Increased manganese uptake by primary astrocyte cultures with altered iron status is mediated primarily by divalent metal transporter

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Abstract:
Neurotoxicity due to excessive brain manganese (Mn) accumulation can occur via occupational exposure to aerosols or dusts that contain extremely high levels (> 1–5 mg Mn/m^3) of Mn, or metabolic aberrations (decreased biliary excretion). Given the putative role of astrocytes in regulating the movement of metals across the blood-brain barrier, we sought to examine the relationship between iron (Fe) status and Mn transport in astrocytes. Furthermore, our study examined the effect of Fe status on astrocytic transferrin receptor (TfR) and divalent metal transporter (DMT1) levels and their relationship to Mn uptake, as both have been implicated as putative Mn transporters. All experiments were carried out in primary astrocyte cultures derived from neonatal rats when the cells reached full confluency (about three weeks in culture). Astrocytes were incubated for 24 h in astrocyte growth medium (AGM) containing 200 μM desferroxamine (ID), 500 μM ferrous sulfate (+Fe), or no compound (CN). After 24 h, 5 min ^{54}Mn uptake was measured and protein was harvested from parallel culture plates for DMT-1 and TfR immunoblot analysis. Both iron deprivation (ID) and iron overload (+Fe) caused significant increases ($p < 0.05$) in ^{54}Mn uptake in astrocytes. TfR levels were significantly increased ($p < 0.05$) due to ID and decreased in astrocytes exposed to +Fe treatments. As expected, DMT-1 was increased due to Fe deprivation, but surprisingly, DMT-1 levels were also increased due to +Fe treatment, albeit not to the extent noted in ID. The decreased TfR associated with +Fe treatment and the increased DMT-1 levels suggest that DMT-1 is a likely putative transporter of Mn in astrocytes.
Keywords: Rat; Astrocytes; Iron; Manganese; Divalent metal transporter; Transferrin receptor

Article:
1. Introduction
Astrocytes maintain brain homeostasis through precise regulation of extracellular constituents. Astrocytes perform several functions that are essential for normal neuronal activity, including glutamate uptake, glutamate release, ionic buffering, and water transport. Manganese (Mn) is one such constituent in which astrocytic control is critical for normal neurological function. The astrocyte efficiently transports Mn, and an astrocyte specific manganoprotein critical for ammonia metabolism, glutamine synthetase (GS), accounts for about 80% of the total brain Mn (Wedler and Denman, 1984). The precise transporter(s) for Mn into astrocytes is unknown; however, it has been suggested that transferrin receptor (TfR) and/or divalent metal transporter (DMT-1) proteins may be important. Because these proteins are linked to iron (Fe) homeostasis and the response(s) to changes in Fe status by astrocytes are relatively unexplored, we sought to examine the effect of varying Fe concentrations on TfR and DMT-1 protein levels in primary astrocyte cultures. Furthermore, we sought to study the effects of Fe deprivation and Fe overload on astrocytic Mn uptake.

Animal studies have demonstrated that iron deficiency (ID) enhances Mn absorption across the gastrointestinal tract independent of body Mn stores (Chandra and Shukla, 1976; Shukla et al., 1976). An inverse association between body Fe stores and Mn absorption has also been demonstrated in humans (Finley, 1999). Competition between Mn and Fe for intestinal absorption (Davis et al., 1992) likely occurs by way of DMT-1 (Gunshin et al., 1997). Functionally, DMT-1 mediates the intestinal uptake of numerous divalent metal cations, and DMT-1 mRNA levels in the duodenum strongly increase in response to Fe depletion (Gunshin et al., 2001). Studies in Caco-2 cells (Tallkvist et al., 2000), an in vitro model of the gastrointestinal epithelium, reveal that

Fe treatment decreases cellular uptake of Fe, Mn, and zinc, suggesting that these metals may utilize the same apical and basolateral transporters. Previous studies from our laboratory showed that dietary ID led to a significant increase in brain regional Mn levels, and that DMT-1 may be involved (Erikson et al., 2004).

