BioMetals **16:** 41–54, 2003.

DMT1: A mammalian transporter for multiple metals

Michael D. Garrick[1,*], Kevin G. Dolan[1], Craig Horbinski[2], Andrew J. Ghio[3], Dennis Higgins[2], Michael Porubcin[4], Elizabeth G. Moore[4], Lucille N. Hainsworth[4], Jay N. Umbreit[4], Marcel E. Conrad[4], Lee Feng[2], Agnieska Lis[2], Jerome A. Roth[2], Stephen Singleton[1] & Laura M. Garrick[1]

[1]*Departments of Biochemistry and* [2]*Pharmacology and Toxicology, SUNY at Buffalo, Buffalo, NY 14214, USA;* [3]*National Health and Environmental Effects Research Laboratory, Environmental Protection Agency, Research Triangle Park, NC 27711, USA;* [4]*USA Cancer Center, University of South Alabama, Mobile, Alabama 36688, USA; *Author for correspondence*

Published on line: September 2002

Key words: copper transport, DCT1, iron transport, manganese transport, Nramp2, SLC11A2

Abstract

DMT1 has four names, transports as many as eight metals, may have four or more isoforms and carries out its transport for multiple purposes. This review is a start at sorting out these multiplicities. A G185R mutation results in diminished gastrointestinal iron uptake and decreased endosomal iron exit in microcytic mice and Belgrade rats. Comparison of mutant to normal rodents is one analytical tool. Ectopic expression is another. Antibodies that distinguish the isoforms are also useful. Two mRNA isoforms differ in the 3′ UTR: +IRE DMT1 has an IRE (Iron Responsive Element) but -IRE DMT1 lacks this feature. The ±IRE proteins differ in the distal 18 or 25 amino acid residues after shared identity for the proximal 543 residues. A major function is serving as the apical iron transporter in the lumen of the gut. The +IRE isoform appears to have that role. Another role is endosomal exit of iron. Some evidence indicts the -IRE isoform for this function. In our ectopic expression assay for metal uptake, four metals – Fe^{2+}, Mn^{2+}, Ni^{2+} and Co^{2+} – respond to the normal DMT1 cDNA but not the G185 R mutant. Two metals did not – Cd^{2+} and Zn^{2+} – and two – Cu^{2+} and Pb^{2+}–remain to be tested. In competition experiments in the same assay, Cd^{2+}, Cu^{2+} and Pb^{2+} inhibit Mn^{2+} uptake but Zn^{2+} did not. In rodent mutants, Fe and Mn appear more dependent on DMT1 than Cu and Zn. Experiments based on ectopic expression, specific antibodies that inhibit metal uptake and labeling data indicate that Fe^{3+} uptake depends on a different pathway in multiple cells. Two isoforms localize differently in a number of cell types. Unexpectedly, the -IRE isoform is in the nuclei of cells with neuronal properties. While the function of -IRE DMT1 in the nucleus is speculative, one may safely infer that this localization identifies new role(s) for this multifunctional transporter. Management of toxic challenges is another function related to metal homeostasis. Airways represent a gateway tissue for metal entry. Preliminary evidence using specific PCR primers and antibodies specific to the two isoforms indicates that -IRE mRNA and protein increase in response to exposure to metal in lungs and in a cell culture model; the +IRE form is unresponsive. Thus the -IRE form could be part of a detoxification system in which +IRE DMT1 does not participate. How does iron status affect other metals' toxicity? In the case of Mn, iron deficiency may enhance cellular responses.

Introduction

This review identifies some of the multiple properties of DMT1, begins to organize them coherently and relates them to functions. DMT1 has four names defined in Table 1. Nramp2 was assigned when it was first found as a DNA sequence with an unknown function, but clearly related by sequence similarity to Nramp1 (Vidal *et al.* 1995). DCT1 was proposed

Table 1. Multiple names for one transporter.

Nramp2	Natural Resistance Associated Macrophage Protein 2
DCT1	Divalent Cation Transporter 1
DMT1	Divalent Metal Transporter 1
SLC11A2	Solute Carrier Family 11, member 2

(Gunshin *et al.* 1997) after mRNA expression cloning made it a likely candidate for the apical, duodenal iron transporter. The name focused on divalent cations because, in addition to demonstrating transport of Fe^{2+}, the authors showed that Zn^{2+}, Cd^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+} and Pb^{2+} elicited conductances (listed in decreasing order of magnitude, respectively) in their oocyte expression assay. Almost instantly the apical iron transport role was established when Fleming *et al.* (1997) identified a G185R mutation in the corresponding gene of the microcytic (*mk*) mouse because it clearly has a defect in uptake of iron from the lumen of the gut (Edwards & Hoke 1972). Soon after, Fleming *et al.* (1998) found the identical mutation accounted for the phenotype of the Belgrade (*b*) rat extending the function of the gene to exit of Fe^{2+} from endosomes during the transferrin (Tf) cycle and leaving little room to doubt the major role of the transporter in intestinal iron uptake. Andrews (1999) agreed that the name should be changed, but proposed DMT1. OMIM (http://www3.ncbi.nlm.nih.gov/omim) now uses SLC11A2, a choice that may turn out to be the final one; but this review will use DMT1 henceforth.

Characterizing DMT1

One approach for examining the function of a transporter is to learn what happens when the transport activity is decreased. The mutations mentioned above (*b* in the rat and *mk* in the mouse), remarkably both G185R, allow investigators to use this approach; i.e., comparison of b/b to $+/b$ and $+/+$ rats and similar comparisons for *mk/mk*, $+/mk$ and $+/+$ mice are controlled experiments essentially by definition. Another approach is ectopic expression or overexpression. Expression studies allow investigators to ask what happens when DMT1 activity is increased or made available where there was no such activity initially. Isoforms of mRNA and/or protein multiply the capabilities for selective expression of many genes.

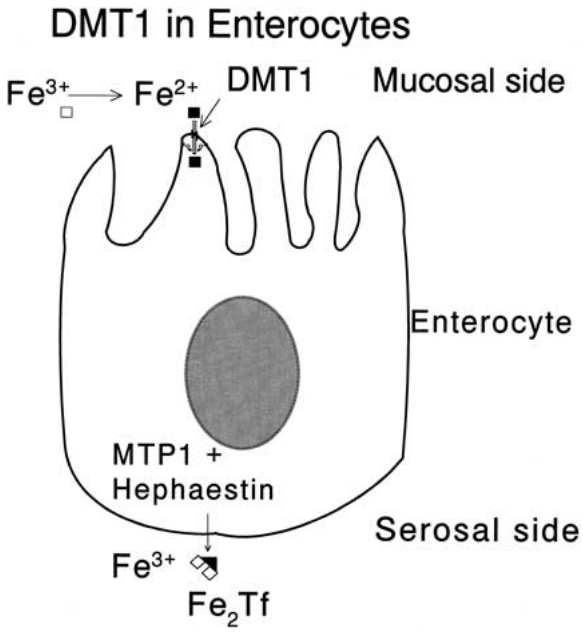

Fig. 1. DMT1 in enterocytes. Squares represent Fe, filled for Fe^{2+} and open for Fe^{3+}. DMT1 is ordinarily present on the luminal side (apical/mucosal – the brush border) where it is responsible for Fe(II) uptake. After transcytosing the cell, Fe(II) exits via MTP1 (aka ferroportin1/Ireg1/SLC11A3) to load Tf (the triangle) probably after oxidation via hephaestin or ceruloplasmin. This exchange may occur on the basolateral side as drawn or could take place perinuclearly.

This review is also a start at addressing DMT1's potential functions in the metabolism of iron, copper and other metals. We begin below with some of the multiple functions.

Multiple functions

Figure 1 provides a model for DMT1 function in duodenal iron uptake via the enterocyte. Fe(III), solubilized by gastric acidity, is reduced to Fe(II) presumably by dcytb (for duodenal cytochrome *b* – McKie *et al.* 2001) or a similar reductase on the apical surface. Fe(II) enters the brush border via DMT1. Because DMT1 may act as a proton symporter, transporting one H^+ for each Fe^{2+} in the oocyte assay (Gunshin *et al.* 1997), one assumes that Fe^{2+} uptake is facilitated by the mildly acidic pH expected in the proximal duodenum. DMT1 is found on the apical surface of the enterocyte (Canonne-Hergaux *et al.* 1999; Trinder *et al.* 2000). This location is consistent with the finding (Knöpfel *et al.* 2000) that divalent cation transport activity is associated with brush border mem-

brane vesicles. Subsequently, iron exits the enterocyte effectively from the basolateral side through MTP1 (aka Ferroportin, IREG1 and SLC11A3 – Abboud and Haile 2000; McKie *et al.* 2000; Donovan *et al.* 2000).

