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Iron Deficient and Manganese Supplemented Diets Alter Metals and Transporters in the Developing Rat Brain

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Manganese (Mn) neurotoxicity in adults can result in psychological and neurological disturbances similar to Parkinson's disease, including extrapyramidal motor system defects and altered behaviors. Iron (Fe) deficiency is one of the most prevalent nutritional disorders in the world, affecting approximately 2 billion people, especially pregnant and lactating women, infants, toddlers, and adolescents. Fe deficiency can enhance brain Mn accumulation even in the absence of excess Mn in the environment or the diet. To assess the neurochemical interactions of dietary Fe deficiency and excess Mn during development, neonatal rats were exposed to either a control diet, a low-Fe diet (ID), or a low-Fe diet supplemented with Mn (IDMn) via maternal milk during the lactation period (postnatal days [PN] 4–21). In PN21 pups, both the ID and IDMn diets produced changes in blood parameters characteristic of Fe deficiency: decreased hemoglobin (Hb) and plasma Fe, increased plasma transferrin (Tf), and total iron binding capacity (TIBC). Treated ID and IDMn dams also had decreased Hb throughout lactation and ID dams had decreased plasma Fe and increased Tf and TIBC on PN21. Both ID and IDMn pups had decreased Fe and increased copper brain levels; in addition, IDMn pups also had increased brain levels of several other essential metals including Mn, chromium, zinc, cobalt, aluminum, molybdenum, and vanadium. Concurrent with altered concentrations of metals in the brain, transport proteins divalent metal transporter-1 and transferrin receptor were increased. No significant changes were determined for the neurotransmitters gamma aminobutyric acid and glutamate. The results of this study confirm that there is homeostatic relationship among several essential metals in the brain and not simply between Fe and Mn.

Key Words: **Iron deficiency; manganese; brain; development; rat; metal transport.**

Iron (Fe) is important for neural functioning, energy metabolism, muscle function, macromolecular biosynthesis, myelination, and cell growth and differentiation (Beard, 2001). One of the most prevalent nutritional concerns worldwide, Fe deficiency affects approximately 2 billion people, predominately infants, children, adolescents, and pregnant and lactating women (Beard, 2001; Felt and Lozoff, 1996). Fe deficiency can impair thermal regulation, immune function, mental function, and physical performance; result in fatigue; and/or cause anemia (Beard, 2001). Fe deficiency can also enhance absorption of divalent metals such as lead, cadmium (Cd), aluminum (Al), and manganese (Mn) (Beard, 2001).

Mn is important in the metabolism of fats, carbohydrates and proteins, immune function, regulation of blood sugars, production of cellular energy, reproduction, bone growth, and oxidation-reduction processes (Aschner, 2000; Gerber *et al.*, 2002). Although rare, Mn deficiency during development can lead to birth defects, seizure disorders, poor bone formation, and impaired fertility; conversely, excess Mn can be toxic (Aschner, 2000; Gerber *et al.*, 2002). Mn neurotoxicity in adults is associated with Parkinson-like symptoms while effects in children are not well characterized. Occupational exposure to toxic levels of Mn in industrial workers results in psychological and neurological disturbances, including extrapyramidal motor system defects (Aschner, 2000; Gerber *et al.*, 2002). A recent case report suggests that the clinical and pathophysiological features of manganism may overlap with idiopathic Parkinson's disease (PD) (Racette *et al.*, 2005).

Infants and children, Fe-deficient individuals, and patients with liver or gastrointestinal (GI) disorders are particularly susceptible to Mn toxicity (Aschner, 2000; Gerber *et al.*, 2002). Neonates accumulate more Mn than adults due to enhanced Mn absorption (70 vs. 3% in adults); an incomplete blood-brain barrier (BBB); and little to no biliary excretion until weaning (Brenneman *et al.*, 2000; Mena, 1974). Further, some intensive care infants and patients and with chronic liver or GI disease receive total parental nutrition (TPN). Patients receiving TPN absorb most administered Mn and cannot efficiently eliminate Mn, resulting in Mn accumulation which increases the risk of Mn toxicity (Agency for Toxic Substances and Disease Registry, 2000; Devenyi *et al.*, 1994; Fell *et al.*, 1996; Ono *et al.*, 1995).

Fe and Mn share physiological valences (+2, +3), ionic radius, transferrin (Tf) binding, and mitochondrial accumulation. Additionally, both Mn and Fe target and accumulate in the basal

The authors certify that all research involving human subjects was done under full compliance with all government policies and the Helsinki Declaration.

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ganglia (Barron *et al.*, 1994; Connor *et al.*, 2001; Malecki *et al.*, 1999b), and Mn can interact with Fe-containing enzymes (Aschner, 2000). Fe and Mn, as well as other essential metals, are regulated within the central nervous system by influx into the brain via Tf and Tf receptors (TfRs), as well as via the divalent metal transporter-1 (DMT-1); as such, there is an inverse relationship between Mn and Fe. Fe deficiency increases Tf and DMT-1, facilitating Mn uptake (Aschner, 2000; Connor *et al.*, 2001; Malecki *et al.*, 1999a). Consequently, Fe deficiency can lead to increased Mn accumulation in the brain and vice versa (Aschner, 2000; Aschner *et al.*, 2002; Connor *et al.*, 2001; Kwik-Uribe *et al.*, 2000; Mena, 1974). Likewise, other essential metals sharing the transporters may be altered by imbalances in Fe and/or Mn. Indeed, Fe deficiency is a known risk factor for metal toxicity, resulting in enhanced absorption and accumulation of divalent metals (Beard, 2001; Chandra and Shukla, 1976; Malecki *et al.*, 1999a; Shukla *et al.*, 1990).

In both experimental rodent models and humans, Fe deficiency during development is associated with abnormal cognition, motor function, neurotransmitter metabolism (dopamine, glutamate, gamma aminobutyric acid [GABA], serotonin) and myelinogenesis, with some persistent effects despite Fe supplementation (Beard *et al.*, 2006; Connor *et al.*, 2001; Felt and Lozoff, 1996; Grantham-McGregor and Ani, 2001; Kwik-Uribe *et al.*, 2000; Pinero *et al.*, 2000). Mice exposed to a Fe-deficient diet throughout gestation, lactation, and post-weaning had decreased grip strength and altered motor activity/learning behaviors. Upon repletion, the mice experienced limited recovery (Kwik-Uribe *et al.*, 1999, 2000). In addition, lasting cognitive impairments (learning disabilities) in children have been associated with an Fe-deficient diet consumed during 6–24 months of life (deUngria *et al.*, 2000). The irreversible effects following Fe deficiency during early development substantiates that development is a critical period during which nonacute disturbances can permanently alter normal brain development and lead to long-term consequences. Further, Mn administration alters behavior, motor function, cognition, and neurotransmitter metabolism (Aschner *et al.*, 2002; Dorman *et al.*, 2000; Kontur and Fechter, 1985). Several studies postulate that Mn-induced neurodegeneration primarily involves GABAergic neurons within the globus pallidus, rather than dopaminergic neurons within the nigra-striatal pathway as in PD (Olanow *et al.*, 1996; Pal *et al.*, 1999; Roth and Garrick, 2003; Verity, 1999).