In the brain, it is known that striatum is a region rich in DMT-1 (Burdo et al., 1999). However, little is known about specific brain cell response(s) to changes in Fe status vis-a-vis DMT-1 expression levels and function. While DMT-1 mRNA (Williams et al., 2000) and protein levels (Lis et al., 2004) have been measured in primary astrocyte cultures, the effects of altering Fe status within the context of modulating Mn transport into these cells remain unexplored.

Recently, it has been shown that ID causes a significant increase in Mn concentrations throughout the rat brain (Erikson et al., 2002, 2004). Glial cells, particularly astrocytes represent a "sink" for brain Mn (Wedler and Denman, 1984), with concentrations 10–50 folds greater than in neurons. Furthermore, astrocytes are critical for the proper functioning of the blood-brain barrier (BBB) (Kimelberg, 1983). Accordingly, understanding potential interactions between Fe status and Mn transport is warranted. Prior cellular studies have shown that Mn toxicity leads to altered Fe status in the brain (Zheng et al., 1999), but the consequences of altering Fe status and its effect on Mn transport in various cell models is relatively unknown. As discussed above, some of these interactions are likely to be mediated by DMT-1 and TfR. The latter is quite abundant in oligodendrocytes compared to neurons or astrocytes (Moos and Morgan, 2002). While past studies have used immunohistochemical techniques to examine whole brain TfR level, a recent study using Western blot analysis and primary astrocyte cultures showed that astrocytes contain TfR and respond to changes in Fe status as in other cell types (e.g., ID causes increased TfR and overload leads to decreased TfR levels) (Hoepken et al., 2004).

The present study was designed to accommodate two major objectives. The first objective was to ascertain the effect(s) of altered Fe status on Mn transport into the astrocyte. We measured Mn transport both by examining radiolabelled Mn (^{54}Mn) uptake and measuring intracellular Mn concentrations in Fe-depleted and -overloaded astrocytes. We used both methods in order to explore a potential alternative to using radiolabelled materials (i.e., if Mn is detectable via atomic absorption spectroscopy than it could be used instead of radioisotopes). The second goal was to examine the effect of changes in Fe status on TfR and DMT-1 levels in primary astrocyte cultures. Our overall hypothesis was that: (1) iron deprivation will cause increased astrocytic Mn transport (i.e., increased intracellular Mn concentrations) and that this increased transport is related to increased levels of DMT1 and/or TfR; (2) iron overload will cause decreased Mn transport into exposed astrocytes due to decreased levels of TfR and perhaps DMT-1.

2. Methods and materials

2.1. Cell cultures

Primary astrocyte cultures were prepared as described by Frangakis and Kimelberg (1984). Briefly, the cerebral hemispheres of newborn Sprague-Dawley rats were removed and meninges were carefully dissected off. The basal ganglia and midbrain were removed and the remaining cortical tissue dissociated with Dispase (Life Technologies, Gaithersburg, MD). Cells were grown in minimal essential medium (MEM), supplemented with 10% horse serum (AGM). The cultures were maintained in a humidified atmosphere of 95% air/5% CO_2 at 37 °C. The media was changed twice weekly. ^{54}Mnuptake experiments and Western blots were performed after 3 weeks in culture, when the cells have formed a confluent monolayer. Immunocytochemically, >95% of the cells stained positively for the astrocytic marker glial fibrillary acidic protein (GFAP).

