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Role of manganese in neurodegenerative diseases

Aaron B. Bowman¹, Gunnar F. Kwakye¹, Elena Herrero Hernández³, and Michael Aschner²

¹Department of Neurology, Vanderbilt Kennedy Center, Center for Molecular Toxicology, Vanderbilt University Medical Center, 465 21st Ave S. Nashville TN 37232-8552

²Department of Pediatrics, Pharmacology, Vanderbilt Kennedy Center, Center for Molecular Toxicology, Vanderbilt University Medical Center, 2215-B Garland Ave. Nashville TN 37232-0414

³Department of Molecular and Medical Genetics, Oregon Health & Science University, 3181 S.W. Sam Jackson Park Rd. Portland, Oregon 97239-3098

1. Mn essentiality and toxicity

Mn is an essential ubiquitous trace element required for normal growth, development and cellular homeostasis [1]. Specifically, Mn is important in bone formation, fat and carbohydrate metabolism, blood sugar regulation, and calcium absorption. In humans and animals, Mn functions as a required cofactor of several enzymes necessary for neuronal and glial cell function, as well as enzymes involved in neurotransmitter synthesis and metabolism [2, 3, 4]. Furthermore, *in vitro* data has implicated Mn in the induction of stellate process formation by astrocytes [5]. Mn exists in various chemical forms including oxidation states (Mn²⁺, Mn³⁺, Mn⁴⁺, Mn⁶⁺, Mn⁷⁺), salts (sulfate and gluconate), and chelates (aspartate, fumarate, succinate). The versatile chemical properties of Mn have enabled its industrial usage in making glass and ceramics, adhesives, welding, paint, gasoline anti-knock additives (methylcyclopentadienyl manganese tricarbonyl (MMT)), and many others. While uncommon, Mn deficiency can contribute to birth defects, impaired fertility, bone malformation, weakness, and enhanced susceptibility to seizures [6, 7]. The routes of Mn exposure are mainly through dietary intake, dermal absorption, and inhalation. Moreover, Mn in the diet is found mostly in whole grains, nuts, and seeds, tea, legumes, pineapple, and beans. Despite its essential role in multiple metabolic functions, excessive Mn exposure can accumulate in the brain and has been associated with dysfunction of the basal ganglia system that causes a severe neurological disorder similar to PD [8].

Mn levels in brain tissue average approximately 1–2 µg/g dry weight. The concentration of Mn in the brain varies across brain regions under excessive exposures. Importantly, the highest Mn levels are found in the globus pallidus in humans and in the hypothalamus in rats [9, 10]. Excessive and prolonged exposure to Mn resulting from occupations such as welding and mining, inhalation of combustion products from the anti-knock agent in fuel (MMT) and highly concentrated Mn concentrations in ground/well water leads to Mn accumulation in the dopamine-rich regions of the basal ganglia. In fact, spectroscopy in rats has demonstrated that mitochondria in the basal ganglia accumulate the highest amount of Mn following exposure [11, 12]. This causes a clinical disorder referred to as manganism that is characterized by a set of extrapyramidal symptoms resembling idiopathic Parkinson's

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disease (IPD) including anorexia, apathy, and muscle and joint pain. Shortly after the onset of these symptoms, patients also exhibit memory loss, compulsive behavior, visual impairment, illusions and delusions, and disorientation, which is clinically referred to as *locura manganica*, or manganese madness [13]. Mn overload affects two vital organs, the brain and lungs; the latter results from inhalation [14, 15]. Elevated Mn levels in the brain have been associated with impairment in iron homeostasis, excitotoxicity, mitochondrial dysfunction, oxidative stress, induction of protein aggregation, and alteration in the homeostatic conditions of other divalent metals that share similar transporter systems with Mn. Although the relationship between increased Mn levels and its disruptive effects on the neurochemistry of neurotransmitters has been debated, elevated Mn has been suggested to alter concentrations of γ -aminobutyric acid (GABA), dopamine, and glutamate neurotransmitters in the brain [7, 16].

1a. Mn biology and tissue homeostasis

Although copper and magnesium can substitute for Mn as a cofactor for some enzymes, a subset of enzymes with roles in neuron and/or glial function only in the presence of Mn. These discrete Mn-binding proteins (manganoproteins) include glutamine synthetase, superoxide dismutase 2 (SOD2), arginase, pyruvate decarboxylase, and serine/threonine phosphatase [10, 17, 18].

Glutamine synthetase (GS), the most abundant manganoprotein, is predominantly expressed in astrocytes, where it converts glutamate to glutamine. Because GS contains four Mn ions per octamer [19], Mn has been proposed to regulate GS activity. In fact, insufficient manganese has been proposed to increase glutamate trafficking, glutamatergic signaling, and excitotoxicity [20]. Furthermore, it has been proposed that the increased susceptibility to seizures observed in individuals with Mn deficiency may be due in part to diminished GS levels and/or activity [21]. SOD2 is a mitochondrial enzyme that detoxifies superoxide anions through the formation of hydrogen peroxide (H_2O_2). Although the concentration of Mn within neurons is low ($<10^{-5}$ M), their high mitochondrial energy demands is correlated with a propensity of increased SOD2 in neurons compared to glia [9, 14]. Furthermore, loss of SOD2 activity increases the susceptibility to mitochondrial inhibitor induced toxicity and causes oxidative stress [22].

Arginase regulates elimination of ammonia from the body by converting L-arginine, synthesized from ammonia, to L-ornithine and urea as part of the urea cycle. Moreover, in the brain, L-arginine is converted to nitric oxide by neuronal nitric oxide synthetase. Proper regulation of arginase promotes neuronal survival by impairing nitric oxide signaling [23, 24]. Pyruvate carboxylase is an essential enzyme required for glucose metabolism that interacts with Mn to generate oxaloacetate, a precursor of the tricarboxylic acid (TCA) cycle [25]. Interestingly, in the brain, pyruvate carboxylase is predominantly expressed in astrocytes [26, 27]. Protein phosphatase 1 is essential for glycogen metabolism, cell progression, regulation synthesis and release of neurotrophins, which promote neuronal survival, and synaptic membrane receptors and channels [28].

