

Globus pallidus: a target brain region for divalent metal accumulation associated with dietary iron deficiency

By: [Keith M. Erikson](#), Tore Syversen, Eiliv Steinnes, Michael Aschner

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Abstract:

Recently, iron deficiency has been connected with a heterogeneous accumulation of manganese in the rat brain. The striatum is particularly vulnerable, for there is a significant negative correlation between accumulated manganese and γ -aminobutyric acid levels. The effect of dietary iron deficiency on the distribution of zinc and copper, two other divalent metals with essential neurobiological roles, is relatively unexplored. Thus, the primary goal of this study was to examine the effect of manipulating dietary iron and manganese levels on the concentrations of copper, iron, manganese and zinc in five rat brain regions as determined with inductively coupled plasma mass spectrometry analysis. Because divalent metal transporter has been implicated as a transporter of brain iron, manganese, and to a lesser extent zinc and copper, another goal of the study was to measure brain regional changes in transporter levels using Western blot analysis. As expected, there was a significant effect of iron deficiency ($P < 0.05$) on decreasing iron concentrations in the cerebellum and caudate putamen; and increasing manganese concentrations in caudate putamen, globus pallidus and substantia nigra. Furthermore, there was a significant effect of iron deficiency ($P < 0.05$) on increasing zinc concentration and a statistical trend ($P = 0.08$) toward iron deficiency-induced copper accumulation in the globus pallidus. Transporter protein in all five regions increased due to iron deficiency compared to control levels ($P < 0.05$); however, the globus pallidus and substantia nigra revealed the greatest increase. Therefore, the globus pallidus appears to be a target for divalent metal accumulation that is associated with dietary iron deficiency, potentially caused by increased transporter protein levels.

Keywords: Rat; Iron deficiency; Brain; Striatum; Zinc; Manganese

Article:

1. Introduction

Iron deficiency (ID) is one of the most prevalent nutritional problems in the world, affecting approximately 2 billion individuals [1]. ID is also a known risk factor for metal toxicity (e.g., cadmium, aluminum, lead) [2–4], with an inverse relationship existing between dietary iron content and the gastrointestinal absorption of a host of other metals. Iron also shares similar absorption mechanisms with essential divalent metals, particularly manganese [5], and a dietary deficiency of one of these minerals in the diet can lead to excess absorption of the other mineral. For example, high iron intakes decrease gastrointestinal manganese absorption, whereas ID leads to increased manganese absorption [6,7]. In addition, rats exposed to high doses of manganese exhibit altered brain iron metabolism [8]. Furthermore, ID is associated with increased manganese accumulation in the brain [9,10] and has a heterogeneous effect on manganese distribution (i.e., because of ID, manganese concentrations were unaltered in the cortex, but increased dramatically in the striatum) [11].

Both manganese and iron transport to extrahepatic tissues, including the brain, is dependent upon transferrin-mediated endocytosis [12,13]. Because ID causes increased brain regional transferrin (Tf) and transferrin receptor (TfR) concentrations in a heterogeneous fashion [14–16], it is likely that ID-associated brain manganese accumulation also occurs in a heterogeneous manner. High levels of manganese in globus pallidus, caudate putamen, and sub-thalamic nuclei have contributed to the vulnerability of the striatum to manganese toxicity [17,18]. Accordingly, a goal of this study was to investigate whether ID is associated with increased

accumulation of manganese, especially in brain regions such as the striatum that inherently contain high manganese levels.

Zinc is an abundant trace metal in the brain, especially within the hippocampus, where its neurobiological role has been extensively studied [19]. Zinc has several neurobiological roles such as modulation of glutamate receptors [20,21] and dopamine transporter functioning [22,23]. Behavioral alterations are associated with zinc deficiency in rodents [24,25]. It is also a critical component for DNA transcription (zinc-finger motif). However, the relationship between brain iron and zinc, particularly during dietary iron deficiency, is not well understood. The few studies that exist examined whole brain levels or the level of ID was not as severe as the current study [10,26]. The current study used a severe ID diet to ensure upregulation of iron dependent transporters across several brain regions known to be sensitive to these alterations [11,15,16]. This design allows us to examine the effect of ID on zinc distribution in the rat brain in a manner similar to previous studies, which examined manganese distribution in this same animal model [11].

