Quantitative Proteomic Analyses of Cerebrospinal Fluid Using iTRAQ in a Primate Model of Iron Deficiency Anemia

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Key Words

Prostaglandin-H2 D-isomerase • Fibronectin • α -1-Antitrypsin • Toll-like receptor 8 • Amyloid β A4 protein • Cerebrospinal fluid • Anemia • Monkey

Abstract

Iron deficiency affects nearly 2 billion people worldwide, with pregnant women and young children being most severely impacted. Sustained anemia during the first year of life can cause cognitive, attention and motor deficits, which may persist despite iron supplementation. We conducted iTRAQ analyses on cerebrospinal fluid (CSF) from infant monkeys (*Macaca mulatta*) to identify differential protein expression associated with early iron deficiency. CSF was collected from 5 iron-sufficient and 8 iron-deficient anemic monkeys at weaning age (6–7 months) and again at 12–14 months. Despite consumption of iron-fortified food after weaning, which restored hematological indices into the normal range, expression of 5 proteins in the CSF remained altered. Most of the proteins identified are involved in neurite outgrowth, migration or synapse formation. The results reveal novel ways in which iron deficiency undermines brain growth and results in aberrant neuronal migration and connections. Taken together with gene expression data from rodent models

of iron deficiency, we conclude that significant alterations in neuroconnectivity occur in the iron-deficient brain, which may persist even after resolution of the hematological anemia. The compromised brain infrastructure could account for observations of behavioral deficits in children during and after the period of anemia.

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Introduction

Iron deficiency affects nearly 2 billion people worldwide [1], with pregnant women and young children most severely impacted due to the increased iron demands associated with these periods. In the USA, infants from low socioeconomic status, especially African-American infants, are particularly at risk for developing iron deficiency anemia (IDA) [2–4].

It has been shown that sustained anemia during the first year of life can cause cognitive, attention and motor deficits, which may persist even after receiving iron supplementation [5–7]. These children were also found to evince differences in the socioemotional domain, including being more fearful, wary, unhappy and fatigued [8]. The findings indicate that IDA not only has immedi-

ate metabolic effects but appears to undermine the normal development of the brain and can disrupt the regulatory set points for several important neurotransmitters [9].

Iron deficiency during this period of rapid neural development has multiple effects on the maturing biochemistry, bioenergetics and morphology of the central nervous system (CNS). Rodent studies have demonstrated that iron deficiency during the nursing period results in decreased dendritic arborization, in association with fewer and less complex interneuronal connections (i.e. decreased synaptogenesis) [10–12]. In addition, the locations and activity of oligodendrocytes in iron-deficient rat pups are altered, resulting in an abnormal composition of white matter and less myelination [13, 14]. Iron is also critical for the biosynthesis and catabolism of the monoaminergic neurotransmitters, impacting both dopamine and norepinephrine. Impairments of motor control, dysregulation of the sleep-wake cycle and poorer learning and memory functions have all been linked to these changes in monoamine levels [7]. Finally, in addition to the traditional findings of disturbed oxidative metabolism and glucose homeostasis [15], recent studies using more sophisticated approaches, such as magnetic resonance spectroscopy, have identified numerous metabolites that are altered in important brain regions, including the striatum and hippocampus [11, 16–18].

There is a continuing need for better biomarkers to enable more systematic assessment of brain iron status during development and to evaluate intervention strategies that attempt to replenish brain iron and remedy the associated neurological deficits. Although serum would be the most convenient source for routine, noninvasive testing, cerebrospinal fluid (CSF) is more likely to provide an accurate reflection of depleted iron stores in the brain [19–21]. Several recent studies have documented that there is a selective prioritization and partitioning of iron across different tissues in the anemic state, and thus hematological indices do not sensitively reflect the full extent of the CNS depletion [19]. One other advantage of CSF as the biological medium for the proteomic approach used in the current research is that CSF is sequestered behind both the blood-brain and brain-CSF barriers. This isolation permits the identification of proteins derived from the parenchyma and specific to iron deficiency within the CNS. Moreover, the CSF used in this project was acquired via taps at the cervical level rather than from the lumbar region, thereby ensuring that the specimen was obtained primarily from the brain. This location also permitted the analyses to be unaffected by the

rostral-to-caudal diffusion gradient in the intrathecal compartment that has been detected for a number of compounds with lumbar taps [22].

In the present study, a well-characterized nonhuman primate model of infancy anemia was employed to generate the iron-sufficient (IS) and iron-deficient subjects [23]. CSF was collected at this time and then again following the normalization of iron status at 1 year of age, due to consumption of an iron-fortified diet. An iTRAQ analysis of the CSF at both time points was utilized to test the hypothesis that the protein profile would be abnormal in the iron-deficient infants and that certain proteins would then remain altered, persisting for several months after the normalization of hematological parameters. Moreover, the analyses provided the opportunity to identify novel proteins that might account for the lingering neural and behavioral deficits that persist after repletion.