Intricate regulation of Mn absorption and tissue specific accumulation is crucial for it to properly regulate these enzymes, so understanding Mn's essential roles and toxicity in the brain requires knowledge of its regulation in the periphery. Three major factors have been postulated to modulate plasma Mn levels. First, given that the main source of Mn is diet, tight regulation of gastrointestinal absorption of Mn is crucial. Second, following Mn absorption and a concomitant increase in plasma Mn levels, transport of Mn to target organs, including the liver, is necessary to prevent Mn-induced toxicity in the periphery. Finally, although the liver detoxifies substances, Mn must be further eliminated from the plasma via shuttling to bile [14]. The absorption of Mn by the gastrointestinal tract is highly dependent

on the quantity of ingested Mn and net accumulated levels in the plasma. *In vivo* experiments in mice and rats have defined the range (1–3.5%) of GI absorption of Mn [29, 30]. While Mn is transported by simple diffusion in the large intestine, Mn is absorbed by active transport in the small intestine [14]. Mn excretion into bile is likely active as well because it depends on concentration gradients [31].

A plethora of plasma proteins or ligands have been implicated as specific Mn carrier proteins, including transglutaminase, beta₁-globulin, album, and transferrin [32, 33]. In fact, approximately 80% of plasma Mn is bound to beta₁-globulin [32]. Despite the demonstration that Mn preferentially binds to albumin in the plasma of both rabbits and humans, emerging evidence has provided evidence for weaker binding of Mn to albumin compared to Cd and Zn [34, 35].

Intracellular Mn²⁺ is sequestered in the mitochondria of the brain and liver via the Ca²⁺ uniporter [36, 37]. Mitochondria are the primary pool of Mn in the cell, however, nuclei have also been implied (remains debatable) to preferentially accumulate this metal [26, 38, 39]. The efflux of mitochondrial Mn²⁺ is mediated preferentially through an active but slow Na⁺-independent mechanism; a Na⁺-dependent mechanism also contributes minimally (reviewed in [10]). This slow Mn efflux has been suggested to account for the net accumulation of Mn in mitochondria. Nevertheless, the cytoplasmic Fe²⁺ exporter, ferroportin-1, has been reported to transport Mn. Interestingly, ferroportin-1 surface localization and protein expression is perturbed following Mn exposure [40].

1b. Mn transporters in the brain

Mn can cross the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCB) through several carriers and in different oxidation states. Given the essential physiological functions of Mn and the neurotoxicity associated with Mn overload, Mn absorption, transport, and tissue levels are stringently regulated. Under normal physiological conditions, Mn is efficiently transported across the blood-brain barrier (BBB) in both the developing fetus and adults [14, 41]. Although Mn transport across the BBB has been actively investigated, which transporter systems are primarily responsible have not been conclusively determined. However, over the past three decades, several Mn transporter systems have been characterized, including active and facilitated diffusion modes of Mn transport [42, 43, 44]. Emerging reports have indicated that Mn can be transported via the divalent metal transporter 1 (DMT1), the transferrin (Tf) receptor (TfR) that mediates trivalent Fe uptake, the divalent metal/bicarbonate ion symporters ZIP8 and ZIP14, various calcium channels, the solute carrier-39 (SLC39) family of zinc transporters, park9/ATP13A2, the magnesium transporter hip14 and the transient receptor potential melastatin 7 (TRPM7) channels/transporters. While the tissue-specific expression of each of the aforementioned Mn transporters is yet to be determined, it is likely that optimal tissue Mn levels are maintained through the involvement of all the above and other unknown Mn transporters. In addition, other cellular processes may regulate the activity of the above transporters in response to Mn deficiency or overload. Of all the above listed polyvalent transporters, DMT1 and TfR are the most extensively documented [44]. Interestingly, a small fraction of plasma Mn is bound to transferrin (Tf), an iron-binding protein, while approximately 80% of plasma Mn is associated with albumin and beta₁-globulin[32].

The divalent metal transporter 1 (DMT1) is a member of the family of natural resistance-associated macrophage proteins (NRAMP) and crucial for the maintenance of essential metal homeostasis in the brain [45, 46, 47]. It is best known for its ability to regulate Fe homeostasis in the gastrointestinal lumen [47]. DMT1 is also known as the divalent cation transporter (DCT1) due to its ability to competently transport divalent metals including Zn²⁺, Mn²⁺, Co²⁺, Cd²⁺, Cu²⁺, Ni²⁺, Pb²⁺, and Fe²⁺ across the plasma membrane into the

cytosol [45, 48]. Both alternative splice isoforms of DMT1 are predominantly localized at the plasma membrane; however, one also localizes to late endosomes and lysosomes while the other localizes to early endosomes [49]. Because only one isoform contains an iron-response element (IRE), subcellular localization depends on Fe concentration [10, 46]. The relative high affinity of DMT1 for Mn has been well established both *in vivo* and *in vitro*. Specifically, mutations in the DMT1 gene of Belgrade rats and microcytic anemia mice result in significant decreases in Mn and Fe tissue levels.[50, 51, 52] Furthermore, magnetic resonance imaging (MRI) in a recent study demonstrated the consistency of the cross-BBB transport mechanism(s) of Mn and Fe, suggesting that they are shared [53]. Finally, DMT1-mediated metal transport across rat brain endothelial cells in culture has been reported to be pH, temperature, and Fe-dependent [54, 55].

The TfR is the major cellular receptor for Tf-bound Fe, but because Tf can also bind trivalent Mn, TfR can also mediate Mn transport. Once Mn^{3+} is internalized through the endocytic pathway, it is reduced to Mn^{2+} and transported through DMT1 to the cytosol. Mn binding to Tf is time-dependent and Tf receptors are also present on the surface of cerebral capillaries [44, 56]. Additionally, the TfR is an active transporter that is pH and Fe-dependent [56]. Both *in vivo* and *in vitro* studies have reported that Mn is efficiently transported via the TfR. For example, a spontaneous mutation in a murine gene linked to the TfR, referred to as hypotransferrinemic, results in a drastic serum TfR deficiency, impairs Mn transport, and disrupts Fe deposition [57, 58]. Interestingly, autoradiography reports have indicated that the TfR is generally localized in gray matter, but not the Fe-abundant white matter tracts in humans and rodents [59, 60, 61].

