REVIEW PAPER

Manganese Flux Across the Blood–Brain Barrier

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Abstract Manganese (Mn) is essential for brain growth and metabolism, but in excess can be a neurotoxicant. The chemical form (species) of Mn influences its kinetics and toxicity. Significant Mn species entering the brain are the Mn^{2+} ion and Mn citrate which, along with Mn transferrin, enter the brain by carrier-mediated processes. Although the divalent metal transporter (DMT-1) was suggested to be a candidate for brain Mn uptake, brain Mn influx was not different in Belgrade rats, which do not express functional DMT-1, compared to controls. Brain Mn influx was not sodium dependent or dependent on ATP hydrolysis, but was reduced by mitochondrial energy inhibitors. Mn and Fe do not appear to compete for brain uptake. Brain Mn uptake appears to be mediated by a Ca uptake mechanism, thought to not be a p-type ATPase, but a store-operated calcium channel. Efflux of Mn from the brain was found to be slower than markers used as membrane impermeable reference compounds, suggesting diffusion mediates brain Mn efflux. Owing to carrier-mediated brain Mn influx and diffusion-mediated efflux, slow brain Mn clearance and brain Mn accumulation with repeated excess exposure would be predicted, and have been reported. This may render the brain susceptible to Mn-induced neurotoxicity from excessive Mn exposure.

Abbreviations

BBB	Blood–brain barrier
BMEC	Brain microvascular endothelial cell
$D_{o/a}$	Octanol/aqueous partitioning coefficient
J	Influx
K_d	Diffusion constant
K_{el}	Apparent elimination rate constant
K_{in}	Influx transfer coefficient
K_m	Michaelis–Menten constant
K_{out}	Brain capillary efflux
MMT	Methylcyclopentadienyl manganese tricarbonyl
Mn	Manganese
MW	Molecular weight
$P_{diffusion}$	Estimated permeability due to diffusion
PS	Permeability-surface area product
S	Surface area of rat brain capillaries
Tf	Transferrin
TfR-ME	Transferrin receptor mediated endocytosis
V_{brain}	Brain distribution volume
V_{max}	Maximum velocity of carrier-mediated transport

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Manganese is an Essential Trace Element that can be a Neurotoxicant

Manganese (Mn) is an essential trace element for normal brain development and function. It is a cofactor for glial-specific glutamine synthetase (that accounts for 80% its brain concentration), superoxide dismutase, pyruvate carboxylase, and other enzymes.

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Manganese can also be a neurotoxicant. This has been reported in workers involved in mining and grinding manganese dioxide, dry-cell battery production, ferromanganese smelting, Mn oxide and salt production, and Mn welding (Chandra et al. 1981; Huang et al. 1989; Ono et al. 2002; Roels et al. 1987, 1992; Sjögren et al. 1990, 1996). Neurotoxicity has also been associated with exposure to Mn-containing fungicides (e.g., maneb) (Ferraz et al. 1988), drinking well water contaminated by buried dry cell batteries (Kawamura et al. 1941), and has been seen in patients who received Mn-containing medical diagnostic contrast agents (e.g., mangafodipir) and long-term parenteral nutrition (Nagatomo et al. 1999). Other Mn exposures include fertilizers, electronics, and the fuel additive methylcyclopentadienyl manganese tricarbonyl (MMT) (Hudnell 1999). Symptoms of manganism include asthenia, adynamia, sialorrhea, fatigability, cephalalgia, sleep disturbances, muscular pain and hypertonia, mask-like face, gait changes, reduced coordination, hallucinations, and mental irritability.

The Chemical Form (Species) of Mn can Influence its Kinetics and Toxicity

The chemical form (species) of metals may influence their absorption, distribution, and toxicity (Yokel et al. 2006). Greater brain Mn concentrations were seen after $MnCl_2$ than MMT or Mn oxide (Gianutsos et al. 1985). Higher striatal Mn concentrations were seen after oral and intratracheal, but not intraperitoneal, $MnCl_2$ than MnO_2 (Roels et al. 1997). More rapid brain uptake resulted from soluble Mn chloride or sulfate than insoluble Mn oxide or phosphate (Dorman et al. 2001a, 1986).

In biological environments Mn most commonly exists as Mn^{2+} and Mn^{3+}. Mn^{2+} is stable in aqueous solutions up to pH ~ 7.5. Mn^{3+} is unstable in aqueous solutions >pH 2 and can undergo disproportionation to Mn^{2+} and Mn^4 unless stabilized as a complex with a ligand. After absorption, some Mn^{2+} oxidizes to Mn^{3+} in blood in 1–5 h (Jursa and Smith 2009; Scheuhammer and Cherian 1985). Ultrafiltration of rat serum suggested >70% of the Mn was protein bound (Rabin et al. 1993). Predominant serum Mn^{2+} species were calculated to be albumin-bound (84%), a hexahydrated ion (6.4%), 1:1 complexes with bicarbonate (5.8%) and citrate (2.0%), and Mn transferrin (Tf) (1%) (Harris and Chen 1994). Size exclusion chromatography suggested 55% of serum Mn was bound to albumin or Tf and 30% to low MW species, of which 3.5 and 94% were Mn citrate and inorganic Mn (Nischwitz et al. 2008). Albumin does not cross the intact blood–brain barrier (BBB). The 1:1 Mn:citrate complex involves coordination bonds between Mn and the central hydroxyl group and the two terminal carboxylates. The central carboxylate group, which is not involved in this complex, may serve as a recognition moiety for a transporter.

Mn^{3+} is quite reactive, rapidly associating with Tf, whereas Mn^{2+} does not (Aisen et al. 1969). It is expected that all Mn^{3+} in plasma is bound to Tf, but it is not known what percent of total plasma Mn is Mn^{3+}. With a MW of $\sim 77,000$ Da, Tf would not be expected to cross membranes in the absence of a carrier. Tf-receptor-mediated endocytosis (TRf-ME) is expressed by the brain microvascular endothelial cells (BMECs) of the BBB (Jefferies et al. 1984), potentially enabling Mn to cross the BBB. Based on the Mn species in blood plasma, candidates for brain Mn uptake are the hydrated Mn^{2+} ion, $Mn(HCO_3)_2$, Mn citrate and Mn Tf.

