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Manganese Neurotoxicity and the Role of Reactive Oxygen Species

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Abstract

Manganese (Mn) is an essential dietary nutrient but excess or accumulations can be toxic. Disease states, like manganism, are associated with overexposure or accumulation of Mn and are due to the production of reactive oxygen species, free radicals and toxic metabolites, alteration of mitochondrial function and ATP production and depletion of cellular antioxidant defense mechanisms. This review focuses on all of the preceding mechanisms and the scientific studies that support them as well as provides an overview of the absorption, distribution, and excretion of Mn and the stability and transport of Mn compounds in the body.

Keywords

Manganese; reactive oxygen species; mitochondrial dysfunction; manganism; DA autoxidation

Introduction

Manganese (Mn) serves as a constituent of metalloenzymes and as an enzyme activator so the absorption and excretion of Mn is tightly controlled in order to maintain stable tissue Mn levels for essential reactions. The most widely known enzyme requiring Mn is manganese superoxide dismutase (MnSOD), whose primary function is detoxification of superoxide free radicals. Mn^{2+} is a central component of some metalloenzymes and an activator of

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many metal-enzyme complexes. Mn activates these enzymes by binding the protein directly or by acting through an intermediate interaction with a substrate, like ATP, to initiate a conformational change and activate enzymatic activity [1]. Mn^{3+} is found in the essential enzymes manganese catalase and MnSOD, both of which break down oxidants using the Mn^{3+} in their reactive catalytic centers. Catalase converts hydrogen peroxide into oxygen and water, aiding in reducing oxidative stress. MnSOD is involved in the dismutation of superoxide in mitochondria to decrease oxidative stress. Oxidative stress is also significant in Mn-induced dopaminergic (DAergic) neurodegeneration. Mn is an essential nutrient and as such it is required as part of a healthy diet; however exposure to excess levels yields toxicity. Mn is available as a nutritional supplement as well as a component in most infant formulas and total parenteral nutrition given to some neonates.

The primary source of Mn toxicity is occupational exposures, which are chiefly inhalational. Workplace exposure to Mn-containing fumes or dusts can be a concern in the iron and steel, ferromanganese, dry-cell battery, welding and smelting industries with the highest concern in the welding industry. Exposure to high levels of Mn can lead to a condition referred to as manganism, characterized by behavioral changes, including slow and clumsy movements, tremors, difficulty walking and facial muscle spasms. Slowed hand movements, irritability, aggressiveness and hallucinations are other symptoms of exposure that may precede manganism, and patients may be asymptomatic for months or years following exposure.

Mn toxicity is mediated, at least in part, by the autoxidation or turnover of intracellular catecholamines, production of free radicals, reactive oxygen species and toxic metabolites, depletion of cellular antioxidant defense mechanisms and alterations of mitochondrial function and ATP production. These mechanisms will be discussed in this review. Evidence supporting Mn-induced cell death through the mechanisms described above will be presented as well as a subsection on the use of mitochondrially-targeted antioxidants as therapeutics.

Mn Stability and Transport

Mn has several valence states; with Mn^{3+} most commonly found in enzymes and Mn^{2+} in the diet [2]. The valence of Mn can be changed within the body and studies suggest that Mn^{3+} is more cytotoxic than Mn^{2+}, due to higher oxidative reactivity [3]. Enhanced oxidative stress induction by Mn^{3+} was supported by a study in rats given either Mn^{2+} or Mn^{3+} [4]. Mn^{2+} does not readily bind to sulfhydryl (-SH) groups or amines, and does not vary in its stability constants for endogenous ligands glycine, cysteine, riboflavin, and guanosine [2].

Mn^{2+} enters the portal circulation from the gastrointestinal tract and binds to plasma alpha2-macroglobulin or albumin. It is then secreted in the bile, and some is oxidized to Mn^{3+} by ceruloplasmin. The plasma carrier protein for Mn^{3+} is transferrin, and Mn^{3+} entry into neurons occurs via transferrin receptor-mediated endocytosis and/or the divalent metal transporter (DMT-1) [5–7]. In the case of Mn toxicity DMT-1 facilitates the accumulation of Mn in DA-rich areas [8]. There are several studies that suggest that Mn can cross the blood-brain barrier through the choline transporter [9, 10], voltage-gated and store-operated calcium channels [11, 12], ionotropic glutamate receptor Ca^{2+} channels [13] and a Mn citrate transporter [14]. Additional evidence points to Mn transport through the Zn transporters, ZIP8 and ZIP14 [15, 16], and a through ATP13A2, a P-type transmembrane ATPase protein [17].