2.2. ^{54}Mn-uptake experiments

Uptake of ^{54}Mn was measured as described by Aschner et al. (1992). Astrocytes (grown on six well plates for 3–4 weeks) were incubated overnight at 37 °C with AGM containing 0 or 200 μM desferoxamine (DFO) or 500 μM ferrous sulfate (+Fe). A subset of cells were exposed to 500 μM ferrous sulfate for 15 min immediately prior to the uptake experiments (15 min +Fe) to assess the effects of acute iron overload on manganese uptake in astrocytes. We hypothesized that the Fe would compete with Mn and lower Mn uptake. The next day, cells were

washed 3 x with HEPES buffer and incubated 5 min with HEPES buffer containing 0.5 μCi ^{54}Mn. The reaction was stopped by aspirating the buffer and washing the cells 4 x with cold (4 °C) 290 mM mannitol buffer containing 0.5 mM calcium nitrate to maintain cell adhesion to the substrate. Cells were solubilized in 2 ml1 M NaOH, and aliquots (750 μl) were neutralized for β-counting with a Beckman LS 3801 liquid scintillation analyzer (Beckman Instruments), and for protein determination (50 μl) with the bicinchonic assay (BCA, Pierce Chemicals). We initially performed uptake experiments with AGM containing 0 or 200 μM desferoxamine (DFO) and incubated the cells for 1, 2, 5 and 15 min. We saw a significant difference in ^{54}Mn uptake between the Fe-deprived (DFO) and control cells beginning at 5 min. While the concentration of Mn entering the cells increased in a time dependent manner, the overall difference in ^{54}Mn uptake between DFO and control was similar at both 5 and 15 min. Therefore, we used the 5 min time point for this pilot study.

2.3. Intracellular Mn concentration analyses

Intracellular Mn concentrations were measured with graphite furnace atomic absorption spectroscopy (Varian AA240, Varian Inc., USA). Astrocytes were cultured in 100 mm plates for 3–4 weeks until fully confluent. One hour Mn uptake (300 μM $MnCl_2$) was measured under normal conditions (CN), Fe deprivation (ID), or Fe overload (+Fe). After 1 h, cells were harvested and protein was measured. Cells were then digested in ultrapure nitric acid (0.1 ml) for 48 h in a sandbath (60 °C), digested tissue was brought to 1 ml total volume with 2% nitric acid and analyzed for Mn. It should be noted that the 300 μM $MnCl_2$ used in this experiment was representative of the concentration of the Mn used in the ^{54}Mn uptake experiment which was calculated based on the specific activity of the radioligand; and during manganese toxicity, this concentration is also considered physiologically relevant in terms of reported concentrations in vivo (Ingersoll et al., 1999; Lai et al., 1999; Roels et al., 1997).

2.4. Western blot analysis

Astrocytes were cultured in 100 mm plates for 3–4 weeks until fully confluent. Cells were incubated as described above and the following day Western blot analysis was utilized to measure DMT-1 and TfR levels. Briefly, cell lysates were sonicated in five volumes (1:5, w:v) of tissue lysis buffer (10 mM Tris–HCl; 1 mM sodium orthovanadate; 1% SDS, pH 7.4). Tissue lysates were centrifuged for 10 min 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).

Following fractionation, proteins were electrophoretically transferred to a nitrocellulose membrane (Protran, BA83, Schleicher and Schuell, Keene, NH) in 20% methanol, 0.1% SDS, 25 mM Tris and 192 mM glycine for 3 h at 60 V. Membranes were blocked with 5% low-fat powdered milk in Tris-buffered saline with Tween (TBST, 0.1% Tween, 150 mM NaCl, 20 mM Tris). DMT-1 was detected using a Goat IGg polyclonal antibody [Santa Cruz, Temecula, CA; DMT-1 antibody is made to a portion of the protein (fourth extracellular domain) and recognizes both the IRE and non-IRE forms of the protein] 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 and 1 h, respectively. Membranes were stripped with Restore Western Blot Stripping Buffer© (Pierce Chemical) and probed for TfR by using a mouse anti-rat IGg monoclonal antibody (Chemicon, Temecula, CA) diluted 1:1000 followed by incubation with a horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody diluted 1:5000 (Santa Cruz, Temecula, CA), both in TBST and 1% milk for 2 and 1 h, respectively. Protein bands were visualized with the Western enhanced chemiluminescence system (Pierce Chemical, Rockford, IL). 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