The zinc- interacting protein 8 (ZIP8) and 14 (ZIP14) proteins are divalent metal/bicarbonate ion symporters known to transport Mn, Zn, and Cd under normal conditions [62, 63]. ZIP8 and ZIP14 are members of the SLC39 family of genes [63, 64] that are glycosylated and expressed on the apical surface of brain capillaries. Mn uptake through ZIP8 or Zip14 is driven by extracellular carbonate (HCO_3^-). The expression of Zip8 and Zip14 in the brain is lower than that in the liver, duodenum, and testis [65]. In addition, voltage-gated Ca^{2+} channels, including L and P-type channels [66], such as ligand-gated Ca^{2+} channels; store-operated Ca^{2+} channels (SOCCs) [67], and the ionotropic glutamate receptor Ca^{2+} channels [68] have been implicated in Mn transport across the BBB.

Over the past decade, there has been increasing interest in the exploration and identification of novel candidate Mn transporters, including Hip14 and the product of the *park9* gene. Emerging experimental data has indicated that huntingtin-interacting protein 14 and 14L (Hip14, Hip14L) mediates transport of Mn^{2+} and other divalent metals (Mg^{2+} , Sr^{2+} , Ni^{2+} , Ca^{2+} , Ba^{2+} , Zn^{2+}) across cell membranes [69, 70]. Hip14 is the mammalian ortholog of the ankyrin repeat protein 1 (Akr1p) that is primarily expressed in neurons of the brain. Hip14 is involved in the palmitoylation of several neuronal proteins including huntingtin (HTT) [49]. In addition, it is required for endo- and exocytosis, as well as targeting of cysteine string protein (CSP) and synaptosomal-associated protein 25 (SNAP25) to the synapse [71, 72]. Hip14 is predominantly expressed in the presynaptic terminal, Golgi apparatus, and vesicular structures localized in the axon, dendrites, and soma of neurons [73]. Biochemical studies including yeast-two-hybrid screens have demonstrated that the interaction between Hip14 and HTT is inversely correlated with the poly-Q length in the HTT protein [72].

Interestingly, Gitler and colleagues have recently reported that the *park9* gene responsible for early-onset Parkinsonism also transports Mn [70]. The *park9* gene encodes a putative P-type transmembrane ATPase (ATP13A2) protein. Although the exact function of *park9* is unknown, it is generally thought to be a shuttle for cations, including Mn, across the cell.

Biochemical studies have demonstrated that the highest and lowest *park9* mRNA levels are localized within the substantia nigra and cerebellum, respectively [74].

Although Mn inhibits the choline transporter at the BBB, it has been suggested that the choline transporter may mediate Mn transport during periods of high throughput. In addition, the choline transporter has a higher affinity for Mn compared to the other metals (Cd^{2+} and Al^{3+}) that it transports [75, 76, 77]. Mn transport across the choline transporter is sodium-independent, carrier-mediated, and saturable [56].

TRPM7 is ubiquitously expressed in vertebrates and functions as an active Ca^{2+} selective transporter and a serine/threonine protein kinase. Furthermore, the kinase activity is important for its metal transport function. Specifically, the transporter operates by regulating intracellular Ca^{2+} levels and Mg^{2+} homeostasis through the creation of an inward current, thus contributing to the establishment of a cellular membrane potential. The relative permeability of cations through TRPM7 has been reported to be as follows: Zn^{2+} , $\text{Ni}^{2+} > \text{Ba}^{2+}$, $\text{Co}^{2+} > \text{Mg}^{2+} > \text{Mn}^{2+} > \text{Sr}^{2+} > \text{Cd}^{2+} > \text{Ca}^{2+}$. Physiological levels of Mg^{2+} and Ca^{2+} are necessary for maintaining the permeability of TRPM7 to Mn^{2+} , Co^{2+} , and Ni^{2+} [56].

The homomeric purinoreceptors, including P2X and P2Y, have been suggested to participate in Mn transport. These receptors are ATP-dependent and ubiquitously expressed on endothelial cells [78, 79, 80]. Purinoreceptors have a relatively lower affinity for Mn than for the other divalent metals they transport ($\text{Ca} > \text{Mg} > \text{Ba} > \text{Mn}$) [56]. Finally, the citrate transporter has also been implicated in Mn transport across the BBB [81].

1c. Methods for detecting Mn in biological specimens

Over the past two decades, several analytical methods have been developed for detecting Mn levels and monitoring Mn homeostasis in biological samples. Most methods require digestion of all organic matrix prior to analysis, a recently developed method has successfully measured trace concentrations of metals without sample digestion [82]. In older methods, the biological sample type determines the method of digestion to be used. For example, blood or saliva may be digested by an ion exchange resin while tissue samples would require acid (nitric or sulfuric) digestion. Regardless of the sample preparation method, exogenous Mn can contaminate biological samples and affect the accuracy of measurements, especially if Mn levels are low. The current methods used for measuring Mn levels in biological specimens include atomic absorption spectroscopy (AAS), atomic emission spectroscopy (AES), inductively coupled plasma-atomic emission spectrometry (ICP-AES) and mass spectrometry (ICP-MS), neutron activation analysis, x-ray fluorimetry, spectrophotometry, and radioactive trace assays.