Unlike zinc, brain regional distribution of copper is not as well understood. As copper plays an important role in dopamine biology through its role as a cofactor for dopamine β -hydroxylase (the enzyme which catalyzes the conversion of dopamine to norepinephrine), dopamine-rich regions such as the midbrain would likely be more abundant in copper than dopamine-sparse regions such as the cerebellum. Ceruloplasmin is a transporter of plasma copper and it also functions as an oxidative enzyme, most notably as ferroxidase, which catalyzes the conversion of cellular iron (Fe^{2+}) to Fe^{3+} -transferrin (*i.e.*, iron mobilization). The effect of ID on brain copper levels is limited to whole brain analysis [10] and analogous to zinc, brain regional effects of ID on copper levels are unknown. Although the interaction between copper and iron status is unexplored, the inverse relationship between copper and zinc levels exists primarily through their interactions with cellular metallothionein (MT) biology [27].

Divalent metal transporter (DMT-1, *a.k.a.* NRAMP-2) is emerging as an important protein for cellular transport of iron and manganese. A recent study demonstrated that ferrous iron and manganese both depend on DMT-1 for proper cellular transport and that the transport of zinc, copper, and ferric iron across the cell membrane is independent from this process, but this remains unexplored in brain tissue [28]. Previous studies from our laboratory showed that dietary ID led to a significant increase in brain regional manganese levels, but the specific mechanism(s) involved in this event is unknown. In the gut, it is assumed that DMT-1 levels are increased during dietary ID favoring increased manganese absorption. In the brain, it is known that striatum is a brain region that is rich in DMT-1 [29]. However, brain regional response of DMT-1 to dietary ID is not known.

The first goal of this study was to examine the effects of dietary iron and manganese manipulations on the brain regional distribution of copper, iron, manganese, and zinc within the brain. The five brain regions examined, cerebellum, caudate putamen, globus pallidus, hippocampus, and substantia nigra were chosen for their responsiveness or lack thereof to ID. For example, it was previously found that the striatum and hippocampus accumulate the highest concentrations of manganese in response to ID, followed by substantia nigra and cerebellum. It is unknown, however, whether and how zinc and copper concentrations are altered in these regions as a consequence of ID.

The final goal of this study was to examine the regional distribution of DMT-1 protein levels in the brains of rats exposed to varying dietary levels of iron and manganese. Measuring DMT-1 levels is important to gain a better understanding of the connection between iron status and manganese accumulation. Finally, there are no data connecting DMT-1 to zinc and copper metabolism in the brain, specifically from a regional perspective.

2. Methods and materials

2.1. Animals

Male 21-day-old Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were randomly divided into four dietary treatment groups: control (CN; 35 mg Fe/kg, 10 mg Mn/kg diet, $n = 6$), control/manganese-supplemented (CNMn+; 35 mg Fe/kg, 100 mg Mn/kg diet, $n = 6$) iron-deficient (ID; 4 mg Fe/kg, 10 mg Mn/kg

diet, $n = 6$), and iron-deficient/manganese-supplemented (IDMn+; 4 mg Fe/ kg, 100 mg Mn/kg diet, $n = 6$). Diets were obtained from Bio-Serv (Frenchtown, NJ) and certified for mineral content. Rats had free access to food and water 24 hours daily, and the lights were turned off between 6 PM and 6 AM. Room temperature was maintained at $25 \pm 1^\circ\text{C}$. After 4 weeks of dietary treatment, the rats were killed and brains removed for mineral and protein analyses. The Wake Forest University School of Medicine Animal Care and Use Committee approved all of the animal procedures.

2.2. Hematological measurements

Hematocrit was measured weekly via blood samples acquired by tail prick. It was determined by centrifugation of blood collected into heparinized microcapillary tubes. Blood samples were collected at the end of the experiment into heparinized tubes; aliquots were used for hematocrit and hemoglobin (procedure no. 525, Sigma Chemical, St. Louis, MO) measurements.

2.3. Brain trace metals

2.3.1. Sample preparation

Brains were dissected into five regions: caudate putamen, globus pallidus, hippocampus, substantia nigra, and cerebellum and wet weight recorded. Formalin, 0.2 mL, was then added and the samples were sent to Trondheim for analysis. Samples were kept on formalin for 12 weeks before analysis. The tissue and formalin were analyzed separately. Some release of metals from the brains to the formalin was noted, but because we did not observe any differences between dietary treatment groups, we report only the tissue content in this manuscript. Ultrapure water (Millipore Milli-Q water purification system) was used throughout. All sample storage containers and other equipment used was of polyethylene or glass and was either soaked for 24 hours in 6 mol/L HNO_3 and then rinsed copiously with ultrapure water or washed in an acid dishwasher (where final rinsing was also done with ultrapure water). Only talc-free (nonpowdered) gloves were used. 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 no visual solid residual remained, and the samples were diluted directly in the Teflon bombs to 57.6 mL with ultra-pure water to achieve a final acid concentration of 0.5 mol/L.