Mn^{2+} and Mn^{3+} have different toxicities (Reaney et al. 2002). Extensive studies have failed to positively identify Mn^{3+} in the mitochondria of PC12 or human neuroteratocarcinoma (NT2) cells, brain, liver, or heart, suggesting Mn^{2+} may be the toxic species (Gunter et al. 2006).

Relevant to Mn efflux from brain to blood, Tf probably does not play a significant role. The concentration of Tf in brain extracellular fluid is <0.25 μM. The Tf metal binding sites may all be occupied by Fe, leaving none available for Mn binding (Bradbury 1997). Most Mn in brain extracellular fluid is a low MW species, probably Mn citrate (Michalke et al. 2007).

The kinetics of Mn re-speciation may influence the availability of Mn species for distribution into or out of the brain. The rate of formation of a Mn chelate complex, such as Mn citrate, should be about 10^{+6} s^{-1}, based on the rate of water exchange for Mn^{2+} ($\sim 10^{+7}$ s^{-1}) (Burgess 1992). The rates of binding of other ligands with metals are usually within a factor of 10 of the water exchange rate. The rate that Mn might exchange a ligand, such as citrate, for another, can be calculated from $K_{eq} = K_{on}/K_{off}$, where K_{eq} (log K) is the equilibrium constant, and K_{on} and K_{off} are the metal binding association and dissociation constants. For Mn citrate, with a K_{eq} of ~ 4 (Petti and Powell 1997) and K_{on} $\sim 10^6$ s^{-1}, K_{off} will be 100 s^{-1}. For an exchange reaction involving Mn and ligands with comparable K_{eq} values, the approach to equilibrium will be a first-order process with a rate constant equal to the sum of the two ligand exchange times (Moore and Pearson 1981), or ~ 200 s^{-1}. This corresponds to a half-life ($t_{1/2}$) of 3 ms. The complete reaction would occur in ~ 5–6 $t_{1/2}$s, or ~ 20 ms. Therefore equilibrium among the Mn^{2+} ion and Mn complexes with citrate and other common ligands in blood plasma should occur within seconds. After oral or intravenous $MnCl_2$ administration nearly all of the Mn was associated with Tf after 30 min, suggesting Mn Tf is the predominant species of Mn in blood plasma (Davidsson et al. 1989).

Mn can Enter the Brain via Several Routes

Brain Mn entry is primarily a result of exchange between blood plasma and brain tissue. Mn can enter the brain from blood plasma (1) by crossing the BMECs into the brain, (2) crossing the choroid plexuses into cerebrospinal fluid and then into the brain, and (3) uptake by the olfactory and trigeminal nerves directly into the brain. This review focuses on the BBB route of brain Mn exchange.

The BBB is a highly restrictive membrane. The anatomical basis includes the tight junctions between BMECs, a basement membrane of collagen and collagen-like protein, pericytes that surround $\sim 30\%$ of the BMECs, and astrocyte foot processes that surrounded $\sim 98\%$ of this complex. The BMECs comprise $\sim 0.1\%$ of the total volume of the brain (Pardridge 2003).

Mn can Enter the Brain across the Blood–Brain Barrier

Some Mn species have been shown to enter the brain. Intracarotid injection of $MnCl_2$ with ^{14}C-sucrose (as a marker of cerebrovascular space) and ^3H_2O (which can enter the brain during a single pass) to rats followed by decapitation after 15 s (single pass of injected solution through the brain) indicated saturable brain Mn uptake (Aschner and Gannon 1994). No difference in brain Mn uptake was seen when Tf was added to the Mn and the mixture immediately injected into the carotid artery. Incubation of Mn with Tf for up to 5 days increased brain Mn uptake (Aschner and Gannon 1994), suggesting oxidation of Mn^{2+} to Mn^{3+} over time, Mn^{3+} complexation with Tf, and Mn Tf brain uptake via TfR-ME. These results suggested brain uptake of two Mn species; Mn Tf and perhaps the Mn^{2+} ion. In rats given an i.v. infusion of $MnCl_2$ to achieve a steady-state arterial blood plasma Mn concentration, the brain Mn transfer coefficient decreased as Mn concentration increased from 0.08 to 78 μM, demonstrating both saturable and non-saturable components (Murphy et al. 1991). Mn uptake into sciatic nerve was similarly mediated by facilitative, saturable process(es) (Wadhwani et al. 1992). Noting that the transfer coefficient for 0.08 μM Mn in plasma was more than two orders of magnitude greater than reported for TfR-ME of Fe and Ga, the authors concluded that non-protein-bound Mn^{2+} would be the predominate species that crosses the BBB, even if it was only 1% of total plasma Mn. In those studies the Mn ion mixed with blood before it entered the brain, so it may have crossed the BBB associated with a ligand from blood plasma. The Micheala–Menten constant (K_m) of Mn influx into the brain was ~ 1 μM (Murphy et al. 1991).

The use of radioisotopic Mn (^{54}Mn) at concentrations below the K_m enables the conduct of studies at physiologically relevant Mn concentrations, and assures that measured Mn is due to the administration of the ^{54}Mn test dose. ^{54}Mn was used as a tracer in most of the above and following studies.

Control over the chemical composition of the fluid flowing through the brain can be accomplished using the in situ brain perfusion technique. A perfusate introduced into a carotid artery is delivered to the brain at a rate sufficient to prevent entry of collateral blood circulation into the perfused hemisphere (Takasato et al. 1984). This technique was used to study $MnCl_2$ brain uptake from bicarbonate-buffered plasma electrolyte solution, plasma/serum, and whole blood perfusates (Rabin et al. 1993). Brain uptake was reported as influx transfer (K_{in}), a measure of clearance from blood into a single brain compartment containing endothelial cells, brain cells, and brain interstitial space. K_{in}, the quotient of uptake space and time, is a measure of the rate of influx of a substance in a given time into a given amount (space) of brain. It reflects the volume of perfusate cleared of substrate which is transferred into mass of brain over a given time period. The Mn K_{in} from blood and plasma was several-fold less than from the electrolyte solution. Addition of albumin, Tf and α_2-macroglobulin to an electrolyte solution reduced brain Mn K_{in} several fold, suggesting the Mn ion entered the brain more rapidly than plasma-protein-bound Mn species. It is known that TfR-ME is quite slow, ~ 2.5 Fe molecules/receptor/min (Cole and Glass 1983). These results, and the results of Aschner and Gannon (1994), who found an increase of brain uptake of Mn incubated with Tf, are not in agreement.