The goal of this study is to assess the neurochemical interactions of dietary Fe deficiency and excess Mn during development by evaluating blood parameters to monitor the extent of Fe deficiency in dams and pups; metals in the brain to assess the relationship of Fe and Mn with other metals; DMT-1 and TfR expression to elucidate the mechanism of metal transport into the brain; and GABA and glutamate because these neurotransmitters are associated with Fe deficiency as well as Mn neurotoxicity.

MATERIALS AND METHODS

Animal treatment. All animals were treated in accordance with ACUC protocols established by Wake Forest University Health Sciences. For a schematic view of the experimental design, refer to Figure 1. Upon arrival, groups of five to seven gestational day (GD) 7 timed pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN) were fed one of three experimental diets (Bio-Serv, Frenchtown, NJ) that varied only in Mn or Fe content: (1) control (CN; 35 mg Fe/kg diet; 10 mg Mn/kg diet); (2) low Fe (ID; 3 mg Fe/kg diet; 10 mg Mn/kg diet); or (3) low Fe with supplemented Mn (IDMn; 3 mg Fe/kg diet; 100 mg Mn/kg diet) (Bio-Serv, Frenchtown, NJ). Human average daily intakes are approximately 1–10 mg Mn/day, and the recommended Mn intake for laboratory rats is 10 mg Mn/kg diet. On postnatal day 4 (PN4), pups born to control dams were pooled and randomly cross-fostered to dams fed one of the two Fe-deficient diets such that initial mean litter weights were approximately equivalent. Pups were exposed to each of these diets via maternal milk from PN4 to PN21. To assess maternal hemoglobin (Hb) during lactation, blood was collected from dams via tail prick on PN4, PN11, and PN21. On PN21, dams were sacrificed using CO_2. Pups were sacrificed via decapitation. Pup brains

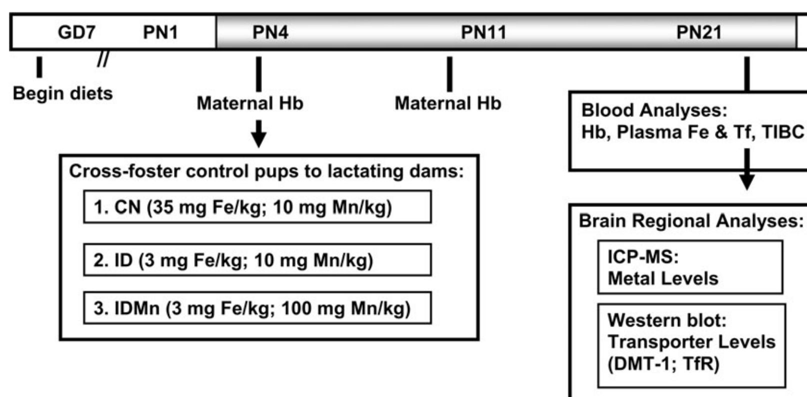

FIG. 1. Experimental design. GD 7 timed pregnant rats were fed one of three diets that varied only in Fe and/or Mn content. Pups were exposed to each of these diets via maternal milk from PN4 to PN21. Maternal Hb was measured on PN4, PN11, and PN21. On PN21, blood was collected from dams and pups and brains were collected from pups. Brain samples were analyzed for several metals, for DMT-1 and TfR expression, and for glutamate and GABA concentrations. See text for experimental details. ICP-MS, inductively coupled plasma mass spectrometry.

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were dissected into five regions (cerebellum, cortex, hippocampus, striatum, and midbrain—including thalamus), wet weights were recorded, and regions immediately were frozen on dry ice, and stored at − 70°C until use. Trunk blood was collected in heparinized test tubes.

Hematological parameters. Blood collected from dams and pups was assayed for measures of Fe-deficiency anemia: Hb, plasma Fe, Tf, and total iron binding capacity (TIBC). Hb was measured colorimetrically by a standard cyanmethemoglobin method (procedure #525, Sigma, St Louis, MO). Whole blood was centrifuged 15 min at 2000 × g to separate cells from plasma. Plasma was frozen at − 20°C until analysis in the Clinical Chemistry Laboratory at Wake Forest University (Dr Zak Shihabi). Plasma Fe was reacted with ferrozine as the color reagent and measured with the AVIDA 1650 (Bayer Corp., Tarrytown, NY). Plasma Tf was measured by turbidimetric immunoassay. TIBC was measured by a colorimetric diagnostic kit (procedure #565, Sigma).

Metal levels via inductively coupled plasma mass spectrometry. To determine metal levels within the brain, regions (cerebellum, cortex, hippocampus, striatum, and midbrain) were lyophilized in glass tubes and sent to the Norwegian University of Science and Technology (Trondheim, Norway) for analysis as described previously (Erikson *et al.*, 2004). Samples were analyzed for several essential trace metals chromium (Cr), cobalt (Co), copper (Cu), Fe, Mn, molybdenum (Mo), vanadium (V), and zinc (Zn) and nonessential metals Al, Cd, magnesium (Mg). Formalin (0.2 ml) was added to the samples 12 weeks before analysis. The samples were digested in sealed Teflon bombs in a microwave oven (Milestone MLS 1200) after 2 ml 65% HNO_3 (Merck, Suprapur) was added. After the microwave digestion the samples were diluted directly in the Teflon bombs to 57.6 ml with ultrapure water (MilliQ, Millipore, Bedford, MA) to achieve a final acid concentration of 0.5mol/l.

High-resolution inductively coupled plasma mass spectrometry analysis was performed using a Thermo (Finnigan) model Element instrument (Bremen, Germany). The Radio Frequency power was 1150 W. The sample was introduced using a CETAC ASX 500 auto sampler (Omaha, NE) with a peristaltic pump (pump speed 1 ml/min). The instrument was calibrated using 0.5mol/l HNO_3 solutions of multielement standards at appropriate concentrations. Internal standards were not used. To check for possible drift in the instrument, a standard solution with known elemental concentrations was analyzed for every 10 samples. In addition, blank samples (0.5mol/l HNO_3, Suprapur) were analyzed for approximately every 10 samples. The samples were analyzed in random order, and the analyst did not know the identification of the samples. All elements were determined in the medium resolution mode ($M/\Delta m = 4000$).

DMT-1 and Tf protein analysis via Western blot analysis. Brain tissues were homogenized in five volumes of cold buffer (10mM Tris, 1% sodium dodecyl sulfate [SDS], 1mM sodium orthovanadate, pH 7.4) using a sonicator with microtip, boiled for 10 min, and centrifuged at 16,000 × g for 10 min at 15°C. Protein concentrations were measured in the supernatants using the bicinchoninic acid method (Pierce Chemical, Rockford, IL). Prepared samples were run through an 8% SDS-polyacrylamide gel electrophoresis gel and subsequently transferred to a nitrocellulose membrane (*Schleicher & Schuell Biosciences*, Keene, NH). Blots were washed and proteins blocked overnight at 4°C in phosphate-buffered saline solution containing 5% dry milk and 0.05% Tween-20. Blots were washed and incubated with primary antibody (DMT-1 [natural resistance-associated macrophage protein]) goat polyclonal (Santa Cruz), 1:400; TfR (CD-71) monoclonal (Dako Corporation, Carpenteria, CA), 1:200; or anti-β-actin monoclonal (Sigma), 1:8000 for 2 h, then with horseradish peroxidase (HRP)–conjugated secondary antibody for 1 h (rabbit anti-goat IgG-HRP [Santa Cruz], 1:1000–2000 for DMT-1; goat anti-mouse IgG [Kirkegaard & Perry Labs, Gaithersburg, MD], 1:1000 for TfR or 1:5000 for actin). Blots were developed with enhanced chemiluminescence reagents and exposed to film. To allow for subsequent reprobing with additional antibodies, blots were washed, stripped with Restore Western blot stripping buffer (Pierce Chemical), and proteins subsequently blocked as before.

