Olfactory uptake of manganese requires DMT1 and is enhanced by anemia

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ABSTRACT **Manganese, an essential nutrient, can also elicit toxicity in the central nervous system (CNS). The route of exposure strongly influences the potential neurotoxicity of manganese-containing compounds. Recent studies suggest that inhaled manganese can enter the rat brain through the olfactory system, but little is known about the molecular factors involved. Divalent metal transporter-1 (DMT1) is the major transporter responsible for intestinal iron absorption and its expression is regulated by body iron status. To examine the potential role of this transporter in uptake of inhaled manganese, we studied the Belgrade rat, since these animals display significant defects in both iron and manganese metabolism due to a glycine-to-arginine substitution (G185R) in their DMT1 gene product. Absorption of intranasally instilled ^{54}Mn was significantly reduced in Belgrade rats and was enhanced in iron-deficient rats compared to iron-sufficient controls. Immunohistochemical experiments revealed that DMT1 was localized to both the lumen microvilli and end feet of the sustentacular cells of the olfactory epithelium. Importantly, we found that DMT1 protein levels were increased in anemic rats. The apparent function of DMT1 in olfactory manganese absorption suggests that the neurotoxicity of the metal can be modified by iron status due to the iron-responsive regulation of the transporter.—Thompson, K., Molina, R. M., Donaghey, T., Schwob, J. E., Brain, J. D., Wessling-Resnick, M. Olfactory uptake of manganese requires DMT1 and is enhanced by anemia. *FASEB J.* 21, 223–230 (2007)**

Key Words: Belgrade rat · iron deficiency

INHALATION OF MANGANESE PROMOTES its deposition in the brain and can lead to neurotoxic effects. Clinical cases of manganism are associated with elevated levels of manganese in the basal ganglia accompanied by neuronal loss and an extrapyramidal movement disorder preceded by psychiatric symptoms (1). Occupational exposures that can result in manganism include metal-working, mining, and battery manufacture as well as pesticide use (2, 3). Recent debate over potentially harmful effects of low level chronic manganese exposures have been sparked by the use of the fuel additive methylcyclopentadienyl manganese tricarbonyl (MMT) (4–6) and the increased environmental burdens resulting from its combustion (7–9).

The route of exposure strongly influences the potential neurotoxicity of manganese-containing compounds (10, 11). Recent studies have suggested that inhaled manganese can enter the rat brain through the olfactory pathway (12–15), but little is known about the molecular factors involved. DMT1 is the major transporter responsible for intestinal iron absorption and its expression is regulated by body iron status (16, 17). Exogenous expression studies have shown that DMT1 mediates uptake of manganese as well as iron (18, 19). To examine the potential role of this transporter in uptake of inhaled manganese, we chose to study the Belgrade (*b*) rat since its DMT1 gene product contains a glycine-to-arginine substitution at codon 185 (G185R; ref 20). This defective allele encodes a protein with diminished activity in iron uptake assays (21–23). Accordingly, Belgrade rats are not only anemic but also display significant defects in manganese metabolism (24). Our results show that absorption of intranasally instilled ^{54}Mn was significantly reduced in Belgrade rats, whereas nasal absorption was enhanced in iron-deficient rats compared to iron-sufficient controls. DMT1 was localized to both the lumen microvilli and end feet of sustentacular cells of the olfactory epithelium and protein levels were increased in anemic rats. The apparent function of DMT1 in olfactory manganese absorption suggests that the neurotoxicity of the metal can be modified by iron status due to the iron-responsive regulation of the transporter.

MATERIALS AND METHODS

Animals and Diets

Animal protocols were approved by the Harvard Medical Area Animal Care and Use Committee. Belgrade rats (*b*/*b*) were

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bred from an original breeding triad obtained from Dr. Michael Garrick (SUNY Buffalo, NY) and were housed in microislolator cages with free access to water and rodent chow.