AAS and ICP-MS are the most common methods used for measuring Mn levels in biological samples. AAS analysis requires aspiration of the sample into a flame or graphite furnace (GFAAS), where a photoelectric detector measures levels of elements. GFAAS is most used for the determination of very low analyte levels in solid samples [83]. ICP-MS and ICP-AES are similarly sensitive methods for measuring levels of multiple elements, including Mn, in both liquid and solid biological specimens. In fact, ICP-AES and ICP-MS can often measure analytes at the part per trillion levels [84]. Both types of samples require manipulation prior to measurement: while measurements of analytes in solid samples require a laser ablation system, liquid samples are introduced into the ICP by a nebulizer for analyte (Mn) measurements. In contrast, neutron activation analysis minimizes potential contamination of biological Mn levels through minimal sample handling and no reagent usage. Furthermore, neutron activation analysis also has a low detection limit; it is capable of accurate detection of analyte concentrations as low as 4ng/g [83]. However, none of the

above Mn detection methods can distinguish between different Mn compounds or oxidation states.

In spite of the several advantages that these Mn detection techniques provide, for example, multi-elemental analysis, excellent specificity, extremely high sensitivity and limited chemical interference, their expense and duration makes them unfeasible for assessing Mn transport kinetics on a large scale. A less expensive, more rapid quantitative approach has recently been developed in which fura-2 is loaded into living cells to assess intracellular metal ion concentrations by rapid and time-dependent quenching of fura-2 fluorescence [48, 85, 86, 87, 88, 89]. This method, still has a very low throughput, prohibiting pharmacological and toxicological concentration-response curve experiments and other experimental approaches requiring multiple samples to be analyzed. However, a newer approach, the cellular fura-2 Mn extraction assay (CFMEA), enables quantitative measurements of extracted Mn levels in multiwell plate format [90, 91]

2. An overview of the role for Mn and other metals in neurodegeneration

In the past decade, there has been a growing interest in understanding the metabolism of neurotoxic metals and their influence on various neurodegenerative diseases, including manganism, Wilson's disease (WD), PD, and Alzheimer's disease (AD). These metals (see below) likely also contribute to Huntington's disease (HD), though fewer studies have investigated the link. Occupational and environmental exposures (see section 1) to neurotoxic metals, including Mn^{2+} , Hg^{2+} , Cu^{2+} , Zn^{2+} , As^{2+} , Cr^{2+} , Pb^{2+} , and Al^{3+} have been associated with neurodegeneration and modulation of the age of disease onset and severity in neurodegenerative diseases. The brain is capable of efficiently regulating these metals under physiological conditions; however, excessive exposure can cause them to accumulate in the brain. The distribution of metals throughout the brain is not uniform, and accumulation in specific brain regions reflects neurotoxicity; for example, Mn accumulation and neurotoxicity in the globus pallidus results in manganism. Alterations in metal homeostasis have been suggested to cause neurodegeneration via association of metals with proteins and subsequent induction of aggregate formation. In addition, metals can cause neurodegeneration through a vicious cycle by disrupting mitochondrial function, which depletes ATP, induces ROS production, and ultimately causes cell death by apoptotic and/or necrotic mechanisms.

Acute exposure of a transgenic *C. elegans* model of PD to Mn has recently been reported to result in degeneration of dopaminergic neurons [92]. Experimental studies in PD postmortem brain tissues have found strong evidence for a link between oxidative stress and PD, specifically, increased Fe levels, markers of oxidative stress and lipid peroxidation in the substantia nigra. Interestingly, pharmacological chelation of elevated Fe levels in an MPTP-induced neurotoxicity model of PD prevents and delays degeneration of midbrain dopaminergic neurons [93]. These studies indicate the potential neurotoxic contributions of Fe and Mn in PD neuropathology. On the other hand, Zn has been proposed to serve as a cofactor for several enzymes and be involved in normal neurological function because it is present at significant levels (10 μ M) in the brain [94]. While some brain Zn is associated with proteins, the neocortex and hippocampus possess a substantial amount of chelatable Zn [94, 95, 96, 97]. The defined physiological functions of Zn are currently unclear, but it has been suggested to help stabilize glutamate-containing vesicles at the synapse of secretory cells [98, 99]. In addition, *in vitro* experimental studies have demonstrated that Zn attenuates NMDA-induced toxicity [100]. However, intracerebroventricular administration of Zn in rats causes epileptic seizure-induced hippocampal neurodegeneration [101].

In fact, other studies have also shown that Mn and other metals (e.g. Cu, Al, Zn) can interact and promote amyloid fibrillogenesis and aggregation of proteins such as prion protein (PrP) and α -synuclein [102]. These proteins bind metals, which contributes to their altered conformational state, solubility, and aggregation [103, 104, 105, 106, 107]. However, *in vitro* analysis of PrP aggregates has demonstrated that Mn can promote aggregation independent of the PrP metal binding site [106]. In AD, Cu has been shown to bind A β with high affinity and modulate its conformational state and peptide length [108, 109]. Other *in vitro* studies have demonstrated that A β interacts with Fe and Zn to promote amyloidogenesis. Interestingly, these findings have been corroborated in AD postmortem brains that show significantly elevated Fe and Zn in the neocortex and amyloid plaque deposits [109]. All these studies indicate that metal interactions with PrP, α -synuclein, and A β proteins can cause cell death by inducing the formation of aberrant and toxic aggregates as well as activating redox cycling via Fenton and Haber-Weiss reactions, which depletes production of cellular antioxidants, including glutathione, thus increasing levels of ROS (HO \cdot , O $_2^{\cdot-}$, or H $_2$ O $_2$). The generation of these highly reactive species can cause oxidative stress that damages lipids, proteins, and DNA and further deplete ATP to cause cell death. These pathophysiological mechanisms, including excitotoxicity, oxidative stress, protein aggregation, mitochondrial dysfunction, and altered metal homeostasis, are strikingly similar to those underlying most common neurodegenerative diseases, including PD, AD, and HD.

3. Manganese exposure and Parkinson's disease

3a. Manganism vs. PD

Manganism was first described in 1837 by Couper [110], who observed five patients working in an ore-crushing facility who presented with muscle weakness, bent posture, whispering speech, limb tremor and salivation (see section 1) [111]. Manganism's psychological symptoms occur early during intoxication and are characterized by hallucinations, psychoses and a myriad of behavioral disturbances. Later, motor deficits develop, encompassing the extrapyramidal system: gait dysfunction with a propensity to fall backward, postural instability, bradykinesia, rigidity, micrographia, mask-like facial expression and speech disturbances [111]. Unlike the resting tremor characteristic of PD, manganism features less frequent and kinetic tremor, if tremor is present at all. Exposure to high Mn levels may also cause dystonias characterized by plantar flexion of the foot, which causes a "cock-walk," and facial grimacing. Noticeably, the symptoms of Mn intoxication, once established, usually become progressive and irreversible, reflecting permanent damage of neurologic structures.

