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REVIEW

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Manganese-induced neurotoxicity: from *C. elegans* to humans

Pan Chen,[a] Sudipta Chakraborty,[a] Tanara V. Peres,[a,b] Aaron B. Bowman[c] and Michael Aschner*[a]

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Manganese (Mn) is one of the most abundant metals on the earth. It is required for normal cellular activities, but overexposure leads to toxicity. Neurons are more susceptible to Mn-induced toxicity than other cells, and accumulation of Mn in the brain results in Manganism that presents with Parkinson's disease (PD)-like symptoms. In the last decade, a number of Mn transporters have been identified, which improves our understanding of Mn transport in and out of cells. However, the mechanism of Mn-induced neurotoxicity is only partially uncovered, with further research needed to explore the whole picture of Mn-induced toxicity. In this review, we will address recent progress in Mn-induced neurotoxicity from *C. elegans* to humans, and explore future directions that will help understand the mechanisms of its neurotoxicity.

Introduction

Manganese (Mn) is a naturally occurring heavy metal that is the 5th most abundant metal in the earth's crust, and the 12th most abundant element overall. Mn typically exists in its natural form in the environment as oxides, carbonates and silicates. Humans are readily exposed to Mn from air, soil and waterways due to natural erosion, as well as from man-made, industrial sources. However, the primary route of human exposure to Mn arises from daily dietary intake. Whole grains, legumes, rice and nuts contain the highest levels of Mn, but it can also be found in chocolate, tea, leafy green vegetables, and some fruits like blueberries.[1] The variety of Mn-containing dietary sources allows humans to easily obtain adequate Mn levels (2.3 mg per day for men, 1.8 mg per day for women).

The necessity of Mn from dietary intake is evident in its vital role in several important physiological processes, including reproduction, development, immune function, digestion, energy metabolism and antioxidant defenses against cellular stress.[2] Mn participates in these physiological processes by acting as a cofactor of multiple enzymes. For example, Mn-containing pyruvate carboxylase (PC) catalyzes the carboxylation of pyruvate to oxaloacetate, acting as a crosstalk between lipid and carbohydrate and metabolism.[3] The arginase enzymes (ARG1/2) also require Mn ions for their function in removing toxic ammonia from the body by converting arginine into urea and ornithine.[4] Outside of its role in energy metabolism, Mn plays a major role in antioxidant functions. A major enzyme in this category is superoxide dismutase 2 (MnSOD or SOD2), a mitochondrial protein that converts superoxide into hydrogen peroxide and O_2. This enzyme is found in the mitochondrial matrix and is thought of as a major antioxidant in the cell that is tightly connected to aging, with decreased SOD2 expression associated with aging processes.[5]

However, excessive Mn may accumulate in the liver, kidney and brain, resulting in various symptoms, including hepatic cirrhosis, dystonia, polycythemia, and hypermanganesemia.[6,7] The neurotoxicity induced by Mn in the brain is termed as "manganism". Patients with manganism present similar symptoms of idiopathic Parkinson's disease (PD), such as bradykinesia and rigidity, reduced response speed, irritability, mood changes.[8] Therefore, Mn is considered as an environmental risk factor for idiopathic PD. However, there are differences between these two. For example, manganism patients tends to have Mn accumulation in GABAergic cells of the globus pallidus and impair GABAergic signaling, while in PD patients, the dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc) undergo neurodegeneration and produce less dopamine (DA)[9] resulting in decreased dopamine release,[10,11] oxidative stress,[12,13] mitochondrial dysfunction[14] and protein dyshomeostasis[15] are the primary concerns induced by excessive Mn levels, which impair normal neuronal function and lead to neurodegeneration. Mn levels are under strict homeostatic regulation, since Mn is required for cellular activities, but can result in toxicity at excessive levels. Mn enters and exists a cell through various Mn transporters. These trans-

[a]*Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY, USA*
[b]*Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil*
[c]*Department of Neurology, Vanderbilt University Medical Center, Nashville, TN, USA*

porters include the importers [the divalent metal transporter 1 (DMT1), the citrate transporter, calcium channels, the choline transporter, dopamine transporter (DAT), zinc transporters ZIP8 and ZIP14, the transferrin receptor (TfR)], and exporters [ferroportin (Fpn), secretory pathway Ca2+-ATPase 1 (SPCA1), ATPase 13A2 (ATP13A2), and solute carrier family 30 member 10 (SLC30A10)].[6] Of note, all these transporters are permeable to other essential metal ions (*e.g.* iron and zinc) or solutes at similar or even higher efficiencies compared with Mn, with the possible exception of SLC30A10 (for which permeability to zinc *versus* Mn has not been reported. Thus a metal selective Mn transporter has yet to be identified. In this review, we will address the neurotoxicity induced by Mn, the symptoms and behavioral alternation, as well as Mn transporters identified in patients and animal models.