Materials and Methods

Animals

The subjects were 13 infant rhesus monkeys (*Macaca mulatta*) born and reared under standardized conditions at the Harlow Primate Laboratory. They were from full-term, singleton pregnancies with normal vaginal deliveries. The focus was on potential biomarkers in the CSF from these offspring (5 IS and 8 IDA), which were determined at weaning age (6–7 months) and then at 12–14 months of age, after they had been consuming solid food independent from the mother.

Diet

This primate model of infant anemia is generated by maintaining adult female monkeys on a controlled diet with moderate levels of iron fortification (225 mg Fe/kg) during pregnancy and lactation (Purina 5LFD). Approximately 20–40% of infant monkeys will typically become anemic when the dams consume this diet. The prevalence is comparable to that found by other laboratories previously with diets containing similar amounts of iron [24, 25]. Ferrous sulfate was used to fortify the diet up to the desired level, and the concentration of other minerals and vitamins was also controlled. Once weaned by 7 months of age, the juvenile monkeys were fed the same diet. A small amount of supplemental fruit was provided in the afternoon as part of the environmental enrichment program, in keeping with federal regulations.

Hematology and Iron Panel

Small blood samples (<4 ml) were obtained via femoral venipuncture to determine the infants' hematological and iron status. Results are presented for two red blood cell parameters with known clinical cutoffs for IDA in monkeys, i.e. mean corpuscular volume (<60 fl for IDA) and hemoglobin (<11 g/dl for IDA), as well as two other indices that were confirmatory of the categorization of an infant as either IS or IDA, i.e. the hematocrit and red blood cell distribution width.

CSF Collection

CSF was collected when the monkeys were 6–7 and 12–14 months of age. Following rapid sedation with ketamine hydrochloride (15 mg/kg body weight), approximately 1.0 ml of CSF was obtained via cervical puncture with a 25-gauge needle and syringe and placed immediately on ice. CSF specimens were spun at low speed in a refrigerated centrifuge to remove any cellular contamination, aliquoted and stored at -60°C . All samples were collected between 09.30 and 11.00 h.

Experimental Design

Experiment 1. CSF from 8 IDA infant monkeys was labeled as follows: pool 1 (equivalent protein amounts from 6- to 7-month-old monkeys ID1, ID2 and ID3), iTRAQ reagent 113; pool 2 (equivalent protein amounts from 6- to 7-month-old ID4, ID5 and ID6), iTRAQ reagent 114; 6-month-old ID7, iTRAQ reagent 115; 6-month-old ID8, iTRAQ reagent 116; pool 1 (equivalent protein amounts from 12-month-old ID1, ID2 and ID3), iTRAQ reagent 117; pool 2 (equivalent protein amounts from 12-month-old ID4, ID5 and ID6), iTRAQ reagent 118; 12-month-old ID7, iTRAQ reagent 119, and 12-month-old CS1 (an IS common sample used in both sets of experiments for comparison), iTRAQ reagent 121.

Experiment 2. CSF from 5 IS infant monkeys was labeled as follows: pool 1 (equivalent protein amounts from 6- to 7-month-old IS1 and IS2), iTRAQ reagent 113; pool 2 (equivalent protein amounts from 6- to 7-month-old IS3 and IS4), iTRAQ reagent 114; 6-month-old IS5, iTRAQ reagent 115; pool 1 (equivalent protein amounts from 12-month-old IS1 and IS2), iTRAQ reagent 116; pool 2 (equivalent protein amounts from 12-month-old IS3 and IS4), iTRAQ reagent 117; 12-month-old IS5, iTRAQ reagent 118, and 12-month-old CS1 (an IS common sample used in both sets of experiments for comparison), iTRAQ reagent 121.

Protein Digestion and Labeling with iTRAQ Reagent

Ten micrograms of each sample were dried under vacuum prior to trypsin digestion. The general protocol for trypsin digestion and iTRAQ labeling of the peptides was similar to that recommended by Applied Biosystems Inc. (ABI, Foster City, Calif., USA) [26]. All reagents were purchased from ABI unless otherwise noted. Briefly, 20 μl of dissolution buffer (0.5 M triethylammoniumbicarbonate, Sigma, St. Louis, Mo., USA), pH 8.5, 1 μl of the denaturant (2% sodium dodecyl sulfate) and 1 μl of reducing reagent [tris-(2-carboxyethyl)phosphine] were added to each sample and incubated at 60°C for 1 h. After the samples had cooled to room temperature, 1 μl of a freshly prepared 84 mM solution of iodoacetamide was added, and the samples were incubated in the dark at room temperature for 30 min. Sequencing grade trypsin (Promega, Madison, Wisc., USA) was reconstituted with 21 μl of resuspension buffer (50 mM acetic acid), and 10 μl were added to each sample. The samples were incubated at 48°C overnight. Each vial of iTRAQ reagent was allowed to equilibrate to room temperature and was reconstituted with 50 μl of isopropanol. The contents of one iTRAQ reagent vial were added to one sample and incubated for 2 h. After the incubation, 100 μl of Milli-Q water were added to each sample to quench the iTRAQ reaction, and the samples were incubated at room temperature for 30 min. All iTRAQ reagent-labeled samples were combined and dried.