Fe^{3+} is the species that binds to Tf; Fe^{2+} must be oxidized to Fe^{3+} first for this binding to occur. The membrane anchored multi-copper ferroxidase hephaestin performs this function (Vulpe *et al.* 1999) or oxidation may also be taken care of by circulating ceruloplasmin (Yoshida *et al.* 1995). How Fe^{2+} reaches MTP1 is unclear, but one possibility (Yeh *et al.* 2000) is that DMT1 is involved in transcytosis. Although most investigators assume that the Fe^{2+} reaches the basolateral surface before delivery to Tf, there is strong evidence that apo-Tf enters from the basolateral surface (Nuñez *et al.* 1997; Nuñez & Tapia 1999; Alvarez *et al.* 2000) to collect the iron. Thus the possibility that DMT1, apo-Tf, MTP1 and hephaestin meet in the middle of the cell (perinuclearly) deserves continuing experimental consideration.

The Belgrade rat (Oates & Morgan 1996; Garrick *et al.* 1997) and the *mk/mk* mouse (Edwards & Hoke 1972) share not only a G185R mutation; but, more importantly in this context, a defect in apical iron uptake. This defect leaves an increased quantity of DMT1 in the b/b enterocyte (Oates *et al.* 2000) that may be improperly localized in the *mk/mk* enterocyte (Canonne-Hergaux *et al.* 2000). One wonders if the residual gastrointestinal iron uptake that allows these rodents to survive is due to alternate pathways reliant on other forms of iron such as Fe^{3+} and heme, or alternate pathways for Fe^{2+} uptake, or 'leaky' activity of the mutant DMT1, or of course – some combination of these possibilities. Studies to learn the basis of the residual uptake and the means by which iron transits the enterocyte in the mutants could be rewarding in providing additional insights into the iron uptake pathways.

Circulating ferric-Tf is responsible for most iron delivery to cells as modeled in Figure 2 (see Ponka 1997 for a review). The trs constitutively endocytoses, assuring endocytosis of Fe-Tf-TfR after Tf binds to the Tf receptor (trs). Vesicles acidify because the v-ATPase pumps H^+ into them. Acidification releases Fe^{3+} from the complex by affecting both the Tf and TfR components cooperatively (Sipe & Murphy 1991; Bali *et al.* 1991). Reduction (Nuñez *et al.* 1990 – one wonders whether the ferrireductase is dcytb or another reductase?) may also facilitate release and is required for Fe^{2+} to exit via DMT1. Acidification (Nuñez *et al.* 1990; Watkins *et al.* 1991) again plays a role in the exit via the symport function. Although some iron may exit to the cytosol and be stored in ferritin, much of it goes to the mitochondrion in erythroid cells, perhaps directly rather than via a cytosolic carrier (Ponka 1997). The *b* mutation prevents iron from reaching the mitochondria in Belgrade rat reticulocytes (Garrick *et al.* 1993a). While the redox capability of the mitochondrion could convert some iron to Fe(III), ferrireductase is present so that Fe^{2+} is still regenerated as a substrate for heme formation. Meanwhile the apo-Tf remains bound to TfR within the endosome, but is released after reaching the cell surface. Although the current view is that release of Tf at the cell surface is facilitated by the prior removal of iron in the endosome and by the higher pH (7.4), Garrick *et al.* (1993b) observed release of Fe_2-Tf from b/b rat reticulocytes after exocytosis. This observation indicates both that Fe(III) remained in the endosome and bound again to its ligand when DMT1 transport was inactive and that the rate with which Fe_2-Tf-TfR releases Tf is readily detected at pH 7.4. These experiments also showed that only about half of the Fe(III) was removed from Fe_2-Tf-TfR during a single Tf cycle in normal ($+/b$) rat reticulocytes.

Garrick *et al.* (1991) observed that b/b rat reticulocytes retained 20% of Tf-dependent Fe uptake activity compared to $+/b$ or $+/+$ reticulocytes. This remaining activity raises a more acute version of the question discussed above for residual GI uptake of iron, i.e., is the remaining activity due to 'leakiness' for the G185R DMT1 mutation or is there an alternative means for some iron to exit the endosome that is not dependent on DMT1?

Another cellular mechanism for iron uptake, non-Tf bound iron uptake (NTBI), also involves DMT1 (Figure 2). Studies on the Belgrade rat (Farcich & Morgan 1992; Garrick *et al.* 1999) show that a high affinity pathway for NTBI uptake is diminished by the *b* mutation. Thus at least one means for NTBI entry into cells depends on DMT1. It is not surprising that the mutation alters this pathway because at least one form of NTBI uptake in mammalian cells is likely to be a generic version of the apical uptake of iron in the enterocyte. The data are consistent with a model where Fe(II) arrives in the endosome and merges there with Tf-dependent iron incorporation.

All three of these functions (apical Fe^{2+} uptake by enterocytes, Fe^{3+} trafficking into cells via the Tf cycle and NTBI uptake into cells) are normally critical in iron homeostasis because iron is lost from the body only in the unregulated process of exfoliation of

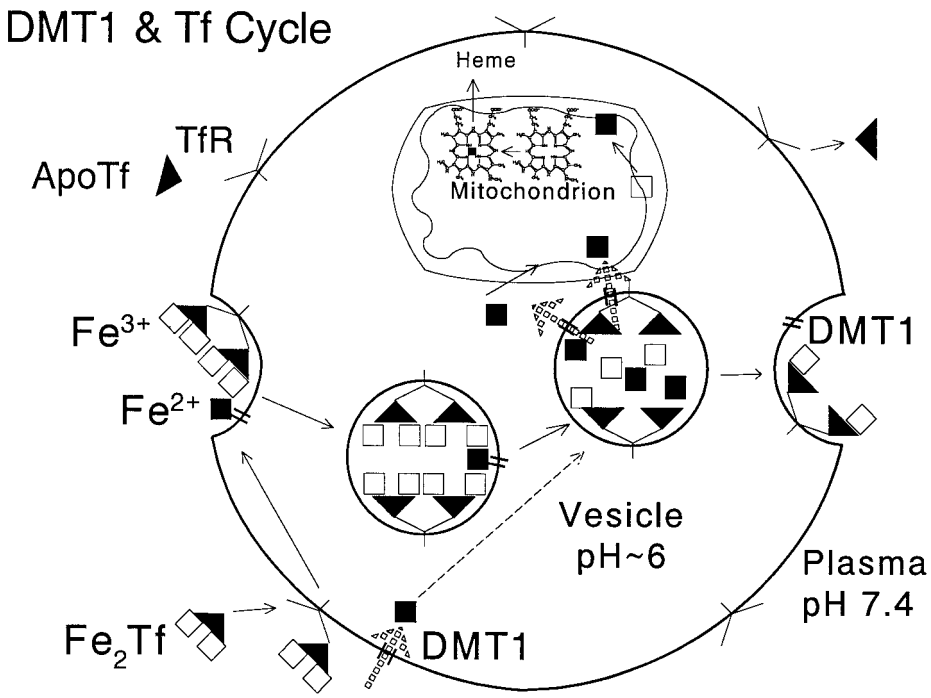

Fig. 2. DMT1 and the Tf Cycle. Again squares represent Fe, filled for Fe^{2+} and open for Fe^{3+}. The triangles are Tf and the Y's are their receptors. Constitutive endocytosis brings the Fe-Tf-TfR complex into vesicles. Although the pH at the cell surface is 7.4, the pH of the endosomes drops to ~ 6.0 as protons are pumped into the vesicles by the v-ATPase. At the lower pH, the Fe-Tf-TfR complex cooperatively releases Fe(III) so that a ferrireductase can reduce it. DMT1 is on the surface of the vesicles so Fe(II) exits, also probably driven by the pH gradient. Fe^{2+} may transit to the mitochondrion via the cytosol and could rely on carriers yet to be identified or it may enter the mitochondrion directly. DMT1, also present on the cell surface, is responsible for a portion of Tf-independent Fe(II) uptake as well.

cells. Enterocyte DMT1 activity increases in hereditary hemochromatosis (Zöller *et al.* 1999, 2001), a disorder in which iron overload is often attributable to a mutation in an HLA gene named *HFE* (for high iron), and in murine models for this disorder in which the *HFE* gene is ablated (Fleming *et al.* 1999; Griffiths *et al.* 2001) or engineered to contain the most common human mutation C282Y (Levy *et al.* 1999). These observations stimulated a clever experimental analysis of the effects of mutations that lead to iron overload in mice in the presence of the G185R (*mk*) DMT1 mutation (Canonne-Hergaux *et al.* 2001). The *mk* mutation was epistatic to the HFE knockout and C282Y knockin, as well as to the β_2-microglobulin knockout (a mouse mutant that also suffers from iron overload reflecting the interaction of the HFE protein with β_2-microglobulin). While these experiments do not demonstrate that DMT1 dysregulation is the primary defect in hereditary hemochromatosis models, they do show that decreased apical iron uptake after DMT1 mutation overcomes any dysregulation and overexpression so that one obtains a chronic iron deficiency phenotype. Thus, a major decrement in DMT1 activity overrides HFE inactivation in any scheme of metabolic and regulatory interactions for iron.