Manganese exposure in humans

The primary route of human exposure to Mn is from daily dietary intake. While the typical adult human ingests <5 mg Mn kg^{-1}, only around 3–5% of ingested Mn is absorbed through the gastrointestinal tract. Radiolabeled ^{54}Mn uptake studies show that for a meal containing 1 mg Mn, adult males absorb 1.35 ± 0.51%, while adult females absorb 3.55 ± 2.11%.[16,17] There is also rapid turnover of ingested Mn, and homeostatic Mn levels are maintained through proper biliary excretion. The majority of excreted Mn is bound to bile salts and other bile components in the liver and secreted into the intestines for elimination in the feces.[16,18] Therefore, patients with hepatic encephalopathy or liver failure are at high risk of Mn poisoning.[19] There is also a small amount of Mn that is reabsorbed in the intestines from the bile, forming an enterohepatic circulation,[20] with some Mn also excreted by the pancreas or *via* urine.[16]

Within the brain, astrocytes are thought to be the major site of Mn accumulation due to the primary localization of glutamine synthetase (GS) within these glial cells. This enzyme is required for the glutamate–glutamine shuffle, and is found bound to 80% of brain Mn, which is a necessary cofactor for its activity.[21] The majority of brain Mn being bound to this enzyme results in the higher accumulation of Mn in astrocytes. The selectivity of Mn accumulation within these cells makes astrocytes an early target of toxicity, despite the fact that they are also more resistant to stressors than neurons.[22,23]

Mn levels are regulated by a tight homeostatic control of intestinal absorption and biliary excretion. Therefore, toxicity in humans from "normal" dietary intake is uncommon. However, some neonatal and infant formulas can be partially composed of a trace element-enriched solution that contains small amounts of Mn. In unhealthy neonates, total parenteral nutrition (TPN) therapy can have detrimental consequences, as younger individuals, absorb and retain higher levels of Mn compared to adults.[24,25] With TPN, intravenous (IV) administration of a Mn-containing solution results in the bypass of the typical regulation *via* intestinal absorption, potentially leading to higher Mn retention and subsequent toxicity. Human milk contains 3–10 μg per l Mn, while TPN solutions without the addition of a trace element solution contain 5.6–8.9 μg per l Mn. While only about 8% of the Mn in human milk is absorbed in neonates, 100% of the Mn in TPN is absorbed from IV administration. Furthermore, the addition of a Mn-containing trace element solution to the TPN administration results in a100-fold greater Mn burden *vs.* neonates consuming human milk.[26] Additionally, manganism has also been seen in addicts using the drug methcathinone, which is created by the oxidation of ephedrine and pseudoephedrine using potassium permanganate. Amateur chemists synthesizing methcathinone illegally will often have extremely high levels of Mn from the potassium permanganate that easily cause damage from intravenous application.[27,28]

In addition to dietary exposure, the general population may also be exposed to high levels of Mn from a variety of man-made sources, including the antiknock agent in unleaded gasoline known as methylcyclopentadienyl Mn tricarbonyl (MMT). Combustion of gasoline containing this additive can result in the release of Mn into the ambient air.[29,30] Moreover, Mn is also found in some fungicides and pesticides, resulting in agricultural workers having a direct source of exposure, while surface runoff could lead to potential contamination of waterways with Mn-containing chemicals.[31] Recent studies have demonstrated the harmful consequences on intellectual abilities in children exposed to high levels of Mn in groundwater.[32] In addition to these environmental sources, humans can be exposed to high levels of Mn from several occupational settings.[33] Manganese is a key component in steel production, and is also found in the manufacturing of batteries, fireworks, ceramics, leather, glass cosmetics and other textiles. In addition to direct contact with steel rods containing high levels of Mn, a major concern for industrial workers (miners, smelters, welders, *etc*.) is inhalation of the dangerous, Mn-containing fumes in these factories that may be concentrated in small spaces.[34]

Manganese-induced neurotoxicity and symptoms

Regardless of the source, excessive Mn exposure can lead to a state of neurotoxic Mn poisoning known as "manganism," a condition first identified by James Couper in 1837 in five industrial workers exposed to high levels of manganese from the use of manganese oxide in the production of chloride for bleaching power. This condition results in irreversible damage to the basal ganglia region of the brain, the same region implicated in the 2nd most common neurodegenerative disorder, Parkinson's disease (PD).[35] PD is marked by the selective loss of dopaminergic (DAergic) cells in the substantia nigra pars compacta (SNpc), often seen with inclusions composed of the protein alpha-synuclein known as Lewy bodies. Cardinal PD motor symptoms include bradykinesia, rigidity, tremors and postural instability.[36] Unfortunately, symptoms do not typically present until nearly 80% of the cells are already lost, making

current treatment options like L-DOPA (L-3,4-dihydroxyphenylalanine, the precursor to dopamine) administration incapable of fully restoring DAergic tone, resulting in only partial symptomatic relief.[37] However, manganism remains a separate entity from PD, with distinctive targets of initial cell death resulting in some differing symptomatology. While bradykinesia and rigidity are still present, tremor is not as apparent in patients suffering from manganism.[9] Unlike PD, dystonia is more highly prevalent, with manganism patients showing a propensity to falling backward.[38] Similar to PD, however, manganism is progressive in nature, with only partial recovery of certain symptoms following elimination of the source of overexposure for an extended period of time.[39]