Mating pairs of female heterozygotes (+/*b*) and male homozygotes (*b*/*b*) were fed an iron-supplemented diet containing 500 ppm iron (Harlan Teklad TD 02385; Harlan Teklad, Madison, WI). Female +/*b* rats were maintained on the iron-supplemented diet prior to and throughout the pregnancy, while all *b*/*b* rats received this diet postweaning as recommended by Garrick *et al.* (25). At postnatal day 6, litters were cross-fostered to F344 Fischer female dams (Harlan Teklad) fed a standard chow diet containing 200 ppm iron (Purina diet #5053; PharmaServe, Framingham, MA). Three experimental groups were established at the time of weaning: *b*/*b* and +/*b* rats fed the iron-supplemented diet and +/*b* rats fed a low-iron diet containing 20 ppm iron (Harlan Teklad TD 99397) to induce iron deficiency.

For DMT1 immunostaining experiments, 21 day old Sprague-Dawley rats (Taconic Farms, Inc., Germantown, NY) were fed standard chow (200 ppm) or iron-deficient diet (20 ppm iron; TD 99397, Harlan Teklad) for 3 wk. After 3 wk, hematocrits were 40.1 and 22.3% for rats fed control and iron-deficient diet ($P<0.05$; $n=4$). Rats were anesthetized with vaporized isofluorothane, snouts were collected, and then processed and fixed for DMT1 staining as detailed below.

Genotyping

Belgrade *b*/*b* rats were identified at birth by their anemic features and their genotype was verified by polymerase chain reaction (PCR). Briefly, forward (5′-TATCCCAAGGTCCCACGGAT) and reverse (5′-GAGGGCCATGATAGTGATGA) primer pairs were used to identify either a single 940 bp product for *b*/*b* rats or an additional 890 bp product for +/b rats (20).

Pharmacokinetics and Tissue Uptake of $^{54}MnCl_2$

Rats were anesthetized with vaporized halothane prior to delivery of radioisotope. For intranasal instillation, $^{54}MnCl_2$ (Perkin Elmer/NEN, Boston, MA) was diluted in PBS to 75 μCi/ml and a volume of 0.2 ml/kg body wt was administered through polyethylene tubing into the right nostril of the rat to a distance of ~10–15 mm as described by Tjälve *et al.* (26). For intravenous injection, $^{54}MnCl_2$ was diluted in PBS to 30 μCi/ml and a volume of 0.5 ml/kg body wt was injected into the lateral tail or penile vein. Blood samples were collected from the tail artery vein at 5, 15, and 30 min and at 1, 2, and 4 h postinstillation or -injection. Blood samples for hematocrit determination were taken at the 5 min time period. Rats were humanely killed 4 h postinstillation or injection. Tissue and blood samples were collected, weighed, and radioactivity measured in a Packard gamma counter (Cobra Quantum, Packard Instrument, Downers Grove, IL). Tissue uptake was calculated as a percentage of the instilled or injected dose of ^{54}Mn. Some dissections were carried out one week postadministration following the same procedures except that after the total brain ^{54}Mn was determined, the brains were microdissected into different regions (cortex, brainstem, hippocampus, basal ganglia, substantia nigra, and cerebellum) to measure ^{54}Mn as described above.