Strong Cation Exchange Chromatographic Fractionation

The pooled iTRAQ-labeled peptide sample was desalted and resuspended in 500 μl of cation exchange buffer (10 mM potassium phosphate in 25% acetonitrile at pH 2.5–3.0) prior to strong cation exchange (SCX) chromatographic fractionation and liquid chromatography (LC)-dual mass spectrometry (MS/MS) analysis using a Sep Pak[®] C18 Pak peptide concentration and desalting trap (Michrom Bioresources, Auburn, Calif., USA). SCX separations were performed on a passivated Waters 600E HPLC system, using a 4.6×250 mm PolySULFOETHYL aspartamide column (PolyLC, Columbia, Md., USA) at a flow rate of 1 ml/min. Buffer A contained 10 mM ammonium formate, pH 2.7, in 20% acetonitrile/80% water. Buffer B contained 666 mM ammonium formate, pH 2.7, in 20% acetonitrile/80% water.

The gradient was buffer A at 100% (0–22 min following sample injection), 0 $\rightarrow$ 40% buffer B (16–48 min), 40 $\rightarrow$ 100% buffer B (48–49 min), then isocratic 100% buffer B (49–56 min), which was switched back to 100% buffer A at 56 min to re-equilibrate for the next injection. The first 26 ml of eluent (containing all flow-through fractions) was combined into 1 fraction, and then 14 additional 2-ml fractions were collected. All 15 of these SCX fractions were dried down completely to reduce volume and to remove the volatile ammonium formate salts, then resuspended in 9 μl of 2% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid and filtered prior to reverse phase C18 nanoflow-LC separation. For 2nd-dimension separation by reverse phase nanoflow LC, each SCX fraction was autoinjected onto a Chromolith Cap-Rod column (150 $\times$ 0.1 mm, Merck) using a 5- μl injector loop on a Tempo LC Matrix-Assisted Laser Desorption/Ionization (MALDI) Spotting system (ABI-MDS/Sciex). Buffer C was 2% acetonitrile and 0.1% trifluoroacetic acid, and buffer D was 98% acetonitrile and 0.1% trifluoroacetic acid. The elution gradient was 95% buffer C/5% buffer D (2 $\mu\text{l}/\text{min}$ flow rate from 0 to 3 min, then 2.5 $\mu\text{l}/\text{min}$ from 3 to 8.1 min), 5 $\rightarrow$ 38% buffer D (8.1–40 min), 38 $\rightarrow$ 80% buffer D (41–44 min) and 80 $\rightarrow$ 5% buffer D (44–49 min; initial conditions). The flow rate was 2.5 $\mu\text{l}/\text{min}$ during the gradient, and an equal flow of MALDI matrix solution was added post-column (7 mg/ml recrystallized α -cyano-hydroxycinnamic acid, 2 mg/ml ammonium phosphate, 0.1% trifluoroacetic acid, 80% acetonitrile). The combined eluent was automatically spotted onto a stainless steel MALDI target plate every 6 s (0.6 μl per spot), for a total of 370 spots per original SCX fraction.

Mass Spectrometric Analysis

After sample spot drying above, 13 calibrant spots (ABI 4700 Mix) were added manually to each plate. MALDI target plates (15 per experiment) were analyzed in a data-dependent manner on an ABI 5800 and 800 MALDI time of flight (TOF)-TOFs. As each plate was entered into the instrument, a plate calibration/MS default calibration update was performed, and then the MS/MS default calibration was updated. MS spectra were then acquired from each sample spot using the updated default calibration, with 500 laser shots per spot and a laser intensity of 3,200. A plate-wide interpretation was then automatically performed, choosing the highest peak of each observed mass-to-charge (m/z) value for subsequent MS/MS analysis. Up to 2,500 laser shots at a laser power of 4,200 were accumulated for each MS/MS spectrum.

Table 1. Proteins identified in experiment 1 at critical false discovery rates

Critical FDR	Number of proteins detected		
	local FDR	global FDR	global FDR from fit
1.0%	50	33	58
5.0%	55	67	65
10.0%	58	70	70

This table demonstrates the stringency of the 5% local false discovery rate (FDR) as compared to the 1% global FDR in experiment 1.