There was no difference in brain Mn 1 h after i.v. injection of Mn in pH 7.4 or 8.6 vehicle, but less brain Mn was seen after 6 days from the pH 8.6 than 7.4 vehicle, and even less when the Mn was Tf bound (Takeda et al. 2000). The difference between the pH 7.4 and 8.6 vehicles is difficult to understand in light of the buffering ability of blood and the rapid ligand exchange of Mn discussed above. The authors suggested Tf-independent brain Mn uptake for a short period. This is supported by observations of no difference in brain Mn in hypotransferrinemic compared to normal mice after i.v. Mn injection (Dickinson et al. 1996; Malecki et al. 1999).

Brain Mn Uptake is Carrier-Mediated

Mn citrate is another plasma Mn species that is a candidate for brain uptake. To assess Mn citrate flux across the BBB and further study the Mn^{2+} ion and Mn Tf, the in situ brain perfusion technique was used to determine their K_{in} values. These three species represent unbound, small ligand-complexed, and plasma protein-bound Mn species. To determine if their brain uptake rates were diffusion or carrier

Table 1 Octanol/aqueous partition coefficient, predicted brain capillary diffusion rate ($P_{diffusion}S$), and brain Mn uptake into the parietal cortex (K_{in}) for three representative Mn species, compared to two substances that very slowly diffuse across the BBB. From (Crossgrove et al. 2003)

Mn species or diffusion marker	Octanol/aqueous partition coefficient	$P_{diffusion}S$ (ml/s/g)	K_{in} (ml/s/g)[c]
Mn^{2+} ion	3.6×10^{-6}	1.5×10^{-5}	13×10^{-5}
Mn citrate	1.0×10^{-5}	1.7×10^{-5}	40×10^{-5}
Mn transferrin	8×10^{-4a}	2.8×10^{-5}	12×10^{-5}
Sucrose		2.9×10^{-5b}	
Dextran	2.1×10^{-3}	4.8×10^{-5}	

[a] The $D_{o/a}$ of Mn Tf was corrected to account for the presence of unbound Mn (19%), which was assumed to be the Mn^{2+} ion

[b] Sucrose $P_{diffusion}$ was directly determined

[c] The capillary depletion (enrichment) method, which separates brain tissue from capillary tissue (Triguero et al. 1990), was used to differentiate Mn reaching brain extracellular fluid from Mn adsorbed onto, or localized within, endothelial cells. The results showed 92, 89, and 75% of the Mn^{2+} ion, Mn citrate, and Mn–Tf were associated with the fraction composed of brain cells and brain extracellular fluid (Crossgrove et al. 2003). The results have been adjusted to account for the fraction of Mn associated with the endothelial cells

mediated, the K_{in} of each Mn species was compared to its estimated rat brain cerebral capillary diffusion rate. This was estimated based on the relationship between MW and the octanol/aqueous partitioning coefficient ($D_{o/a}$) (Levin 1980). The permeability × surface area product ($P_{diffusion}S$) was then calculated from the product of diffusion and rat brain capillary surface area (240 cm²/g) as described by Smith (1989). $P_{diffusion}S$ has the same units as K_{in} and K_{out} (ml/s/g brain), measures of influx and efflux at the BBB, respectively.

The calculated rat brain capillary diffusion rates of these three Mn species, sucrose, and dextran are shown in Table 1. The $P_{diffusion}S$ values for the three Mn species, sucrose, and dextran were similar and very small, suggesting diffusion of all three Mn species through the BBB would be very slow. In fact, sucrose and dextran would be expected to diffuse more rapidly through the BBB than the Mn^{2+} ion and Mn citrate in the absence of a carrier.

The K_{in} values of the Mn^{2+} ion, Mn citrate, and Mn Tf were determined by in situ brain perfusion in nine brain regions (frontal, parietal, and occipital cortex; cerebellum; caudate; hippocampus; thalamus/hypothalamus; midbrain/colliculus; and pons/medulla). They ranged from 5 to 13, 3 to 51, and 2 to 13 × 10⁻⁵ ml/s/g, respectively. The K_{in} for Mn ion influx exceeded that predicted for diffusion in the parietal cortex and hippocampus, but not caudate; for Mn citrate the K_{in} exceeded that predicted for diffusion in six brain regions, including the caudate (Crossgrove et al. 2003). As the caudate is part of the basal ganglia, which is involved in manganism and a site of Mn accumulation in those conditions, the results suggest carrier-mediated Mn citrate uptake may contribute to Mn accumulation. A significantly greater K_{in} than the calculated diffusion rate suggests carrier-mediated processes enable brain Mn uptake. Within a given brain region, the K_{in} of Mn citrate was generally higher than Mn–Tf and the Mn^{2+} ion (Crossgrove et al. 2003; Murphy et al. 1991; Rabin et al.

1993). As noted above, thermodynamic modeling suggests 40% of the Mn available to cross the BBB is the Mn^{2+} ion, while ~15% is Mn citrate. With ~one-third the concentration and with an uptake rate three times faster, Mn citrate brain influx would be comparable to the Mn^{2+} ion, suggesting it is a major Mn species that enters the brain. For the Mn–Tf species, the finding of no difference in brain Mn in hypotransferrinemic compared to normal mice after i.v. Mn injection suggests it does not play a major role in brain Mn uptake (Dickinson et al. 1996; Malecki et al. 1999).

The K_{in} of the Mn^{2+} ion and Mn citrate decreased in the presence of increasing, and very large, amounts of the Mn^{2+} ion and Mn citrate, respectively, although not in a concentration-dependent manner (Crossgrove et al. 2003). This is consistent with a non-saturable component of Mn uptake from blood (Murphy et al. 1991). The flow rate dependency of the Mn^{2+} ion influx rate was determined because flow-rate dependent uptake is a property of some carrier-mediated uptake systems, but not diffusion. Brain Mn K_{in} was not different at brain perfusion rates of 10 compared to 20 ml/min, showing Mn brain uptake was not rapid enough to be limited by the rate of perfusate flow through the brain (Crossgrove et al. 2003).