All experiments were conducted in five astrocyte preparations (separate culture dates) 1–2 replicates (Western blot analysis), or 3–6 replicates per treatment (uptake experiments). The data were analyzed with the SPSS system v11.2 statistical analysis package (SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) was performed to test for treatment-dependent effects. For the time-course study, we utilized one-way repeated

measures ANOVA to test for treatment-dependent effects within each time point. Dunnet's procedure was used to evaluate mean differences compared to control. The alpha level for the analyses was set at $p < 0.05$.

3. Results

3.1. ^{54}Mn uptake and intracellular Mn concentrations

Iron deprivation (ID) caused a significant increase in ^{54}Mn uptake at 5 and 15 min compared to control ($p < 0.01$) (Fig. 1a). Because there was no difference in ^{54} Mn uptake at 5 and 15 min in the control cells (Fig. 1a), subsequent studies were performed at 5 min to minimize possible effects of efflux. Astrocytes incubated with 200 μM DFO (ID) and 500 μM ferrous sulfate (+Fe) for 24 h displayed significantly increased ($p < 0.05$) ^{54}Mn uptake (Fig. 1b). Both Fe-depleted (ID) and - overloaded (+Fe) astrocytes displayed an equally significant increase ($p = 0.002$) in intracellular Mn levels compared to control cells (Fig. 2). This effect was evident after 1 h of incubation with $MnCl_2$.

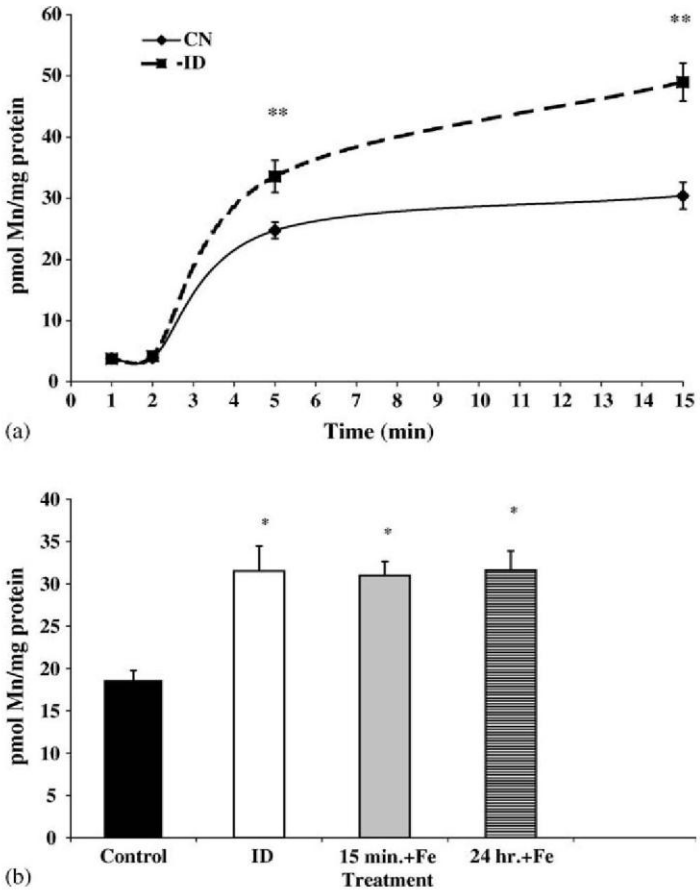

Fig. 1. (a) 1, 2, 5 and 15 min ^{54}Mn uptake in primary rat astrocyte cultures exposed to 24 h incubation with astrocyte growth media (control) or 200 μM DFO (ID). One-way repeated measures ANOVA revealed that iron deprivation (ID) caused a significant increase in ^{54}Mn uptake at 5 and 15 min compared to control ($p < 0.01$) as indicated by asterisks (**), but that within the control treatment, ^{54}Mn uptake did not significantly increase beyond the 5 min time point. (b) 5 min ^{54}Mn uptake in primary rat astrocyte cultures exposed to 24 h incubation with astrocyte growth media (control), 200 μM DFO (ID), or 500 μM ferrous sulfate (+Fe). One-way ANOVA followed by Dunnet's procedure revealed that all treatments caused a significant change, $p < 0.05$, in Mn uptake compared to control as indicated by asterisk (*).