Mn Administration, Distribution and Excretion

Adults consume between 0.7 and 10.9 mg of Mn per day [18] with normal ranges of Mn at 4–15μg/L in blood, 1–8μg/L in urine and 0.4–0.85μg/L in serum. Mn complexes with other substances, resulting in low plasma and tissue concentrations, making it difficult to assess overexposure [19]. Mn^{2+} is more readily cleared than Mn^{3+}. High iron diets have been shown to suppress Mn absorption, aiding in protection from toxicity, and deficiencies in other essential nutrients, such as magnesium (Mg), may increase the toxicity of Mn [20–24]. It has also been shown that DMT-1 and Mn levels increase with low brain iron concentrations [25]. Mn can readily cross both the blood-brain and placental barriers.

The route of exposure governs the distribution, metabolism, and neurotoxicity of Mn [26, 27]. Only a small fraction of ingested Mn is normally absorbed into the body and in response to high dietary Mn, gastrointestinal absorption is reduced [28]. Several studies have shown that animals with high dietary Mn have increased liver metabolism and excretion of Mn into bile and pancreatic fluids [29–33]. This suggests that ingested Mn is tightly regulated probably due to its essentiality in the diet. In addition to the influence of other nutrients, genetics and disease states can also influence Mn concentrations in the body. In one study of individuals with chronic hepatic encephalopathy, elevated blood Mn concentrations and brain MRI changes consistent with elevated Mn levels have been reported [34]. Following inhalational exposure, increased Mn absorption from the respiratory tract, slower blood clearance of absorbed Mn, and direct olfactory or trigeminal presynaptic nerve endings transport aid in more efficient delivery of Mn to the brain [26].

Suarez and colleagues set out to determine if Mn^{3+} bound to transferrin (MnTf) is responsible for mediating the neurotoxicity of Mn. They compared Mn bound to pyrophosphate (MnPPi) and MnTf in human neuroblastoma cells (SH-SY5Y). Parameters studied were mitochondrial dehydrogenase activity, cell growth and survival. MnPPi was more toxic than MnTf in their cell line. Membrane permeability was significantly altered in cells exposed to Mn PPi, whereas MnTf did not change membrane permeability significantly. They concluded that while both complexes inhibited mitochondrial enzyme activity, MnPPi was more toxic in affecting morphology, cell number and general membrane permeability [35].

In a study using adult rats, the biological half-life of Mn in the brain was between 51 and 74 days [36]. In humans given intravenous ^{54}Mn tracer doses, an elimination half-time of 53 days was reported from the brain [37]. In accordance, a study in macaque monkeys given $MnCl_2$ via an implanted subcutaneous osmotic minipump reported an elimination half-time of 53 days [38]. This group also exposed two cynomolgus monkeys to aerosolized $^{54}MnCl_2$ via an endotracheal tube and observed an estimated half-life of elimination of greater than 220 days from the head [38]. The slow elimination time may be due to the elimination of Mn from the skull and not from the brain itself. Neonatal rats given Mn orally throughout lactation had Mn concentrations similar to controls when assessed at PND 73, suggesting that the half-life of elimination from young adult rat brain occurs prior to PND 73 [39], which is consistent with human findings [37]. They also found that brain delivery of inhaled Mn is influenced by particle solubility as $MnSO_4$ was more rapidly cleared from the rat lung than Mn phosphate or Mn tetroxide [39].

Cotzias and colleagues (1968) reported that Mn elimination was dose-dependent in miners exposed to Mn dusts [37]. Absolute amounts of Mn absorbed from Mn-containing dusts are unknown. Two additional studies showed enhanced biliary Mn excretion following inhalation [40, 41]. Smaller particles can also directly enter the blood stream through the

gastrointestinal epithelial lining following mucociliary elevator clearance from the respiratory tract [2].