Influx (J; velocity of brain uptake) was calculated as the product of PS and Mn concentration. Mean J vs. Mn concentration was plotted and fit to kinetic models involving 0, 1, and 2 Michaelis–Menten terms (below), where J_0, J_1, and J_2 represent fits of the same J data set, K_d is the diffusion constant, C is total Mn concentration, V_{max} is the maximum velocity of the putative transporter(s), K_m is the Michaelis-Menten constant of saturable uptake, and $K_d * C$ represents the non-saturable component(s) of uptake. Sucrose and dextran were included in the calculations for comparison since they were used as vascular markers that exhibit poor membrane permeability, crossing the BBB by diffusion.

$$J_0 = K_d * C$$

$$J_1 = V_{max} * C/[K_m + C] + K_d * C$$

$$J_2 = V_{max2} * C/[K_{m2} + C] + V_{max} * C/[K_m + C] + K_d * C$$

Seven of nine Mn citrate-treated (including caudate) and 2 of 9 Mn^{2+} ion-treated brain regions (caudate and thalamus/hypothalamus) were best fit by J_1. The remaining regions were best fit by J_0, the diffusion-only model of brain Mn uptake. These results provided further evidence of carrier-mediated brain Mn influx of the Mn^{2+} ion and Mn citrate (Crossgrove et al. 2003). It is noteworthy that the caudate, one of the brain regions involved in manganism, showed evidence of non-diffusional Mn uptake of both the Mn^{2+} ion and Mn citrate and a K_{in} for Mn citrate influx that exceeded the prediction for diffusion (above).

Mn^{2+} ion uptake into BMECs was temperature dependent from 4 to 37°C. Uptake was plotted against temperature to generate the activation energy from the Årrhenius equation, which was 27 kJ/mol. This is consistent with values reported for ion channels (18–34 kJ/mol) (Liu and Ambudkar 2001).

Further evidence of carrier-mediated uptake of Mn citrate was obtained from its rate of uptake into immortalized murine brain endothelial-derived b.End5 cells. Uptake normalized to cell volume, assumed to be 2 μl/mg protein (Edlund and Halestrap 1988), showed intracellular Mn citrate was ~10-fold greater than predicted by diffusion (Yokel and Crossgrove 2004).

Manganese is Transported Across Several Non-blood–brain-Barrier Membranes

The above results suggest brain influx of the Mn^{2+} ion, Mn citrate, and Mn–Tf are carrier-mediated processes. Mn flux across other membranes is carrier mediated. There is likely to be active Mn transport by the liver into bile. The carrier has not been identified (Schramm and Brandt 1986) other than its independence from glutathione (Sugawara et al. 1994); Curtis Klaassen, personal communication). Manganese is actively transported into and out of astrocytes. Although the transporter(s) has not been identified, efflux was stimulated by $MnCl_2$ introduction into the cell medium suggesting trans-stimulation, "consistent with efflux and influx mediated by the same system" (Aschner et al. 1992). Ferret and human erythrocyte Mn uptake was thought to be mediated by the Na/Ca exchanger and Ca channels, respectively (Frame and Milanick 1991; Lucaciu et al. 1997). Mn flux across Caco-2 cells was much faster from the apical than basolateral side (Leblondel and Allain 1999). The authors found evidence for temperature dependence, competition of Mn uptake by Ca, inhibition by

Ca channel blockers, Na-dependence, and ATP-independence, concluding that Mn uptake from the gastrointestinal tract may by mediated by the same type channel as involved in Ca uptake. Transporters mediating Mn flux across some of these non-BBB membranes have been identified, including Ca channels at pre-synaptic nerve endings and motor nerve terminals, a Na/Mg anti-porter in erythrocytes, and an active Ca uniporter in mitochondria (Takeda 2003). Owing to the essential nature of Mn, it is not surprising that it distributes across the placenta into umbilical cord blood and into the fetus; and is excreted in breast milk, absorbed by suckling offspring, and appears in their organs (Miller et al. 1987; Nishimura and Inaba 1983; Onoda et al. 1978; Rossipal 2000; Tsukamoto et al. 1987). Placental transfer seems to increase in the latter stages of gestation (Onoda et al. 1978; Tsukamoto et al. 1987). Although the TfR and DMT-1 are expressed in human placenta (Chong et al. 2005; Georgieff et al. 2000; Leong and Lonnerdal 2005; Vandewalle et al. 1985), there are no reports that identify the mechanism(s) of Mn transport across the placenta or mammary epithelial cells. These sites and mechanisms of Mn transport across membranes, and those discussed below, are shown in Fig. 1.

Mechanisms Mediating Brain Mn Influx

There was no significant increase of brain Mn in humans from pre-mature infants to aged adults. This suggests the brain has an efficient homeostatic mechanism for normal Mn exposure (Markesbery et al. 1984).

Considering both the essentiality of Mn in the brain and its potential to damage the brain when present in excess, one might expect Mn brain influx and efflux to be carrier mediated, as all organisms have systems for the export of toxic cations (Ketchum 1999). There are numerous facilitative diffusion carriers and active transporters at the BBB (Yokel 2006). However, not all BBB carriers have been identified. It was estimated that only ~50% of the functional BBB transporters had been discovered (Pardridge 2003).