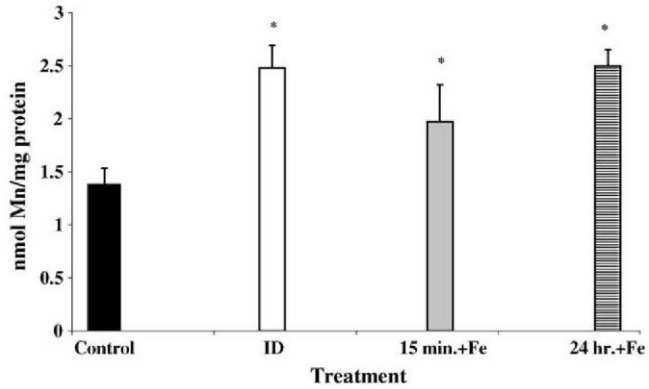

Fig. 2. Intracellular manganese concentrations in primary rat astrocyte cultures exposed to 24 h incubation with astrocyte growth media (control), 200 μM DFO (ID), 500 μM ferrous sulfate (+Fe) or 15 min incubation with media containing 500 μM ferrous sulfate. One-way ANOVA followed by Dunnet's procedure revealed that ID, and both Fe+ exposures caused a significant increase in intracellular manganese concentration, $p < 0.05$, compared to control cultures as indicated by asterisk (*). All astrocyte cultures were incubated with $MnCl_2$ for 1 h to assess intracellular manganese concentrations (i.e., "cold" Mn uptake).

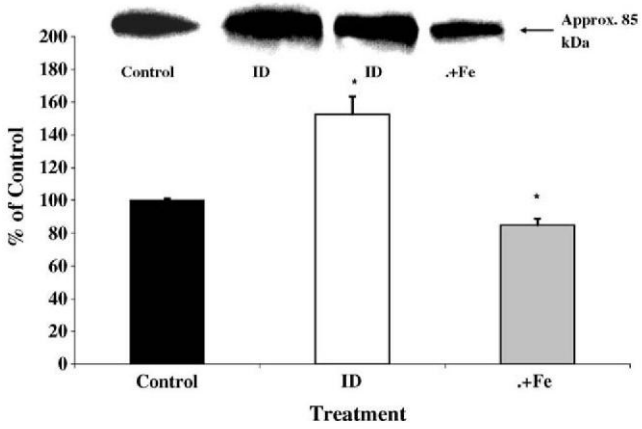

Fig. 3. Transferrin receptor (TfR) protein levels in primary rat astrocyte cultures exposed to 24 h incubation with astrocyte growth media (control), 200 μM DFO (ID), or 500 μM ferrous sulfate (+Fe). One-way ANOVA followed by Dunnet's procedure revealed that ID caused a significant increase and +Fe caused a significant decrease, $p < 0.05$, in TfR levels compared to control as indicated by asterisk (*).

3.2. Transferrin receptor levels and divalent metal transporter

- Transferrin receptor (TfR): Iron deprivation (ID) caused a significant increase ($p < 0.05$), and Fe overload (+Fe) caused a significant decrease ($p < 0.05$) in astrocytic TfR protein levels compared to control (Fig. 3).
- Divalent metal transporter (DMT-1): Iron deprivation (ID) caused a significant increase ($p < 0.05$), in astrocytic DMT-1 protein expression levels compared to control (Fig. 4). Iron overload (+Fe) increased DMT-1 protein levels compared to CN levels. Although this increase was not statistically significant ($p = 0.09$) it is indicative of a trend towards significance.