Mn is transported to the liver, conjugated to bile and then passed to the intestine for fecal excretion. Reabsorption through the enterohepatic circulation is possible and Mn can also be detected in small amounts in urine, sweat and breast milk [42]. Absorption, distribution and excretion following dermal exposure have not been documented.

Brain Mn Targets

The distinct neurological effects seen with Mn toxicity are due to its preferential uptake by the brain. Entry of Mn to the brain occurs in one of three ways: (1) from the nasal mucosa to the brain olfactory bulb through olfactory neural connections; (2) from the blood through capillary endothelial cells of the blood-brain barrier; and (3) from the blood to the cerebral spinal fluid via the choroid plexuses [43]. In the cell, Mn is found primarily in the mitochondria in both neurons and astrocytes, in the nucleus and in synaptosomes [44–47]. In the brain, the striatum, globus pallidus and substantia nigra are reported target sites, with the globus pallidus as the primary site for Mn accumulation and toxicity [19, 48]. In addition to cell death, several studies have reported pathologic changes in the globus pallidus [48–50]. The damage has been reported to be the result of dis-inhibition of inputs to the globus pallidus [51] and the high rate of oxidative phosphorylation in this region may also account for its susceptibility. The DAergic system is a major target of Mn. Various studies, even those dating back as early as the 1920's documented the susceptibility of the globus pallidus to Mn-induced cell death [52–55]. More currently, a study of rhesus monkeys chronically exposed to Mn dusts reported decreased levels of dopamine (DA) in several brain regions, including the caudate and globus pallidus [56]. In another study, rhesus monkeys that received Mn through intravenous injection exhibited gliosis of the globus pallidus and substantia nigra as well as an extrapyramidal syndrome, a condition reflective of deregulated DA release and reuptake [49]. Two independent studies in primates, using either intravenous injection or inhalation showed widespread Mn accumulation in the brain via T1-weighted MRI [57, 58]. In the inhalational studies, Dorman and colleagues, observed Mn accumulation in the globus pallidus ranging from 1.6 to 6.0 fold relative to controls [57]. Following intravenous injection, Guilarte reported 5 fold increases in Mn in the globus pallidus [58]. Mn preference for the DAergic system was also corroborated by a study that linked Mn exposure in welders to a higher prevalence of Parkinsonian symptoms and reduced [^{18}F]fluoro-L-DOPA (FDOPA) in asymptomatic welders [59].

Manganism and PD share in their etiology common cellular mechanisms such as preferential accumulation of Mn within mitochondria resulting in oxidative stress [60], and selective DAergic neurotoxicity [61] (Table 1). Manganism commonly occurs in response to acute Mn exposures, whereas PD likely reflects long-term exposure to relatively low Mn exposures. Manganism features less frequent and kinetic tremor, or no tremor vs. patients with PD. Acute high Mn exposures also lead to dystonias and a "cock-walk" with symptoms becoming progressive and irreversible. In addition to affecting the basal ganglia, manganism is also known to affect other brain regions, including the cortex and hypothalamus and at the morphological level leading to neuronal loss and reactive gliosis in the globus pallidus and substantia nigra pars reticulata (SNpr) in the absence of Lewy bodies, which are a hallmark of PD. Furthermore, in manganism, damage to the striatum (caudate nucleus and putamen) and subthalamic nucleus may occur, while the substantia nigra pars compacta (SNpc) is generally spared whilst PD is predominantly characterized by neuronal loss in the SNpc.

Mn and autoxidation of catecholamines and other neurotransmitters

The effects of Mn on DA metabolism have received considerable attention due to preferential accumulation of Mn in the substantia nigra, globus pallidus and striatum, all DA-rich brain regions that are sensitive to oxidative injury. The free radicals that originate from DA autoxidation have been postulated to cause degeneration of DAergic cells [62–64]. Mn^{3+} is a much more potent oxidizing agent than Mn^{2+} and has been shown to readily oxidize DA, leading to higher concentrations of damaging DA quinone products [65, 66]. Mn-induced toxicity resulted from the production of hydrogen peroxide and DA quinone and the depletion of DA due to its oxidation [66]. This study also tested ascorbic acid and thiamine as potential antioxidants and results showed that they completely inhibited DA oxidation however they did so in both the absence and presence of Mn, suggesting a complicated mechanism [66]. The oxidization of DA by Mn^{3+}, which does not require oxygen, results in decreased overall DA. The resulting quinone can initiate superoxide radicals by the reduction to the semiquinone, by NADH or NADPH-dependent flavoproteins, which is then readily oxidized by molecular oxygen to form superoxide radicals. The reaction, which is irreversible, can be prevented by NADH, GSH, and ascorbic acid [67]. The mitochondria are import cellular targets in Mn-induced apoptosis in DAergic cells [44].