The Hypothesis that DMT-1 Mediates Brain Mn Uptake

One of the carriers reported to be expressed at the BBB is the divalent metal (cation) transporter DMT-1 (aka: DCT-1, Nramp2, and SLC11A2). It is a high affinity, non-transferrin-bound, membrane transporter with non-selective affinity for divalent cations, including Mn^{2+} (Gunshin et al. 1997). Mn^{2+} was a substrate at pH 5.5 when DMT-1 was transfected into oocytes (Gunshin et al. 1997). It has
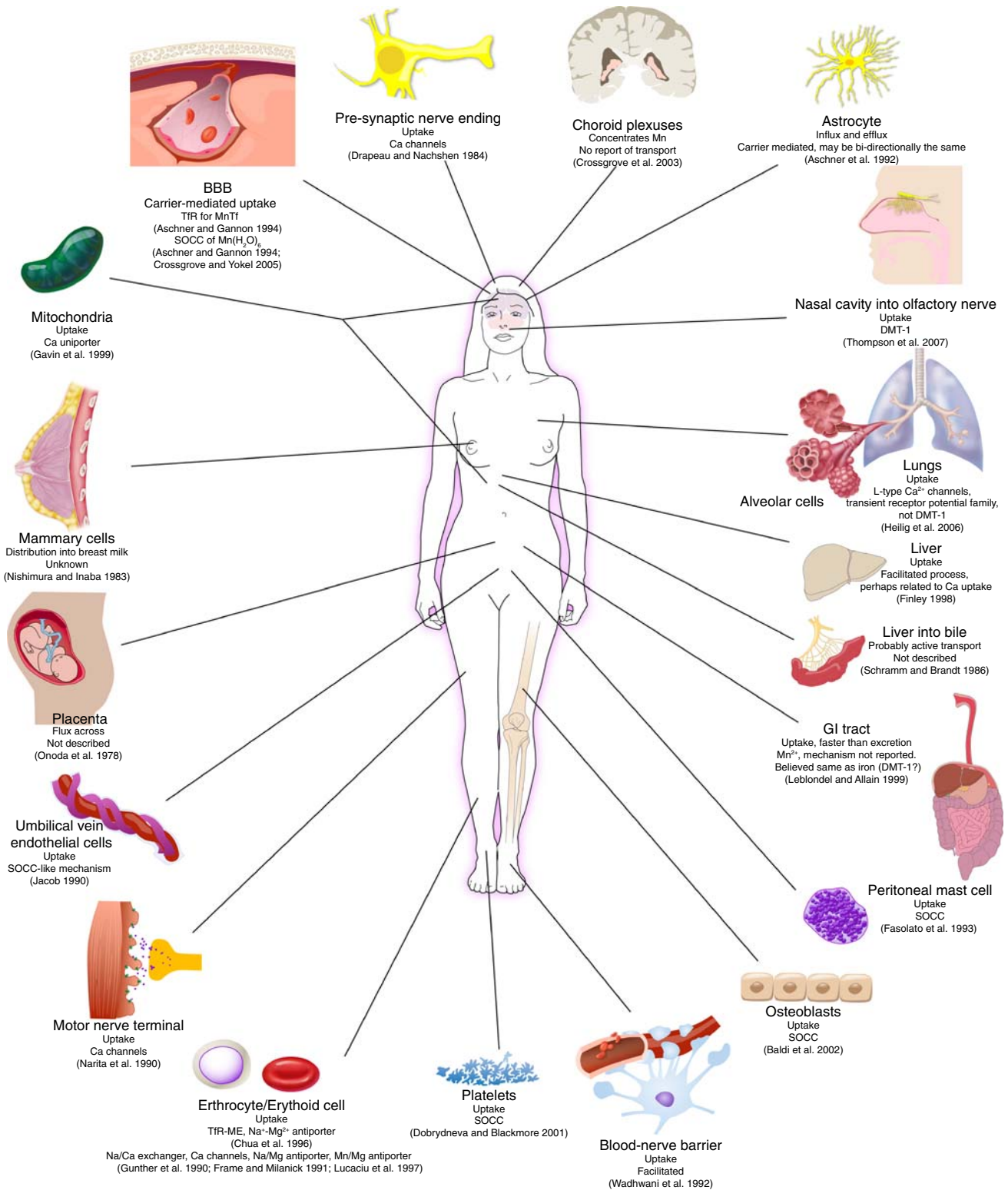
Fig. 1 Sites where Mn flux across mammalian membranes has been described, the direction of the flux (line 2), flux mechanism(s) when identified (line 3), and reference(s)

four isoforms (Roth 2009). DMT-1 and the TfR are up-regulated by Fe deficiency (Garcia et al. 2007). DMT-1 may work in conjunction with TfR-ME trivalent cation uptake into endosomes, in which the pH is dropped to ~ 5.5 by V-ATPase, the metal released from Tf, and reduced to a divalent cation. DMT-1 is a H^+ symporter,

transporting one H^+ and one divalent cation in the same direction (Sacher et al. 2001), with greatest activity between pH 5.5 and 6.5 (Picard et al. 2000). A DMT-1 antibody decreased Mn uptake by erythroleukemia-like K562 cells (Conrad et al. 2000).

DMT-1 was identified at the BBB in the normal and Belgrade rat (Burdo et al. 2001; Burdo et al. 2003). It was suggested to mediate direct cellular uptake of Fe at the plasma membrane as well as efflux of Fe from the endosome after its release from Tf, as discussed in the previous paragraph (Fleming et al. 1998; Garrick et al. 1999). This is shown in Fig. 2 of (Roth 2009). A subsequent study did not find DMT-1 expression in monkey BMECs, but in astrocyte foot processes (Wang et al. 2001), whereas further work by this group reported DMT-1 in developing rat brain BMECs (Siddappa et al. 2003; Siddappa et al. 2002). Others were unable to verify DMT-1 expression in normal (post-natal day 1 to adult) or Belgrade rat BMECs or neonatal rat brain (Ke et al. 2005). An Fe efflux transporter expressed by BMECs ferroportin (aka: Ireg, MTP1, and SLC11A3) has been suggested to mediate the export of divalent cations across the abluminal membrane of BMECs (Wu et al. 2004). However, another group failed to detect ferroportin in BMECs (Burdo et al. 2003).

The effect of pH on Mn^{2+} ion uptake into bovine BMECs (bBMECs) was assessed from media buffered to pH 6.4, 6.9, 7.4, and 7.9. Primary bBMECs are a classical model of the BBB (Audus et al. 1996). Mn uptake positively correlated with pH (Crossgrove and Yokel 2005). Increased Mn^{2+} ion uptake in the presence of reduced H^+ concentration is not consistent with a H^+-dependent carrier. Studies were conducted with RBE4 cells, an immortalized cell line derived from rat BMECs, which were grown to confluence on porous filters in the absence and presence of astrocyte-conditioned medium (Fitsanakis et al. 2006; Fitsanakis et al. 2005). The filters were suspended between two fluid compartments. RBE4 cells do not form tight junctions even when co-cultured with astrocytes (Françoise Roux, personal communication, May 2000), so may not be a good model for studies of transendothelial transport. The integrity of the tight junctions was not reported for these studies, although it was demonstrated that addition of astrocyte-conditioned medium significantly reduced Mn flux across the cells (Fitsanakis et al. 2005). In these cells, Mn flux inversely correlated with pH, from 7 to 7.8, suggesting Mn flux may be related to H^+ currents (Fitsanakis et al. 2006, 2005).