2.3.2. Analytical procedures

HR-ICP-MS analysis was performed using a Thermo (Finnigan) model Element instrument (Bremen, Germany). The RF power was 1150 W. The sample was introduced using a CETAC ASX 500 autosampler (Omaha, NE, USA) with a peristaltic pump (pump speed 1 mL/min). The instrument is equipped with a concentric Meinhard nebulizer (Golden, CO, USA) connected to a Scott spray chamber, and a quartz burner with a guard electrode. The nebulizer argon gas flow rate was adjusted daily to give a stable signal with maximum intensity for the nuclide ^{115}In .

The instrument was calibrated using 0.5 mol/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 (0.5 mol/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 four elements were determined in the medium resolution mode ($M/\Delta m = 4000$). The relative standard deviation was generally 10% or better for all elements. The reagent blanks were nearly constant and negligible (<1%) for Cu and Zn. For Mn and Fe, they were of the order of 5% and 10% of the concentrations in sample solutions, respectively.

2.4. Divalent metal transporter levels

Western blot analysis was used to measure DMT-1 levels in the brain regions examined. Brain regions were sonicated in 5 volumes (1:5, w:v) of tissue lysis buffer (10 mmol/L Tris-HCl; 1 mmol/L sodium orthovanadate; 1% SDS, pH 7.4). Tissue lysates were centrifuged for 10 minutes at 10,000 X g to remove cellular debris, and

the protein content of the resultant supernatant was determined with the bicinchoninic acid method (Pierce Chemical, Rockford, IL). An aliquot of 100 µg of protein was concentrated from the lysis buffer by organic extraction. Sample volumes were brought up to 400 µL with water and an equal volume of methanol (400 µL) was added, followed by 100 µL of chloroform. Samples were vortexed for 20 seconds and centrifuged at 14,000 X g for 3 minutes. The upper layer was removed and discarded. An additional 300 µL of methanol was added to each sample and they were again vortexed and centrifuged. The supernatant was removed and the pellet was air-dried. Each pellet was then dissolved in 100 µL 2% SDS and heated to 65° C.

A quantity of 5 µL of 5x loading buffer (50% glycerol; 10% SDS, 0.25 mol/L Tris pH 6.8) and DTT (final concentration 100 mmol/L) were added to the extracted proteins and the samples were boiled for 10 minutes. Bromophenol blue (1 µL of a 50% w/v solution) was added and proteins were resolved by denaturing SDS-PAGE with a 5% stacking and 8% resolving acrylamide gels in a 0.1% SDS, 25 mmol/L Tris, 192 mmol/L glycine buffer. After fractionation, proteins were electrophoretically transferred to a nitrocellulose membrane (Protran, BA83, Schleicher & Schuell, Keene, NH) in 20% methanol, 0.1% SDS, 25 mmol/L Tris and 192 mmol/L glycine for 3 hours at 60 V.

Membranes were blocked with 5% low-fat powdered milk in Tris-buffered saline with Tween (TBST, 0.1% Tween, 150 mmol/L NaCl, 20 mmol/L Tris). DMT-1 was detected using a Goat IgG polyclonal antibody (Santa Cruz, Temecula, CA) diluted to 1:500 followed by incubation with a horseradish peroxidase conjugated rabbit anti-goat secondary antibody diluted to 1:3000 (Santa Cruz, Temecula, CA), both in TBST and 1% milk for 2 hours and 1 hour, respectively. Protein bands were visualized with the Western enhanced chemiluminescence system (Pierce Chemical). The autoradiograms were quantified by densitometry scanning in conjunction with the TINA v2.09e computer program (Raytest USA, Inc., Wilmington, NC).

2.5. Statistical analysis

The data were analyzed with the SAS system for Windows version 6.12 statistical analysis package (SAS Institute, Cary, NC). Data were examined for normality of distribution and presence of outliers. Box plot analysis was used to test for outliers. Repeated-measures analysis of variance with repeated-measures factors (brain regions) and between-groups factors (dietary treatment) was used to test for interactions between dietary treatments and brain regions for trace metal data. Dunnett's procedure was used to evaluate mean differences of treatments compared to control diet. The α level for the analyses was $P < 0.05$.