Amino acid concentrations via high-performance liquid chromatography. Glutamate and GABA concentrations were measured as previously in our lab (Erikson *et al.*, 2002) with high-performance liquid chromatography (HPLC) and fluorescence detection. Brain regions were pulverized in liquid nitrogen and stored at − 80°C until extraction. Amino acids were extracted from ~10 to 15 mg of frozen tissue powder with 0.4 ml of 4°C methanol (MeOH). Homoserine was added to each sample as an internal standard. Homogenates were vortexed and centrifuged at 2000 rpm for 10 min, and the supernatants were dried at 37°C under a stream of dry nitrogen. The dried samples were stored at − 20°C until analysis. Several concentrations of amino acid standards were processed to quantify unknowns. Amino acid standards also were added to pooled brain extracts and processed in parallel as an external standard.

MeOH-reconstituted samples were reacted with OPT reagent (100 mg *O*-pthaldialdehyde in 0.5 ml MeOH, 100 μl 2-mercaptoethanol in 1.9 ml of 0.4 M sodium borate, pH 9.5) for 2 min and injected into a gradient HPLC system (Gilson Model 201) using an autoinjector (Gilson 401 and 231). A C_{18} reverse-phase column (Vydac; 0.46 × 15 cm, 5 μm) was used with fluorometric detection (Gilson 121) (excitation 305–395 nm, emission 430–470 nm). The mobile phase was 0.1M sodium acetate, pH 6.2, containing 0.1mM ethylenediaminetetraacetic acid and increasing concentrations of MeOH (15–50%); flow rate was 1.3 ml/min. Retention times for glutamate and GABA were 3.2 and 18.1 min, respectively. Proteins were measured in the pellets using the Lowry *et al.* (1951) method and content (nmol/mg protein) was determined from the internal standards correcting for recovery for each sample.

Statistical analysis. Data were analyzed using NCSS software (Kaysville, UT). Data are presented as means ± SEM and considered significant at $p < 0.05$. The litter is the statistical unit. For body and brain weight data and blood data, litter averages were analyzed; for all other measures, one male and one female from each litter were used. For statistics where gender was collapsed due to no significant treatment × gender interaction, the sample size (n) equals two times the litter number to account for one male and one female from each litter. Outliers that were ± 2 SD from the mean were omitted from statistical analyses. Treatment effects in the brain were analyzed by global analysis of variance (ANOVA) incorporating all factors: dietary treatment, gender (male, female), brain region (cerebellum, cortex, hippocampus, midbrain, striatum), and measurement (Hb, plasma Fe, Tf, and TIBC; metal level; DMT-1 or TfR protein expression; glutamate or GABA concentration). Films containing DMT-1 and TfR blots were scanned into PhotoShop and band densities were measured using TINA program software. To normalize band densities across Western blots, bands from treated pups were analyzed as a percent of the control band on each blot. To verify consistent protein loading among gels, some blots were probed for actin and the ratio of DMT-1 or TfR:actin was compared to the analyzed values. There were no statistical differences between density percent control and their ratio with actin, so analyses are presented for percent control of each blot. Three-way ANOVA (treatment × region × gender) tested for interactions between dietary treatments and measurement (metal level; transporter expression; amino acid concentration) in each brain region with gender as a factor. If the global ANOVA indicated a significant interaction (treatment × region × gender, treatment × region, or treatment × gender), data were analyzed by one-way (treatment) or two-way (treatment × region, or treatment × gender) ANOVA with Tukey-Kramer multiple comparison *post hoc* test used to evaluate differences in individual means within the larger comparison groups.

RESULTS

Animal Growth

Although initial pup body weights were equivalent at PN4 ($p < 0.3$), by PN21 body weights of pups maternally exposed to the ID or IDMn diets were 72 or 80% ($p < 0.0001$) of control pup weights, respectively. Only ID pups had significantly lower brain weights as well (90% of control, $p < 0.04$). Body weights

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were affected more than brain weights such that the brain to body weight ratios for both treatment groups were significantly increased ($p < 0.002$) by approximately 20% compared to the control group. Thus, the nutritional deficits related to the ID and IDMn diets resulted in smaller pups with brain sparing in the IDMn group.

Hematological Parameters

Both treatment diets (ID and IDMn) resulted in decreased Hb and Fe and increased Tf and TIBC in both dams and pups. Dams fed the ID or IDMn diets had decreased Hb (13–45% decrease) throughout the treatment period compared to the control pups, although statistical significance was achieved only at PN11 ($p < 0.05$) and PN21 ($p < 0.0001$) for the ID group (27% decrease both days) and only at PN21 (45% decrease, $p < 0.0001$) for the IDMn group. ID dams had reduced plasma Fe (90% decrease, $p < 0.0001$) and increased plasma TIBC and Tf (87–103% increase, respectively, $p < 0.0002$) (Fig. 2a). Unfortunately, IDMn dams were used for another study so plasma Fe, Tf and TIBC were not determined. Alterations in hematological parameters were similar in pups raised by dams fed ID or IDMn diets: decreased Hb (40–55% decrease, $p < 0.0001$) and plasma Fe (73–82% decrease, $p < 0.0001$) concurrent with increased plasma Tf and TIBC (~20% increase, $p < 0.0001$ for both), compared to controls (Fig. 2b). Hb was statistically significantly lower ($p < 0.0001$) in ID pups (55% decrease) compared to IDMn pups (40% decrease) but this was the only significant difference observed between the treatment groups. Raw hematological control values are provided in the figure legend of Figure 2 for dams and PN21 pups in all groups. There were no gender differences for hematological measurements (no treatment × gender interaction), so litter averages combined both males and females.

Brain Metal Levels

Several metals were measured to assess the specificity of metal alterations in the brain. Pups maternally exposed to the ID diet had statistically significantly decreased brain Fe (30% decrease, $p < 0.0001$) concurrent with increased Cu (15% increase, $p < 0.0005$) levels, compared to controls. Surprisingly, these were the only metals statistically significantly altered in the ID pups. On the other hand, in the IDMn exposed pups, the changes in brain metals were widespread: decreased Fe (20% decrease, $p < 0.001$) and increased Co (200% increase, $p < 0.002$), Cr (180% increase, $p < 0.0001$), V (170% increase, $p < 0.005$), Zn (140% increase, $p < 0.0001$), Al (140% increase, $p < 0.002$), Mn (50% increase, $p < 0.0001$), Mo (30% increase, $p < 0.02$), and Cu (15% increase, $p < 0.002$) compared to controls (Fig. 3). Although there were treatment × region interactions for Mn ($p < 0.0001$), Cr ($p < 0.01$), Co ($p < 0.0001$), and Mo ($p < 0.005$), there was not a consistent target region. There were no gender differences for metal levels (no treatment × gender interaction), so analyses combined males and females.