Desole and colleagues (1994) observed that 22mg Mn/kg/day administered orally in 6-month-old rats resulted in increased concentrations of the DA oxidation product, DOPAC, and uric acid but overall DA levels were unchanged. Higher doses of Mn significantly decreased concentrations of DA, DOPAC, glutathione and ascorbic acid and increased uric acid concentrations in the striatum compared to controls [68]. These results provided support for Mn oxidization of DA.

In a *C. elegans* study investigating the effects of Mn on intra and extracellular DA, investigators showed that Mn causes a dose-dependent degeneration of DAergic neurons, which required the presence of the reuptake transporter, DAT-1. Knockdown of this transporter abolished loss of GFP-fluorescence in Mn-induced DAergic GFP-labeled neurons. Toxicity was prevented by loss of tyrosine hydroxylase function and loss of the vesicular monoamine transporter, which normally aids DAergic neurons in releasing DA at the synaptic cleft, there was increased tolerance to Mn indicating that DA synthesis is required for DAT-1- dependent Mn toxicity and that extracellular DA, and not intracellular DA, is involved in Mn neurotoxicity [69].

Mn-induced DA oxidation and cell death of neurons in the globus pallidus is likely the result of dis-inhibition of inputs to the globus pallidus [51] and the high rate of oxidative phosphorylation in this region. Although the globus pallidus accumulates a higher concentration of Mn than other regions, it is important to note that other parts of the brain that have lower accumulation may still be vulnerable and neurotransmitters other than DA can be affected. Mn has been shown to cause reduced glutamine synthetase (GS) in the globus pallidus, which is important for conversion of glutamate to glutamine [70]. Additionally, a handful of studies in rodents have shown increased glutamate levels in the brain after exposure to Mn [71–73]. Another study looking specifically at brain regions showed increased glutamate in frontal cortex but decreased glutamate in the globus pallidus [74]. Glutamate transporters have been shown to be decreased in *in vitro* studies [22] and primates showed increased mRNA expression and decreased protein of two glutamate transporters and decreased GS in multiple brain regions [70]. Multiple studies looking at γ-aminobutyric acid (GABA) have presented conflicting data [71–73, 75–78], leaving the exact role of GABA in Mn toxicity somewhat unclear. For example, several groups using rodents showed Mn induced increases in striatal GABA levels [71–73, 77, 79, 80] however,

decreases in GABA levels in the striatum and frontal cortex were also reported by several groups [76, 81]. Still other studies have reported no effect on Mn on GABA levels [75, 82]. A short-term study in Welders showed no effect on Mn fumes on GABA located in DAergic regions of the brain [83]. MRI studies of presymptomatic smelters exposed to Mn showed Mn accumulation in globus pallidus and elevated GABA levels in the thalamus and adjacent basal ganglia via MRI [84]. Contradictory conclusions about the role of GABA are likely due to differences in route and duration of exposure, age at time of exposure and/or the contribution and alteration of other neurotransmitter systems.

Reactive oxygen species

Mn is known to enhance production of reactive oxygen species (ROS), which contributes to Mn toxicity. ROS is the name commonly given to highly reactive oxygen radicals and peroxides generated by a number of mechanisms within cells. The most common of these ROS are superoxide radical, hydrogen peroxide and hydroxyl radical. Common sites of production of these ROS include the mitochondrial electron transport chain (ETC), NADPH oxidases, enzymatic activation of cytochrome p450, xanthine oxidase and cyclooxygenase 1 and 2; however, for most types of cells the greatest production is by the mitochondrial ETC [85–88]. In mitochondria the superoxide radical ($O_2^{-\bullet}$) is generally the initial ROS species formed. Superoxide radical can be converted into hydrogen peroxide (H_2O_2) by superoxide dismutase. In the mitochondrial matrix MnSOD catalyzes this reaction, while in the intermembrane space and cytosol it is catalyzed by copper/zinc superoxide dismutase (Cu/Zn SOD). In the presence of iron or other transition metals such as Mn, H_2O_2 can be converted into the very reactive hydroxyl radical ($OH^{\bullet}$) [89].