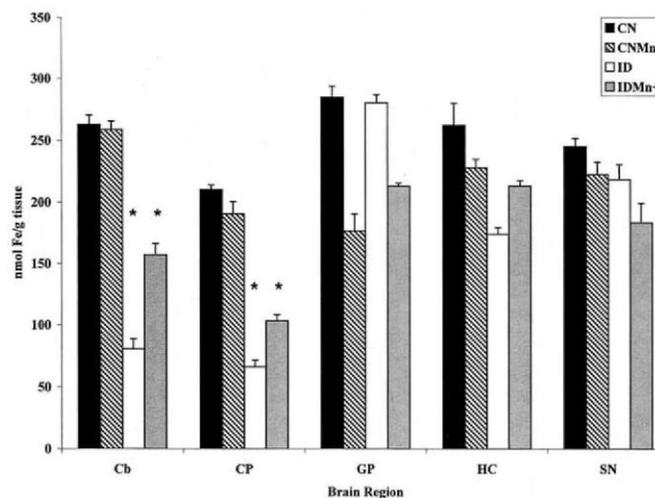


Fig. 1. Iron concentrations in five brain regions of control (CN), control/manganese-supplemented (CNMn+), iron-deficient (ID), and iron-deficient/manganese-supplemented (IDMn+) rats. Brain regions examined were cerebellum (Cb), caudate putamen (CP), globus pallidus (GP), hippocampus (HC), and substantia nigra (SN). Data are expressed as mean \pm SEM. There was a significant effect of ID (ID and IDMn+) on lowering iron concentrations in the Cb and CP as indicated by the asterisks (* $P < 0.05$).

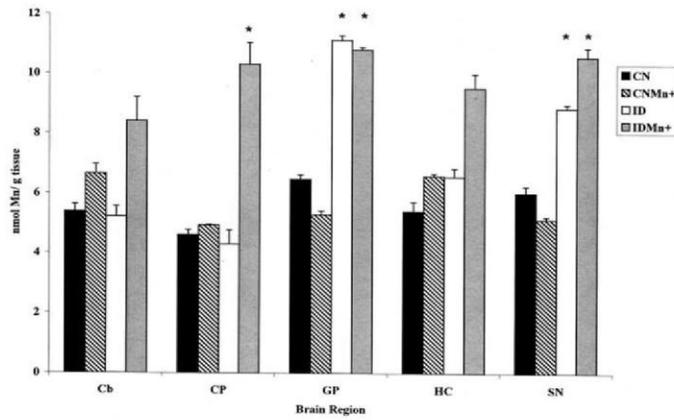


Fig. 2. Manganese concentrations in five brain regions of control (CN), control/manganese-supplemented (CNMn+), iron-deficient (ID), and iron-deficient/manganese-supplemented (IDMn+) rats. Brain regions examined were cerebellum (Cb), caudate putamen (CP), globus pallidus (GP), hippocampus (HC), and substantia nigra (SN). Data are expressed as mean \pm SEM. There was a significant effect of ID on increasing manganese concentrations in the GP and SN as indicated by the asterisks ($*P < 0.05$). There was also a significant interaction between dietary iron and manganese levels with the IDMn+ diet causing elevated manganese levels, the most pronounced being in the CP ($P < 0.05$).

3. Results

3.1. Trace metals

3.1.1. Iron

There was an overall main effect of ID on decreasing brain iron ($P < 0.05$) (Fig. 1). Within brain regions, both ID diets (ID and IDMn+) caused significantly decreased iron concentrations in the cerebellum and caudate putamen compared to control ($P < 0.05$).

3.1.2. Manganese

There was an overall main effect of ID on increasing brain manganese ($P < 0.05$) (Fig. 2). Within brain regions, the rats fed the ID diets (ID and IDMn+) showed increased manganese concentrations in globus pallidus and substantia nigra compared to control rats ($P < 0.05$). In the caudate putamen, only the rats fed the IDMn+ diet had significantly increased Mn concentrations compared to CN ($P < 0.05$).

3.1.3. Zinc

There was no effect of dietary iron or manganese manipulations on overall brain zinc concentrations (Fig. 3). Within brain regions, only the globus pallidus displayed a significant effect of dietary treatment, with the ID diet causing over a 2-fold increase in zinc concentration ($P < 0.05$).