Data Analysis

The software used for data acquisition was ABSciex TOF/TOF Series Explorer™. The software used for protein identification and quantitation was Protein Pilot™ 4.0 software (Software Revision Number 148085, Applied Biosystems) with the integrated Paragon™ search algorithm 4.0.0.0 (Revision Number 148083, Applied Biosystems). The iTRAQ fragment tolerance was set to 0.2 Da. The data analysis parameters were set as follows: sample type, iTRAQ (peptide labeled); Cys alkylation, iodoacetamide; digestion, trypsin; instrument, 5800; species, *M. mulatta*; ID focus: biological modifications and amino acid substitutions; database search, NCBI nr rhesus monkey of 20101117 sequences, plus 156 common lab contaminants; effort, thorough; maximum missed cleavages, 2; false discovery rate analysis, yes; user-modified parameter files, no; bias correction, auto, and background correction, yes. Identified proteins were grouped by the software to minimize redundancy. All peptides used for the calculation of protein ratios were unique to the given protein or proteins within the group, and peptides that were common to other isoforms or proteins of the same family were ignored. The protein confidence threshold cutoff is 1.3 (unused ProtScore) with at least one peptide with 95% confidence. Average iTRAQ ratios were obtained from at least 3 experiments using like samples (either IDA or IS and at either 6 or 12 months). In addition, the only protein IDs accepted had a 'local false discovery rate' estimation of no higher than 5%, as calculated from the slope of the accumulated decoy database hits by the Proteomics System Performance Evaluation Pipeline [27]. We choose to utilize the 5% local false discovery rate versus the 1% global false discovery rate because that value was shown to provide more stringent results (tables 1, 2).

Statistical Analysis

Hematological differences between IS and IDA infants were confirmed with analyses of variance, considering iron status as a between factor and infant age as a repeated measure. Protein profiles were compared between IS and IDA infants utilizing the Mann-Whitney U test with α set at $p < 0.05$.

Table 2. Proteins identified in experiment 2 at critical false discovery rates

Critical FDR	Number of proteins detected		
	local FDR	global FDR	global FDR from fit
1.0%	86	86	88
5.0%	87	91	92
10.0%	87	102	99

This table demonstrates the stringency of the 5% local false discovery rate (FDR) as compared to the 1% global FDR in experiment 2.

Results

Hematology

Based on the hematology tests, 8 infant monkeys were classified as anemic (IDA) at weaning age and 5 were IS when the first CSF specimens were collected (table 3). By 1 year of age, the hematological parameters of the formerly IDA infants had returned to the normal range and were no longer significantly different from the yearlings that had been IS across the whole first year of life (fig. 1).

Effect of Age on the CSF Protein Profile of the IDA Monkeys (Experiment 1)

A database search of MS/MS spectra identified 46 proteins at the 95% confidence level, and the complete list is provided in appendix A in the online supplementary materials (for all online supplementary material, see www.karger.com/doi/10.1159/000341919). The total protein scores are presented in order from highest to lowest. Among the proteins detected, 21 proteins were identified with one unique peptide with a minimum peptide confidence of 95%. The false positive peptide rate calculated from the reversed database search results was between $0.01 > p > 0.001$.

For protein quantification, peak areas for iTRAQ tag peaks (113–121 m/z) were determined, and after normalization of sample quantities using median ratios (Paragon algorithm search, ProteinPilot 3.0 software), differential expression between the CSF samples of IDA infants at weaning age (113, 114, 115 and 116 m/z) and an internal standard (121 m/z) or subsequently at 1 year of age (117, 118 and 119 m/z) and an internal standard (121 m/z) were determined. Differentially expressed proteins in the CSF between the two age points were identified using non-parametric statistical tests, and 2 proteins were identified

Table 3. Mean $\pm$ SE and individual hematological values for IDA and IS infant monkeys from which CSF was collected for the iTRAQ analyses

		Sex	Weaning age				One year of age			
			MCV, fl	Hgb, g/dl	Hct, %	RDW, %	MCV, fl	Hgb, g/dl	Hct, %	RDW, %
IDA monkeys										
Mean ± SE			54.8 ± 0.5 ^a	10.5 ± 1.7 ^b	32.7 ± 1.2 ^b	29.5 ± 2.3 ^a	69.4 ± 1.1	12.6 ± 0.3	38.8 ± 0.9	13.9 ± 0.3
ID1	M		55.2	11.8	35.8	25.7	64.5	12.0	35.8	13.7
ID2	M		55.7	11.4	35.1	33.8	71.3	13.1	39.8	13.5
ID3	M		61.0	10.6	32.7	20.3	68.4	12.6	37.8	14.2
ID4	M		54.2	9.8	30.0	34.7	70.6	12.5	36.7	13.2
ID5	M		50.4	9.5	31.0	26.8	69.2	12.6	38.2	15.1
ID6	M		45.5	9.2	28.5	40.5	67.2	13.5	40.2	13.5
ID7	M		59.1	12.7	38.4	26.0	69.0	12.8	38.5	13.1
ID8	F		56.9	9.3	30.8	28.4	75.0	14.5	43.6	15.2
IS monkeys										
Mean ± SE			68.6 ± 1.9	12.9 ± 0.2	37.7 ± 0.5	13.3 ± 0.2	69.2 ± 2.1	12.5 ± 0.2	37.6 ± 0.5	13.4 ± 0.4
IS1	M		64.9	12.5	38.3	14.0	64.8	12.6	37.3	12.9
IS2	M		65.5	13.0	38.8	12.7	66.5	12.6	38.5	12.2
IS3	M		66.6	12.2	36.4	13.3	66.6	12.2	35.9	14.3
IS4	M		74.5	12.9	38.6	13.0	75.5	12.9	39.0	13.1
IS5	M		71.3	12.1	36.5	13.4	72.7	12.1	37.2	14.3

