin and phospholipid content of the brain (57,58). Thus, in the present study, increased NAA levels may represent decreased utilization of the compound for myelination. A similar mechanism may also be responsible for the elevated phosphorylethanolamine levels in the iron-deficient group in the present study (Fig. 3). As a precursor of phosphatidylethanolamine, a major phospholipid in the brain (59), phosphorylethanolamine levels decrease with the onset and progression of myelination in humans and rats (37,60). Hypomyelination is postulated to be responsible for the persistent abnormalities in the auditory and visual evoked potentials demonstrated in human infants with early iron deficiency (61,62).

In summary, the present study suggests that perinatal iron deficiency significantly altered the neurochemical profile of the developing hippocampus. The ability to sequentially track neurochemical changes reliably as they unfolded attests to the sensitivity of high field ^{1}H NMR spectroscopy for evaluating regional brain development under typical and adverse conditions. Because it is noninvasive, the method may be extended for similar studies in human infants. Changes in various metabolites suggest that multiple iron-dependent biochemical pathways may be involved in perinatal iron deficiency and compound its adverse effects on neurodevelopment. Their reversibility with iron rehabilitation, as well as their effects on neuronal function and behavior, has yet to be studied. Nevertheless, the findings of the present study may provide plausible biochemical explanations for the electrophysiologic and neurodevelopmental abnormalities observed in human infants with perinatal brain iron deficiency.

ACKNOWLEDGMENT

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