Proton transport

As cited above, Gunshin *et al.* (1997) observed that DMT1 also transported protons with a stoichiometry of one proton per divalent cation, postulating that the proton gradient associated with the mildly acidic pH of the proximal duodenum was the driving force for metal uptake. Similarly Fleming *et al.* (1998) pointed out that a proton gradient based on vesicle pH compared to cytosolic pH (or mitochondrial pH) could provide a driving force for iron exit from endosomes. If DMT1 also participates in some NTBI uptake, there is a question as to what provides the driving force. In some situations, there might be a pH gradient into the cell, although this possibility is relatively unlikely. One should keep in mind, however, that, although a pH of 6 is equivalent to $[H^+] = 1\ \mu$M, a pH of 7.4 is equivalent to $[H^+] \sim 40$ nM not 0. Thus, sufficient

protons are available at the pH where one presumes NTBI uptake occurs to allow for proton uptake stoichiometric with Fe uptake into the cell but where is there a proton gradient to be a driving force for a symport function? One must conclude that more work is necessary before it is compelling that DMT1 is a divalent metal/proton symporter although it is safe to state that DMT1 usually transports Fe(II) and H^+ in an associated fashion.

Years ago, we (Garrick *et al.* 1990a, b) reported that Tf-containing b/b reticulocyte endosomes had a higher pH than $+/b$ or $+/+$ endosomes. Possibly this elevated pH reflects the G185R mutation diminishing Fe flux without decreasing proton flux, perhaps even freeing the proton flux from a rate-limiting Fe flux so that the protons can leave endosomes faster than normal. This speculation is essentially equivalent to arguing that the mutation has led to a proton leak. It is also consistent with recent studies (Chen *et al.* 1999; Sacher *et al.* 2001) suggesting that DMT1 transport of Fe^{2+} and H^+ can be dissociated.

Ectopic expression

Gunshin *et al.* (1997) expressed a DMT1 mRNA construct in *Xenopus* oocytes to determine whether it had Fe^{2+} uptake activity. They then showed that 7 other metals elicited a conductance under similar conditions and postulated that it was a transporter for them as well. Using DMT1 cDNA constructs in pMT2 to transfect HEK293T cells, we (Fleming *et al.* 1998) demonstrated that expression of rat wild type DMT1 leads to increased Fe^{2+} uptake whereas expression of the G185R mutant yielded minimal activity. Su *et al.* (1998) similarly examined the effect of several other mutations on expression and found that each mutation decreased or eliminated Fe^{2+} uptake.

Garrick & Dolan (2002) characterized both Fe^{2+} and Mn^{2+} uptake in the HEK293T cell transfection assay. Uptake was time and concentration dependent with K_d values for both metals approximately 1 μM. Maximal uptake occurred at pH 6.0, but cell toxicity limited assays at lower pH and there was still detectable uptake at pH 7.4. Nonradioactive Mn^{2+} inhibited $^{59}Fe^{2+}$ uptake and nonradioactive Fe^{2+} inhibited $^{54}Mn^{2+}$ uptake, each with a K_i of approximately 1 μM. Mn^{2+} uptake was so severely affected by the G185R mutation that no residual activity remained.

In principle, ectopic expression can be used to learn which other metals can be taken up via DMT1. Conrad *et al.* (2000) also used HEK293T cell transfection to verify that uptake of $^{59}Fe^{2+}$ and $^{54}Mn^{2+}$ was stimulated by the wild type G185 rat DMT1 construct, but not by the mutant R185 construct. Surprisingly, the same assay showed that DMT1 did not transport $^{59}Fe^{3+}$ and $^{65}Zn^{2+}$. Consistent with the data from ectopic expression, Tandy *et al.* (2000) report that $^{65}Zn^{2+}$ uptake into Caco2 cells is unaffected by an antibody to the 4th extracellular domain of DMT1 under conditions where Fe uptake is blocked. Ferguson *et al.* (2001) also could not associate DMT1 activity in rat kidney with Zn^{2+} transport. Thus three series of findings are in apparent contradiction to the claim of Gunshin *et al.* who actually demonstrated a Zn^{2+}-dependent conductance in oocytes after DMT1 expression. This conflict suggests that the conductance is elicited by Zn without Zn actually traversing the oocyte membrane. The studies of Sacher *et al.* (2001) are consistent with this interpretation; their data demonstrate that Zn^{2+} can elicit a proton conductance although $^{65}Zn^{2+}$ is not actually transported in oocytes expressing DMT1. Although Zn^{2+} does not appear to be transported by DMT1, unpublished data associated with the study by Conrad *et al.* (2000) further reveal that Ni^{2+} and Co^{2+} are also transported (Table 2).

The ability of one metal to inhibit uptake of another metal also provides insights into DMT1 functionality, particularly if inhibition is competitive. Fitting this criterion, Ni^{2+} and Co^{2+} compete with $^{54}Mn^{2+}$ uptake with K_i in the low μM range. Cu^{2+} and Pb^{2+} are similarly also competitors although we were unable to test their uptake because radiolabeled forms were not available. Cu^{2+} was actually a challenge. Conrad *et al.* (2000) report that it too does not compete effectively with Fe^{2+}; however, the analysis was performed at pH 7.4 to represent physiological conditions. When analysis was repeated at pH 6.0, Cu^{2+} inhibited $^{54}Mn^{2+}$ uptake with K_i in the low μM range. Zn^{2+} is much less effective, so either it does not bind to DMT1 or it binds with considerably lower affinity. Current information on which metals are potentially transported by DMT1 is summarized in Table 2.

Separate pathways for Fe^{3+} and Fe^{2+} uptake

One way for exogenous Fe^{3+} to enter cells is by encountering a surface reductase such as dcytB (McKie *et al.* 2001) prior to uptake as Fe^{2+} via DMT1 shown for enterocytes in Figure 1. Is this route, however,

Table 2. DMT1 interaction with metals.

Metal	Conductance[a]	Uptake[b]	Competition[c]	Tissue/mutation[d]
Fe^{2+}	Yes	Yes	Yes	Yes
Fe^{3+}	No	No	No	NA[e]
Zn^{2+}	Yes	No[e]	No	No
Mn^{2+}	Yes	Yes	Yes	Yes
Ni^{2+}	Yes	Yes	Yes	Untested
Co^{2+}	Yes	Yes	Yes	Untested
Cu^{2+}	Yes	Untested	Yes[f]	No
Cd^{2+}	Yes	Untested[g]	Yes[h]	Untested
Pb^{2+}	Yes	Untested	Yes	Untested

[a]In the *Xenopus* oocyte expression assay (Gunshin *et al.* 1997).
[b]In the HEK293T cell expression assay (Conrad *et al.* 2000; Garrick & Dolan 2001; and our unpublished data).
[c]Competes with $^{54}Mn^{2+}$ uptake in the HEK293T cell expression assay (Conrad *et al.* 2000; Garrick & Dolan 2001; and our unpublished data). Also see note f below.
[d]Deficient in liver of *mk/mk* mice (Conrad *et al.* 2000 – only tissue tested) or multiple tissues of *b/b* rats (Garrick *et al.* 1997 for Fe; and our unpublished data for Mn).
[e]Tandy *et al.* (2000) also observed a lack of proton driven Zn^{2+} uptake and no sensitivity to anti-DMT1 that blocked iron uptake in Caco2 cells. Sacher *et al.* (2001) confirmed the conductance assay, but also showed that $^{65}Zn^{2+}$ uptake was not stimulated in oocytes. Following up on the former paper, Yamaji *et al.* (2001) find that Zn exposure increases the expression of DMT1 in Caco2 cells.
[f]Although Conrad *et al.* (2000) observed little competition with K562 cells at pH 7.4, we (unpublished) find that $K_i \sim 1\ \mu M$ at pH 6.0. See also note g.
[g]Elisma & Jumarie (2001) detect DMT1 dependent $^{109}Cd^{2+}$ uptake in Caco2 cells at pH 5.5 (using Fe^{2+} competition as a criterion), but not at pH 7.4. Zn^{2+}, in contrast, competes with $^{109}Cd^{2+}$ uptake at pH 7.4 but not pH 5.5 supporting both a separate transport system for Zn^{2+} and Cd^{2+} as well as shared use of DMT1 by Fe^{2+} and Cd^{2+}.
[h]Olivi *et al.* (2001) also used HEK293 cells for ectopic expression of DMT1 to see a stimulation of $^{109}Cd^{2+}$ and $^{55}Fe^{2+}$ uptake. This group also observed competition between Cd^{2+} and Fe^{2+} uptake into MDCK cells and marshaled several arguments to attribute this competition to DMT1 as the transporter. Tallkvist *et al.* (2001) also see $^{109}Cd^{2+}$ uptake in Caco2 cells and argue that DMT1 is the transporter based on Fe competition.