MCV = Mean corpuscular volume; Hgb = hemoglobin; Hct = hematocrit; RDW = red blood cell distribution width.

^a Significantly lower in anemic infants at 6–7 months of age, $p < 0.001$; ^b significantly lower in anemic infants at 6–7 months of age, $p < 0.02$.

Fig. 1. Hematological parameters in infant monkeys. This figure summarizes the iron-related hematological parameters of the rhesus monkeys assessed in this study. The mean corpuscular volume (MCV) and hemoglobin (Hgb) values clearly demonstrate the anemic status of some infants at weaning age, which then resolved by 1 year of age. Mean $\pm$ SE values for the individual data presented in table 1 are shown.

that met significance ($p \leq 0.05$). As shown in table 4, both proteins (apolipoprotein E and proactivator polypeptide-like isoform) identified using iTRAQ were higher in the CSF samples from anemic infants at the weaning age.

Effect of Age on the CSF Protein Profile in the IS Monkeys (Experiment 2)

A database search of MS/MS spectra identified 74 proteins at the 95% confidence level, and the ordered list of total protein scores from high to low is provided in

Table 4. Effect of age on protein expression in the CSF of IDA and IS infant monkeys

Protein	Mean fold change from 6 to 12 months	p value
<i>IDA monkeys</i>		
Apolipoprotein E	2.35	0.029
Proactivator polypeptide-like isoform 7	7.48	0.029
<i>IS monkeys</i>		
Transferrin	0.73	0.050
Clusterin	0.62	0.050
Amyloid β A4 precursor-like protein 1 isoform 1	0.88	0.050
Pro-SAAS-like protein	0.52	0.050

SAAS = Proprotein convertase subtilisin/kexin type 1 inhibitor.

appendix B (online suppl. materials). Among the proteins detected, 36 proteins were identified with one unique peptide with a minimum peptide confidence of 95%. The false positive peptide rate calculated from the reversed database search results was between $0.01 > p > 0.001$.

For protein quantification, peak areas for iTRAQ tag peaks (113–118 and 121 m/z) were determined, and after normalization of sample quantities using median ratios (Paragon algorithm search, ProteinPilot 3.0 software), differential expression between CSF from the IS infants at 6 months (113, 114 and 115 m/z) and an internal standard (121 m/z) or between the CSF from the IS yearlings (116, 117 and 118 m/z) and an internal standard (121 m/z) were determined. Differentially expressed proteins between 6–7 months and 1 year of age were identified, and 4 proteins met statistical significance ($p \leq 0.05$). As shown in table 4, all of the identified proteins (transferrin, clusterin, amyloid β A4 precursor-like protein 1 and pro-SAAS-like protein) were lower at weaning age than at 1 year of age.

Effect of Iron Status on CSF Protein Profiles at Weaning Age

In order to examine the effect of iron status on the CSF profile at 6–7 months of age, the outputs from the two iTRAQ experiments were reduced to a table of reporter ion intensities relative to the intensity of the internal standard (121 m/z) for each peptide across the multiplexed samples constituting each run. We identified 11 proteins

Table 5. Effect of iron status on protein expression in the CSF of infant monkeys at weaning age

Protein	Mean fold difference between IDA and IS	p value
Prostaglandin-H2 D-isomerase	0.25	0.029
AAT isoform 4	0.33	0.037
Mamu IgG-rh1 heavy chain	0.53	0.029
Hemopexin-like	0.85	0.029
Gelsolin isoform 18	0.57	0.037
TLR8	0.45	0.029
Amyloid β A4 protein-like	1.80	0.029
Complement factor H-like isoform 3	1.17	0.029
Chromobox protein homolog 6 isoform 1	1.78	0.029
Hypothetical protein LOC 706413	1.38	0.029
Fibronectin	4.14	0.029

that were common to both IS and IDA samples that met statistical criteria for significance ($p \leq 0.05$). As shown in table 5, 6 proteins were lower in the CSF of the IDA infants [prostaglandin-H2 D-isomerase, α -1-antitrypsin (AAT) isoform 4, Mamu IgG-rh1 heavy chain, hemopexin, gelsolin and toll-like receptor 8 (TLR8)]. Five proteins (amyloid β A4 protein, complement factor H, chromobox protein homolog 6, hypothetical protein LOC 706413 and fibronectin) were found to be at higher concentrations in the CSF of the IDA infants when compared to CSF samples of the IS infants.