While PD initially targets the DAergic cells of the SNpc, Mn preferentially accumulates in and damages the GABAergic cells of the globus pallidus and corpus striatum,[9,40,41] although high concentrations of Mn have been found in striatum and hippocampus of rats after chronic exposure.[42] Consequently, the major difference lies in the fact that one condition destroys cells responsible for dopamine production, while the other targets cells responsible for GABA production. Distinguishing between the two conditions relies heavily on a variety of biomarkers and tests specific for each condition. Diagnostics using magnetic resonance imaging (MRI) techniques can visualize the increased signal intensities in the globus pallidus in T1-weighted images, though they will disappear within six months to a year of removing the source of Mn exposure. Moreover, a positron emission tomography (PET) scan can also distinguish between manganism and PD: manganism patients show a normal scan, while PD patients show reduced striatal uptake of the radioactively labeled analog of the dopamine precursor DOPA (18-fluorodopa).[9,43] Another potential biomarker for manganism was recently identified using voxel-based morphometry (VBM). The study found that compared to healthy control subjects, welders chronically exposed to Mn possess decreased brain volumes in the globus pallidus and cerebellum that correlate with cognitive and motor deficits.[44] Another key difference between manganism and PD is the lack of effectiveness of L-DOPA treatment for manganism, contrary to PD.[45] Instead, treatment with the metal chelator EDTA (ethylenediaminetetraacetic acid) and sodium *para*-aminosalicylic acid (PAS) has shown to be beneficial in some cases,[2,46] though this option may be most beneficial before the condition has progressed too far.

The association between occupational exposure to Mn-containing fumes (*e.g.*, those experienced by welders) and Parkinsonism remains controversial in the literature.

A cross-sectional study conducted in 2006 found a higher prevalence of Parkinsonism in Alabama welders compared to age-matched control subjects.[47] The same group recently published another study showing that Mn-exposed welding workers had similar scores (>15) on a commonly used questionnaire for PD motor evaluation (UPDRS3, or Unified Parkinson's Disease Rating Scale motor subsection 3) compared to newly diagnosed, untreated idiopathic PD (IPD) patients.[48] Yet, other studies have not found an increased risk for PD in welders. A 2012 Danish study[49] and a 2005 study using data from movement disorder clinics[50] found no positive association between PD and welding. However, the former study relied on hospital contacts, and the latter study relied on specialty clinic surveys to define Parkinsonism, compared to clinical examinations and/or the UPDRS3. The discrepancies in these studies may be due to differences in defining PD in their cohorts, as well as varying welding exposures to Mn.

The association between non-occupational Mn exposure and Parkinsonism is also unclear. A 2009 study from a mining district in Mexico found attention impairments in a population where a majority of participants were exposed to Mn in ambient air at levels higher than the recommended EPA guidelines for non-occupational environments (>0.05 μg m^{-3}).[51] Similarly, a study on the general population living near a ferromanganese refinery in Ohio found slight, subclinical impairments in postural balance upon chronic exposures to Mn in ambient air.[52] Moreover, a recent 2013 study on a population living close to a manganese processing plant found decreased olfactory function compared to a population living far from the plant.[53] Yet, other studies have found minimal effects of Mn on the general population. A study on the role of MMT from gasoline combustion compared garage mechanics *vs.* blue-collar workers and found no significant difference between the two groups in whole blood Mn concentrations, with no obvious health problems.[54] Moreover, a more recent study found limited evidence for any association between ambient metal exposure in adults and the risk of PD using a nurses' cohort and the Environmental Protection Agency's (EPA) Air Toxics data.[55] More studies must be done to investigate the long-term effects of chronic, low-dose Mn exposure to the general human population, be it from gasoline combustion or other non-occupational sources found in ambient air.

Though the literature remains disputed in the connection between environmental Mn exposure and Parkinsonism, the molecular mechanisms behind both PD and manganism share several key processes. A major hallmark of both conditions is increased oxidative stress. In PD, the selectivity of DAergic cell loss brings to question whether dopamine oxidation-induced ROS (reactive oxygen species) production is responsible for the cell death. Recent studies have noted the possible role of polymorphisms in certain proteins involved in the dopamine pathway, such as the vesicular monoamine transporter type 2 (VMAT2),[56] which buffers free, cytoplasmic DA that would otherwise generate ROS. A more recent study found reduced vesicular DA uptake and VMAT2 binding in isolated, striatal synaptic vesicles containing dopamine from autopsied PD brains compared to controls after correcting for DA nerve terminal loss.[57] Similar to dopamine oxidation in PD, high Mn levels can result in increased oxidative stress through a variety of mechanisms. Mn can directly inhibit complexes of the electron transport chain in the mitochondria that are responsible for ATP production. This results in both the leakage of damaging free radicals, as well as ATP depletion in the cell.[12] Recent *in vitro* evidence using the human neuroblastoma SH-SY5Y cell line has found Mn-induced changes at

the DNA level, with increased accumulation of DNA single strand breaks and oxidized thymine bases. However, pre-treatment with antioxidants could rescue these signs of oxidative damage, further supporting the role of Mn in increasing oxidative stress in human cells.[58] Moreover, Mn-exposed Gli3 cells (a human astrocyte line) show a loss in mitochondrial membrane potential and caspase-9 activation, with concomitant alterations in mitochondrial fission and fusion protein levels resulting in enhanced fragmentation.[59] Manganese has also been shown to affect glutamate transporter levels and overall glutamate neurotransmission, resulting in cell death from glutamate excitotoxicity, a phenomenon also seen in PD.[60] Another hallmark of both conditions is increased protein aggregation, with dopamine oxidation as a potential modifier of protein aggregation states in PD.[61] Similarly, Mn can induce aggregation of the protein alpha-synuclein[62] that is found aggregated in Lewy body inclusions in a majority of PD cases. The crosstalk is further evident by the fact that Mn itself can also enhance DA oxidation.[63]