the only way that Fe^{3+} can enter cells? Conrad *et al.* (1999) have provided evidence that Fe^{3+} uptake occurs via the IMP (Integrin/Mobilferrin/Paraferritin) pathway. One cannot test for concurrent uptake of Fe(II) and Fe(III) because the two species contaminate each other when used simultaneously. One can, however, use Mn(II) or another divalent cation as a surrogate for Fe(II) uptake by DMT1. Conrad *et al.* (2000) started with immunoblot evidence that DMT1 exists in K562 cells to test whether all Fe uptake is via DMT1 or whether another pathway is responsible for Fe^{3+} uptake. While nonradioactive Fe^{3+} competed effectively with $^{59}Fe^{3+}$ uptake, it had no effect on uptake of labeled Cu^{2+} or Zn^{2+}. Lack of inhibition supports separate uptake paths for Cu^{2+} or Zn^{2+} compared to Fe^{3+}, so one or all of these metals is not reliant on DMT1 for uptake. Nonradioactive Zn^{2+} inhibited $^{65}Zn^{2+}$ uptake and $^{64}Cu^{2+}$ uptake but not $^{59}Fe^{3+}$ uptake nor $^{59}Fe^{2+}$ uptake. This series of results argues in a similar fashion that Cu^{2+} and Zn^{2+} share a common uptake pathway but that Zn does not share it with Fe^{3+} nor with Fe^{2+}. Most critically, nonradioactive Mn^{2+} decreased $^{59}Fe^{2+}$ uptake but not $^{59}Fe^{3+}$ uptake. This result is consistent with Fe^{2+} and Mn^{2+} sharing the DMT1 uptake pathway, but Fe^{3+} relying on a separate pathway. One might argue that both divalent cations prevent access of Fe^{3+} to a ferrireductase or prevent the product of the reductase from following a channelized access to DMT1 but the data covered below diminish the strength of this argument.

Antibody to the 4th extracellular domain of human DMT1 should and does block DMT1 dependent Fe^{2+} uptake and Mn^{2+} uptake by K562 cells, but the antibody does not block Fe^{3+} uptake (Conrad *et al.* 2000). Conversely, antibody to β_3-integrin blocks Fe^{3+} transport, but not Fe^{2+} transport; and tiron, a chelator of Fe^{3+}, also prevents Fe^{3+} transport without affecting Fe^{2+} uptake. While one might try to rescue a

single DMT1-dependent pathway by arguments about close cooperation between the reductase and DMT1 for some of these data, the combination forces us to recognize that there is a separate Fe^{3+} uptake pathway that does not rely on DMT1 and that some cells (here the K562 line) have insufficient ferrireductase to drive much Fe^{3+} into the DMT1 transport pathway. One hopes that other groups will provide evidence bearing on the existence and potential usage of the IMP pathway but the effect of the antibody to β_3-integrin is consistent with the postulated pathway even though more evidence (not rhetoric) should be brought to bear to support or deny its existence. Recently, Umbreit *et al.* (2002) complicate this picture by reporting that they detect both DMT1 and mobilferrin in immunoprecipitated or purified paraferritin, indicating a way that the two pathways may intersect.

Tissue metal densities

Garrick *et al.* (1997) showed that multiple tissues of *b/b* rats were Fe deficient. Conrad *et al.* (2000) also examined the tissue density of 4 metals in livers from *mk/mk* mice compared to +/*mk* littermates (Table 2). They found that *mk/mk* livers were profoundly Fe deficient and significantly Mn deficient, but Zn levels were comparable to those of controls and Cu levels were actually higher (but without achieving significance). In *b/b* rats (our unpublished data), Mn deficiency occurs not only in the liver, but also in the spleen, kidney and duodenum, tissues not reported in the *mk* studies. The heart and lung, however, did not have significant differences but the absence of statistical significance might be due to the small number of rats studied. Chua and Morgan (1997) also observed that some tissues of the Belgrade rat were Mn deficient.

Isoforms of DMT1 mRNA and protein

Gunshin *et al.* (1997) called attention to an IRE within the 3′ UTR of their clone of rat DMT1 because the IRE's interaction with IRP's (Iron Regulatory Proteins) might cause DMT1 mRNA to be stable when the intracellular labile iron pool level is low yet destabilized when the labile iron pool level is higher. Fleming *et al.* (1998), however, cloned a cDNA in which the IRE was absent and noted that its absence required a non-IRE/IRP mode of regulation. The initial 543 residues appear to be in common for the two forms of the protein although the C-terminal 18 or 25 amino acid residues differ as a result of splicing a different exon at this region. Lee *et al.* (1998) subsequently reported similar ±IRE mRNA isoforms for human DMT1. Recently, Hubert and Hentze (2001) have drawn attention to the 5′ end of the mRNA and protein. There they find an alternative exon to exon 1 of human DMT1 so they have dubbed the new sequence 1A (the old becoming an untranslated 1B and the next exon remaining as 2). Exon 1A predicts 29 additional residues for human DMT1 or 31 for rat DMT1. Ironically, this exon is actually present in Gunshin *et al.*'s (1997) original cDNA sequence. Perhaps translation *in silico* was inhibited in 1997 by the lack of a canonical ACC immediately before the ATG that should encode the first M. Now it is possible to consider 4 alternative splice isoforms (Figure 3). Technically the alternatives at the C-terminus are due to alternative polyadenylation and those at the N-terminus are probably due to alternative promoters but the end result is different exons in the mRNA and protein. The tissue localization and functional significance of the 4 isoforms remain to be reported, but the presence of a candidate NLS (nuclear localization signal – Figure 4) in 1A's N-terminus is suggestive of different functions and differences in the relative content of the 4 isoforms as one compares tissues (below). The expectation that they have different promoters also has potential regulatory significance. Exon 1A was actually detected because Hubert & Hentze (2001) did not observe the expected responses to iron levels in reporter constructs that contained the IRE in the 3′UTR. This challenge encouraged them to focus on the 5′ end of the mRNA.

Several groups have begun to examine whether the ±IRE species are differentially expressed and whether they localize differently within cells (Canonne-Hergaux *et al.* 1999; Tabuchi *et al.* 2000; Beaumont *et al.* 2000; Roth *et al.* 2000). The N-terminal isoforms were detected so recently that they are still predicted translations at the time of this review with only a brief mention of tissue distribution for the mRNAs that start with exon 1A or 1B (Hubert & Hentze 2001). Canonne-Hergaux *et al.* (1999) infer that the +IRE form is the main species in mouse duodenum based on differences in staining patterns with an antibody to a common epitope compared to what they observed with an antibody that is specific for the -IRE form. This +IRE form is enriched in the apical two thirds of villi where enterocytes should be mature and iron absorptive and is primarily localized in the brush border. They also conclude that +IRE DMT1 increases in