Effect of Iron Status on CSF Protein Profiles in Monkeys at One Year of Age

Similar comparisons were conducted on the outputs from iTRAQ experiments run on CSF from yearling monkeys. The data were reduced to a table of reporter ion intensities relative to the intensity of the internal standard (121 m/z) for each peptide across the multiplexed samples constituting each run. We identified 12 proteins common to the CSF from IS and IDA monkeys that met statistical criteria for significance ($p \leq 0.05$). As shown in table 6, 10 proteins were lower in the CSF of the yearling monkeys that had previously been iron-deficient (apolipoprotein E, prostaglandin-H2 D-isomerase, AAT isoform 4, apolipoprotein A-I, clusterin, TLR8, fibulin-1, protein kinase C-binding protein NELL2, serpin peptidase inhibitor, clade A and proactivator polypeptide-like isoform 7). At the same time, when compared to the iTRAQ data for monkeys that had been continuously IS,

Table 6. Effect of prior iron status at weaning age on the protein expression in the CSF of yearling monkeys

Protein	Mean fold difference between IDA and IS	p value
Apolipoprotein E	0.41	0.050
Prostaglandin-H2 D-isomerase	0.34	0.050
AAT isoform 4	0.42	0.050
Apolipoprotein A-I isoform 3	0.38	0.050
Clusterin	0.71	0.050
TLR8	0.54	0.050
Fibulin-1 isoform 2	0.49	0.050
Amyloid β A4 protein-like, partial	1.60	0.038
Protein kinase C-binding protein NELL2 isoform 2	0.66	0.050
Serpin peptidase inhibitor, clade A, member 3	0.66	0.038
Proactivator polypeptide-like isoform 7	0.15	0.050
Fibronectin	2.74	0.050

there were 2 proteins that were elevated in the CSF of the former IDA monkeys (amyloid β A4 protein and fibronectin).

Discussion

Our study shows that CSF can be utilized to demonstrate the significant effect that iron status, as well as age, has on critical biochemical pathways in the CNS. The iTRAQ methodology was chosen because it not only identifies proteins of interest but also permits accurate quantification, allowing for key comparisons in a systematic manner. A previous study of IDA in infant monkeys using two-dimensional analyses [19] revealed that a distinct CSF protein profile persisted for over 4 months after the period of anemia and was still evident in infants that received 2 months of ferrous sulfate treatment. The current results confirm and extend those findings. Because of the sensitivity of the iTRAQ techniques, we have now identified 5 additional proteins that remain persistently altered in the CSF. These proteins are prostaglandin-H2 D-isomerase, AAT, TLR8, amyloid β A4 precursor protein and fibronectin. Prostaglandin-H2 D-isomerase, AAT and TLR8 were decreased in the CSF both during and subsequent to the anemic period, whereas amyloid β A4 precursor protein and fibronectin remained increased through 1 year of age, even though the monkeys were

consuming fortified solid foods and now evinced normal hematological values (fig. 2). A number of these proteins are likely to be associated with the behavioral deficits that have been observed in iron-deficient children [8, 28, 29].

Although we are unaware of other studies in the literature that have utilized iTRAQ methodology in either infant monkeys or humans, we know that iTRAQ studies using adult human CSF have, in fact, identified many more proteins than our study [30–32]. One reasonable explanation for the more limited number of identified proteins compared to adult human CSF studies could be the lower total protein concentration in infant monkey CSF. Total protein concentrations in normal human adults range from 300 to 700 ng/ μ l [33]. Protein concentrations recovered from the CSF of infant monkeys were considerably lower (approximately 100–200 ng/ μ l). These differences in protein concentration could reflect adult versus infants or species differences. Our data compare favorably to the only other published results for protein concentrations in monkey CSF [34], where CSF from adult monkeys with HIV-1-associated neurological disorders was examined and a similar protein concentration (146 proteins) was identified from 10 μ g of total protein using MS analyses.