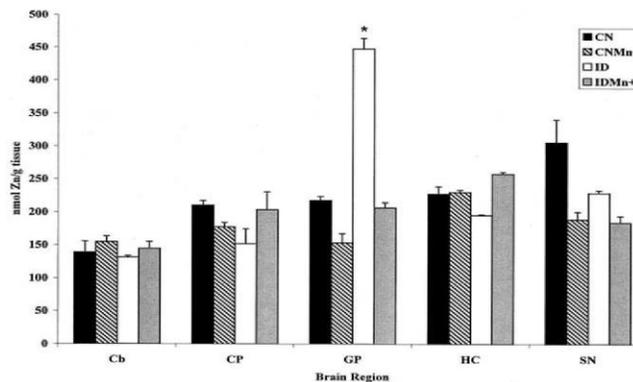


Fig. 3. Zinc concentrations in five brain regions of control (CN), control/manganese-supplemented (CNMn+), iron-deficient (ID), and iron-deficient/manganese-supplemented (IDMn+) rats. Brain regions examined were cerebellum (Cb), caudate putamen (CP), globus pallidus (GP), hippocampus (HC), and substantia nigra (SN). Data are expressed as mean \pm SEM. There was a significant effect of ID on increasing zinc concentration in the GP, as indicated by the asterisk ($*P < 0.05$). This effect was limited to the ID diet alone and was absent in all other brain regions tested.

3.1.4. Copper

There was no effect of dietary iron or manganese levels on overall brain copper concentrations (Fig. 4), nor were there any statistically significant effects of diet within brain regions. However, there was a statistical trend for low dietary iron to cause increased copper concentration in the globus pallidus ($P = 0.08$).

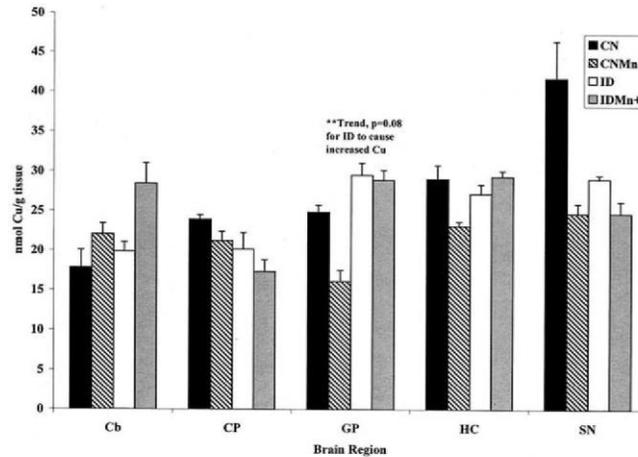


Fig. 4. Copper concentrations in five brain regions of control (CN), control/manganese-supplemented (CNMn+), iron-deficient (ID), and iron-deficient/manganese-supplemented (IDMn+) rats. Brain regions examined were cerebellum (Cb), caudate putamen (CP), globus pallidus (GP), hippocampus (HC), and substantia nigra (SN). Data are expressed as mean \pm SEM. Although there was no statistically significant effects of ID on copper concentration across brain regions, there was a statistical trend ($P < 0.08$) for ID to increase copper in the GP.

3.1.5. Iron/manganese ratio

Iron concentrations were plotted against manganese concentrations in each brain region to examine the relationship between these two metals (Fig. 5). All five brain regions exhibited a decreased iron/manganese ratio, which is indicative of an inverse relationship between iron and manganese concentrations in brain tissue ($P < 0.05$).