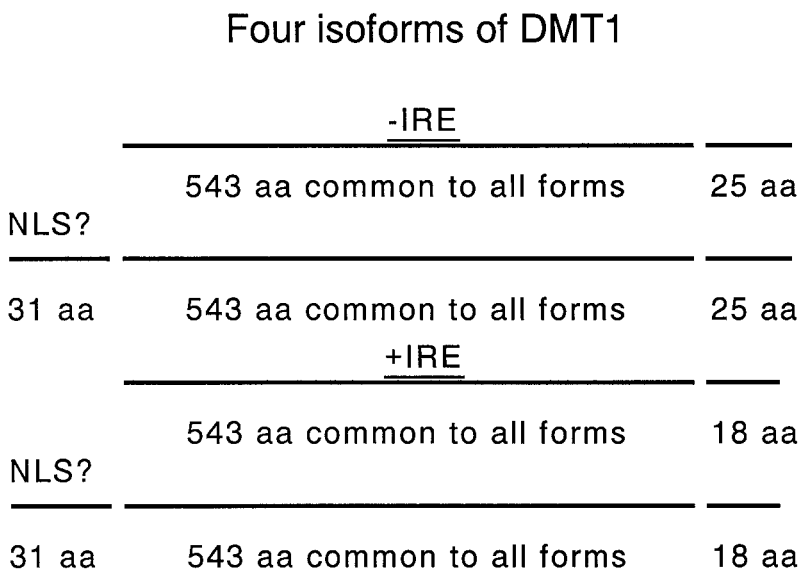

Fig. 3. Four isoforms of rat DMT1. Two C-terminal variants exist: The -IRE, associated with the absence of an IRE in the 3′ UTR of the mRNA and the +IRE, associated with its presence (Fleming *et al.* 1998; Lee *et al.* 1998). Two N-terminal variants were identified more recently: One originates with an MV sequence as originally reported (Gunshin *et al.* 1997; Fleming *et al.* 1998) and one should start with 31 amino acid residues proximal to that sequence (Hubert & Hentze 2001). The longer peptide species has a potential NLS motif within it.

```
                  M  R  K  K  Q  L  K  T  E  A        A  P  H  C  E  L  K
Hs AGTTCTGCACCATGAGGAAGAAGCAGCTGAAGACGGAGGCA------GCTCCACACTGTGAACTAAA
Rn CCACGCGTCCGATGGGGAAGAAGCAGCCGAGGGCAGCAGCAAGTGCTGCTCCAAACTGTGAGCTAAA
                  M  G  K  K  Q  P  R  A  A  A  S  A  A  P  N  C  E  L  K

      S  Y  S  K  N  S  A  T  Q  V  S  T  M  V  L  G  P  E  Q  K  M  S
Hs ATCATATTCTAAGAACTCAGCCACTCAGGTATCCACCATGGTGCTGGGTCCTGAACAGAAGATGTCA
Rn ATCCTATTCTAAGAGCACAGATCCTCAGGTATCTACCATGGTGTTGGATCCTGAAGAAAAGATTCCA
      S  Y  S  K  S  T  D  P  Q  V  S  T  M  V  L  D  P  E  E  K  I  P
```

Fig. 4. Potential NLS in DMT1 containing exon 1A. Human (*Hs*) and rat (*Rn*) mRNA sequences are depicted with their translations above and below the respective mRNAs. The expected NLS are underlined; the transition from exon 1A to exon 2 is double underlined and the alternative initiating Met codons and residues are bold. The proximal Met codon should be used when exon 1A is present while the distal Met codon is used when the untranslated exon 1B is present.

iron deficiency. The data are consistent with the +IRE form as the major apical iron transporter given that the rodent mutants show that DMT1 has such a function (as discussed above for Figure 1) and that the -IRE isoform simply is not present in sufficient quantity.

The kidney has more DMT1 than the intestinal track (Canonne-Hergaux *et al.* 2000), but there the -IRE species predominates. Indeed the kidney has the most DMT1 mRNA of any tissue with the brain next (Beaumont *et al.* 2000). The -IRE isoform is present in larger quantity than the +IRE species in most tissues except the duodenum, liver and bone marrow (Beaumont *et al.* 2000). Canonne-Hergaux *et al.* (2001) have also estimated that murine reticulocytes contain a greater fraction of -IRE DMT1 than +IRE. Their claim can be reconciled with the mRNA quantitations (Beaumont *et al.* 2000) either by arguing that the mRNA data reflect nonerythroid marrow to an extent that accounts for the difference or by arguing that mRNA levels and protein proportions do not correlate. If the reticulocyte proportions are confirmed, then they would support the interpretation that the -IRE isoform is the usual transporter for exit of iron from endosomes.

Nuclear localization of -IRE DMT1

Roth *et al.* (2000) considered the hypothesis that intracellular localization of ±IRE DMT1 would be different reflecting the potential for distinct modes of

regulation associated with the presence or absence of the IRE in the 3′ UTR. Different functions for the two proteins was another expectation. Both PC12 (pheochromocytoma) cells, a rat line with a neural phenotype after treatment with nerve growth factor (Green & Tischler 1982) or Mn (Lin *et al.* 1993), and primary cultures of sympathetic neurons contained both mRNA isoforms. Antibodies that reacted with the two distinct C-termini each detected a 65 kD protein as the main DMT1 species in immunoblots, but a second species at ∼67 kD was usually noted with the -IRE antibody while one at ∼91 kD was often detected with the +IRE antibody. Confocal fluorescent microscopy of PC12 cells immunostained with the antibodies confirmed differential localization by showing that the +IRE DMT1 was present on the cell surface, in neurites (but little in growth cones) and in puncta within the cytosol representing intracellular vesicles; while the -IRE form was highly expressed on the plasma membrane and neurites, much more prominent in growth cones but less apparent within the cytosol. Most notably, however, the -IRE antibody stained strongly throughout the nuclei of PC12 cells while +IRE DMT1 was absent from nuclei. When we examined primary cultures of sympathetic neurons from superior cervical ganglia of neonatal rats, the differential localization was equally striking and selective nuclear expression of the -IRE species even more pronounced.

Although absence of the +IRE stain from the nucleus served as a control for the prominent staining for the -IRE form there, it was necessary to test for confounding cross reactions or the possibility that only a C-terminal fragment of the isoform was in the nucleus. Fractionating PC12 cells into nuclear and cytosolic membrane fractions, then testing the fractions by immunoblotting, revealed that the nuclear -IRE DMT1 was not only intact but actually slightly larger than the cytosolic fraction while the nuclear fraction did not contain the +IRE form, serving as a control that indicated that cytosolic membranes had not contaminated it. We estimated the cytosolic -IRE DMT1 to be ∼65 kD and the nuclear form to be ∼67 kD. This difference is consistent with the presence of a potential NLS in the conjunction of exon 1A and exon 2 and suggests that a proximal peptide adds ∼3 kD to the size. The immunizing peptides blocked the specific reaction of each antibody and transfection of HEK293T cells with constructs expressing -IRE DMT1 (Roth *et al.* 2000) and the +IRE form (unpublished) confirmed the specificity of the two immunoaffinity absorbed antibodies. Neither the endogenous ±IRE DMT1 nor the ectopically expressed isoforms were found in the nucleus of the HEK293T cells.

Because -IRE DMT1 was present in the nucleus in PC12 cells, primary cultures of sympathetic neurons and human medulloblastoma cells but not in HEK293T cells and Hep2G hepatoma, it was important to ask about intracellular localization of ±IRE DMT1 in neurons isolated from the central nervous system (CNS). Figures 5 and 6 show results for striatal and hippocampal neurons, respectively. Although the characteristic morphology of the two types of CNS neurons is distinct, they stain in very similar patterns. MAP2, shown for comparison, selectively labels neurites and soma. The +IRE DMT1 shares these locales and also appears in punctate cytosolic vesicles, but it is relatively excluded from the nucleus in CNS neurons too. The -IRE DMT1 is also present in neurites, at the cell surface and in punctate cytosolic vesicles, but staining for it is most intense in nuclei. These results demonstrate that nuclear localization of -IRE DMT1 is also characteristic for CNS-derived neurons. Immunohistochemical staining of sections of rat brains verifies that this localization also occurs *in situ* (not shown).

Nuclear staining of the -IRE species appears to be prominent in neuronal cells (Roth *et al.* 2000; Garrick *et al.* 2001) but also occurs in other types of cells. It can also be detected with an antibody that recognizes a common epitope and is not abolished by the G185R mutation (Garrick *et al.* 2000). These data indicate that there are aspects of iron homeostasis and the metabolism of other divalent metals that remain to be understood. Perhaps this transporter sequesters a metal in the nucleus or carries one there. In doing so it may signal an aspect of metal status or it may serve as a signal of metal status itself.

The -IRE isoform of DMT1 is not the only protein involved in iron metabolism that is expressed in the nucleus. Gurgueira & Meneghini (1996) identified an ATP-dependent iron transport system in rat liver nuclei. H-ferritin has been found in the nucleus of avian corneal epithelium (Cai *et al.* 1997) where its presence protects against iron-promoted UV damage to DNA after UV irradiation (Cai *et al.* 1998). Cai & Linsenmayer (2001) recently demonstrated that the ferritin undergoes translocation to the nucleus. Convincing evidence shows that the H-chain of ferritin (or a very similar protein) reaches the nucleus and has DNA binding properties for a sequence that is reminiscent of an IRE (Broyles *et al.* 2001). Very recently IRPs were also detected in the nucleus (White *et al.* 2001). One