Immunohistochemistry

Tissue processing and staining was performed according to Goldstein and Schwob (27) and Holbrook *et al.* (28) for epithelial markers and according to Canonne-Hergaux *et al.* (29) for DMT1. Rabbit DMT1 antisera (N-terminal) was a kind gift of Dr. Philippe Gros (McGill University, Montreal, QC, Canada). Briefly, after fixation, the rat nasal regions were separated from surrounding tissue and decalcified in 0.5 mM EDTA at pH 7.0, cryoprotected with 30% sucrose, embedded in O.C.T. compound (Tissue-Tek, Hatfield, PA), and frozen in liquid nitrogen until cryo-sectioned (8 μm). Labeling of olfactory epithelial markers SUS-4 (mouse anti-SUS4, 1:40; ref 27) or anti-human neuronal protein HuC/HuD (mouse anti-HuC/D, 1:500; Molecular Probes/Invitrogen, Carlsbad, CA) was detected using Cy-3 anti-mouse antisera (Jackson ImmunoResearch Laboratories, West Grove, PA); the flouresceine tyramide signal amplification (TSA) system (PerkinElmer) was used to detect rabbit anti-DMT1 (1:2500). Detection of rabbit anti-DMT1 and rabbit anti-cytokeratin double labeling was achieved using the TSA System (PerkinElmer) for rabbit anti-DMT1 (1:2500) and rabbit anti-cytokeratin (1:250) using Cy-3 labeled anti-rabbit antisera (Jackson ImmunoResearch Laboratories) for the latter (30). To quantify the levels of DMT1 in control and iron-deficient rats, anti-DMT1 (1:250) was visualized using peroxidase-based immunohistochemistry with swine anti-rabbit IgG (1:50) and rabbit anti-PAP (1:100; Dako North America, Carpinteria, CA). Infrared imaging (21 μm resolution; Odyssey Infrared Imaging System, Li-Cor, Lincoln, NE) of nickel chloride-enhanced 3,3′-diaminobenzidine staining was performed using Odyssey v1.2 image analysis software. Antibody (Ab) controls performed for all of the immunohistochemical experiments showed no staining, confirming specificity of detection.

Analytical Procedures

Results are mean ± SEM. Pharmacokinetic results and the distribution of radioisotope among brain regions were evaluated by multivariate ANOVA (MANOVA) using the general linear model procedure (SAS statistical software, SAS Institute, Cary, NC). *P* values for tissue ^{54}Mn uptake were determined by F test. Comparisons of hematocrit values were evaluated by MANOVA. Statistical significance was considered to be $P < 0.05$.

RESULTS

Pharmacokinetics of ^{54}Mn absorption

Three experimental groups of rats were established for our study: homozygous Belgrade weanlings fed an iron-supplemented diet (*b*/*b*), a cohort of heterozygote siblings fed the same iron-supplemented diet (iron-replete +/*b*), and a cohort of heterozygote siblings fed an iron-deficient diet (iron-deficient +/*b*). After 3 wk on this diet, hematocrits for *b*/*b*, iron-replete +/*b*, and iron-deficient +/*b* were 32.2, 42.9, and 23.2%, respectively ($n=5$, $P<0.001$). The iron deficiency of *b*/*b* rats results from impaired intestinal iron absorption due to loss of DMT1 activity; therefore, the iron-deficient +/*b* cohort was established as a control for the state of anemia. To monitor manganese absorption from the nasal cavity to the blood, $^{54}MnCl_2$ was intranasally instilled, and blood radioisotope levels were determined from 5 min to 4 h postinstillation. The pharmacokinetics of metal absorption varied significantly among the three experimental groups (**Fig. 1*A***), with markedly reduced uptake by *b*/*b* rats. By comparison,

iron-deficient $+/b$ controls showed enhanced absorption relative to both b/b rats and iron-replete $+/b$ controls. Levels of ^{54}Mn remaining in circulation at 4 h for b/b, iron-deficient $+/b$ and iron-replete $+/b$ rats were 0.022, 0.115, and 0.065% of instilled dose, respectively.

To evaluate the potential contribution of ^{54}Mn clearance from the blood to peripheral tissues after nasal metal absorption, blood isotope levels were also determined after intravenous injection of the radioisotope. The pharmacokinetics for *iv*-injected ^{54}Mn did not differ among the three groups (Fig. 1*B*). At the 4 h time point, however, b/b and $+/b$ control blood levels were statistically different with more ^{54}Mn remaining in the blood (0.380 *vs.* 0.137 and 0.169% for iron-supplemented and iron-deficient $+/b$ controls, respectively). The pharmacokinetic data are consistent with a key role for DMT1 in manganese tissue uptake in that more ^{54}Mn would remain in the blood of Belgrade rats over time due to impaired transport activity. We can infer from these data that the pharmacokinetics of intranasally-instilled manganese (Fig. 1*A*) must largely reflect ^{54}Mn absorption across the olfactory epithelium into circulation.