To test the hypothesis that DMT-1 may mediate brain Mn uptake, the K_{in} values of the Mn^{2+} ion and Mn–Tf were determined in homozygous and heterozygous Belgrade rats, compared to Wistar rats, from which Belgrade rats were derived. The homozygous recessive Belgrade rat (b/b) expresses a form of DMT-1 that is not functional for cation

uptake, whereas their heterozygous littermates (+/b) are phenotypically normal. Mn uptake into reticulocytes, intestinal Mn absorption, olfactory nerve Mn uptake (Thompson et al. 2007), and Mn concentration in liver and kidney were lower in b/b than +/b or Wistar rats. Endogenous brain Mn was lower (5 and 25%), but not significantly, in adult b/b than +/b or Wistar rats 2 h after iv injection of Mn as Mn–Tf or in serum. Brain Mn was lower in 15-day-old rats after Mn injection and adult rats after both Mn species (Chua and Morgan 1997).

Using the in situ brain perfusion technique, uptake of the Mn^{2+} ion and Mn–Tf were determined into the nine brain regions noted above of b/b, littermate +/b, and control rats. No statistically significant differences in K_{in} values were seen. Nor were there significant differences in the percentage of Mn in the isolated brain capillaries among the b/b, +/b, and Wistar rats following Mn^{2+} ion or Mn–Tf perfusion (Crossgrove and Yokel 2004). This is consistent with the lack of difference in the amount of Fe in BMECs among b/b, +/b, and Wistar rats (Burdo et al. 1999). These results suggest DMT-1 does not have an essential role in brain Mn uptake. The Health Effects Institute Health Review Committee opined that "In experiments to identify the transporters for Mn influx, the investigators convincing ruled out the involvement of the metal transporter DMT-1…" (Yokel and Crossgrove 2004, p. 67). However, it has been suggested that these studies were not sufficient to exclude a role for DMT-1 in Mn transport at the BBB; that the rate-limiting step may be the transfer of Mn out of the cell, for which the optimal pH may be near 7 (Roth 2006) and that the potential for adaptation and compensation due to DMT-1 deficiency may be relevant (Au et al. 2008). Another possibility might be that DMT-1 expression is sufficient (albeit very low) in the Belgrade rat to mediate Mn transport. There is considerable debate about the role of DMT-1 in Mn distribution, particularly across the BBB. Further work to definitively determine the location of DMT-1 expression at the BBB would help to clarify the controversy whether or not it plays a role in brain Mn uptake. Other approaches will also be necessary to resolve this controversy.

Missense mutations of DMT-1, such as that of the Belgrade rat and mk/mk mouse, are evidently not expressed in the human. However, a missense mutation of the HFE gene is seen in 90% of hereditary hemochromatosis patients. The HFE protein complexes with transferrin receptors, lowering Fe uptake (Griffiths et al. 2000). Therefore, enhanced TfR-ME Mn uptake might occur in this population. The finding that dihydropyridine L-type voltage sensitive calcium channel blockers prolong Fe transport by DMT-1 suggests a possible interaction between these systems for Mn uptake (Ludwiczek et al. 2007).

To determine if Mn^{2+} ion uptake was mediated by a Na-dependent process, its uptake into bBMECs from media

in which 50 or 100% of the Na was replaced with choline or lithium was determined. Mn uptake was not inhibited by Na replacement. In contrast, Mn uptake was significantly increased from a choline-containing media (Crossgrove and Yokel 2005). Similarly, replacement of Na by choline did not reduce Mn flux across RBE4 cells (Fitsanakis et al. 2006, 2005).

The energy dependence of Mn^{2+} ion uptake was assessed as its uptake into bBMECs in the presence of metabolic inhibitors, specifically: DNP, which releases H^+ gradients and causes Ca^{2+} release from mitochondria to prevent the driving force of ATP production (Lynch and Deth 1984); azide, which inhibits complex IV in the electron transport chain (Cheng et al. 2001); and 2-deoxyglucose, which is metabolically inactive and cannot enter into the glycolysis pathway (Cheng et al. 2001). These inhibitors reduced the conversion of MTT (by a mitochondrial reductase) to its formazan product to $\sim 60\%$ of control, but did not significantly affect Mn^{2+} ion uptake. These results suggest Mn^{2+} ion uptake is not directly dependent on a source of energy mediated by ATP hydrolysis (Crossgrove and Yokel 2005). However, concentrative uptake relies on an energy source or electron transfer. The pH dependence of Mn uptake suggested that it might be driven by an electromotive force, such as an H^+ or cation anti-porter. In contrast, mitochondrial energy inhibition produced by cyanide, rotenone, or oligomycin significantly reduced Mn flux across RBE4 cells, suggesting Mn uptake is an energy-dependent active process (Fitsanakis et al. 2006; 2005).

There are known interactions between Fe and Mn. High Fe intake reduced oral and lung Mn absorption (Davis et al. 1992). Over 7% of Mn was orally absorbed by Fe-deficient humans versus 3% by normal individuals (Mena et al. 1969). Mn absorption increased in young women who consumed a low-Fe diet (Finley 1999). Fe depletion generally increased brain Mn in rats, although it decreased hippocampal Mn. In contrast, Fe deficiency did not increase brain Mn in post-natal day 21 rats (Garcia et al. 2007). However some studies also showed increased brain Mn in Fe-loaded rats (Chua and Morgan 1996; Erikson et al. 2002, 2004; Fitsanakis et al. 2008, 2009; Kwik-Uribe et al. 2000).

To assess a Fe–Mn interaction at the BBB, rats were i.v. infused with Fe(OH)dextran for 6 h followed by carotid artery $MnCl_2$ injection and killed 15 s later. Brain Mn inversely correlated with the Fe dextran dose (Aschner and Aschner 1990). A further test of common processes that mediate brain Fe and Mn uptake was conducted in rats using the in situ brain perfusion technique and perfusates void of, or containing, antioxidants and Fe^{2+}. Iron, in the absence or presence of antioxidants, did not significantly decrease brain Mn uptake. To the contrary, the non-significant trend was for Fe to increase brain Mn uptake

(Yokel and Crossgrove 2004). Depletion of Fe (by inclusion of the Fe chelator desferrioxamine in the culture medium) and Fe supplementation (by incubation of monocultures in Fe dextran) increased Mn flux into RBE4 cells (Fitsanakis et al. 2005, 2006). The authors suggest the increased uptake could be due to a Mn–dextran complex, mediated by a mechanism similar to Mn citrate uptake. These results suggest brain Mn uptake is not mediated by a carrier that transports both Fe and Mn into the brain, such as DMT-1, unless the transporter's V_{max} is much larger than the concentrations of Fe and Mn tested.