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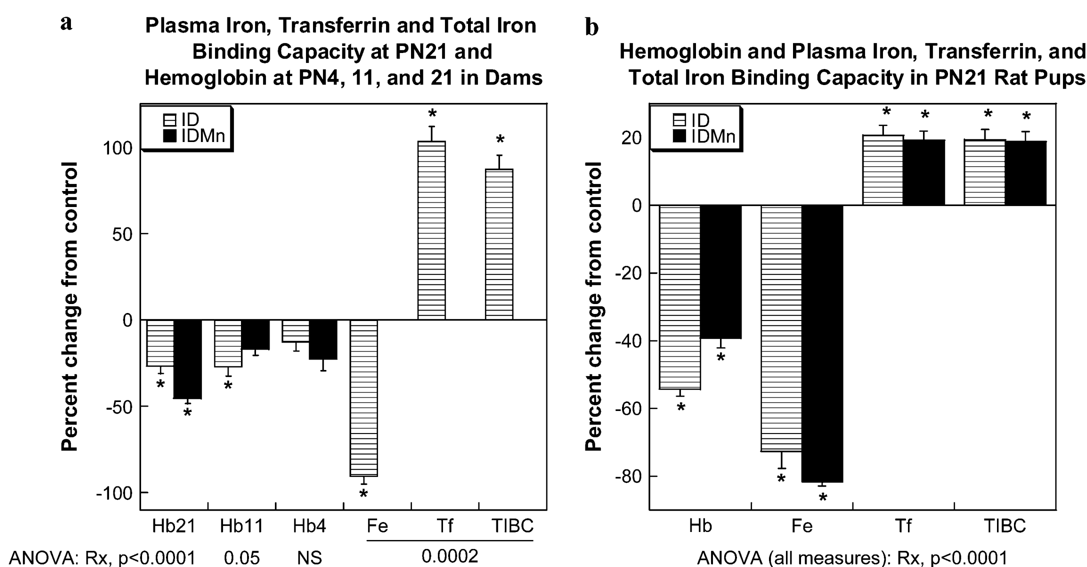

FIG. 2. Hb and plasma Fe, Tf, and TIBC in dams and rat pups on PN21. Data represent means ± SEM of group averages ($n = 5$–7 dams; 10–14 pups, 2× litter since males and females were averaged separately). Hematological data are shown as a percent change from control values for (a) dams and (b) pups. *Indicates statistically significant difference compared to control values. (a) Maternal Hb was decreased during the lactation period (significantly on PN21 for both the ID and IDMn groups, and significantly on PN11 for the ID group). In ID dams, plasma Fe decreased while Tf and TIBC increased compared to controls. Raw data values for maternal controls were as follows (mean ± SD): Hb21: 14.5 ± 2.5 g/dl; Hb11: 15.1 ± 2.7 g/dl; Hb4: 14.8 ± 2.5 g/dl; Fe: 161 ± 26 μg/ml; Tf: 406 ± 56 mg/dl; TIBC: 250 ± 52 μg/dl. (b) For both diets, pup Hb and Fe decreased while Tf and TIBC increased compared to pups reared by control dams. Raw data values for offspring controls were as follows: Hb21: 8.1 ± 1.3 g/dl; Fe: 215 ± 23 μg/ml; Tf: 49.7 ± 27.5 mg/dl; TIBC: 308 ± 33 μg/dl.

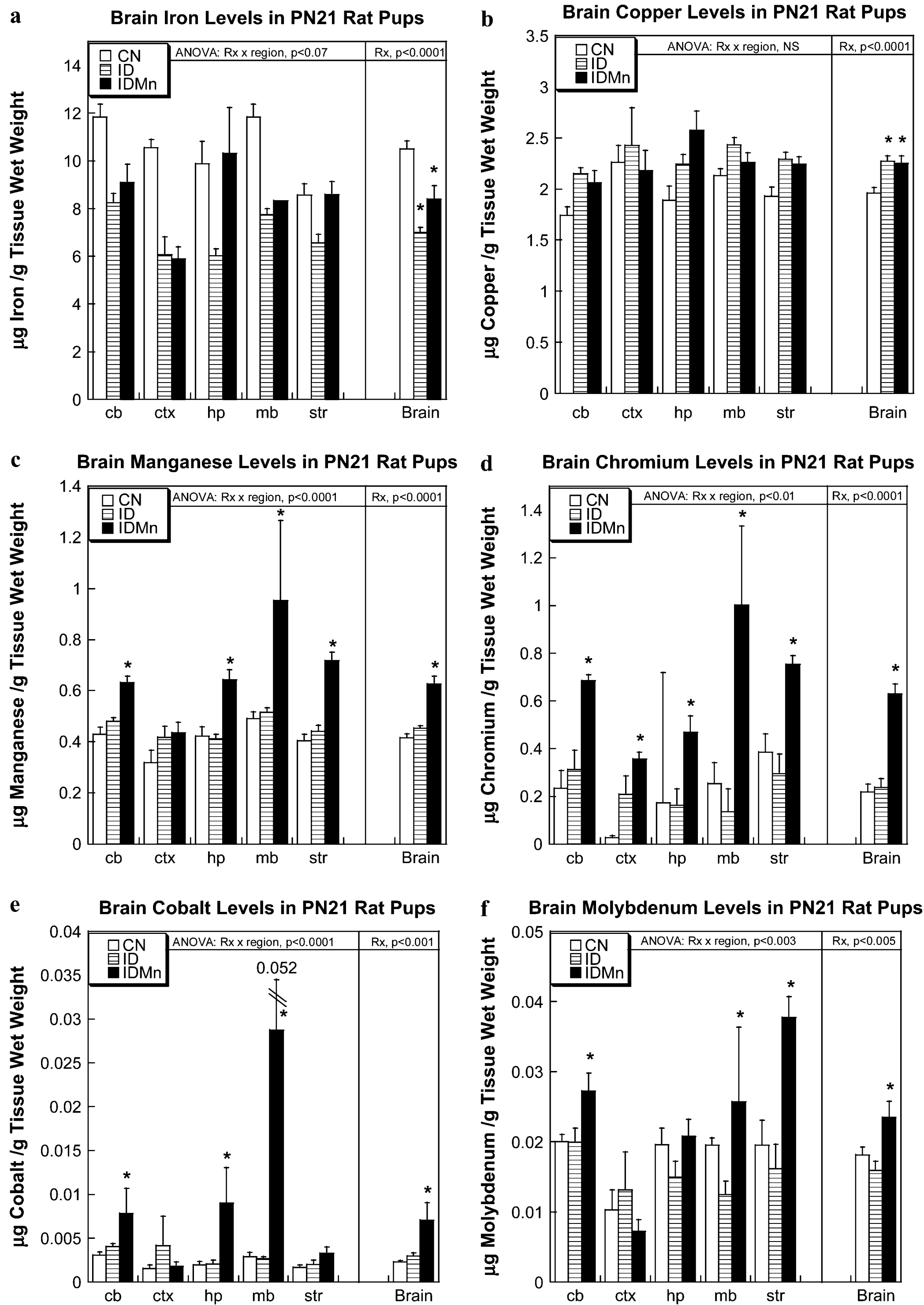

FIG. 3. Brain metal levels (µg metal/g tissue wet weight) in PN21 rat pups. Data represent means ± SEM of group averages ($n = 4$–7 per region per sex; some regions and metals had fewer sample values analyzed statistically due to technical errors or outliers). Values for overall "brain" effects were determined by collapsing regional values together. *Indicates statistically significant difference compared to control values. (a) Fe decreased overall in the brains of ID and IDMn pups. (b) Cu increased in the brains of ID and IDMn pups. (c) Mn, (d) Cr, (e) Co, and (f) Mo increased overall in IDMn brains and in regions marked with an asterisk (treatment × region interaction). (g) Zn, (h) Al, and (i) V increased in the brains of IDMn pups.