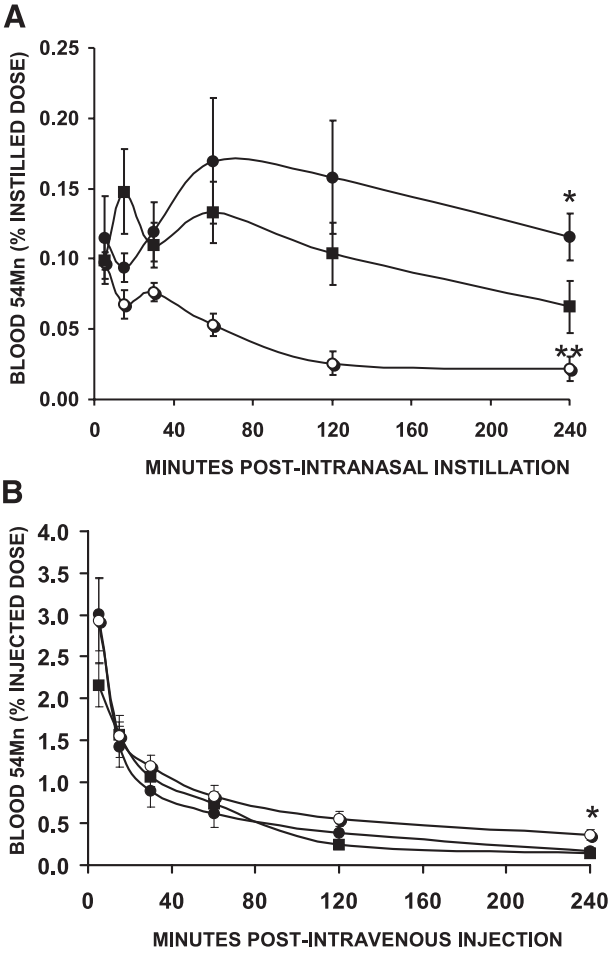

Figure 1. Vascular kinetics of intranasally instilled (*A*) and i.v. injected (*B*) ^{54}Mn. Anemic $+/b$ rats (filled circles) were fed an iron-deficient diet (20 ppm iron) while iron-replete $+/b$ rats (filled squares) and Belgrade b/b rats (open circles) were fed iron-supplemented diet (500 ppm iron) for three weeks postweaning. Blood ^{54}Mn levels are expressed as percent of instilled dose or injected dose. Each point is mean ± SEM of 5–8 rats. *A)* $*P < 0.05$ (iron-replete $+/b$ *vs.* anemic $+/b$); $**P < 0.01$ (b/b *vs.* anemic $+/b$ rats). *B)* $*P < 0.05$ (b/b *vs.* anemic $+/b$ and b/b *vs.* iron-replete $+/b$).

Distribution of ^{54}Mn between regions of the brain

To determine the pattern of manganese deposition in the brain, rats were instilled or injected with ^{54}Mn, and then analyzed 14 d later. Following intranasal installation, b/b rats accumulated less radioisotope in the basal ganglia and hippocampus compared with iron-deficient $+/b$ controls (**Fig. 2*A***). In contrast, after intravenous injection, iron-deficient $+/b$ rats accumulated less ^{54}Mn in all brain areas compared to either b/b or iron-supplemented $+/b$ rats (Fig. 2*B*).

Cytolocalization of DMT1 in the olfactory epithelium

Immunohistochemical experiments were carried out to characterize the distribution of DMT1 in the nasal cavity. Intense immunoreactivity was detected throughout the rat olfactory epithelium (OE; **Fig. 3**). The OE is comprised of sustentacular cells, mature and immature neurons, and basal cells. Sustentacular cells are non-neuronal supporting cells with microvilli rather than cilia, which are more typical of airway respiratory epithelial cells. A series of staining experiments revealed that DMT1 immunoreactivity overlapped extensively with SUS-4, a marker for sustentacular cells (27), in the region of sustentacular cell microvilli and end foot processes near the basal lamina (**Fig. 4*A–C***). Cytokeratin immunoreactivity, an identifier of basal cells (28), revealed that DMT1 staining surrounded cytokeratin positive cells, where sustentacular end feet reside (Fig. 4*D–F*). DMT1 did not colocalize with the neuronal Ab anti-Hu (Fig. 4*G–I*). DMT1 immunoreactivitiy was also found in the mucociliary complex (MC). Manganese superoxide dismutase (SOD) (31, 32) and the metal binding protein lactoferrin (33) have also been detected in the MC region.