The interaction between Mn toxicity and PD-associated proteins has recently become a focus of studies in humans. In the first study of its kind, Aboud and colleagues differentiated fibroblasts into human induced pluripotent stem cell (hiPSC)-derived early neural progenitor cells (NPCs) from a patient carrying a mutation in PARK2/parkin and a control subject. Though no difference in Mn cytotoxicity or mitochondrial fragmentation was found between the subjects, but increased Mn-dependent ROS generation was found in the NPCs carrying the parkin deletion.[64] Furthermore, the increased ROS occurred in the face of decreased Mn accumulation in the PARK2/parkin mutant cells. In contrast, Roth and colleagues found that human lymphocytes (immune cells that lack dopamine) from patients expressing mutated parkin show increased mitochondrial dysfunction from Mn exposure compared to control lymphocytes. However, they do not exhibit any difference in Mn-induced cell death or Mn accumuation.[65] Taken together these data suggest differential influences of this PD genetic risk factor on Mn neurotoxicity in neuronal *versus* non-neuronal models.

Mn-induced neurotoxicity in rodent cell culture models

In addition to clinical studies, rodent models are also widely used for the study of the mechanisms underlying Mn-induced neurotoxicity. High concentrations of Mn may activate a series of intracellular molecular events that lead to apoptosis in various cell lines including rat pheochromocytoma (PC12, a DAergic neuron model) cells,[66–69] rat astrocytoma C6 cells,[70] rat mesencephalic cells (MES 23.5) overexpressing human α-synuclein.[71] Mn accumulation in the mitochondria is a primary cause of cellular toxicity, which results in mitochondrial dysfunction,[14] oxidative stress[12,13,58,72] and apoptosis.[66–68,70,71,73–76] In rat primary astrocyte culture, the accumulation of Mn in mitochondria caused dissipation of the inner membrane potential which may be at the origin the toxic effect of Mn on astrocytes, leading to oxidative stress, dysregulation of intracellular signaling pathways (increased phosphorylation of ERK, a mitogen activated protein kinase, MAPK) and cleavage of caspase-3 causing apoptotic cell death.[12–14,72–77] Metabolism was also affected in PC12 cells exposed to Mn by the downregulation of mitochondrial glutaryl-CoA dehydrogenase (GCDH).[74] Mn also induces production of H_2O_2 and activates an antioxidant response in rat PC12 cells that involves the upregulation of heme oxygenase 1 (HO-1).[73] The induction of HO-1 expression is regulated by binding of the transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2) to the antioxidant responsive element (ARE) and it has been shown before that Mn exposure activates this pathway in PC12 cells.[78] Evidence shows that mitochondria isolated from rat brain are susceptible to Mn in the form of inhibition of mitochondrial aconitase activity. Mitochondrial aconitase is an Fe containing enzyme that catalyzes the interconversion of L-citrate and isocitrate in the tricarboxylic acid cycle, thus it plays an important role in energy production, while the cytoplasmic aconitase regulates cellular Fe homeostasis and may also influence mitochondrial aconitase activity. *In vivo*, chronic Mn exposure *via* i.p. injections in rats caused a disruption of total brain aconitase activity (both cytoplamic and mitochondrial), which may be due to altering the binding of Fe and/or L-citrate to the active site of the enzyme. The effect of Mn on aconitase activity exemplifies the contribution of Fe dyshomeostasis to Mn toxicity.[75] Collectively, these studies show that mitochondrial dysfunction is a key event in the neurotoxicity of Mn.

In *C. elegans*, Mn specifically targets DAergic neurons, rather than GABAergic neurons. The hermaphrodite worms have eight DAergic neurons, including two pairs of cephalic (CEP) neurons, a pair of anterior deirid (ADE) neurons in the head, and a pair of postdeirid (PDE) neurons in the tail; male animals have additional DAergic neurons in the tail.[79] These neurons can be visualized by expressing $P_{dat\text{-}1}$::GFP, which drives GFP expression in DAergic neurons specifically. When DAergic neurons undergo neurodegeneration, they usually show puncta, shrunken soma, loss of dendrites and cell body in the 4 CEPs and 2 ADEs in hermaphrodites.[79] Benedetto *et al.* first compared Mn induced neurotoxicity in different types of neurons (including DAergic, GABAergic, cholinergic and chemosensory neurons) and found that only DAergic neurons undergo neurodegeneration, and other neurons are not affected.[13,80] This phenotype is dependent on dopamine transporter DAT-1, as DAergic neurons do not degenerate in *dat-1* deletion mutant worms upon Mn exposure. However, worms carrying *dat-1* mutations are hypersensitive (with decreased survival rate) to Mn treatment, as well as worms carrying a triple knockout of all three DA receptors (DOP-1, DOP-2 and DOP-3). On the other hand, worms carrying loss-of-function mutations in tyrosine hydroxylase (*cat-2*) and vesicular monoamine transporter 2 (*cat-1*) are hyper resistant to Mn exposure.[13] DAT-1 and the DOPs are involved in clearance of synaptic DA; CAT-2 and CAT-1 are responsible for endogenous DA production and DA packing for synaptic release, respectively. Therefore, these results