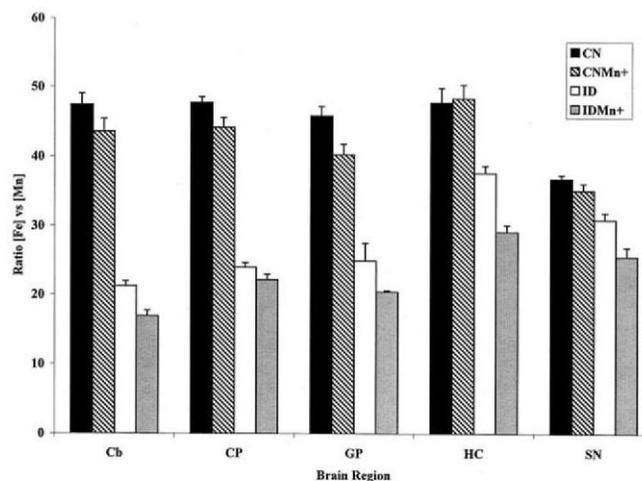


Fig. 5. Iron:manganese ratio in five brain regions of control (CN), control/manganese-supplemented (CNMn+), iron-deficient (ID), and iron-deficient/manganese-supplemented (IDMn+) rats. Brain regions examined were the cerebellum (Cb), caudate putamen (CP), globus pallidus (GP), hippocampus (HC), and substantia nigra (SN). Data are expressed as mean \pm SEM. There was a significant effect ($P < 0.05$) of ID on decreasing the iron:manganese ratio in all brain regions tested.

DMT-1 Western blot analysis

Western blot analyses in each brain region revealed that some brain regions were dramatically affected by ID (*i.e.*, increased DMT-1 levels as in hippocampus, globus pallidus, and substantia nigra) (Fig. 6), whereas no significant changes in DMT-1 protein levels were observed in the cerebellum and cortex (data not shown).

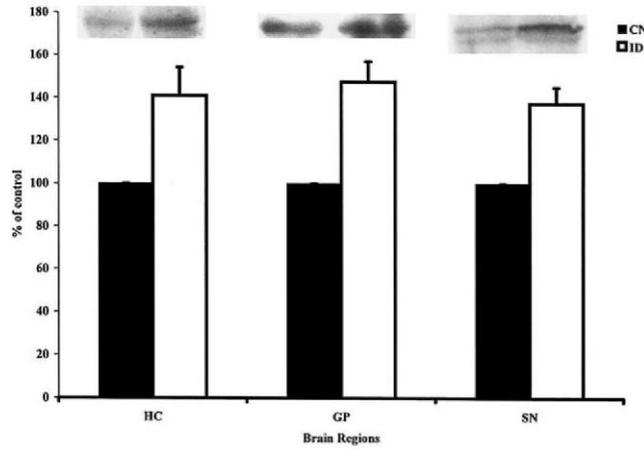


Fig. 6. Western blot analysis of divalent metal transporter (DMT-1) protein levels in three brain regions of control (CN) and iron-deficient (ID) rats. Brain regions examined were globus pallidus (GP), hippocampus (HC), and substantia nigra (SN). Data are expressed as percentage of control levels. Increased DMT-1 levels occurred in all three regions, with the most pronounced change occurring in the globus pallidus.

4. Discussion

This study investigated the effects of dietary iron and manganese manipulation on brain regional copper, iron, manganese, and zinc levels. To our knowledge, this is the first time that such a study has been undertaken. Several novel findings have emerged from this study, the first being that ID had a significant effect on divalent metal concentrations in the globus pallidus. Manganese and zinc concentrations were significantly elevated in the globus pallidus due to ID, and there was a statistical trend ($P = 0.08$) for elevated copper in this region. This brain region is known for its high concentration of manganese, particularly after exposure to high doses of manganese. It is believed to be the primary area of manganese-induced neurotoxicity (manganism) [18]. Its responsiveness to ID was recently elucidated [11] with respect to manganese accumulation. However, no studies precede our findings of increased zinc and copper levels in this seemingly vulnerable region. Our data would suggest that ID-induced increased DMT-1 levels in the globus pallidus may be responsible, at least in part, for this specific accumulation of manganese, zinc, and copper.

Zinc homeostasis is essential for proper neurological functioning, especially for glutamatergic modulation [20,21]. The globus pallidus is important for motor control via its GABAergic and glutamatergic innervations. The role of zinc in pallidal functioning is unknown; however, decreased or increased zinc concentrations would seriously impact this region. It has been shown that zinc uptake can occur at the postsynaptic site [20] and that zinc release occurs from glutamatergic neurons [30]. Previous studies suggest a disruption of glutamate metabolism occurs due to ID [11,31], specifically in cortex and striatum. Therefore, our finding of significantly increased zinc levels in the globus pallidus due to ID may be indirectly caused by a disturbance in glutamate biology. Another possible cause of this ID-associated accumulation of zinc in this region revolves around transport protein(s) and is discussed below.