Though generally described as toxicity to the basal ganglia, manganism is known to affect other CNS region, such as the cortex and hypothalamus [112]. Human manganism at the morphological level is characterized by neuronal loss and reactive gliosis in the globus pallidus and substantia nigra pars reticulata (SNpr) without Lewy bodies, the intraneuronal protein aggregates that distinguish PD [112]. Though rarely reported, damage to the striatum (caudate nucleus and putamen) and subthalamic nucleus may occur, while the substantia nigra pars compacta (SNpc) [113] is less likely to be affected. In contrast, idiopathic PD is predominantly characterized by neuronal loss in the SNpc [114].

3b. Human exposure to Mn and relationship to PD

A recent editorial [116] proposed that radiotracer imaging techniques should be used to investigate the integrity of the dopaminergic system in asymptomatic workers exposed to Mn and highlights the need to assess motor signs in addition to cognitive and behavioral symptoms. PET with [18F]FDOPA [117] in a small study with very high average blood Mn in workers and gender imbalance among groups showed that active and asymptomatic

welders have presynaptic nigrostriatal dopaminergic dysfunction with different anatomical localization from that generally observed in PD, affecting the caudate more than the putamen. The welders also had significantly lower Unified Parkinson's Disease Rating Scalesubsection 3 scores than those of the control group, indicating that their occupation led to motor impairment.

3c. α -synuclein and Mn-related protein aggregation

Mn, and certain other essential and toxic metals, can directly increase fibril formation by α -synuclein. Though α -synuclein's function is still unclear, these fibrils form the intracytoplasmic inclusions (Lewy bodies and Lewy neurites) found in idiopathic Parkinson's disease, dementia with Lewy bodies (DLB) and multiple system atrophy (MSA), disorders classified as synucleinopathies. It is known that both genetic and environmental factors affect synuclein pathology (reviewed by Eller and Williams, 2011) [40]. Thus, Mn seems to act jointly with α -synuclein in inducing neuronal cell death [119]. It has also been suggested that some metals, including Mn, can act synergistically, even at low concentrations, with certain herbicides to promote α -synuclein misfolding and aggregation [120].

Mn also increases the expression of α -synuclein *in vitro* [121, 122], and chronic Mn exposure leads to aggregation of α -synuclein *in vivo* in neurons and glial cells of non-human primates [123]. A genetic interaction between α -synuclein and PARK9 has been reported in yeast and because PARK9, which could encode a metal cation transporter, seems to protect cells from Mn toxicity, this could provide a mechanism linking genetic and environmental causes of neurodegeneration [70]. Various mechanisms mediated by Mn could converge on α -synuclein *in vivo*, potentially linking Mn to Parkinson's disease [124]. Overexpression of α -synuclein in human cells seems to facilitate Mn-induced neurotoxicity through activation of the transcription factor NF- κ B, the kinase p38 MAPK, and apoptotic signalling cascades, thus possibly playing a role in dopaminergic cell death [125]. It has also been recently suggested that chronic exposure to Mn could decrease striatal dopamine turnover in transgenic mice expressing human α -synuclein [126].

3d. Mitochondrial dysfunction, Mn and PD

Studies of postmortem PD brains show damage to the SN consistent with generation of reactive oxygen and nitrogen species (ROS, RNS), characterized by lipid peroxidation, protein oxidation, 3-nitrotyrosine formation, DNA oxidation, DNA breaks, and a decrease in the activities of the ROS scavenging enzymes glutathione peroxidase and superoxide dismutase. ROS and RNS lead to mitochondrial damage, and mitochondrial dysfunction has been observed in a plethora of PD models, including 1-methyl-4-phenylpyridinium ions (MPP⁺) [the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)], rotenone, and 6-OHDA. Mitochondria have been posited to play a central role in the etiology of both PD and manganism, which has raised several hypotheses, namely, that (1) dopaminergic neuron mitochondria may be selectively vulnerable to toxins that cause mitochondrial dysfunction; (2) neurons within the SN produce endogenous mitochondrial toxin(s); or (3) SN mitochondria inherently possess defects in enzymes, such as complex I, that cause impaired energy metabolism. It is noteworthy that overexpression of wild type human α -synuclein in *C. elegans* increases vulnerability to mitochondrial complex I inhibitors, such as rotenone, fenperoximate, pyridaben and stigmatellin [127]. α -Synuclein overexpression has also been recently shown to enhance Mn-induced neurotoxicity via the NF- κ B-mediated pathway [125] in a mesencephalic cell line (MES 23.5). Mn also induces the overexpression of α -synuclein in PC12 cells via ERK activation [121], and chronic exposure to Mn decreases striatal dopamine turnover in human α -synuclein transgenic mice [126].

PD and Mn-induced neurotoxicity are analogous in numerous mechanistic ways at the mitochondrial level. Interestingly, the primary storage site for intracellular Mn²⁺ is within the mitochondria, where it is taken up via the Ca²⁺ uniporter [38, 128]. Both MPP⁺ (a model toxin for experimental PD) and Mn activate heme oxygenase-1 [129], resulting in oxidative mitochondrial damage. Mitochondrial impairment, oxidative stress and increased α -synuclein aggregation have been linked in both Mn exposure and various experimental PD models [130, 131, 132]. A link between familial PD-related genes and Mn is also well established; for example, Mn treatment is known to upregulate ER-stress factors, including parkin. Notably, *parkin* mutations are associated with early onset PD and the E3 ubiquitin ligase it encodes is neuroprotective and is up-regulated upon oxidative stress [133]. Parkin also upregulates PINK1 [134], the first protein shown to directly link mitochondrial abnormalities to a PD phenotype.