Iron-responsive expression of DMT1 in the olfactory epithelium

Increased DMT1 levels observed in anemic animals are thought to be due to the presence of an iron-responsive element (IRE) in the 3′ untranslated region of certain transcripts (16, 17). To determine whether DMT1 expression in the olfactory epithelium is regulated by iron status, we compared staining intensity for the protein expressed in Sprague-Dawley rats fed a normal (200 ppm) or iron-deficient (20 ppm) diet. Quantified by infrared analysis of nickel chloride enhanced 3.3′-diaminobenzidine staining, anemic rats had 1.5- to 2.5-fold greater DMT1 levels where a striking increase in staining for DMT1 was detected in the sustentacular end-feet (**Fig. 5**).

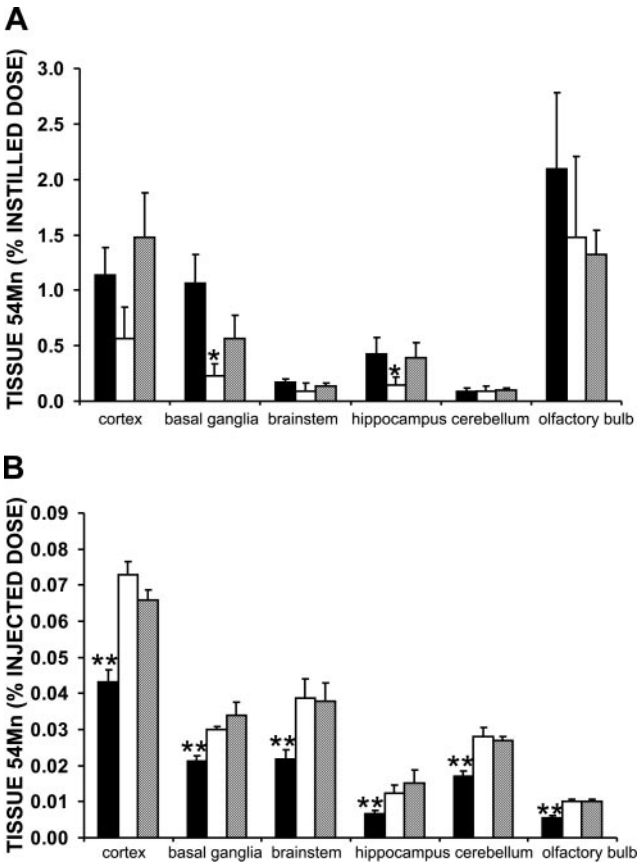

Figure 2. Brain microdissections of intranasally-instilled (*A*) and i.v.-injected (*B*) ^{54}Mn. Anemic $+/b$ rats (solid bars) were fed an iron-deficient diet (20 ppm iron) while iron-replete $+/b$ rats (hatched bars) and Belgrade b/b rats (open bars) were fed iron-supplemented diet (500 ppm iron) for 3 wk postweaning. Two weeks after intranasal instillation or iv injection, rats were humanely killed and brains were collected for microdissection. Tissue ^{54}Mn levels are expressed as percentage of instilled dose or injected dose. Each point is mean ± SEM of 5–8 rats. *A)* $*P < 0.05$ (*b/b vs.* iron-replete $+/b$ and *b/b vs.* anemic $+/b$). *B)* $**P < 0.05$ (iron-replete $+/b$ *vs.* b/b and iron-replete $+/b$ *vs.* anemic $+/b$).