This study is consistent with both rat and mouse models of early iron deficiency that documented persistent neurochemical and behavioral alterations even after the iron deficiency is corrected. In the mouse model, chronic marginal iron intake during early development has been shown to disrupt brain iron levels, motor and cognitive performance, dopamine metabolism and myelin composition [35–37]. Similarly, in the rat model of early iron deficiency, deficits in monoamine metabolism, myelin synthesis and energy metabolism and alterations in the neurochemical profiles have been demonstrated [17, 38–41]. In the rat model of early iron deficiency, the persistence of deficits in the monoamine pathways is dependent on the timing of the iron deficiency [42]. In nonhuman primates, the timing of the iron deficiency insult has also been shown to be critical to the development of certain behaviors. Prenatal iron deficiency led to increased impulsive behavior, whereas postnatal iron deficiency resulted in more passive behavior [43]. Monkeys that experience IDA during development also perform more poorly on a cognition test (reversal learning) even after the systemic iron deficiency has been corrected [44], consistent with the current report of prolonged alterations in the CSF proteome.

There were 5 proteins that were altered in the CSF despite the replenishment of iron. One protein identified by

Fig. 2. Venn diagram of CSF protein alterations in IDA infants. This figure summarizes the significant protein changes identified in the CSF of anemic infant monkeys. The left oval identifies protein differences at weaning age (6–7 months) during the period of anemia; the right oval portrays those proteins altered at 1 year of

age. The overlapping region highlights CSF proteins that were altered at 6–7 months and remained abnormal at 1 year despite correction of the underlying iron deficiency. Arrows reflect the direction of change.

our iTRAQ study that was also altered in our previous study [19] was prostaglandin-H2 D-isomerase, which is also referred to as prostaglandin D2 (PGD2) synthase (PGDS) and functions to catalyze the conversion of prostaglandin H2 to PGD2. PGDS is produced in the leptomeninges and choroid plexus, and the enzymatic product PGD2 is a neuromodulator as well as a trophic factor in the CNS. PGD2 has potent somnogenic effects and has been implicated in the regulation of non-rapid eye movement sleep [45, 46]. Thus, it may be involved in and reflect the sleep disturbances and fatigue frequently experienced by anemic individuals. Sleep disruption also contributes to cognitive impairments, deficits in attention and increased emotional reactivity [47–49]. There is some evidence that attention problems comparable to attention deficit disorder are associated with iron deficiency [29]. Because PGDS levels remained decreased in the iron-challenged infant monkey, despite the correction of iron-related deficits in the blood compartment, it suggests that further research should be conducted on the sleep/wake

cycles and activity patterns in previously anemic children.

A second protein downregulated in the CSF was AAT. AAT is a protease inhibitor that belongs to the serpin superfamily and is a serum trypsin inhibitor. It protects tissues from enzymes produced by inflammatory cells, and its concentration can rise with acute inflammation. Decreased levels of AAT, similar to those found in our study of anemic infant monkeys, have been described in genetic AAT deficiency, which is caused by mutations in the AAT gene [50]. Classic AAT deficiency increases an individual's vulnerability to environmentally induced liver and lung disease [51–54]. However, two types of AAT polymorphisms are associated with a milder form of the disease, an increased age of onset and decreased symptom severity. Individuals with these genotypes have a higher prevalence of mood disorders, specifically anxiety and bipolar disorders [55]. In addition, younger carriers of these AAT polymorphisms often present with attention deficit problems or learning disabilities [56]. In ad-

dition to its role in regulating inflammation, AAT is involved in iron homeostasis by inhibiting the interaction between transferrin and its receptor [57] as well as by binding precursor molecules for hepcidin [58], which could alter a cell's ability to retain iron. Because the CSF levels of AAT remained low despite improvements in the blood indices, it is likely that iron-related dysfunction persists within the CNS. This conclusion is supported by our other research on infant monkeys, which demonstrated that dopaminergic activity is lower and norepinephrine activity is elevated subsequent to the period of anemia [9]. In addition, CSF from anemic monkeys was found to have an enhanced chemoattraction for iron when examined in vitro with an endothelial cell model of the blood-blood barrier, which we believe is due to elevated levels of iron-poor transferrin and decreased levels of hepcidin (in preparation).

Several proteins identified as remaining altered despite improvements in peripheral iron status are involved in neuronal migration, outgrowth and signaling. One particularly important protein is TLR8. Although TLR8 was first described as playing a crucial role in pathogen recognition and innate immunity [59], it is also involved in neurite outgrowth and the regulation of neuronal apoptosis [60]. TLR8 is highly expressed during development and is present in the growth cones of cultured cortical neurons [60]. While TLR8 expression declines normally in the adult brain, it can be upregulated during certain pathologies, suggesting that it has a function in the regulation of axonal dynamics. Our iTRAQ analyses revealed a sustained decrease in TLR8 expression in spite of the resolution of the peripheral anemia. Such a persistent decrease may impact neurite outgrowth and contribute to the abnormal axonal transport that has been described in the rat and mouse models of anemia [61, 62]. MRI evaluations of children with autism, a developmental disorder involving pervasive deficits in social proficiency and communication, have indicated that many have an early period of brain overgrowth followed by abnormally slow growth in some brain regions and premature cessation of growth in others [63]. It is possible that aberrations in the regulation of neuritogenic pathways induced by early iron deficiency recreate a milder version of these abnormalities in similar socioemotional, cognitive and motor domains.