4. Mn and HD

Huntington's disease (HD) is a progressive neurodegenerative disorder that begins late in life, with a median age of onset at 39, and was first described by George Huntington in 1872. HD is inherited in an autosomal dominant pattern, and its prevalence is approximately 5 in 100,000 worldwide and 1 in 10,000 in the United States [135]. HD is characterized by motor impairment, cognitive deterioration, emotional disturbance, and psychiatric deficits. Motor symptoms begin with chorea, postural imbalance, dystonia, incoordination, and oculomotor apraxia and progress to akinesia at later stages. HD patients also exhibit emotional disturbances, including depression, temper, apathy, and irritability, and cognitive deficits in short-term memory, attention, and learning. In fact, it has been reported that the cognitive and emotional features of HD precede motor symptoms by several years [136, 137]. HD is caused by an expansion in the glutamine-encoding triplet repeat (CAG) of the normal *HTT* gene [76, 135, 138]; having more than 36 repeats is pathogenic. Importantly, it has been reported that there is an inverse correlation between age of disease onset and repeat length [139]. Furthermore, the longer the CAG repeat length the greater influence repeat length has over determining age of onset, but for repeat lengths less than 50, only about 44% of the variation in age of onset is due to repeat length [140]. In contrast, individuals with at least 60 repeats invariably exhibit symptoms by 20 years of age. Environmental factors (i.e. non-familial effects) are suspected to contribute to a substantial portion of the residual variance in age of onset [140].

4a. A role for environmental factors in HD

Over a decade after the identification of the causative HD mutation, there have been conflicting reports linking complete or incomplete penetrance of HD to triplet repeat expansion length. Environmental factors have also been suggested to contribute to the residual variation in age of onset, perhaps even more so than genetic factors [140, 141]. Moreover, Gómez-Esteban and others have implicated environmental influences that modify age of disease onset and clinical presentation in monozygotic twin studies, as twins have the same number of repeats [142, 143, 144, 145]. Such twin studies, however, fail to reveal the nature of the environmental factors involved. Furthermore, animal models of HD have provided further support for the influence of environmental factors on HD onset and progression [141, 146].

4b. Links between HTT function and metals

There are several known functional links between the *HTT* gene and metals. For example, wildtype *HTT* is important for iron metabolism and oxidative energy production, as evidenced by the decreased hemoglobin and altered iron endocytosis in *Htt* deficient zebrafish [147]. In fact, Fe and Cu levels are elevated in the corpus striatum of postmortem

brain tissue from HD patients and animal models [148, 149]. In addition, HD postmortem brains exhibit alterations in Mn-dependent enzyme activity [3]. Furthermore, animal models have also demonstrated an increase in microglial ferritin, an intracellular iron storage protein [150]. Interestingly, Fox and colleagues have reported that wildtype Htt protein interacts with Cu and decreases its solubility [151]. Finally, the formation of inclusion bodies by CAG expansion in mutant Htt protein fragments may be associated with iron-dependent oxidative events [152]. All these studies strongly suggest that wildtype Htt is necessary for proper metal homeostasis in the brain.

4c. A role for altered metal homeostasis and toxicity in HD neuropathology

The clinical progression of HD is associated with elevated Fe and Cu in the corpus striatum [148, 149]. Several Mn-dependent enzymes, including arginase, glutamine synthetase, pyruvate decarboxylase, and Mn superoxide dismutase 2 (SOD2) are altered in human HD postmortem brains and toxic models of HD [3, 22, 153, 154, 155, 156]. Also, data from animal models of HD have demonstrated a significant increase in microglial ferritin (an intracellular iron storage protein) levels [150]. Interestingly, Fox et al. have recently reported that the Htt protein interacts with Cu and decreases the solubility of wildtype Htt protein [151]. However, the cellular effects of Cu or other metal ions on Htt function, proteolytic processing to generate N-terminal fragments, aggregation of fragments, and formation of mutant Htt inclusion bodies remain unknown. Finally, emerging evidence indicates that inclusion bodies formed by CAG expansion in mutant Htt protein fragments are associated with iron-dependent oxidative events, opening the possibility that other redox-reactive metal ions, such as Mn, may influence polyglutamine aggregation [152]. In essence, several studies have suggested that oxidative stress, mitochondrial dysfunction, excitotoxicity, and alterations in iron homeostasis are crucial steps in both Mn neurotoxicity and HD neuropathology. Importantly, chronic exposure to Mn in animal models leads to its significant accumulation in the striatum, providing an opportunity for Mn and HTT to interact within the neurons most vulnerable to HD pathology. Given the strong association between protein aggregation, metal ions and neurodegeneration, it is highly rational to speculate that metals may modulate HD pathophysiology.

4d. Discovery of a disease-toxicant interaction between HD and Mn exposure

To identify metals that share pathophysiological mechanisms with HD and thus potentially modify the age of disease onset, severity, and neuropathology, Bowman and colleagues performed a disease-toxicant screen using a striatal cell line model of HD [157]. They tested the potential of Mn²⁺, Fe³⁺, Cu²⁺, Zn²⁺, Pb²⁺, Cd²⁺, Co²⁺, and Ni²⁺ to modify cell survival in an established knock-in immortalized murine striatal cell line model of HD that expresses either wildtype HTT (*STHdh*^{Q7/Q7}) or the polyQ-expanded form of the protein (*STHdh*^{Q111/Q111}) [158, 159, 160, 161, 162, 163, 164]. This study revealed a novel gene-environment interaction between expression of mutant HTT and Mn. Specifically, acute Mn exposure of the cultured striatal cells unexpectedly decreased the vulnerability of mutant expressing cells (*STHdh*^{Q111/Q111}) to Mn cytotoxicity compared to wild-type (*STHdh*^{Q7/Q7}) [165]. Furthermore, total intracellular Mn levels following Mn exposure by GFAAS in *STHdh*^{Q7/Q7} and *STHdh*^{Q111/Q111} cells were significantly lower in mutant than wild type cells. Moreover, the mutant HTT–Mn interaction was corroborated *in vivo* using the YAC128Q mouse model of HD; these mice accumulated less Mn in the striatum than wild-type animals following subcutaneous Mn injections [165, 166]. Furthermore, basal Mn levels were significantly lower in mutant than wild-type cells [165], as was Mn uptake as indicated by CFMEA [91]. Importantly, the discovery of a disease-toxicant interaction between glutamine-expanded HTT protein and Mn establishes the significance of deciphering how mutant HTT protein modulates Mn transport and transporter systems to elicit decreased susceptibility to Mn toxicity.