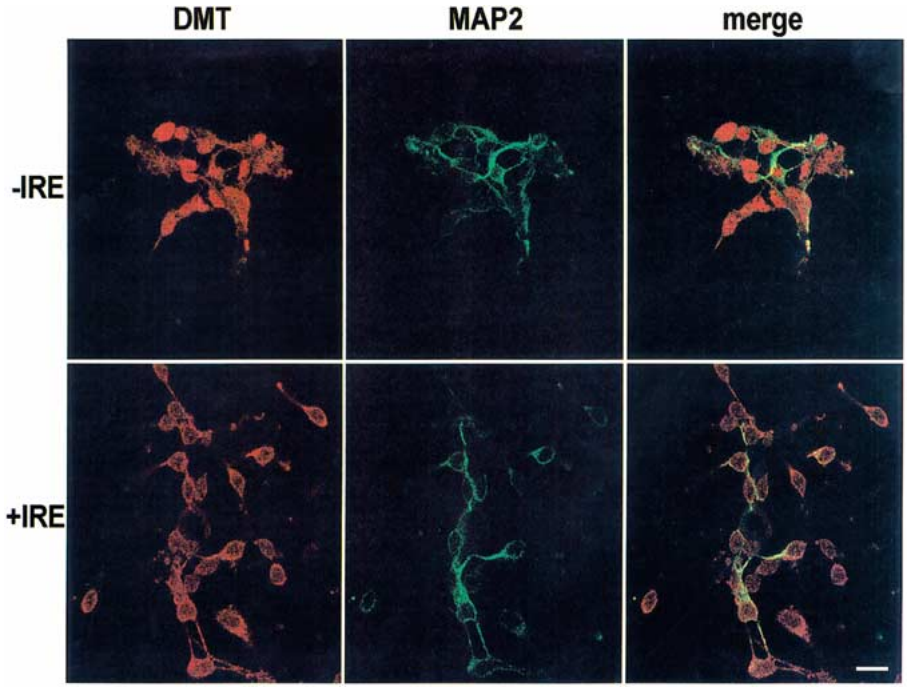

Fig. 5. DMT1 localization in rat striatal neurons. Striatal neurons from 15 day rat embryos were cultured for two days. They were then fixed, permeabilized and reacted with rabbit antibody to the -IRE or the +IRE species of rat DMT1 and with a murine monoclonal antibody to MAP2, a neurite marker. Secondary staining was with rhodamine labeled goat anti-rabbit IgG and fluorescein conjugated goat anti-mouse IgG. The confocal fluorescent micrograph shows one μm optical sections through a plane that contained neuronal nuclei. The bar in the bottom right panel is 10 μm. Panel labels designate those stained for $\pm$IRE DMT1 (red), MAP2 (green) and the merged image (yellow or orange indicating colocalization).

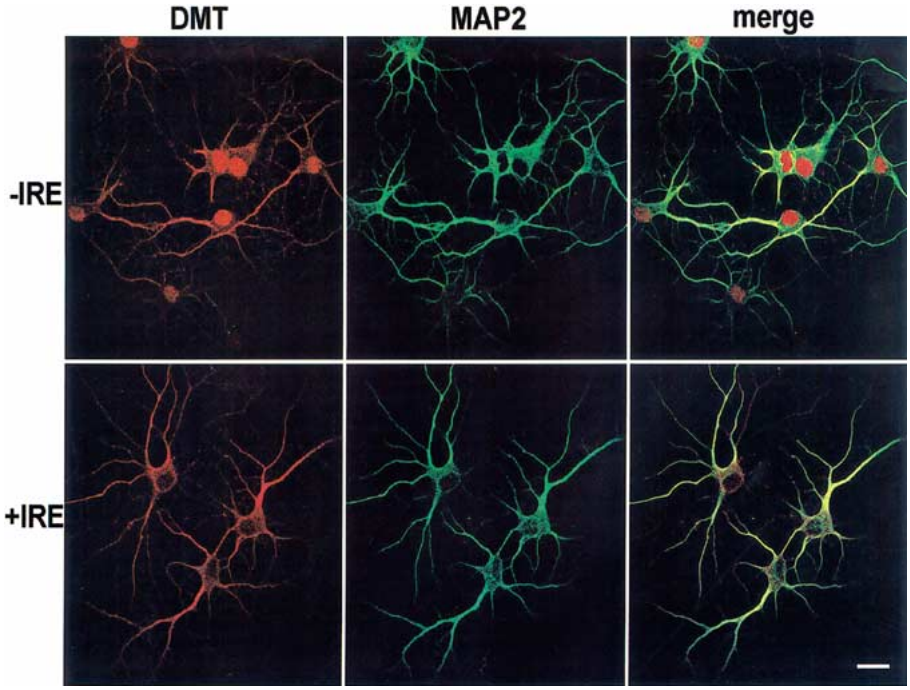

Fig. 6. DMT1 localization in rat hippocampal neurons. Primary cultures of hippocampal neurons were established from embryonic rat pups. After three days they were fixed, permeabilized and reacted with antibodies and confocal fluorescent micrographs obtained as in the legend to Figure 5.

such occurrence could represent an experimental artifact, a misguided activity or some form of accidental engulfment by the nucleus, but the detection of multiple proteins in the nucleus with each ordinarily playing an important role in iron metabolism strongly suggests that a new paradigm relevant to iron homeostasis is required.

Possible protective role for -IRE DMT1

As outlined above, the capacity of DMT1 to transport iron and its ubiquitous expression make it a likely candidate for NTBI uptake of iron in peripheral tissues. We tested the hypothesis that NTBI uptake by airway epithelial cells is associated with DMT1 and that exposure to iron can increase DMT1 mRNA, protein expression, and transport of this metal. The pattern of regulation in these cells (Wang *et al.* 2002) leads to the suggestion that this species might be protective for these cells. BEAS-2B cells, a human bronchial epithelial line immortalized by SV-40 T antigen expression, served as a model for some of the experiments. BEAS-2B cells readily took up iron from ferric ammonium citrate (FAC) from the medium. In the presence of an internalized calcein, uptake from FAC quenched the fluorescent signal indicating intracellular transport of the metal. Uptake characteristics suggested that the Fe^{3+} was reduced to Fe^{2+} and subsequently taken up by DMT1. Exposure to FAC led to a selective, concentration dependent increase in -IRE DMT1 mRNA as detected by quantitative RT-PCR. Immunoblots with isoform specific antibodies revealed that the expressed protein species exhibited the same response patterns, i.e., the +IRE form was unaffected by FAC exposure, but -IRE DMT1 increased with increased FAC. This increase was accompanied by elevated iron uptake. Immunohistochemistry revealed increased -IRE isoform in the rat lung epithelium after instillation of FAC. Comparable to mRNA and protein increases, iron transport was elevated after pre-treatment of BEAS-2B cells with iron-containing compounds. Exposure, however, of respiratory epithelial cells to oil fly ash, a particle with significant biological effect associated with the metal content, did not increase -IRE DMT1 (both mRNA and protein) but rather decreased it; while +IRE DMT1 (both mRNA and protein) demonstrated no change. Identical results were obtained with exposure of the cells to vanadyl sulfate.

We conclude that airway epithelial cells increase both mRNA and expression of -IRE isoform of DMT1 following exposure to iron. The increase results in an elevated transport of iron and its probable detoxification by these cells. Transcriptional regulation of DMT1 by specific metals may contribute to iron uptake and metal detoxification by these cells in the respiratory tract. Vanadium, however, had an opposite effect of diminishing DMT1. This may assist in understanding elevations in lung infections following exposures to vanadium in air pollution particles. These changes are consistent with the rationale that airways cells serve as a gateway for entry of metals into the body in a potentially toxic fashion. We suggest that the increase is adaptive, perhaps allowing potentially toxic exposures to iron to be managed by NTBI uptake into a compartment where iron can be stored in ferritin to minimize its toxicity. This rationale can be tested in b/b rats where one would predict that the deficit in DMT1 function would lead to increased toxicity. Another possibility would be that NTBI iron uptake via DMT1 puts airways tissue at greater risk by allowing the contents of the cells to be more directly exposed to the toxic substance. In the latter scenario b/b rats would exhibit diminished toxicity. If DMT1 is irrelevant, then they would be equally susceptible. Preliminary results (unpublished) favor the first protective hypothesis.

Mn toxicity and DMT1 in PC12 cells

Shortly before identification of the G185R mutation in DMT1 as responsible for the Belgrade phenotype, Chua & Morgan (1997) showed that b/b rats were Mn deficient. This observation combined with those reviewed above under ectopic expression and tissue metal densities indicates that DMT1 plays a major role in Mn homeostasis. The majority of research and clinical studies on the toxic actions of Mn have focused on its neurological effects because exposure to chronic high levels of the metal produces pronounced and irreversible extrapyramidal dysfunction resembling the dystonic movements associated with Parkinson's disease. The evidence for these neuorological effects and some studies on PC12 cells have recently been reviewed (Roth *et al.* 2002b).

PC12 cells possess much of the biochemical machinery associated with dopaminergic neurons making them useful as a model for neurotoxicity. Mn, like NGF, induces neuronal differentiation of PC12 cells but Mn-induced differentiation is dependent on its interaction with the cell surface integrin recep-

tors and basement membrane proteins, vitronectin or fibronectin. Similar to NGF, Mn-induced neurite outgrowth is dependent on the phosphorylation and activation of the MAP kinases, ERK1 and 2, but unlike NGF, Mn is cytotoxic. Although many apoptotic signals are stimulated by Mn, cell death is caused ultimately by disruption of mitochondrial function leading to loss of ATP. RT-PCR and immunoblotting studies suggest that a majority of the uptake of Mn into PC12 cells depends on DMT1. Alterations in Fe levels are, therefore, likely to influence the biological actions of Mn in PC12 cells since both are transported via DMT1.