4. Discussion

We showed for the first time that astrocyte cultures exposed to both an Fe-deprived (depleted) and Fe-overloaded media increase Mn transport and intracellular concentrations (Figs. 1b and 2). Atomic absorption spectroscopy (AAS) possesses adequate sensitivity to measure intracellular Mn concentrations. This provides a viable alternative to using radiolabelled Mn in metal transport studies with sufficient sensitivity to study uptake kinetics in cultured cells. The absolute concentrations were different (about 100 folds higher for the AAS analyzed cells compared to the ^{54}Mn studies). This difference in concentrations is likely due to the shorter

incubation time for the radiolabelled experiments (5 min) compared to the "cold Mn" experiments (1 h), but could also be due to the endogenous Mn levels which the AAS ("cold Mn") experiments measured and the radiolabelled experiments did not analyze. In either case, the measured increase in Mn concentrations due to Fe deprivation/overload was consistent between the two methods (50–70% rise). Both TfR and DMT-1 protein levels rose significantly during Fe deprivation (depletion) and are likely involved in the observed increased Mn transport. However, the precise involvement of these two proteins and the proportionality of the enhanced transport that may be ascribed to each of the astrocytic transporter proteins will have to await additional studies, where transporter expression can be modulated by anti-sense or siRNA (silencing mRNA). The uptake of Mn in astrocytes exposed to a Fe enriched media is less clear (see below).

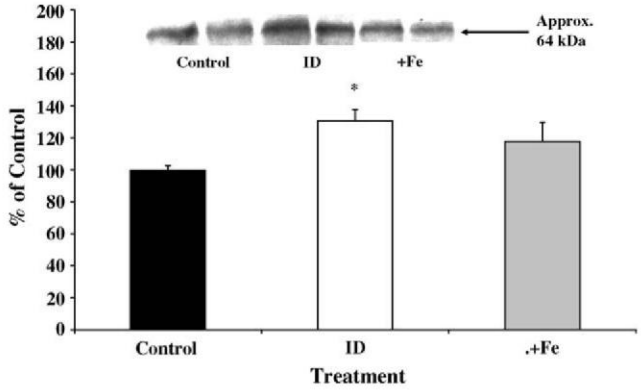

Fig. 4. Divalent metal transporter (DMT-1) protein levels in primary rat astrocyte cultures exposed to 24 h incubation with astrocyte growth media (control), 200 μM DFO (ID), 500 μM ferrous sulfate (+Fe). One-way ANOVA followed by Dunnet's procedure revealed that ID caused a significant increase, $p < 0.05$ in DMT-1 levels compared to control as indicated by asterisk (*). Iron overload, Fe+ increased DMT-1 protein levels compared to control, although this increase was not statistically significant, $p = 0.09$.

One would hypothesize that TfR and DMT-1 levels would decrease when exposed to Fe for 24h. While TfR levels decreased slightly in Fe exposed astrocytes, DMT-1 levels unexpectedly increased (Fig. 4). This finding may be due to the non-specific polyclonal antibody used to detect DMT-1 levels. The antibody used in this study indiscriminately detects all four isoforms of DMT-1. This is because it was directed at detecting an internal region on the protein. Whereas the isoforms differ in their N- and C-terminal residues the antibody used in this study would not be expected to reveal any isoform-specific changes. Two of the isoforms are translated from mRNA that possess an iron response element (IRE+) and the other two do not (IRE-); therefore, two isoforms of DMT-1 are responsive to changes in cellular Fe status and two lack this response (see Roth and Garrick, 2003 for review). Accordingly, it is likely that the antibody used in this study was indiscriminate in its detection of DMT-1 isoforms (i.e., measuring changes in Fe responsive and non-responsive DMT-1). We chose this antibody for this pilot study because it would allow us to assess general DMT-1 levels in primary astrocyte cultures that were exposed to varying Fe status in order to manipulate Mn uptake. We had hypothesized that ID would lead to increased levels and Fe overload would cause decreased levels; however, in retrospect, it will behoove us to use more specific antibodies in our future studies in order to resolve this limiting factor.