indicate that synaptic DA, but not intracellular DA, plays a key role in the DA-dependent Mn-induced toxicity.[13] Recently, Bornhorst and Chakraborty identified a few PD genes (α-synuclein, parkin and DJ-1) that are involved in Mn-dependent DAergic neurodegeneration. They found that deletion of parkin (*pdr-1* in worms) or DJ-1 (*djr-1.1* in worms) alone does not significantly alter DAergic neurodegeneration upon Mn treatment, when compared with control worms.[80] However, overexpression of α-synuclein rescues DAergic neurodegeneration in *pdr-1* deletion mutants, but not *djr-1.1* mutants.[80]

Mn transporters: regulation of Mn levels

Mn is required for cellular activities, but it also results in toxicity at excessive levels, therefore its levels need to be strictly regulated. Mn moving in and out of cells is controlled by Mn transporters, including DMT1, SLC30A10, ZIP8 and ZIP14, citrate transporter, calcium channels, choline transporter, DAT, ATP13A2, TF/TfR, Ca^{2+} uniporter, SPCA1, and Na^{+} -independent mechanisms.[6] The primary transporters being studied include DMT1, SLC30A10 and Tf/TfR. It is noteworthy, that all these transporters also mobilize either iron or zinc across cell membranes, and that both iron and zinc have been substantiated as environmental modifiers of PD and or parkinsonism.[82–85] The similar molecular features of both PD and manganism call into question whether proteins involved in Mn homeostasis may also be connected to PD. Interestingly, recent evidence has started investigating the role of various Mn transporters in promoting neurodegeneration.

Divalent metal transporter 1 (DMT1)

The primary Mn importer is known as DMT1. In addition to Mn^{2+}, this protein transports a variety of divalent metals, including iron (Fe^{2+}). Increased expression of DMT1 has been found in the SNpc of PD patient brains and is also associated with enhanced Fe accumulation.[86] However, other linkage studies have failed to find a connection between DMT1 polymorphisms and PD, despite finding a haplotype in the DMT1 gene that may confer increased risk in a Chinese population.[87] As Mn and Fe share this transport mechanism, a tight interplay between the two metals can lead to conditions of neurodegeneration. Low Fe levels, such as iron-deficiency anemia, can be a risk factor for Mn accumulation.[88] DMT1 has an iron-responsive element (IRE) that results in upregulation and stabilization of the protein in conditions of low Fe levels. Consequently, this upregulation can result in increased Mn uptake, as blood Mn concentrations are increased in humans with Fe deficiency.[89] Interestingly, sleep disorders are common in PD, including restless leg syndrome (RLS),[90–92] a disease that has been linked to DMT1 polymorphism cases that also present with anemia.[93] Finally, it is critical to note the convergence on the basal ganglia of both high DMT1 expression (and subsequent Mn uptake) and PD-associated DAergic cell death. Therefore, altered Mn homeostasis would have a significant impact on this region, as Mn accumulation can subsequently potentiate dopamine oxidation to cause overt cell death in the basal ganglia.

In vivo models for the study of DMT-1-dependent Fe and Mn homeostasis include the iron-deficient Belgrade rat[94] and the microcytic anemia (mk) mouse,[95–97] both of which are characterized by loss-of-function of DMT-1 due to a glycine to arginine substitution at position 185. It has been demonstrated that the levels of Mn and Fe are concomitantly reduced and Mn transport into reticulocytes, kidney and brain and across the wall of the duodenum is impaired, indicating the role of DMT-1 in Fe and Mn transport and homeostasis.[98] Furthermore, experiments with the Belgrade rat also indicated DMT-1 dependent Mn transport across the olfactory epithelium.[98] The exchange of Mn from plasma to the brain occurs across the BBB or the blood-CSF barrier (BCB).[99] Mn transport across the BBB is likely to involve various transporters. Using a model of rats with deficient DMT-1 expression (b/b Belgrade rat), Crossgrove and Yokel suggested that DMT-1 may not be the main transporter for Mn across the BBB.[100] In this study, the uptake of Mn in the brain of rats with deficient DMT-1 protein (b/b) was similar to +/b heterozygous Belgrade rats and wild type (WT) Wistar rats, suggesting that other mechanisms are involved in Mn transport into the brain,[100] including the zinc transporters ZIP8 and ZIP14. ZIP8 and ZIP14 have higher affinity for Mn than other metals,[101,102] *e.g.* the order of affinity for ZIP8 is $Mn^{2+} > Hg^{2+} \gg Pb^{2+} = Cu^{2+} = Zn^{2+} = Cs^{2+}$.[101] Although primarily expressed in the liver and lung,[103] ZIP8 and ZIP14 are also expressed in the nasal respiratory epithelium and olfactory receptor neurons, where Mn from inhaled dust can be directly absorbed into the blood or into the brain.[104] This may explain why industrial workers (miners and welders) are more susceptible to develop manganism, as they inhale fumes containing high levels of Mn. However, other studies have reported Mn accumulation in the CNS is increased with elevated levels of DMT-1 during iron deficiency, supporting the hypothesis that DMT1 is involved in the uptake of Mn.[40,105] These discrepancies could be due to the different protocols used in Mn exposure, which vary widely amongst studies from *in situ* brain perfusion of ^{54}Mn[100] to Mn supplemented diet combined or not with Fe deficient diet.[40,105]