Accordingly, the effects of the Fe chelator, desferrioxamine (DfO), on Mn-induced PC12 cell death and neuronal differentiation were assessed (Roth *et al.* 2002a). Mn-induced cell death was increased when PC12 cells were exposed to DfO (10 μM). DfO also stimulated Mn-induced neuronal differentiation by enhancing the phosphorylation of both ERK1 and 2. DfO enhancement of Mn-induced cell death was not related to apoptosis since the apoptotic signal, caspase 3, was attenuated approximately 50% in cells co-treated with DfO. In contrast, DfO stimulated Mn-induced suppression of mitochondrial function as evidenced by a greater loss of ATP production. Since sequestration of iron by DfO would be expected to lead to increased transport of Mn^{2+}, studies were performed to determine whether Fe^{2+} could compete with Mn^{2+} for transport into PC12 cells. Results reveal that Fe^{2+} inhibited $^{54}Mn^{2+}$ (10 nM) transport with an IC_{50} value of approximately 20 μM. In addition, coincubation of DfO with Mn in PC12 cells resulted in increased expression of both the +IRE and -IRE forms of DMT1. In conclusion, these results demonstrate that Fe status is likely to have a direct effect on the uptake and biological actions of Mn and probably other heavy metals that are transported by DMT1.

Acknowledgements

Research results from the authors' laboratories were supported by grants from the NIH, USDA, NSF and EPA.

References

Abboud S, Haile DJ. 2000 A novel mammalian iron-regulated protein involved in intracellular iron metabolism. *J Biol Chem* **275**, 19906–19912.

Alvarez-Hernandez X, Smith M, Glass J. 2000 Apo-transferrin is internalized and routed differently from Fe-transferrin by Caco-2 cells: a confocal microscopy study of vesicular transport in intestinal cells. *Blood* **95**, 721–723.

Andrews NC. 1999 The iron transporter DMT1. *Int J Biochem Cell Biol* **31**, 991–994.

Bali PK, Zak O, Aisen P. 1991 A new role for the transferrin receptor in the release of iron from transferrin. *Biochem* **30**, 324–328.

Beaumont C, Bourgeois M, Martin ME, Tchernitchko D. 2000 Expression of the two isoforms of the iron transporter Nramp2/DMT1. *Blood* **96**, 227a.

Broyles RH, Belegu V, DeWitt CR, Shah SN, Stewart CA, Pye QN *et al.* 2001 Specific repression of β-globin promoter activity by nuclear ferritin. *Proc Natl Acad Sci USA* **98**, 9145–9150.

Cai CX, Linsenmayer TF. 2001 Nuclear translocation of ferritin in corneal epithelial cells. *J Cell Sci* **114**, 2327–2334.

Cai CX, Birk DE, Linsenmayer TF. 1997 Ferritin is a developmentally regulated nuclear protein of avian corneal epithelial cells. *J Biol Chem* **272**, 12831–12839.

Cai CX, Cai CX, Birk DE, Linsenmayer TF. 1998 Nuclear ferritin protects DNA from UV damage in corneal epithelial cells. *Mol Biol Cell* **9**, 1037–1051.

Canonne-Hergaux F, Gruenheid S, Ponka P, Gros P. 1999 Cellular and subcellular localization of the Nramp2 iron transporter in the intestinal brush border and regulation by dietary iron. *Blood* **93**, 4406–4417.

Canonne-Hergaux F, Fleming MD, Levy JE, Gauthier S, Ralph T, Picard V *et al.* 2000 The Nramp2/DMT1 iron transporter is induced in the duodenum of microcytic anemia mk mice but is not properly targeted to the intestinal brush border. *Blood* **96**, 3964–3970.

Canonne-Hergaux F, Levy JE, Fleming MD, Montross LK, Andrews NC, Gros P. 2001 Expression of the DMT1 (NRAMP2/DCT1) iron transporter in mice with genetic iron overload disorders. *Blood* **97**, 1138–1140.

Chen X, Peng J, Nelson N, Hediger MA. 1999 Uncoupled currents mediated by the H+-dependent divalent metal-ion transporters DCT1 and SMF1. *FASEB J* **14**, A397.

Chua A, Morgan EH. 1997 Manganese metabolism is impaired in the Belgrade laboratory rat. *J Comp Physiol B* **167**, 361–369.

Conrad ME, Umbreit JN, Moore EG. 1999 Iron absorption and transport. *Am J Med Sci* **318**, 213–229.

Conrad ME, Umbreit JN, Moore EG, Hainsworth LN, Porubcin M, Simovich MJ *et al.* 2000 Separate pathways for cellular uptake of ferric and ferrous iron. *Am J Physiol Gastrointest Liver Physiol* **279**, G767–G774.

Donovan A, Brownlie A, Zhou Y, Shepard J, Pratt SJ, Moynihan J *et al.* 2000 Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. *Nature (London)* **403**, 776–781.

Edwards JA, Hoke JE. 1972 Defect of intestinal mucosal iron uptake in mice with hereditary microcytic anemia. *Proc Soc Exp Biol Med* **141**, 81–84.

Elisma F, Jumarie C. 2001 Evidence for cadmium uptake through Nramp2: Metal speciation studies with Caco-2 cells. *Biochem Biophys Res Commun* **285**, 662–668.

Farcich EA, Morgan EH. 1992 Uptake of transferrin-bound and nontransferrin-bound iron by reticulocytes from the Belgrade laboratory rat: comparison with Wistar rat transferrin and reticulocytes. *Am J Hematol* **39**, 9–14.

Ferguson CJ, Wareing M, Ward DT, Green R, Smith CP, Riccardi D. 2001 Cellular localization of divalent metal transporter DMT-1 in rat kidney. *Am J Physiol Renal Physiol* **280**, F803–F814.

Fleming MD, Trenor CI, Su MA, Foernzler D, Beier DR, Dietrich WF *et al.* 1997 Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. *Nature Genet* **16**, 383–386.

Fleming MD, Romano MA, Su MA, Garrick LM, Garrick MD, Andrews NC. 1998 Nramp2 is mutated in the anemic Belgrade (b) rat: evidence of a role for Nramp2 in endosomal iron transport. *Proc Natl Acad Sci USA* **95**, 1148–1153.

Fleming RE, Migas MC, Zhou XY, Jiang JX, Britton RS, Brunt EM *et al.* 1999 Mechanism of increased iron absorption in murine model of hereditary hemochromatosis: increased duodenal expression of the iron transporter DMT1. *Proc Natl Acad Sci USA* **96**, 3143–3148.

Garrick LM, Gniecko K, Hoke JE, Al-Nakeeb A, Ponka P, Garrick MD. 1991 Ferric-salicyaldehyde isonicotinoyl hydrazone, a synthetic iron chelate, alleviates defective iron utilization by reticulocytes of the Belgrade rat. *J Cell Physiol* **146**, 460–465.

Garrick LM, Gniecko K, Liu Y, Cohan DS, Grasso JA, Garrick MD. 1993a Iron distribution in Belgrade rat reticulocytes after inhibition of heme synthesis with succinylacetone. *Blood* **81**, 3414–3421.

Garrick LM, Dolan KG, Romano MA, Garrick MD. 1999 Non-transferrin-bound iron uptake in Belgrade and normal rat erythroid cells. *J Cell Physiol* **178**, 349–358.

Garrick MD, Gniecko K, Liu Y, Eckert B, Grasso JA, Garrick LM. 1990a Elevated endosomal pH in Belgrade rat reticulocytes. *J Cell Biol* **111**, 82a.

Garrick MD, Gniecko K, Liu Y, Cohan D, Eckert BS, Grasso JA *et al.* 1990b Defective endosomal acidification in Belgrade rat reticulocytes. *Blood* **76**, 8a.

Garrick MD, Gniecko K, Liu Y, Cohan DS, Garrick LM. 1993b Transferrin and the transferrin cycle in Belgrade rat reticulocytes. *J Biol Chem* **268**, 14867–14874.

Garrick M, Scott D, Walpole S, Finkelstein E, Whitbred J, Chopra S *et al.* 1997 Iron supplementation moderates but does not cure the Belgrade anemia. *BioMetals* **10**, 65–76.

Garrick MD, Horbinski C, Dolan KG, Feng L, Burdo J, Connor J *et al.* 2000 Factors associated with nuclear localization of the -IRE isoform of DMT1 (Nramp2/DCT1). *Blood* **96**, 227a.