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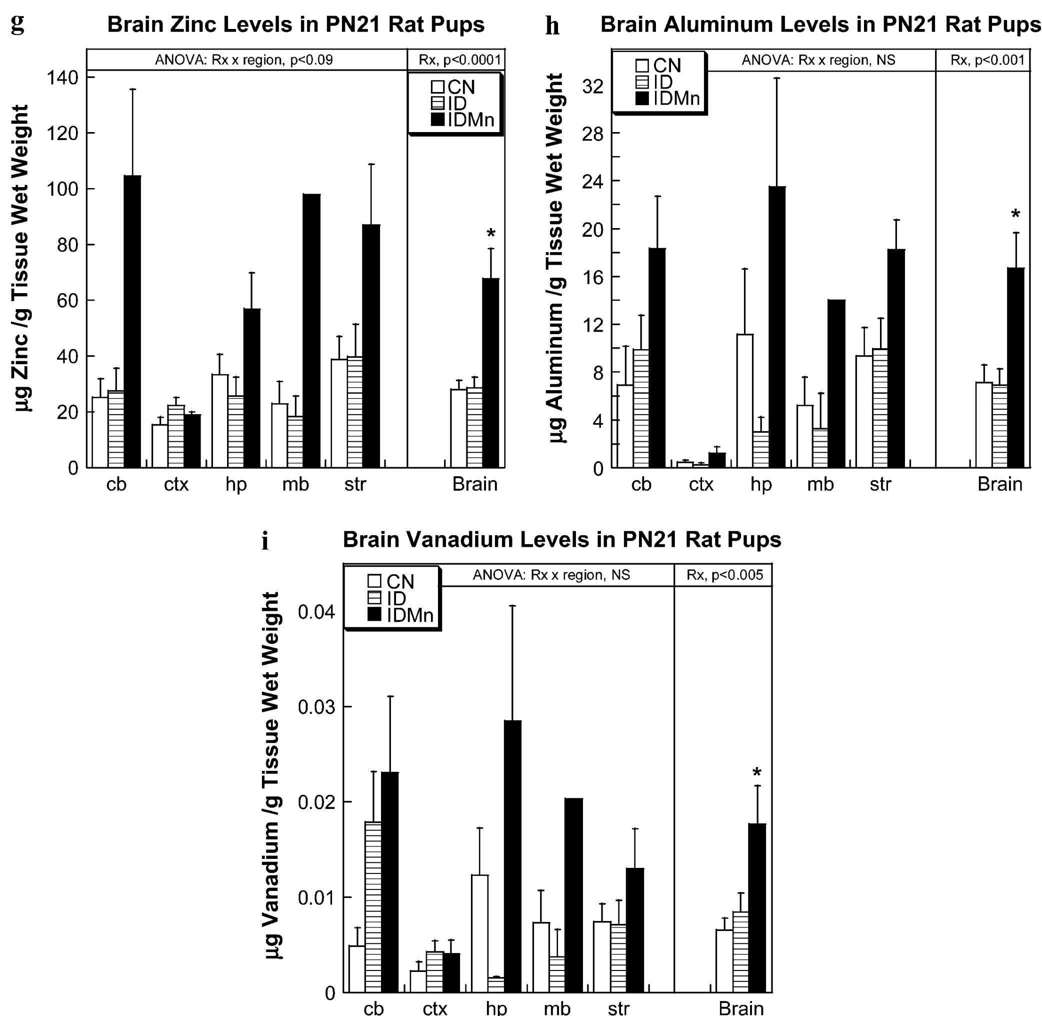

FIG. 3. *Continued*

Brain DMT-1 and TfR Expression

Neurotoxic outcomes depend upon the rate and extent of metal accumulation into the brain, thus we investigated metal regulation in the brain by measuring the expression of DMT-1 and TfR in each brain region at PN21 via Western blot analysis (Fig. 4). Overall, DMT-1 and TfR expression increased by approximately 10–15% in brains from pups maternally exposed to the ID or IDMn diets compared to control pups (DMT-1: $p < 0.05$, and TfR: $p < 0.005$). Since there was no treatment $\times$ region interaction, a susceptible region could not be identified for either transporter. There was not a significant gender effect on DMT-1 expression (no treatment $\times$ gender interaction), so results were combined from males and females. Conversely, there was a significant gender effect on TfR expression for the ID exposed pups ($p < 0.05$) with females contributing all of the treatment effect; TfR expression was increased approximately 15% in females ($p < 0.005$) and was not significantly affected in males (data not shown for gender).

Brain Amino Acid Concentrations

Although GABA and glutamate levels were measured, no significant alterations were determined for either amino acid ($p > 0.5$ for each measure; data not shown). The greatest change observed was less than a 10% decline in GABA levels in the IDMn group compared to controls but this was not significant.

DISCUSSION

The essential metals, calcium (Ca), Co, Cu, Fe, Mg, Mn, and Mo, selenium (Se), and Zn, serve critical roles in physiological functions (Smith *et al.*, 1997). Unlike conventional toxicants that raise concern in excess, essential metals may adversely affect biological processes if there is a deficiency or a surplus and imbalances are associated with neurological diseases. Fe deficiency enhances Mn accumulation (Aschner, 2000; Connor

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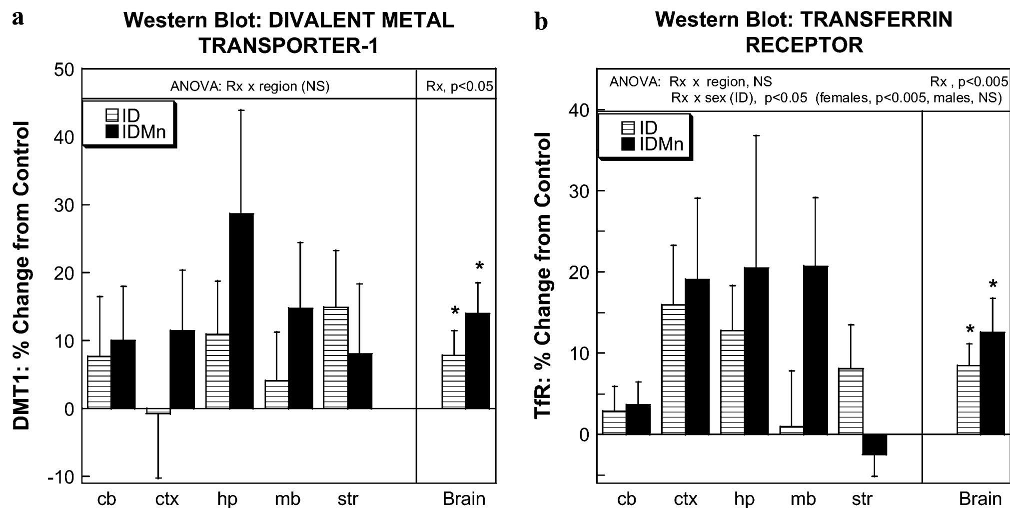