In *C. elegans*, Au *et al.* identified 3 DMT1 homologs named as suppressor of mitochondria import function (smf)-1, 2 and 3. In WT N2 worms, acute exposure (30 minutes) to Mn led to vacuoles in the main epithelia, epidermis, excretory cell and intestine, and resulted in developmental delay and rod-like phenotype (death).[106] *smf-1* and *smf-3* deletion mutant worms are highly resistant to Mn exposure, indicated by higher survival rates when compared with N2 worms; deletion of *smf-2* results in hyper-sensitivity to Mn exposure.[106] These data indicate that *smf-1* and *smf-3* functions as Mn importers as their mammalian homolog DMT1, while *smf-2* acts the opposite as an Mn exporter. Moreover, worms carrying *smf-1* and *smf-3* deletion mutants take up significantly less Mn, and *smf-2* mutant worms have significantly more Mn accumulated in the body,

when compared with N2 worms.[106] These transporters have a strong expression in the intestine and a weaker expression in the head and tail neurons.[106] *smf-1* and *smf-2* have confirmed expression in DAergic neurons,[81] which allows investigation of their function in DAergic neurons.

Transferrin (Tf)/transferrin receptor (TfR)

While DMT1 is the primary divalent Mn transporter, Tf/TfR is responsible for transport of trivalent Mn. A Tf-dependent mechanism for Mn transport in rat PC12 cells has been described before.[108] Recently, Gunter and colleagues have developed an approach to study the Tf-dependent Mn transport independently of other Mn transport mechanisms. Using mouse hippocampal (HT22) and striatal (STHdhQ7/Q7) neurons, they have demonstrated that Mn can be conjugated to Tf when in the Mn^{3+} state and then be transported into neurons *via* the Tf receptor (TfR) analogously to Fe^{3+}. The binding of a fluorescent label (Alexa green) to the Mn^{3+}Tf complex enabled the authors to follow the endocytic transport of Mn^{3+}Tf into neuronal cells and into the region of the mitochondrial network using confocal microscopy.[141] Although Mn^{3+} is a strong oxidizing agent and its accumulation inside the cells could contribute to oxidative damage, the trivalent state is only found at trace amounts, and Mn^{2+} is the prevalent state in which Mn is found within the cells.

Given the chemical similarities between Fe and Mn and their shared mechanisms of transport,[73–76] it was hypothesized that Fe supplementation would protect from Mn accumulation in the brain. This hypothesis was tested in rats given Fe deficient or Fe supplemented diet combined with low dose Mn intravenous injections. Data from magnetic resonance imaging (MRI) and graphite furnace atomic absorption spectroscopy (AAS) indicated that even with Fe supplementation, Mn accumulated in different regions of the rat brain. This effect can be explained by the action of another important element in the mechanism of Fe/Mn transport: ferroportin. This cytoplasmic protein is responsible for the export of Fe to the extracellular space (plasma) and can be degraded in the event of Fe overload. Thus, lower levels of extracellular Fe may result in increased Mn accumulation *via* Tf/TfR.[109] Fpn has also shown affinity for Mn[110] and to reduce Mn accumulation, thus attenuating Mn toxicity *in vitro*. Additionally, mice acutely exposed to Mn *via* subcutaneous injection show increased protein level of Fpn in cortex and cerebellum *in vivo*.[116]

Solute carrier family 30 member 10 (SLC30A10)