Mn^{2+} has the same charge and relative size as Ca^{2+}, suggesting a Ca carrier might mediate brain Mn uptake. There is evidence for Mn–Ca interactions. Ca inhibitors, reduced temperature, and divalent cations, including Ni, reduced Mn uptake into human hepatocarcinoma (Hep-G2) cells (Finley 1998). Mn has been extensively used as a surrogate for Ca in uptake studies because it enters cells by the same pathway as Ca influx (Ambudkar et al. 1992). Ca cycles include the release of endoplasmic Ca stores into cytosol during Ca oscillations which are produced by a variety of events. Sarco-endoplasmic reticulum Ca^{2+} ATPases (SERCAs) pump intracellular free Ca from the cytoplasm into the endoplasmic reticulum or out of the cell (plasma membrane Ca pump) to recover from the flood of cytosolic Ca. When intracellular stores are Ca depleted, an unknown signal opens the plasma membrane store-operated Ca permeable channel (SOCC) to allow Ca entry from extracellular fluid. A role for a Ca uptake process mediating brain Mn uptake was supported by the finding that the Ca concentration in the uptake medium of in situ brain perfusion experiments negatively correlated with Mn^{2+} ion influx into rat brain (Crossgrove and Yokel 2005).

The p-type (plasma membrane) ATPases are channels linked with ATP hydrolysis that pump protons across the cell membrane, including Na/K-ATPase and Ca-ATPase. Ouabain, a selective Na/K-ATPase inhibitor, did not inhibit Mn^{2+} ion uptake into bBMECs (Crossgrove and Yokel 2005). Ca-ATPase is a plasma membrane Ca pump located on BCECs (Carafoli and Brini 2000). Ca^{2+}/Mn^{2+}-p-type-ATPases are quite Mn-specific secretory pathways; however, their expression in BMECs has apparently not been investigated (Xiang et al. 2005). Vanadate, a non-specific inhibitor of p-type ATPases, including Ca-ATPase (Tiffert and Lew 2001), inhibited Mn uptake into bBMECs (Crossgrove and Yokel 2005).

The hypothesis that brain Mn^{2+} ion uptake is mediated by a Ca^{2+}-ATPase was further tested using the in situ brain perfusion technique. Vanadate or p-hydroxyhippuric acid, a specific inhibitor of Ca^{2+}-ATPase (Jankowski et al. 2001), was included in the perfusate. In this experimental procedure, vanadate increased, rather than decreased, Mn K_{in} (Crossgrove and Yokel 2005). p-Hydroxyhippuric acid did

not significantly decrease brain Mn uptake (Crossgrove and Yokel 2005). These results fail to support a role for a Ca^{2+}-ATPase in brain Mn uptake.

The gating of SOCCs is controlled by sarcoplasmic reticulum Ca pump function. It has not been specifically identified but is thought to be homologous to the transient receptor potential (TRP) gene family, e.g., TRP7 (Riccio et al. 2002). The Mn ion enters rat peritoneal mast cells, osteoblast-like cells, and human platelets through SOCC (Baldi et al. 2002; Dobrydneva and Blackmore 2001; Fasolato et al. 1993a, b) and human umbilical vein endothelial cells via a mechanism blocked by Ni and inversely related to extent of fullness of an internal Ca store (Jacob 1990). Based on the use of several of the below pharmacological probes, Mn uptake from the lung was attributed to L-type Ca channels and a member of the TRP family (Heilig et al. 2006). Evidence of SOCCs in brain endothelial cells has been reported, which mediate lead uptake (Kerper and Hinkle 1997a, b; Kim et al. 2004).

To determine if brain Mn uptake occurs via a SOCC, the K_{in} of the Mn^{2+} ion was determined from perfusates containing nifedipine and verapamil, inhibitors of L-type voltage-gated Ca channels, which are expressed by BMECs (Bossu et al. 1992); $La(NO_3)_3$, an inhibitor of a variety of Ca channels; or $NiCl_2$, which inhibits Ca flux through plasma membrane channels. None of these agents significantly decreased Mn brain uptake (Crossgrove and Yokel 2005). However Ni^{2+} profoundly reduced Mn uptake into bBMECs, suggesting a role for Ca channels in Mn uptake (Crossgrove and Yokel 2005). Ni has been shown to inhibit receptor-operated Ca channels and SOCC (Cui and Dannies 1992; Kukkonen et al. 2001). Ni is thought to be a rather specific Ca channel inhibitor, as it did not appear to be a substrate for the channels it blocks (Jones and Sharpe 1994; Shibuya and Douglas 1992). Pre-treatment with agents that inhibit SERCA ATPase to cause release and depletion of intracellular Ca stores (cyclopiazonic acid and thapsigargin; (Kerper and Hinkle 1997b)) significantly increased Mn uptake into bBMECs (Crossgrove and Yokel 2005). Thapsigargin was shown to increase Mn entry into rat parotid acinar cells (Ambudkar et al. 1992), thymic lymphocytes (Mason et al. 1993), and a non-excitable clone of human embryonic kidney cells (HEK293) (Kerper and Hinkle 1997a). These results suggest a possible role of SOCCs in Mn uptake. The pH dependence of brain Mn uptake is also consistent with a role for SOCC in brain Mn influx, as store-operated Ca influx into microglia was inhibited by low extracellular pH (Khoo et al. 2001). These results are consistent with the proposed uptake of Mn from the gastrointestinal tract by processes that mediate Ca uptake (Leblondel and Allain 1999).

A putative carrier for Mn citrate was suggested to be the monocarboxylate transporter (MCT), an H^+ co-transporter.

At dilute Mn and citrate concentrations and pH ~ 7.35 the central carboxylate of the 1:1 Mn:citrate complex is non-coordinated and negatively charged, therefore an organic anion. These are the conditions of brain extracellular fluid, blood plasma, and the in situ brain perfusate. Other suggested candidates for Mn citrate transport are members of the organic anion transporter polypeptide (Oatp) or ATP-binding cassette (ABC) superfamilies.