DISCUSSION

Increased absorption and retention of manganese in the brain of iron-deficient rats have been well documented (34–37). In human subjects, correlations between elevated manganese levels in the blood and lower serum ferritin levels (38), lower dietary intake of nonheme iron (39), and higher levels of circulating soluble transferrin receptor (40) have been reported. In early studies, Mena *et al.* (41) found that iron-deficient men and women absorbed more manganese than control subjects, as measured by whole body counts after ingestion of $^{54}MnCl_2$. This relationship between iron and manganese is now thought to arise from the fact that they share at least one transporter, DMT1(42). *In vitro*, DMT1 has been shown to interact with both metals (16). Over-expression of DMT1 in cultured cells results in increased uptake of iron and manganese (43) and incubation with anti-DMT1 Ab blocks uptake of ferrous iron and manganese (18). *In vivo*, manganese (II) and iron (II) are thought to compete for uptake via DMT1 at the apical surface of duodenal enterocytes. This model is strongly supported by studies of the Belgrade rat, which displays impaired iron (20–23) and manganese (24) metabolism due to its defective DMT1 allele (20).

DMT1 levels are dramatically upregulated in enterocytes of iron-deficient rats (20, 29). Two of the known DMT1 mRNA isoforms contain IREs and are therefore potentially subject to regulation by iron responsive proteins (IRPs) (44). The idea that iron-responsive regulation of manganese uptake occurs is consistent with the animal and human studies noted above that demonstrate iron deficiency will increase intestinal manganese absorption (13, 38, 39, 45, 46). In this study, we directly examined the hypothesis that DMT1 mediates uptake of manganese across the olfactory epithelium. Our pharmacokinetic studies demonstrate that absorption of ^{54}Mn from the nasal cavity to the blood is lower in b/b rats with impaired DMT1 function. Moreover, iron deficiency results in enhanced circulating levels, and this functional effect correlates with increased expression of DMT1 in olfactory epithelium.

Excess manganese can influence cognitive function and behavior. Manganese, like iron, is required for proper development and brain function (47). The relationship between iron deficiency and manganese absorption is of particular interest because develop-

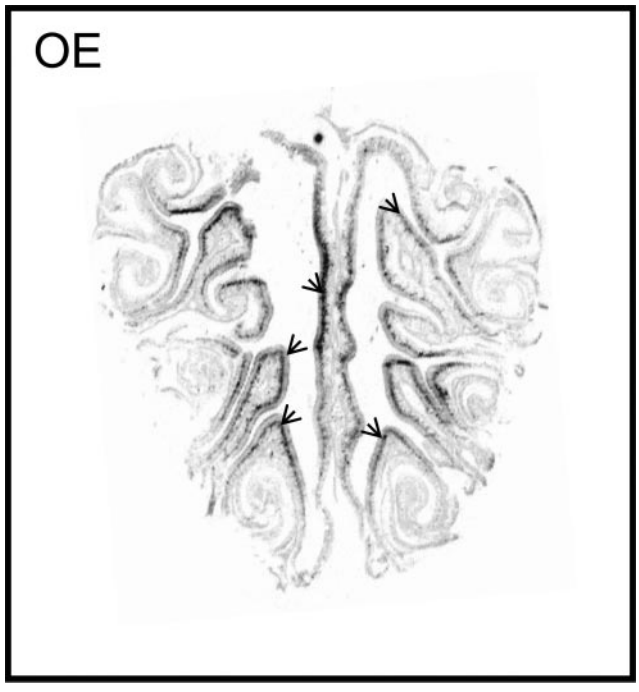

Figure 3. DMT1 immunoreactivity in the rat olfactory epithelium. Infrared imaging (Li-Cor Odyssey) was used to detect nickel chloride enhanced 3′3-diaminobenzidine staining for DMT1 immunoreactivity using a modified protocol of Canonne-Hergaux *et al.* (27, 29) in tissue processed according to the methods of Goldstein and Schwob (27). DMT1 staining (arrows) is found throughout olfactory epithelial regions of the nasal concha.