A possible reason that astrocytes dramatically increase Mn uptake upon Fe exposure (Fe addition to the media) is that these cells possess the ability to respond to increases in extracellular (media) divalent metal concentrations, and, therefore, increased Mn uptake is a byproduct of this response. TfR and DMT-1 are both associated with the foot processes of astrocytes (Burdo et al., 1999; Malecki et al., 1999; Roth and Garrick, 2003; Zheng et al., 2003) and play an integral role in maintaining extracellular concentrations of Mn and Fe in the brain. It is reasonable to assume that astrocytes would have mechanisms that respond to sudden changes in Fe status in a different manner than other cells (e.g., enterocytes) whose role is facilitating the absorption and transport of this metal. In other words, astrocytes have the responsibility for maintaining tight control over extracellular milieu including metals, thus these cells need to respond to sudden and drastic changes in metal concentrations and this responsibility is unique to these cells. Since this study was carried out at short time points (<24 h), it represents an "acute" response. Therefore, it would be interesting to see whether the same

types of responses for DMT-1 and TfR are inherent to longer exposure time (>48 h) in the presence of Fe overload.

While this study did not examine other potential mechanisms of Mn transport, it has been suggested that some candidate transporters are a monocarboxylic transporter, voltage-regulated calcium channels (Yokel and Crossgrove, 2004), and/or possibly an anion exchange transporter. It is quite clear that Mn transport into, and within the brain has both Fe-dependent (e.g., the effects of Fe deprivation on astrocytic Mn uptake), as well as Fe-independent characteristics (e.g., the effects of Fe overload on astrocytic Mn uptake) associated with it. From a biological relevance point, it is logical that Mn transport into astrocytes could occur independently of Fe status, primarily due to its critical role as a cofactor for the enzyme glutamine synthetase.

There is growing evidence that DMT-1 is involved in brain Mn delivery (Roth et al., 2000). In the microcytic anemia (mk) mouse and the phenotypically similar Belgrade (b) rat (Fleming et al., 1997, 1998; Su et al., 1998), orthologous mutations (glycine 185 to arginine) in the DMT-1 gene result in significantly reduced dietary Fe absorption. The role of the defective DMT-1 allele in the transport of Mn across the blood-brain barrier has been recently evaluated in homozygous Belgrade (b/b) rats that exhibit hypochromic anemia, and heterozygous (+/b) Belgrade rats (Chua and Morgan, 1997). Plasma clearance and uptake by the central nervous system after intravenous injection of radioactive ^{54}Mn bound to transferrin or mixed with serum have demonstrated that plasma clearance of Mn-transferrin was much slower than Mn-serum, but both were faster than the clearance of Fe-transferrin. Uptake of ^{54}Mn, as well as ^{59}Fe by the brain was less in b/b than +/b rats, suggesting that the defective DMT-1 allele affects the metabolism of both metals, and that Mn and Fe might share DMT-1 transporters in the blood-brain barrier (Chua and Morgan, 1997).

In conclusion, Mn transport into astrocytes is significantly affected by Fe status. Both Fe deprivation and Fe overload caused significant increases in Mn uptake and the resulting rise of intracellular Mn concentrations. While the majority of studies suggest that Mn and Fe compete for the same carrier, it should be noted that Mn and Fe transport from the plasma to the brain has been postulated to be synergistic rather than competitive in nature, and that excessive intake of Fe plus Mn may accentuate the risk of tissue damage caused by one metal alone (Chua and Morgan, 1996). These studies suggest that Mn dosimetry is complex and multiple pathways may be involved in the delivery and the regulation of Mn into astrocytes, and likely other cells as well.

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