FIG. 4. Protein expression of DMT-1 and TfR. Data represent means ± SEM ($n = 3$–5 per region per sex) and are shown as a percent change from control values. Values for overall ''brain'' effects were determined by collapsing regional values together. *Indicates statistically significant difference compared to control values. Protein expression of (a) DMT-1 and (b) TfR was increased overall in the brain of pups maternally exposed to the ID and IDMn diets. There was a treatment × gender interaction for TfR, with females contributing toward to the significant overall increase in TfR expression.

et al., 2001; Erikson *et al.*, 2002; Kwik-Uribe *et al.*, 2000; Mena, 1974) and likewise, Mn treatment alters Fe homeostasis (Aschner *et al.*, 1999). In a classic study, Mena (1974) reported increased entrance of ^{54}Mn into brains of anemic rats, and fourfold higher ^{54}Mn entrance in the brains of neonatal rats compared to adults. On the other hand, $MnCl_2$ treatment (6 mg/kg/day, ip, 30 days) in male rats resulted in decreased plasma Fe and increased cerebrospinal fluid Fe, indicating a shift to the BBB compartment and altered Fe homeostasis (Aschner *et al.*, 1999). Still, the view that Fe and Mn are inversely related is a simplistic one. Metal uptake into brain is known to be critically influenced by diet, and can also cross-interact so that a deficiency or excess in one metal can influence the uptake and concentration of other metals (Smith *et al.*, 1997). Fe deficiency is a risk factor for metal toxicity (e.g., Mn, Cd, Al) such that an inverse relationship exists between dietary Fe and absorption of a plethora of other metals (e.g., Mn, Zn, Cu) (Erikson *et al.*, 2002, 2004).

We confirm this cross-interaction in the current study. Not only is Fe decreased in neonatal rats reared by dams fed an Fe-deficient diet, Cu is also increased; by altering two metals (Fe and Mn), brain Al, Co, Cr, Cu, Mo, Mn, V, and Zn increased while Fe decreased. Similarly, Cu increased in the brains of Fe-deficient rats exposed to Mn (Malhotra *et al.*, 1984).

Correlations in the cross-interaction of essential metals, not limited to the brain, also can be observed with other dietary metal alterations. In a parallel study, a Mn supplemented diet led to decreased brain Fe and increased brain Mn, Cr, and Zn (Garcia *et al.*, 2006). Except for Zn, this effect is supported by an earlier study; rats injected with $MnCl_2$ accumulated Mn and Cu concurrent with decreases in Fe and Zn in the brain (Chen *et al.*, 2002). Following dietary Cd supplementation in rats, liver mineral content was modified according to Cd level: Fe, Mg, and Se decreased while Cu, Zn, and Mn increased with increasing Cd levels (Noel *et al.*, 2004), indicating an interplay among these metals and supporting the relationship observed in the current study among Fe, Cu, Zn, and Mn in the brain. Further, retention of both ^{54}Mn and ^{59}Fe was 8–10% lower in weanling mice fed a high Al diet, suggesting that high dietary A1 during development alters the ability of nursing mouse pups to retain absorbed Fe and Mn, resulting in lowered tissue Mn and Fe concentrations (Golub *et al.*, 1996a).

The alterations in the hematological parameters are consistent with changes observed in blood from Fe-deficient individuals (decreased Hb and Fe; increased Tf and TIBC) (Beard, 2001; Connor *et al.*, 2001). Although we measured only blood Fe in the current study, brain metal alterations may be mirrored in the blood and other tissues. Paralleling the current study, Yokoi *et al.* (1991) reported that rats fed a Fe-deficient diet had decreased Hb and blood and brain Fe; further, hematocrit and blood Ca and Zn were decreased concurrent with increased blood Mg and Cu and brain Mn.

Fe and Mn compete for the same carrier transport system; both TfR and DMT-1 regulate influx into the brain (Aschner, 2000; Connor *et al.*, 2001; Malecki *et al.*, 1999a; Roth and Garrick, 2003). This is not a simplistic relationship as several metals appear to be regulated together. DMT-1 is a nonspecific divalent metal transporter and has been implicated as a transporter of brain Fe and Mn, and to a lesser extent Zn, Cu, Co, Cd, and Ni (Garrick *et al.*, 2003; Gunshin *et al.*, 1997; Zheng *et al.*, 2003). Likewise, Tf binds several metals, potentially acting as a transport agent for Fe, Mn, Zn, and Cr as well as Cu, Co, Cd, V, and Al (Aschner and Aschner, 1991; Golub *et al.*, 1996b).

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Not surprisingly, protein expression of DMT-1 and TfR were elevated in the brains of neonatal rats reared by dams fed the ID or IDMn diets. In a similar study in our lab using young adult rats, transporter protein in the brain increased due to Fe deficiency compared to control levels (Erikson *et al.*, 2004). Moreover, in PC12 cells, decreased Fe in the cell media was associated with increased Mn uptake and toxicity via upregulation of DMT-1 (Roth *et al.*, 2002). The treatment × gender interaction noted in TfR expression of ID reared pups is not completely unfounded. Erikson *et al.* (2001) found that a 6 week Fe-deficient diet in young adults (PN21–63) affects dopamine biology more dramatically in males than in females, while Kwik-Uribe *et al.* (2000) also reported gender-related changes following Fe deficiency throughout development.

Combined with the nonspecific transport of several metals by DMT-1 and TfR, the web of interaction becomes more complex when considering that other transport systems cannot be excluded. Thus, what was once a simplistic view of Fe and Mn being inversely related due to competing DMT-1 and TfR-mediated transport is extended to include alterations in an array of other essential metals (Cr, Co, Mo, Zn, Cu, and V). It is challenging to speculate why so many metals accumulate in the brains of IDMn rats, while only Fe and Cu were affected in ID brains. Since there was not a significant difference in the upregulation of DMT-1 or TfR between ID and IDMn groups, the accumulation of some metals is not due solely to the upregulation of these transporters. In a parallel study, a high Mn diet decreased Fe and increased Mn, Cr, and Zn (Garcia *et al.*, 2006). It is possible that in the presence of Mn, combined with low Fe, there is upregulation of other transporters besides DMT-1 and TfR on which other metals gain access to the brain.