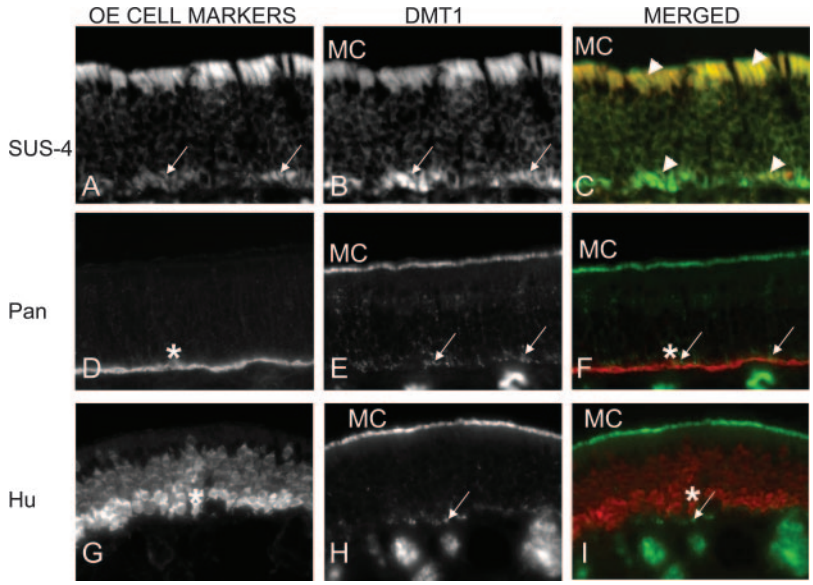

Figure 4. Colocalization of DMT1 with olfactory epithelial markers. Sustentacular cells were labeled with anti-SUS-4 (arrows *A,C*), basal cells with anti-cytokeratin (asterisks *D,F*), and neurons with anti-Hu (asterisks *G,I*) (40X magnification). Colocalization of SUS-4 (red) with DMT1 (green) is noted in *C* (arrowheads). DMT1 immunoreactivity is also detected in the mucociliary complex (MC).

mental and behavioral problems are observed in children with iron deficiency anemia (48, 49). Accumulating evidence indicates children with high manganese also have impaired cognitive function (50–56). Exposure to high manganese in drinking water and diet is relatively rare, and delivery of manganese to the liver after intestinal absorption via portal circulation creates a protective barrier against the toxicity of metal by providing for first pass clearance of excess metal into bile. However, entry of airborne manganese circumvents this protective clearance mechanism. High levels of airborne manganese are common in occupational settings of mining, manganese ore processing, dry battery manufacture, and organochemical fungicide use (2, 3). In 1958, Cotzias (57) reviewed several hundred cases of manganese intoxication and concluded that severe neurological systems resulted from inhalation of dust or fumes containing this metal, providing the first description of "manganese madness" or *locura manganica*. Thus, an important finding from our study was that after intranasal instillation, *b/b* rats accumulated less radioisotope in the basal ganglia and hippocampus, key areas known to be affected by manganese neurotoxicity. In contrast, after intravenous injection, iron-deficient *+/b* rats accumulated less ^{54}Mn in all brain areas compared to either *b/b* or iron-replete *+/b* rats. The latter results agree with previous reports that DMT1 is not involved in the blood-brain transport of manganese (45) and further show that under anemic conditions, uptake of manganese from circulation to the brain is significantly reduced. The greater ^{54}Mn levels observed particularly in the basal ganglia of anemic *+/b* rats provide a further indication that iron status can modify the distribution and therefore the potential neurotoxicity of inhaled manganese. Overall, brain isotope levels of intranasally instilled rats were >10-fold higher than intravenous-injected animals, supporting the idea that inhalation promotes greater brain manganese uptake, possibly because the nasal cavity can act as a reservoir to prolong metal exposure to a given dose and that direct olfactory transport of ^{54}Mn to the brain occurs (12–15). Evidence from our study shows that DMT1 function can directly contribute to these effects.