More recently, alterations in another protein involved in Mn homeostasis have been found in humans with Parkinsonism. For the first time, evidence for an inherited deficiency in Mn metabolism has been tied to mutations in the gene encoding the plasma membrane zinc (Zn) transporter SLC30A10, with affected families presenting with hypermanganesemia and Parkinsonism. SLC30A10 is a membrane-embedded protein with 6 transmembrane domains (TMDs) that contain similarities in sequence homology to that of other Mn transporters.[111] Further establishing SLC30A10 as a Mn transporter, WT (not mutated) human *SLC30A10* expression was found to rescue the Mn-sensitive yeast mutant *pmr1*.[112] These findings collectively establish SLC30A10 as a novel Mn exporter in humans, though further studies are needed to determine its precise subcellular localization and temporal expression patterns. Families carrying *SLC30A10* mutations show signs of adult-onset Parkinsonism and early-onset dystonia (UPDRS3 scores around 27), with high Mn levels in the blood and urine. These individuals also presented with increased signal intensities in the globus pallidus in T1-weighted MRI images.[111,112] Interestingly, chelation therapy with disodium calcium edetate ($CaNa_2$-EDTA) over time was able to normalize the high Mn levels, resulting in improvement of the clinical symptoms, as well as reduction of pallidal MRI intensities.[111–114] Moreover, in the first postmortem study of a patient carrying a homozygous SLC30A10 mutation, researchers found predominant cell death in the globus pallidus of the basal ganglia, with decreased SLC30A10 protein levels and elevated Mn levels in this same brain region. However, this group disagrees with others that have found blood Mn concentrations to be reflective of brain Mn levels, as this patient showed only a slight increase in blood Mn levels before his death, while postmortem brain tissue showed extremely elevated levels.[107] Our data showed that WT SLC30A10 is localized on cell surface and the mutants in the endoplasmic reticulum (ER) in HeLa cells; expression of WT SLC30A10 enhances Mn efflux, increases viability of HeLa cells upon Mn exposure and protects GABAergic AF5 neural progenitor cells and primary midbrain neurons from Mn-induced neurodegeneration.[115] Together, these studies collectively identify SLC30A10 as a novel Mn exporter in humans, that when mutated, can result in Parkinsonism and manganism pathophysiology.

In *C. elegans*, whole tissue overexpression of human SLC30A10 significantly improves the survival rate of worms after Mn exposure, when compared with the control; however, expression of the L89P SLC30A10 does not; when expressed in DAergic neurons specifically, WT SLC30A10 protects against Mn-induced DAergic neurodegeneration; subsequent studies identified that the L89P mutant loses its function most likely due to mislocalization in the cytoplasm, as WT SLC30A10 is localized on the cell membrane.[115] The results are consistent with the clinical research and cell culture studies, confirming SLC30A10 is an Mn exporter.

Behavioral studies on Mn-induced neurotoxicity

The main phenotypic characteristic of Mn intoxication is motor impairment due to the accumulation of Mn in the basal ganglia.[40] The selectivity of Mn towards the basal ganglia structures is confirmed by motor damage observed in several

models of exposure to Mn *in vivo*.[117–119] Animal models have been used to study behavioral outcomes in Mn toxicity.[120] Motor and cognitive function have been assessed in rodent models using a variety of behavioral tests: open-field,[121–123] rotarod,[124–127] beam walking,[128,129] grip strength and fatigue,[121] staircase test,[130] single pellet reaching,[128,129] Morris water-maze,[131,132] and step-down inhibitory avoidance task.[129] Kern and Smith reported that preweaning Mn-exposed rats exhibited an enhanced locomotor response to d-amphetamine challenge in the open-field test.[133] Preweaning Mn exposure also induced deficit in motor coordination in the rotarod and increased levels of isoprostanes in the striatum that were reversed by the antioxidant Trolox.[118,124] In adult mice tested on the rotarod, a decrease in coordination and impaired motor learning caused by Mn treatment might be modulated in part by increased expression of DA D_2-like receptors in the striatum.[126]

Dodd *et al.* exposed aged C57BL/6 mice to $MnCl_2$ subcutaneously (s.c.) and found that increasing the concentration of Mn in the striatum was accompanied by locomotor deficit in the open-field test, which is extensively used to assess behavioral responses such as locomotor activity, hyperactivity, and exploratory behaviors.[121] Recently, the same group reported that s.c. exposure to $MnCl_2$ followed by MPTP administration in mice caused attenuation of the motor behavioral effects (open-field and grip strength) induced by either compound alone.[134] In adult Sprague-Dawley rats exposed to $MnCl_2$ intraperitoneally (i.p.), electrophysiological alterations in the GP and STN were accompanied by reduced locomotor activity in the open-field; reduced time spent on the rotarod before falling from the apparatus (motor coordination test); reduced time spent in the open arms of the elevated plus maze (anxiety test); and increased immobility time in the forced swim test (depressive-like behavior).[125]

One of the main causes of Mn intoxication is exposure in occupational settings, such as mining or welding, dry battery manufacture, and organochemical fungicide use. To address this issue, models of inhalation or intranasal instillation of Mn have been extensively used. Intranasal administration of Mn in rats led to impaired spatial memory in a modified version of the Morris water maze (MWM) test, with altered levels of monoaminergic neurotransmitters.[132] A correlation was found between the neurotransmitter levels and MWM performance.[132] Intratracheal exposure to Mn nanoparticles resulted in Mn accumulation in the brain and reduced the percentage of ambulation and rearing, while local activity and immobility increased in the open-field test.[135] Furthermore, chronic inhalation of Mn in mice resulted in impaired motor skill evaluated in the single-pellet reach task, which requires the execution of a complex motor sequence to reach a food pellet through a narrow slot, as well as short- and long-term spatial memory impairment evaluated in the step-down inhibitory avoidance task.[129] Effects of inhalation of Mn were also tested in the beam-walking test, which measures the animal's ability to traverse a narrow beam (3 mm) to reach an enclosed safety platform. Mice exposed to Mn initially displayed hyperactive behavior verified by decreased latency to cross the beam, but with further exposures to Mn, the mice became weaker and displayed postural instability and akynesia. The integrity of the nigrostriatal dopaminergic system is important for the completion of this task, and the authors also show decreased tyrosine hydroxylase staining in the SNpc.[128]