5. Mn and Amyotrophic Lateral Sclerosis (ALS)

Mn overload has also been implicated in ALS. This link was first described by Voss, who documented a Mn smelter who developed occupational manganism and bulbar ALS in Germany [167]. Subsequently, a Mn miner also affected by occupational manganism and showing some neurological signs of motor neuron disease was reported in Cuba and recovered after treatment [168]. ALS also occurred among Mn miners in Guam [169], and has been observed among welders, as indicated by small and large epidemiological studies in these workers [170, 171, 172, 173]. ALS arising in a patient affected by liver cirrhosis, a condition known to imply Mn overload due to impaired biliary excretion of the metal [174], has also been observed [175]. Mn overload has been reported in pathological and analytical studies of Guamanian and sporadic ALS cases [176, 177, 178, 179, 180]; the last two studies also showed an increase in MnSOD levels in motor neurons of affected subjects. Indeed, environmental data from the Western Pacific endemic foci of ALS, including the Kii peninsula in Japan, support a role for Mn in its prevalence in these areas [181, 182] and contribute to what is generally called “the mineral hypothesis” of the ALS/Parkinson’s Dementia Complex (ALS/PDC).

The alternative “vegetal hypothesis” [183, 184, 185] focuses on cycads, which require high amounts of Mn [186]. However, high levels of Mn have been recently documented in the leaves of Guamanian *Pandanus tectorius*, a plant traditionally used for food, fiber and medicine [187, 188]. Considering that several plants, particularly species in the Western Pacific, hyperaccumulate Mn [189, 190], the two major environmental hypotheses on ALS/PDC could actually converge instead of being mutually exclusive. The recent detection of genetic variants of two melastatins, *TRPM2* and 7, in Guamanian ALS/Parkinson’s Dementia Complex patients [198, 199, 200] appears interesting, as TRPM7 is strongly activated by Mn [201] and Guam is known to be a Mn-rich environment [188]. Therefore, these melastatins could mediate the accumulation of Mn in Guamanian ALS/PDC patients.

A considerable percentage of ALS patients show T1-weighted hyperintensity at MRI, a neuroradiological sign compatible with Mn overload [191, 192], along the motor system [193, 194, 195, 196, 197], but to date, no studies have directly quantified Mn levels in these brain areas in ALS patients. Further, Mn overload induces apoptosis (reviewed by [202]), which contributes to motor neuron disease [203].

Finally, ALS [204] has been occasionally reported among subjects affected by Mn-ephedrone syndrome, a severe motor disorder [205] thought to be mostly due to severe manganism developing in drug abusers who inject themselves intravenously. While to date there is not definitive proof that Mn can cause ALS, these observations collectively indicate that in certain conditions, the metal could cause or trigger motor neuron disease in some individuals, through a mechanism that could involve members of the melastatin ion channel family.

6. Mn and prion diseases

A growing body of literature indicates that Mn triggers misfolding and aggregation of the PrP *in vitro*, and that animals and/or humans with prion disease show increased Mn levels in blood, brain and liver [206, 207, 208, 209]. The PrP influences Mn uptake and protects against Mn-induced oxidative stress and apoptosis [210]. Many observations suggest that Mn overload could play a role in prion diseases, and the main ones are summarized here. Mn increases intracellular PrP levels [211] and induces PrP misfolding and proteinase-resistance [212] at micromolar dosages and physiological pH [104]. High Mn levels are detected in the central nervous system and blood of humans and animals affected by prion diseases [206, 207, 209]. Mn also causes the Prionics[®] test to indicate the presence of

transmissible spongiform encephalopathies (TSE)- related PrP^{Sc} under UVA irradiation/ reducing conditions [213]. Brain MRI of a Creutzfeldt-Jakob disease (CJD) patient has detected T1-weighted hyperintensity in the globi pallidi, compatible with Mn overload [214].

Evidence from responses to treatment also seems to support the Mn/prion disease connection: the metal chelator EDTA reverses Mn-induced aggregation of the prion protein *in vitro* [107] and CDTA, another polyaminocarboxylic chelator with strong affinity for Mn, significantly increases survival of mice inoculated with the human-derived, mouse-adapted, prion strain M1000 [215]. The link between Mn and prion disease has been recently comprehensively reviewed [216]. Additionally, both Mn overload and prion diseases cause MAPK activation and apoptosis [217, 218]. To date there is no definitive proof that Mn overload can trigger prion disease, as the observed high Mn levels in organs and tissues of affected subjects and animals could simply be an epiphenomenon of prion disease and whether Mn triggers PrP misfolding *in vivo* is uncertain. Nonetheless, these multidisciplinary data do not exclude a causal relationship between Mn and prion disease.

Studying other disorders possibly associated with prion disease could prove useful to detect whether essential metal imbalances, particularly those involving Fe, Cu, and Mn, could be involved. At least one CJD patient has simultaneously presented with hepatic encephalopathy, which is known to induce Mn overload (Camacho-Muñoz, Hernández-Ramos and Ortega-Martínez De Victoria L 2001) [219]. Similarly, a case of sporadic CJD recently came to our attention in which the patient was clinically diagnosed with hemochromatosis (not genetically confirmed) (EHH, unpublished data). Hemochromatosis involves not only Fe overload, which may be related to prion disease [220], but also excess Mn [206]. This observation could support a possible relationship between hemochromatosis and prion disease [206]. Very recently, a decrease in cerebrospinal fluid transferrin has been suggested to be a diagnostic marker of prion diseases [221], which could also harmonize with the above hypothesis, as Mn-transferrin is one of the forms in which the metal accesses the brain (review by Yokel, 2009).