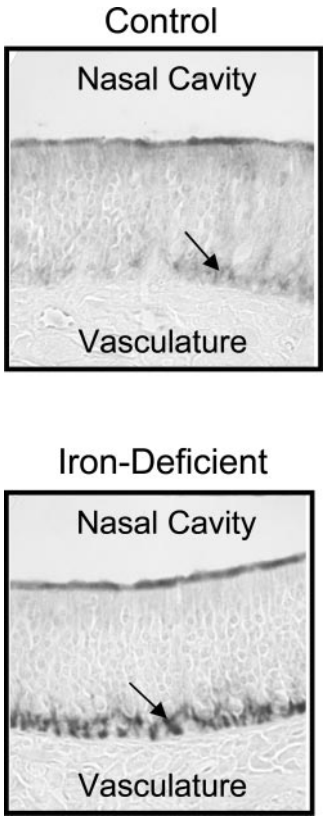

Figure 5. DMT1 staining in control and iron-deficient rats. Weanling Sprague Dawley rats were fed an iron-deficient diet (Harlan Teklad TD 99397) or standard chow (Purina 5061) for 3 wk. Olfactory DMT1 staining intensity was increased in iron-deficient rats. Region that includes the sustentacular cell end feet (arrows) shows greatest increase in iron deficiency for DMT1 staining.

The function of DMT1 in the mammalian olfactory pathway was heretofore unknown. Immunohistochemical experiments showed that DMT1 staining overlapped extensively with markers for sustentacular cells in the region of lumenal microvilli and end foot processes near the basal lamina. Both apical and basolateral DMT1 staining have been reported for intestinal epithelial cells (29, 46). The presence of DMT1, particularly in the apical membrane of sustentacular cells, is consistent with the functional role in manganese uptake from the nasal cavity across the olfactory epithelium supported by our instillation studies. Other studies characterizing the transport of manganese to the brain by the olfactory route have indicated that such uptake can lead to neurotoxicity (12, 43, 56, 57). Interestingly, DMT1-related proteins are associated with taste and smell behaviors in insects (58–62), suggesting additional functions in olfaction for DMT1-mediated metal uptake.

Enhanced absorption of intranasally instilled manganese in anemic rats was associated with increased DMT1 expression in olfactory epithelium, perhaps due to IRP-IRE regulation due to iron deficiency. However, the increased staining was predominantly found in the region of the sustentacular end-feet near the basal lamina. The functional consequence of increased expression of DMT1 in this region and the relationship between neuronal function and manganese transport is uncertain at this time and requires future investigation. It is worth noting that sustentacular cells require iron for the incorporation of heme into enzymes of the cytochrome P450 family, which are highly concentrated in this cell type (58).

Our combined data suggest that manganese neurotoxicity can be modified by iron status due to iron-responsive regulation of DMT1 and the function of this transporter in manganese absorption across the olfactory epithelium. Arguments have been raised that rats are not an appropriate model for human exposure to manganese since they are obligatory nose breathers and their nasal passages are larger in size relative to body mass. However, despite the differences in size, shape, and breathing mode between rats and humans, the percentage of air flow that reaches their olfactory mucosa is comparable, suggesting that potential absorption of airborne metals by rats and humans would be similar (31, 59). The relationship between iron deficiency and olfactory absorption of manganese raises critical concerns about the risks associated with exposures to airborne manganese in populations that are vulnerable to iron deficiency anemia. Today's improved work conditions have resulted in far fewer clinical cases of occupational manganese neurotoxicity, but in a recent study focusing on workers from a manganese alloy plant, Mergler *et al.* (60) noted subtle neurological defects associated with higher levels of blood manganese in the non-"occupationally" exposed reference population. This observation prompted a more detailed community-based survey of the area where the manganese alloy plant had closed. In their follow-up analysis, this group found that neurological effects resulting from chronic manganese exposure vary considerably on an individual basis, falling into what these authors termed a "continuum of dysfunction" and giving rise to early, subtle changes at lower exposure levels (61). Based on this model, we speculate that iron status could be a critical determinant of one's susceptibility to airborne manganese intoxication. A troubling prediction of our hypothesis is that neurological complications of poor iron status could be compounded by an increased vulnerability to manganese exposure. Thus, children with iron deficiency anemia may be at particular risk for neurotoxic effects of airborne manganese. Future work is necessary to better define those risks and to determine the precise role for DMT1 in the olfactory pathway of metal absorption. FJ

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