In *C. elegans*, Mn specifically targets DAergic neurons.[13] behaviors related to DAergic signaling can be used to study Mn-induced neurotoxicity. These assays include basal slowing response,[136] ethanol preference,[137] area-restricted searching[138] and tap withdrawal response.[139] Basal slowing response is the most commonly used behavior assay to test DAergic function. The nematode requires DA to sense bacteria and regulate its locomotion (body bends). Well-fed young adult worms on plates without bacteria move at a high speed, but slow down and start feeding when they encounter a bacteria lawn. This locomotion is quantified as the number of body bends in the anterior region of worms in a 20 second interval, with the change (Δ) of body bends per 20 s between plates with and without bacteria representing the integrity of the DAergic system. When DAergic neurons are impaired, DA production is decreased and the Δ becomes smaller. For example, in tyrosine hydroxylase (*cat-2*) mutants, Δ is almost eliminated,[136] indicating depleted DA production. Recently, we found that expressing WT or L89P SLC30A10 alone in DAergic neurons does not alter the Δ value when compared with the controls; after an acute Mn exposure, WT SLC30A10 has a significantly increased Δ value when compared with the control, while the L89P mutant does not.[115] The other three DA related behavioral assays have not been applied to study Mn-induced DAergic neurotoxicity yet, partially due to the specificity of neurotransmitter involved or requirement for special equipment. Ethanol preference requires both DA and serotonin;[137] area-restricted searching requires both DAergic and glutamatergic signaling;[138] tap withdrawal response requires functional DA and a computer-controlled tapping and tracking system.[139]

Conclusions

Mn is an essential trace metal for humans. It acts as a co-factor for multiple enzymes (such as hydrolases, lyases, glutamine synthetase, the arginase, Mn-SOD and Mn-containing pyruvate carboxylase), and plays an important role in reproduction, development, immune function, digestion, energy metabolism and antioxidant defenses against cellular stress.[6] However, as the 5th most abundant metal in the earth's crust, and the 12th most abundant element overall, the wide availability of Mn also renders the possibility of excessive exposure, which results in toxicity in humans. Workers in mining, welding, and smelting industries; individuals residing in the vicinity of Mn associated industries; patients with a dysfunctional biliary system; infants and children that receive Mn-containing supplements; as well as people with iron-deficiency are all at a higher risk of suffering from Mn-induced toxicity. Mn is considered an environmental risk factor for PD, as excessive Mn accumulation in the brain causes PD-like symptoms,

termed as manganism. Moreover, patients with manganism may also suffer from hepatic cirrhosis, dystonia, polycythemia, and hypermanganesemia.[6] However, there are still distinct differences between manganism and PD. PD usually results from DAergic neurodegeneration in the SNpc, while Mn initially targets GABAergic neurons in the globus pallidus,[111–113] though there are studies showing that Mn also causes DAergic neurodegeneration in rodents.[140] Another key difference is that patients with manganism benefit from EDTA and PAS treatment,[2,46] but do not response to L-DOPA treatment, which is a very common treatment strategy for PD.[45]

In order to maintain an optimal level of intracellular Mn, various importers and exporters help tightly regulate the transport of Mn. Among them, the best studied is DMT1, a Mn/Fe importer primarily localized on the plasma membrane. DMT1 has a strong expression in the basal ganglia of the brain, with increased levels seen in PD patients,[86] potentially increasing the susceptibility of this region to Mn accumulation and related neurotoxicity. Recently, a very interesting Mn transporter, SLC30A10, was identified to be directly associated with Mn neurotoxicity. Mutations in *SLC30A10* result in hepatic cirrhosis, dystonia, polycythemia, and hypermanganesemia, concomitant with high Mn brain levels.[112] Our recent studies in *C. elegans* confirm that SLC30A10 is a Mn exporter localized on the cell membrane, and mutants lose their protection against Mn due to mislocalization.[115] The mechanism of Mn-induced neurotoxicity remains unclear. However, elevated oxidative stress, mitochondrial dysfunction and disruption of protein homeostasis are the major consequences promoting cell toxicity and neurodegeneration upon Mn exposure. Mice, rats and *C. elegans* provide useful animal models to study Mn-induced neurotoxicity, in addition to human clinical study and cell culture. The rodents exposed to excessive Mn show phenotypes similar to the symptoms seen in patients with manganism, with the availability of multiple behavioral assays to study different brain areas affected by Mn exposure. *C. elegans* also provides a very simple nervous system for studies, allowing powerful visualization of individual neurons (*e.g.*, DAergic neurodegeneration) in real time. Moreover, the basal slowing response, ethanol preference, area-restricted searching and tap withdrawal response provide useful assays to test the function and integrity of DAergic neurons in *C. elegans*. In the future, identifying more genetic factors associated with manganism will improve our understanding of Mn-induced neurotoxicity. Meanwhile, compound screens should be carried out to look for small molecules to alleviate Mn-induced toxicity, with the aim of these studies focused on improving the health of those who suffer from manganism and who are at high risk of Mn toxicity.

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