Garrick MD, Dolan KG. 2002 An expression system for a transporter of iron and other metals. In: D. Armstrong (ed.): *Ultrastructure and Molecular Biology* stet *for oxidants and Antioxidants, Methods in Molecular Biology, vol. 196*. Totawa: Humana; pp. 141–154.

Garrick M, Horbinski C, Dolan K, Higgins DL, Feng L, Lis A *et al.* 2001 Isoforms of divalent metal transporter (DMT1 aka DCT1 or Nramp2): what and where. *BioIron (Cairns)*, 014.

Greene LA, Tischler AS. 1982 PC12 pheochromocytoma cultures in neurobiological research. *Adv Cell Neurobiol* **3**, 373–414.

Griffiths W, Sly WS, Cox TM. 2001 Intestinal iron uptake determined by divalent metal transporter is enhanced in hfe-deficient mice with hemochromatosis. *Gastroenterology* **120**, 1420–1429.

Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF *et al.* 1997 Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature (London)* **388**, 482–488.

Gurgueira SA, Meneghini R. 1996 An ATP-dependent iron transport system in isolated rat liver nuclei. *J Biol Chem* **271**, 13616–13620.

Hubert N, Hentze MW. 2001 Regulation of the iron transporter DMT1 in Caco2 cells. *BioIron (Cairns)*, P146.

Knöpfel M, Schulthess G, Funk F, Hauser H. 2000 Characterization of an integral protein of the brush border membrane mediating the transport of divalent metal ions. *Biophys J* **79**, 874–884.

Lee PL, Gelbart T, Halloran C, Beutler E. 1998 The human Nramp2 gene: characterization of the gene structure, alternative splicing, promoter region and polymorphisms. *Blood Cells Mol Dis* **24**, 199–215.

Levy JE, Montross LK, Cohen DE, Fleming MD, Andrews NC. 1999 The C282Y mutation causing hereditary hemochromatosis does not produce a null allele. *Blood* **94**, 9–11.

Lin W, Higgins D, Pacheco M, Aletta J, Perrini S, Marcucci KA *et al.* 1993 Manganese induces spreading and process outgrowth in rat pheocromocytoma (PC12) cells. *J Neurosci Res* **34**, 546–561.

McKie AT, Marciani P, Rolfs A, Brennan K, Wehr K, Barrow D *et al.* 2000 A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. *Mol Cell* **5**, 299–309.

McKie AT, Barrow D, Latunde-Dada GO, Rolfs A, Sager G, Mudaly E *et al.* 2001 An iron-regulated ferric reductase associated with the absorption of dietary iron. *Science* **291**, 1755–1759.

Nuñez MT, Gaete V, Watkins JA, Glass J. 1990 Mobilization of iron from endocytic vesicles. The effects of acidification and reduction. *J Biol Chem* **265**, 6688–6692.

Nuñez MT, Nuñez-Millacura C, Beltran M, Tapia V, Alvarez HX. 1997 Apotransferrin and holotransferrin undergo different endocytic cycles in intestinal epithelia (Caco-2) cells. *J Biol Chem* **272**, 19425–19428.

Nuñez MT, Tapia V. 1999 Transferrin stimulates iron absorption, exocytosis, and secretion in cultured intestinal cells. *Am J Physiol Cell Physiol* **276**, C1085–C1090.

Oates PS, Morgan EH. 1996 Defective iron uptake by the duodenum of Belgrade rats fed diets of different iron contents. *Am J Physiol Gastrointest Liver Physiol* **270**, G826–G832.

Oates PS, Thomas C, Freitas E, Callow MJ, Morgan EH. 2000 Gene expression of divalent metal transporter 1 and transferrin receptor in duodenum of Belgrade rats. *Am J Physiol Gastrointest Liver Physiol* **278**, G930–G936.

Olivi L, Sisk, J, Bressler, J. 2001 Involvement of DMT1 in uptake of Cd in MDCK cells: Role of protein kinase C. *Am J Physiol Gastrointest Cell Physiol* **281**, C793–C800.

Ponka P. 1997 Tissue-specific regulation of iron metabolism and heme synthesis: Distinct control mechanisms in erythroid cells. *Blood* **89**, 1–18.

Roth JA, Horbinski C, Feng L, Dolan KG, Higgins D, Garrick MD. 2000 Differential localization of divalent metal transporter 1 with and without iron response element in rat PC12 and sympathetic neuronal cells. *J Neurosci* **20**, 7595–7601.

Roth JA, Feng L, Dolan KG, Lis A, Garrick MD. 2002a Effect of the iron chelator, desferrioximine, on manganese-induced toxicity of rat pheochromocytoma (PC12) cells. *J Neurosci Res*, **68**, 76–83.

Roth JA, Horbinski C, Higgins D, Lein P, Garrick MD. 2002b Mechanism of manganese-induced rat pheochromocytoma (PC12) cell death and cell differentiation. *NeuroToxicology* **81**, in press.

Sacher A, Cohen A, Nelson N. 2001 Properties of the mammalian and yeast metal-ion transporters DCT1 and Smf1p expressed in *Xenopus Laevis* oocytes. *J Exp Biol* **204**, 1053–1061.

Sipe DM, Murphy RF. 1991 Binding to cellular receptors results in increased iron release from transferrin at mildly acidic pH. *J Biol Chem* **266**, 8002–8007.

Su MA, Trenor CI, Fleming JC, Fleming MD, Andrews NC. 1998 The G185R mutation disrupts functions of the iron transporter Nramp2. *Blood* **92**, 2157–2163.

Tabuchi M, Yoshimori T, Yamaguchi K, Yoshida T, Kishi F. 2000 Human NRAMP2/DMT1, which mediates iron transport across endosomal membranes, is localized to late endosomes and lysosomes in HEp-2 cells. *J Biol Chem* **275**, 22220–22228.

Tallkvist J, Bowlus CL, Lönnerdal, B. 2001 DMT1 gene expression and cadmium absorption in human absorptive enterocytes. *Toxicol Lett* **122**, 171–177.

Tandy S, Williams M, Leggett A, Lopez JM, Dedes M, Ramesh B *et al.* 2000 Nramp2 expression is associated with pH-dependent iron uptake across the apical membrane of human intestinal Caco-2 cells. *J Biol Chem* **275**, 1023–1029.

Trinder D, Oates PS, Thomas C, Sadleir J, Morgan EH. 2000 Localisation of divalent metal transporter I (DMT1) to the microvillus membrane of rat duodenal enterocytes in iron deficiency, but to hepatocytes in iron overload. *Gut* **46**, 270–276.

Umbreit JN, Conrad ME, Hainsworth, LN, Simovich M. 2002 The ferrireductase paraferritin contains divalent metal transporter as well as mobilferrin.

Vidal SM, Belouchi A, Cellier M, Bewaty B, Gros P. 1995 Cloning and characterization of a second human NRAMP gene on chromosome 12q13. *Mammal Genome* **6**, 224–230.

Vulpe CD, Kuo YM, Murphy TL, Cowley L, Askwith C, Libina N *et al.* 1999 Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. *Nature Genet* **21**, 195–199.

Wang X, Ghio AJ, Yang F, Dolan KG, Garrick MD, Piantadosi CA. 2002 Iron uptake and Nramp2/DMT1/DCT1 in human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol*, in press.

Watkins JA, Nunez MT, Gaete V, Alvarez O, Glass J. 1991 Kinetics of iron passage through subcellular compartments of rabbit reticulocytes. *J Membr Biol* **119**, 141–149.

White KN, Graham DC, Bomford A. 2001 Nuclear localisation of iron regulatory proteins. *Biochem Soc Trans* **30**, A38.

Yamaji S, Tennant J, Tandy S, Williams M, Srai SKS, Sharp P. 2001 Zinc regulates the function and expression of the iron transporters DMT1 and IREG1 in human intestinal Caco-2 cells. *FEBS Lett* **507**, 137–141.

Yeh KY, Yeh M, Watkins JA, Rodriguez PJ, Glass J. 2000 Dietary iron induces rapid changes in rat intestinal divalent metal transporter expression. *Am J Physiol Gastrointest Liver Physiol* **279**, G1070–G1079.

Yoshida K, Furihata K, Takeda S, Nakamura A, Yamamoto K, Morita H *et al.* 1995 A mutation in the ceruloplasmin gene is associated with systemic hemosiderosis in humans. *Nature Genet* **9**, 267–272.

Zöller H, Pietrangelo A, Vogel W, Weiss G. 1999 Duodenal metal-transporter (DMT-1, NRAMP-2) expression in patients with hereditary haemochromatosis. *Lancet* **353**, 2120–2123.

Zöller H, Koch RO, Theurl I, Obrist P, Pietrangelo A, Montosi G *et al.* 2001 Expression of the duodenal iron transporters divalent-metal transporter 1 and ferroportin 1 in iron deficiency and iron overload. *Gastroenterology* **120**, 1412–1419.