7. Mn and Alzheimer's disease (AD)

Chronic Mn treatment of macaques [222] induces up-regulation of amyloid-like protein 1, confirmed by immunohistochemistry, and diffuse amyloid-beta plaques in the frontal cortex, potentially supporting a link between advanced-stage manganese and dementia, as occasionally reported [223]. However, this study involved a limited sample and some variability in age, Mn exposure, dosage, and treatment duration. Also, these animals were repeatedly anesthetized to allow intravenous injections and neuroradiological studies [224], raising the possibility that general anesthesia could have altered gene expression. However, the potential link between Mn and Alzheimer's disease [123] appears of great interest and deserves to be investigated further.

8. Future directions

Studies over the past several decades have greatly improved understanding of the health risks associated with exposure to Mn and its signs and refined understanding of Mn transport and molecular mechanisms of cellular neurodegeneration. Several Mn transporters have been identified and the complex interrelationship between Mn and Fe, as well as other divalent metals, has been elucidated. Neurotoxic mechanisms common to Mn and other mitochondrial poisons have also been identified. Yet while manganese and PD, as well as other neurological disorders, display distinct neurological symptoms during their early phases, their multiple striking similarities at the clinical, physiological, cellular, and molecular levels suggest that their etiologies share common neurodegenerative pathways. As

reviewed herein, Mn may play a role in many neurodegenerative disorders, including PD, HD, AD, ALS, and prion disease, all of which rely on similar intracellular mechanisms involving oxidative stress, mitochondrial impairment, and protein aggregation. Future studies will lead to better understanding of the many facets of Mn homeostasis, the interplay between genes and Mn insult, and the molecular mechanisms of Mn-induced neurodegeneration.

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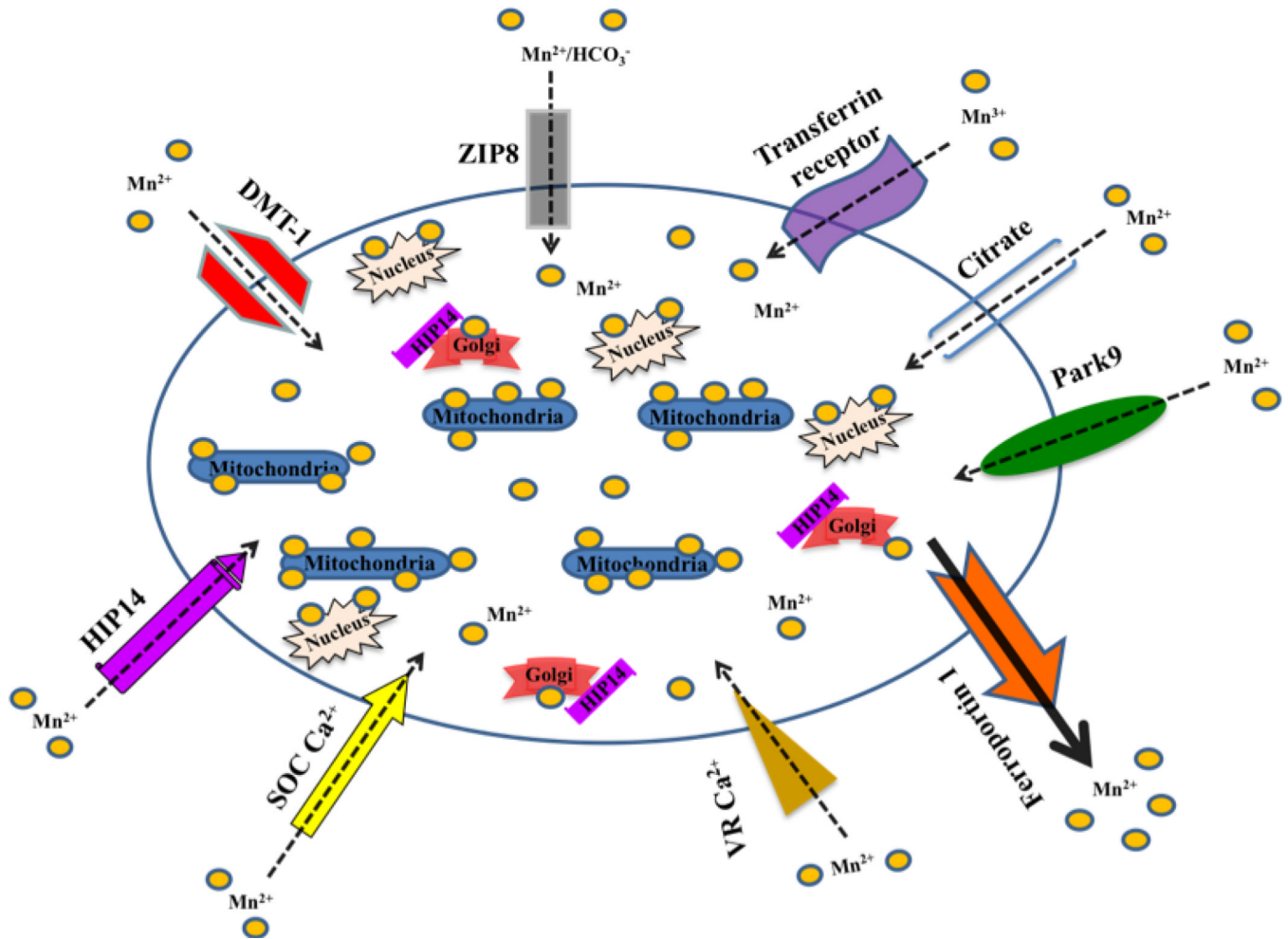


Figure 1. Identified and putative Mn transporters

These illustrated Mn transporters have been demonstrated to facilitate Mn trafficking (uptake, storage, efflux) between the extra- and intra-cellular milieu. Each of these transporter proteins has also been implicated in the transport of other metals.