A recent set of studies raised the potential that Mn transport across the BBB occurs in the form of a Mn-citrate complex, found in plasma at small concentrations (Crossgrove *et al.*, 2003; Yokel and Crossgrove, 2004). The Mn-citrate, as a tridentate complex, possesses a noncoordinated central carboxylate recognition moiety which is likely a substrate for the organic anion transporter or a monocarboxylate transporter (MCT; (Crossgrove *et al.*, 2003; Yokel and Crossgrove, 2004). Candidates for the transport of the Mn-citrate complex are MCT and/or members of the organic anion transporter polypeptide or adenosine 5′-triphosphate-binding cassette superfamilies. However, the relevance of this transporter to *in situ* Mn transport remains to be fully elucidated and as pointed out by Crossgrove *et al.* (2003; Yokel and Crossgrove, 2004), the most likely candidate for mediating Mn transport across the BBB under physiological conditions is DMT-1. Also worth considering are the ZIP transporter proteins, members of the solute-carrier-39 (Slc39) metal-transporter family. Several ZIP proteins have been implicated in divalent metal transport, including Zn, Fe, Mn, and Co. ZupT, for example, is a cytoplasmic membrane protein related to the ZIP family of transporters and has broad substrate specificity in cell studies (e.g., Zn, Fe, and Co) (Grass *et al.*, 2005). Another recent study (He *et al.*, 2006) implies ZIP8 as a Mn transporter. In mouse, fetal fibroblast ZIP8 has high affinity for Mn (He *et al.*, 2006). The K_m of 2.2μM for Mn^{2+} is close to physiological concentrations and within the same range determined in many cell lines or tissues. However, it has yet to be tested whether either transporter is physiologically prominent in Mn transport across the BBB. One has to be cautious in extrapolating into the intact animal because the ability of this protein to transport a particular cation does not necessarily imply that this protein is physiologically relevant. Thus, whether Slc39 and organic transporters function in Mn transport remains to be tested under physiologically relevant conditions. It seems that to date the strongest evidence based studies on Mn imply a physiological role for the transport of Mn both by TfRs and DMT-1. Definitive studies to assess other protein functions (Slc39, MCT) in physiological roles have yet to be carried out. Mn efflux out of the brain is slow and perhaps associated with simple diffusion as it did not efflux from the brain more rapidly than sucrose or dextran (Yokel, 2002). Nevertheless, although knowledge on metal efflux from brain is scarce, it cannot be discounted as a contributing factor in the net accumulation of metals in the brain. More studies are needed to assess the interactions between different metals with the aforementioned transporters vis-à-vis their efflux.

Fe deficiency and excess Mn exposure each alter behavior, motor function, cognition, and neurotransmitter metabolism (e.g., dopamine, glutamate, GABA, and serotonin) (Beard, 2001; Chandra *et al.*, 1982; Erikson *et al.*, 2002; Li, 1998). Although glutamate and GABA levels were measured, no significant changes were observed following either Fe-deficient diet (± Mn supplementation), in contrast to increased GABA levels in PN21 rats following a high Mn diet (Garcia *et al.*, 2006).

In conclusion, although Fe and Mn are inversely related, the results of this study demonstrate that several metals (Al, Co, Cr, Cu, Fe, Mo, Mn, V, and Zn) are co-regulated with Fe and/or Mn. The effects in brain metal levels correlate with enhanced DMT-1 and TfR expression but future research should encompass other potential transporters responsible for the changes in an array of metals in the brain.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

REFERENCES

Agency for Toxic Substances and Disease Registry, A. (2000). Toxicological profile for manganese (Update). U.S. Department of Health and Human Services, Public Health Service, Atlanta, GA.

Aschner, M. (2000). Manganese in health and disease: From transport to neurotoxicity. In *Handbook of Neurotoxicology* (E. Massaro, Ed.), pp. 195–209. Humana Press, Totowa, NJ.

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Aschner, M., and Aschner, J. (1991). Manganese neurotoxicity: Cellular effects and blood-brain barrier transport. *Neurosci. Biobehav. Rev.* **15,** 333–340.

Aschner, M., Shanker, G., Erikson, K., Yang, J., and Mutkus, L. A. (2002). The uptake of manganese in brain endothelial cultures. *Neurotoxicology* **23,** 165–168.

Aschner, M., Vrana, K. E., and Zheng, W. (1999). Manganese uptake and distribution in the central nervous system (CNS). *Neurotoxicology* **20,** 173–180.

Barron, T. F., Devenyi, A. G., and Mamourian, A. C. (1994). Symptomatic manganese neurotoxicity in a patient with chronic liver disease: Correlation of clinical symptoms with MRI findings. *Pediatr. Neurol.* **10,** 145–148.

Beard, J. L. (2001). Iron biology in immune function, muscle metabolism and neuronal functioning. *J. Nutr.* **131,** 568S–580S.

Beard, J. L., Felt, B., Schallert, T., Burhans, M., Connor, J. R., and Georgieff, M. K. (2006). Moderate iron deficiency in infancy: Biology and behavior in young rats. *Behav. Brain Res.* **170,** 224–232.

Brenneman, K. A., Wong, B. A., Buccellato, M. A., Costa, E. R., Gross, E. A., and Dorman, D. C. (2000). Direct olfactory transport of inhaled manganese ($^{54}MnCl_2$) to the rat brain: Toxicokinetic investigations in a unilateral nasal occlusion model. *Toxicol. Appl. Pharmacol.* **169,** 238–248.

Chandra, S., and Shukla, G. (1976). Role of iron deficiency in inducing susceptibility to manganese toxicity. *Arch. Toxicol.* **35,** 319–323.

Chandra, S. V., Malhotra, K. M., and Shukla, G. S. (1982). GABAergic neurochemistry in manganese exposed rats. *Acta Pharmacol. Toxicol.* **51,** 456–458.

Chen, M. T., Yiin, S. J., Sheu, J. Y., and Huang, Y. L. (2002). Brain lipid peroxidation and changes of trace metals in rats following chronic manganese chloride exposure. *J. Toxicol. Environ. Health A* **65,** 305–316.

Connor, J., Menzies, S., Burdo, J., and Boyer, P. (2001). Iron and iron management proteins in neurobiology. *Pediatr. Neurol.* **25,** 118–129.

Crossgrove, J. S., Allen, D. D., Bukaveckas, B. L., Rhineheimer, S. S., and Yokel, R. A. (2003). Manganese distribution across the blood-brain barrier: I. Evidence for carrier-mediated influx of manganese citrate as well as manganese and manganese transferrin. *Neurotoxicology* **24,** 3–13.

deUngria, M. Rao, R., Wobken, J. D., Luciana, M., Nelson, C. A., and Georgieff, M. K. (2000). Perinatal iron deficiency decreases cytochrome c oxidase (CytOx) activity in selected regions of neonatal rat brain. *Pediatr. Res.* **48,** 169–176.

Devenyi, A., Barron, T. F., and Mamourian, A. (1994). Dystonia, hyperintense basal ganglia, and high whole blood manganese levels in Alagille's syndrome. *Gastroenterology* **106,** 1068–1071.

Dorman, D. C., Struve, M. F., Vitarella, D., Byerly, F. L., Goetz, J., and Miller, R. (2000). Neurotoxicity of manganese chloride in neonatal and adult CD rat following subchronic (21-day) high-dose oral exposure. *J. Appl. Toxicol.* **20,** 179–187.

Erikson, K., Jones, B., Hess, E., Zhang, Q., and Beard, J. (2001). Iron deficiency decreases dopamine D1 and D2 receptors in rat brain. *Pharmacol. Biochem. Behav.* **69,** 409–418.

Erikson, K., Shihabi, Z. K., Aschner, J., and Aschner, M. (2002). Manganese accumulates in iron-deficient rat brain regions in a heterogeneous fashion and is associated with neurochemical alterations. *Biol. Trace Elem. Res.* **87,** 143–156.

Erikson, K. M., Syversen, T., Steinnes, E., and Aschner, M. (2004). Globus pallidus: A target brain region for divalent metal accumulation associated with dietary iron deficiency. *J. Nutr. Biochem.* **15,** 335–341.

Fell, J. M. E., Meadows, N., Khan, K., Long, S. G., Milla, P. J., Reynolds, A. P., Quaghebeur, G., and Taylor, W. J. (1996). Manganese toxicity in children receiving long-term parenteral nutrition. *The Lancet* **347,** 1218–1221.