The hippocampus is thought to be the brain region that is richest in zinc. However, in this current study, the substantia nigra had the highest concentration of zinc (Fig. 3) when comparing control levels across regions. This could reflect a difference in dissection techniques, or it could be related to the analytical technique used to measure the zinc concentrations (*i.e.*, ICP-MS is a highly sensitive tool and could detect concentrations that are undetectable via other techniques such as ^{65}Zn uptake methods). Finally, it should be noted that statistical differences in zinc concentration across regions is fully attributable to levels in the globus pallidus, and if the zinc concentration in the ID treated rats in the globus pallidus is removed from the analysis, no statistical differences exist between brain regions.

Divalent metal transporter (DMT-1) levels increased in most brain regions due to ID compared to controls. This effect was most apparent in the globus pallidus, where ID caused a 60% increase in DMT-1 protein. Apart from the substantia nigra, this was the most dramatic response to ID in terms of DMT-1 expression. It is likely that DMT-1 is involved in the accumulation of manganese [28] and, to a lesser extent zinc [32], in the globus pallidus of ID rats. It is known that transferrin receptor (TfR) levels increase due to ID in a brain region-specific manner [16]. Although globus pallidus was not specifically examined in this prior study [16], it is probable that TfR increases and that in conjunction with DMT-1, manganese accumulates in a region-specific manner. This partnership between TfR and DMT-1 in the transport of brain manganese is a possible explanation for the heterogeneous accumulation of manganese due to ID. That is, in some brain regions, TfR levels increase more dramatically (*e.g.*, caudate putamen) and may be responsible for most of the accumulated manganese due to ID, whereas in other regions (globus pallidus), DMT-1 levels may be the primary conduit. Furthermore, this differential transport mode could interact with the metabolism of other divalent metals in the brain during ID (*i.e.*, brain regions where DMT-1 levels are more readily increased upon ID are more likely to accumulate copper, zinc, or other divalent metals, particularly if these metals are in excess).

Although DMT-1 has been implicated in the transport of zinc and copper into the brain, more specific transporters exist for these two metals. Ctr1 is important for copper transport in the brain [33], whereas ZnT3 has been shown to have a role in brain zinc transport [34]. The effect of ID on these proteins is unknown. Furthermore, the distribution of these proteins in the brain has not been examined, including conditions in which the nutritional status is compromised. It is possible that, as with the putative partnership between TfR and DMT-1, a regulatory interaction exists between Ctr1, ZnT3, and DMT-1, causing specific accumulation to occur in the globus pallidus.

The ratio of brain regional iron *versus* manganese concentrations reveals that ID leads to a significantly lower ratio across brain regions. In other words, significantly decreased iron and/or increased manganese prevail in all regions because of ID. Interestingly, the brain regions that were affected most dramatically by ID in terms of decreased iron levels (cerebellum and caudate putamen) differed from those in which manganese levels significantly accumulated due to ID (globus pallidus and substantia nigra). An exception was that the IDMn+ dietary treatment caused a significant accumulation of manganese in the caudate putamen compared to control, an effect that was not evident due to ID diet alone. In fact, there was a significant interaction between dietary levels of iron and manganese ($P = 0.0127$). This is evidenced by the increased manganese concentration in the IDMn+ treated rats in all of the brain regions except the globus pallidus. By expressing these concentrations as the “iron:manganese ratio,” it is quite clear that there is an inverse relationship between these two divalent metals in the brain, specifically the globus pallidus (Fig. 5).

This study shows for the first time that ID-induced accumulation of divalent metals occurs predominantly in the globus pallidus. Our understanding of the tendency of the globus pallidus to accumulate divalent metals caused by ID has been extended from manganese to include zinc. Increased zinc accumulation may be the result of altered glutamate biology in the cortex and/or striatum [11,31] or it may be linked to increased transporter protein (DMT-1) caused by ID, which in turn may lead to altered glutamate biology. In future studies, this relationship between ID-associated zinc accumulation and glutamate should be examined from a pharmacological aspect (*e.g.*, NMDA antagonist administration to block glutamate-associated zinc release). These studies should also include the measurement of other zinc transporter proteins (*e.g.*, ZnT3) in ID and control rat brain regions. The iron/manganese ratios across dietary treatments and within brain regions revealed that ID significantly increased manganese levels in all the examined regions. The relationship between iron and zinc expressed as iron/zinc ratios (data not shown) did not reveal the same correlation as did iron/manganese ratios. It is clear that there is an inverse relationship between iron and manganese concentrations on a regional basis in the brain. This is not the case for iron and zinc, although in the globus pallidus there is a yet to be defined effect of ID on zinc concentration.

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