Mn citrate uptake was assessed in b.End5 cells that were shown to express MCT-1 (Yokel et al. 2002). Uptake was greater from pH 6.9 than pH 7.4 media, suggesting an H^+-dependent uptake mechanism (Yokel and Crossgrove 2004), consistent with the possibility that MCT-1 mediates Mn citrate uptake. To determine if the Mn citrate carrier is a member of the family of organic anion transporters, rats were perfused, during in situ brain perfusion, with Mn citrate in the near absence of Na (a potential co-substrate of some transporters) and in the presence of quercetin (which inhibits several transporters, including MCT-1) (Poole and Halestrap 1993). Low Na had no significant effect (Yokel and Crossgrove 2004). Quercetin significantly, but incompletely, reduced Mn citrate brain uptake (Yokel and Crossgrove 2004). The incomplete inhibition by quercetin suggests that either the quercetin concentration was insufficient to block all the transporters or that more than one carrier is responsible for Mn citrate brain uptake.

Mn Brain Efflux Rates and Mechanisms

Efflux of Mn from cells and the brain has received very little attention. Work in vitro showed Mn efflux from cultured chick glial cells (Wedler et al. 1989). As noted above, Mn is actively transported out of astrocytes (Aschner et al. 1992). Glial cell Mn efflux would not result in Mn efflux out of the brain.

One of the few methods to determine the rate of brain efflux was described by (Kakee et al. 1996). In this method the rate of brain Mn efflux (K_{out} or Cl_{efflux}, a brain efflux rate term comparable to K_{in}) is calculated from the volume of distribution of the test substance in the brain (V_{brain}) and its rate of disappearance from the brain after discrete microinjection (K_{el}). K_{el} is determined from the brain efflux index (BEI), which is the percentage of substance injected into the brain that has effluxed from brain to blood across the BBB. An "impermeable reference compound" that very slowly diffuses across the BBB during the time course of the experiments, serving the same function as $[^{14}C]$-sucrose in the in situ brain perfusion technique, is included in the brain injectate to quantify its volume. This method enables control of the chemical species of test substance introduced into the brain.

To determine the rate of brain Mn efflux, V_{brain} was determined for the Mn^{2+} ion, Mn citrate, and Mn–Tf from uptake into parietal Par2 slices in vitro. Brain slice Mn concentration increased over time to a significantly greater extent than [^{14}C]-sucrose for each Mn species, and was concentrative. To determine BEI, the Mn^{2+} ion or Mn citrate and [^{14}C]-sucrose or [^{14}C]-dextran were co-injected (in 0.2 μl) into rat parietal cortex. The rats were terminated at various times. Nearly 99% of the Mn remaining in the brain was in the central 1/3 of the hemisphere ipsilateral to the injection, indicating very little distribution from the injection site within the brain. Graphs of BEI results, as 100-BEI (%) versus time, an indication of the percentage of Mn remaining in the brain after its injection, showed a small increase of the injected Mn remaining in the brain over time, compared to co-injected ^{14}C-sucrose or ^{14}C-dextran. As 100-BEI increased over time after Mn^{2+} ion and Mn citrate injection, K_{el} and K_{out} could not be calculated. The 100-BEI values after the injection of these two Mn species into the brain were very similar, believed to be due to rapid equilibration to the same Mn species, as noted in the discussion of Mn re-speciation above. Mn species, calculated by Dr. Wesley Harris, University of Missouri-St. Louis, based on the composition and concentration of ligands in cerebrospinal fluid, suggested they would be predominantly the Mn^{2+} ion (56%), Mn citrates (20%), and Mn phosphate (15%), consistent with the quantification of Mn species in human CSF (Nischwitz et al. 2008).

The slightly increasing 100-BEI Mn values over time are consistent with the slightly slower predicted diffusion rate of Mn than sucrose or dextran (Table 1). These results suggest brain Mn efflux is mediated by diffusion. A physiologically based pharmacokinetic model that described the results of three studies of Mn uptake in rats had a half-life of brain Mn influx several-fold shorter than the half-life of brain efflux, consistent with more rapid brain Mn influx than efflux (Nong et al. 2008).

The conclusion that brain Mn efflux may not occur via active transport was challenged in a White Paper by Taylor et al. of the Ethyl Corporation, suggesting an alternative explanation might be Mn uptake by astrocytes, reducing the pool of available Mn for efflux from the brain. The Health Effects Institute's Health Review Committee noted that even if that was true, the Mn would remain in the brain, and the Mn taken up by the astrocytes might contribute to the neurotoxic effects of Mn (HEI response January 3, 2005).

As a result of the observation of higher brain Mn concentration in aceruloplasminemic than wild mice and prior suggestions that brain ceruloplasmin facilitates Fe efflux by oxidation of Fe^{2+} to Fe^{3+}, the authors speculated that ceruloplasmin may play a role in brain Mn efflux (Jursa and Smith 2009). This hypothesis could be tested.

The findings that brain Mn influx is more rapid than efflux is consistent with slower Mn elimination from the brain than other compartments, and brain Mn accumulation during repeated/continuous exposure. After injection, the decrease of Mn over time was slower from the brain than from most tissues (Drown et al. 1986). Little decrease of cerebral Mn was seen in the monkey up to 278 days after its peripheral injection (Dastur et al. 1971). The half-life of Mn in 16 rat brain regions was estimated to range from 52 to 74 days (Takeda et al. 1995), compared to whole body Mn elimination half-lives of 2.4–3.8 and 25–32 days and averages of 6 and 43 days in rats (Dorman et al. 2001a, b). A single i.v. injection of Mn in three humans showed whole body and head biological half-lives of 15–37.5 and 37–62 days, respectively (Cotzias et al. 1968). After inhalation, the half-life of Mn elimination from the brain of two macaque monkeys was 223 to 267 days versus three phases of elimination with half-lives of <1, 12–27, and 94–187 days from the chest area (Newland et al. 1987). Increased brain Mn concentration was seen following repeated Mn exposures (Gianutsos et al. 1985), suggesting steady-state conditions were not met.

In summary, the results from several groups show evidence of the presence of BBB carriers that mediate the influx of Mn^{2+} ion, Mn citrate, and Mn–Tf into the brain. Each of these carriers would increase the rate of brain Mn entry over that provided by diffusion. Evidence has been provided for Mn–Tf and Mn^{2+} ion uptake across the BBB mediated by TfR-ME and SOCC. The role of DMT-1 remains controversial. There is evidence against, but no direct evidence supporting, its role. The results from the only study of brain Mn efflux suggest it is diffusion mediated. In light of carrier-mediated brain influx but not efflux, it would be anticipated that the half-life of Mn in the brain may be greater than other compartments. Several reports have shown this. It would be expected that repeated, excessive Mn exposure might result in brain Mn accumulation over time, which has been shown.

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