Amyloid precursor protein (APP) was increased in the CSF even after the anemia resolved. APP has been extensively studied because of its role in the biogenesis of A β and the neuropathology of Alzheimer's disease (AD), and more recently it has been associated with fer-

roxidase activity [64] and potentially the main mechanism for iron export from neurons [65]. Our data may suggest that elevated APP in the CSF could reflect increased turnover of APP as a compensatory mechanism for neurons to retain more iron. The increased APP in CSF is also consistent with alterations in neuronal migration as it is involved in neurite outgrowth as well as synaptic structure/function and cell targeting [66]. During neuronal differentiation, higher APP levels have been reported [67, 68], and pronounced increases in APP levels were detected in brain homogenates, growth cones and synaptosomes during times of increased synapse formation [69]. Recently, it was reported that plasma from children with severe autism and aggressivity contains increased levels of total soluble APP [70] and soluble APP α [71]. This finding has led researchers to hypothesize that increased levels of APP in the CNS during development result in accelerated migration of precursor cells in the cortex and suppression of cell adhesion support, and that this dysregulation contributes to the brain overgrowth observed in autism [63, 72]. Given the current data on APP changes in the CSF during iron deficiency and the neurological interest in CSF concentrations of A β as a biomarker in AD, the contribution of brain iron status to the changes in CSF A β may be important. There is considerable evidence for a loss of brain iron homeostasis in AD [73]. Although the amount of iron is increased in the brains of individuals with AD, the bioavailability of this iron to neurons has been questioned given the amount of iron bound up in neuritic plaques [73], and it may also be retained in neurons as a result of loss of ferroxidase activity of APP [64]. Another possible connection between brain iron status and the emergence of AD in the older individual can also be drawn from our finding that apolipoprotein E was altered in the CSF during the period of frank anemia at 6–7 months of age. The combination of an apolipoprotein E4 mutation and a variant form of the HFE gene (which is also involved in iron regulation) will significantly lower the age of onset of AD [74]. The observation that iron deficiency impacts APP expression may add additional iron-related stress to neurons.

A third upregulated protein was fibronectin. Fibronectin is a high-molecular weight glycoprotein found in the extracellular matrix (ECM) which binds to membrane-spanning receptor proteins called integrins [75] and also binds ECM components including collagen, fibrin and heparan sulfate proteoglycans (e.g. syndecans). Its primary functions are to promote adhesion and phagocytosis [76, 77]. In the CNS, the ECM provides directional cues

for neuronal development [78] and is a critical mediator in the development and regeneration of the sensory nervous system [79]. Dynamic changes in ECM composition occur during maturation and help to facilitate neuronal migration in the developing brain. Therefore, the upregulation of fibronectin further suggests that neurite outgrowth in the developing brain is significantly and chronically altered by iron deficiency and provides new evidence for the essential involvement of iron in neuritogenic pathways.

Conclusion

iTRAQ analyses were conducted on the CSF of infant monkeys to identify differential protein expression as a result of early iron deficiency. Two limitations of this current study are the small number of monkeys that were evaluated and the need for further validation of the findings. Nevertheless, the expression of 5 proteins appeared to remain altered at two age points despite the consumption of fortified foods after weaning, a diet that was adequate to restore hematological indices into the normal range, and one protein (PGDS) was also altered in a previous report in a similar model using a different technical approach. These findings thus confirm reports of a preferential prioritization of iron to peripheral tissues, including red blood cells, liver and muscle, before the restoration of the low iron reserves in the CNS [80]. The majority of the identified proteins with altered concentration in the CSF are involved in neuritogenic pathways (i.e. neurite outgrowth, migration or synapse formation), and one protein, APP, has a direct role in iron retention by neurons. Based on these changes, it is reasonable to conclude

that iron deficiency will undermine brain growth and result in aberrant neuronal migration and connections. Taken together with gene expression data from rodent models of iron deficiency, we conclude that iron deficiency causes significant alterations in neuroconnectivity in the maturing brain, and some persist even after resolution of the systemic iron deficiency. The compromised brain infrastructure could account for the observations of behavioral deficits in children during and after the period of anemia. Moreover, these findings confirm the long-lasting effects of iron deficiency that have been detected upon histological and neurochemical examination of the brain, which may also establish a predisposition for adult neurodegenerative disorders later in the life span. The data indicate that the prevailing strategies deemed adequate to rescue iron deficiency on the basis of correcting hematologic parameters are likely inadequate for addressing the full impact of iron deficiency on the brain.

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