Felt, B., and Lozoff, B. (1996). Brain iron and behavior of rats are not normalized by treatment of iron deficiency anemia during early development. *J. Nutr.* **126,** 693–701.

Garcia, S., Gellein, K., Syversen, T., and Aschner, M. (2006). A manganese-enhanced diet alters brain metals and transporters in the developing rat. *Toxicol. Sci.* **92,** 516–525.

Garrick, M. D., Dolan, K. G., Horbinski, C., Chio, A. J., Higgins, D., Porubcin, M., Moore, E. G., Hainsworth, L. N., Umbreit, J. N., *et al.* (2003). DMT1: A mammalian transporter for multiple metals. *Biometals* **16,** 41–54.

Gerber, G. B., Leonard, A., and Hantson, P. (2002). Carcinogenicity, mutagenicity and teratogenicity of manganese compounds. *Crit. Rev. Oncol. Hematol.* **42,** 25–34.

Golub, M., Han, B., and Keen, C. (1996a). Iron and manganese uptake by offspring of lactating mice fed a high aluminum diet. *Toxicology* **109,** 111–118.

Golub, M. S., Han, B., and Keen, C. L. (1996b). Aluminum alters iron and manganese uptake and regulation of surface transferrin receptors in primary rat oligodendrocyte cultures. *Brain Res.* **719,** 72–77.

Grantham-McGregor, S., and Ani, C. (2001). A review of studies on the effect of iron deficiency on cognitive development in children. *J. Nutr.* **131,** 649S–668S.

Grass, G., Franke, S., Taudte, N., Nies, D. H., Kucharski, L. M., Maguire, M. E., and Rensing, C. (2005). The metal permease ZupT from Escherichia coli is a transporter with a broad substrate spectrum. *J. Bacteriol.* **187,** 1604–1611.

Gunshin, H., Mackenzie, B., Berger, U., Gunshin, Y., Romero, M., Boron, W., Nussberger, S., Gollan, J., and Hediger, M. (1997). Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* **388,** 482–488.

He, L., Girijashanker, K., Dalton, T., Reed, J., Li, H., Soleimani, M., and Nebert, D. (2006). ZIP8, member of the solute-carrier-39 (SLC39) metal-transporter family: Characterization of transporter properties. *Mol. Pharmacol.* **70,** 171–180.

Kontur, P., and Fechter, L. (1985). Brain manganese, catecholamine turnover, and the development of startle in rats prenatally exposed to manganese. *Teratology* **32,** 1–11.

Kwik-Uribe, C., Golub, M., and Keen, C. (1999). Behavioral consequences of marginal iron deficiency during development in a murine model. *Neurotoxicol. Teratol.* **21,** 661–672.

Kwik-Uribe, C. L., Golub, M. S., and Keen, C. L. (2000). Chronic marginal iron intakes during early development in mice alter brain iron concentrations and behavior despite postnatal iron supplimentation. *J. Nutr.* **130,** 2040–2048.

Li, D. (1998). Effects of iron deficiency on iron distribution and γ-aminobutyric acid (GABA) metabolism in young rat brain tissues. *Hokkaido J. Med. Sci.* **73,** 215–225.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–270.

Malecki, E., Devenyi, A., Barron, T., Mosher, T., Eslinger, P., Flaherty-Craig, C., and Rossaro, L. (1999a). Iron and manganese homeostasis in chronic liver disease: Relationship to pallidal T1-weighted magnetic resonance signal hyperintensity. *Neurotoxicology* **20,** 647–652.

Malecki, E. A., Devenyi, A. G., Beard, J. L., and Connor, J. R. (1999b). Existing and emerging mechanisms for transport of iron and manganese to the brain. *J. Neurosci. Res.* **56,** 113–122.

Malhotra, K. M., Murthy, R. C., Srivastava, R. S., and Chandra, S. V. (1984). Concurrent exposure of lead and manganese to iron-deficient rats: Effect on lipid peroxidation and contents of some metals in the brain. *J. Appl. Toxicol.* **4,** 22–25.

Mena, I. (1974). The role of manganese in human disease. *Ann. Clin. Lab. Sci.* **4,** 487–491.

Noel, L., Guerin, T., and Kolf-Clauw, M. (2004). Subchronic dietary exposure of rats to cadmium alters the metabolism of metals essential to bone health. *Food Chem. Toxicol.* **42,** 1203–1210.

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Olanow, C., Good, P., Shinotoh, H., Hewitt, K., Vingerhoets, F., Snow, B., Beal, M., Calne, D., and Perl, D. (1996). Manganese intoxication in the rhesus monkey: A clinical, imaging, pathologic, and biochemical study. *Neurology* **46,** 492–498.

Ono, J., *et al.* (1995). Manganese deposition in the brain during long-term total parenteral nutrition. *J. Parenter. Enteral Nutr.* **19,** 310–312.

Pal, P. K., Samii, A., and Calne, D. B. (1999). Manganese neurotoxicity: A review of clinical features, imaging and pathology. *Neurotoxicology* **20,** 227–238.

Pinero, D., *et al.* (2000). Variations in dietary iron alter brain iron metabolism in developing rats. *J. Nutr.* **130,** 254–263.

Racette, B., Antenor, J., McGee-Minnich, L., Moerlein, S., Videen, T., Kotagal, V., and Perlmutter, J. (2005). [18F]FDOPA PET and clinical features in parkinsonism due to manganism. *Mov. Disord.* **20,** 492–496.

Roth, J. A., and Garrick, M. D. (2003). Iron interactions and other biological reactions mediating the physiological and toxic actions of manganese. *Biochem. Pharmacol.* **66,** 1–13.

Roth, J. A., Horbinski, C., Higgins, D., Lein, P., and Garrick, M. D. (2002). Mechanisms of manganese-induced rat pheochromocytoma (PC12) cell death and cell differentiation. *Neurotoxicology* **23,** 147–157.

Shukla, A., Agarwal, K., and Shukla, G. (1990). Effect of latent iron deficiency on the levels of iron, calcium, zinc, copper, manganese, cadmium, and lead in liver, kidney, and spleen of growing rats. *Experientia* **46,** 751–752.

Smith, Q., Rabin, O., and Chikhale, E. (1997). Delivery of metals to brain and the role of the blood-brain barrier. In *Metals and Oxidative Damage in Neurological Disorders* (J. Connor, Ed.). pp. 113–130. Plenum Press, New York.

Verity, M. A. (1999). Manganese neurotoxicity: A mechanistic hypothesis. *Neurotoxicology* **20,** 489–498.

Yokel, R. (2002). Brain uptake, retention, and efflux of aluminum and manganese. *Environ. Health Perspect.* **110,** 699–704.

Yokel, R., and Crossgrove, J. (2004). Manganese toxicokinetics at the blood-brain barrier. *Res. Rep. Health Eff. Inst.* **119,** 7–58.

Yokoi, K., Kimura, M., and Itokawa, Y. (1991). Effect of dietary iron deficiency on mineral levels in tissues of rats. *Biol. Trace Elem. Res.* **29,** 257–265.

Zheng, W., Aschner, M., and Ghersi-Egea, J. F. (2003). Brain barrier systems: A new frontier in metal neurotoxicological research. *Toxicol. Appl. Pharmacol.* **192,** 1–11.

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