The seven known sites of superoxide production in mitochondria are: the flavin site in complex I (site IF), the ubiquinone-binding sites in complexes I and III (sites IQ and $IIIQ_0$, respectively), glycerol 3-phosphate dehydrogenase, the electron transferring flavoprotein:Q oxidoreductase of fatty acid beta-oxidation (ETFQOR) and pyruvate and α-ketoglutarate dehydrogenases [90]. Most of the work on mitochondrial production of ROS has focused on superoxide production at complexes I [91–97] and III [92, 93, 95, 98]. All of the mitochondrial sites produce $O_2^{-\bullet}$ in the matrix space while site IIIQo and glycerol 3-phosphate can also produce $O_2^{-\bullet}$ in the intermembrane space; i.e., the space between the inner and outer mitochondrial membranes [90, 96]. Of these mitochondrial sites those associated with ubiquinone binding at complexes I and III in the ETC have the largest maximal rates of $O_2^{-\bullet}$ production [90]. A simple, intuitive view of $O_2^{-\bullet}$ production by the ETC would picture electrons moving between ETC complexes and between binding sites such as flavins, iron-sulfur proteins and cytochromes within each complex. Some of these sites would be near the protein-lipid interface between the ETC complex and the phospholipid of the membrane. Because molecular oxygen (O_2) partitions to about 3 times higher concentration within the phospholipid membrane than in the aqueous portions of the cell [94], this O_2 occasionally captures an electron from a nearby binding site in the ETC complex and forms $O_2^{-\bullet}$.

Mitochondrial $O_2^{-\bullet}$ production has been shown to increase as the membrane potential ($\Delta\psi$) or electrochemical proton gradient ($\Delta\mu_H$) increases [92, 93, 95, 96]. ATP production by oxidative phosphorylation or mild uncoupling decreases these gradients and significantly decreases ROS production. Other factors that increase mitochondrial ROS production include uptake of cations such as Ca^{2+} and Mn^{2+} [99–102], induction of the mitochondrial permeability transition (MPT) [103, 104], inhibition of the ETC [90, 94], and hyperglycemia [105].

While it is not known definitively where normal mitochondrial $O_2^{-\bullet}$ production occurs *in vivo* [90], this would generally take place during ATP production and, therefore, at decreased $\Delta\psi$ and $\Delta\mu_H$. Under these conditions, $O_2^{-\bullet}$ production probably occurs at most of the mitochondrial sites with most of the $O_2^{-\bullet}$ produced at complexes I and III [90, 94].

Most of the mitochondrial production of $O_2^{-\bullet}$ occurs in the mitochondrial matrix with only a small fraction being produced in the intermembrane space [90, 94]. Furthermore, since $O_2^{-\bullet}$ is a charged species, it does not easily penetrate membranes. However, the $O_2^{-\bullet}$ in the matrix is quickly converted into H_2O_2 by Mn SOD, and H_2O_2 readily crosses the membrane [94]. Therefore, the H_2O_2 produced from mitochondrial matrix $O_2^{-\bullet}$ can readily move into the intermembrane space, the cytosol and even the nucleus of the cell.

Each of the three primary ROS species, $O_2^{-\bullet}$, H_2O_2 and $OH^{\bullet}$ can cause damage to nucleic acids, proteins and phospholipids within mitochondria and within cells [106, 107]. Mitochondrial DNA is particularly sensitive to ROS damage because of the lack of protective histones in mitochondria and the lack of repair mechanisms such as those found in the nucleus [106, 107]. Furthermore, mitochondrial ROS can contribute to induction of the mitochondrial permeability transition (MPT), which can, in turn, induce apoptosis [95, 108–112]. This will be discussed in more detail below.

While we do not have experimental evidence for the mechanisms through which intramitochondrial Ca^{2+} or Mn increase ROS production, we know that intramitochondrial Ca^{2+} activates a series of sites within the mitochondrion which increase the rate of ATP production by oxidative phosphorylation including activation of three dehydrogenases in the TCA cycle which catalyze increased production of NADH [113–115]. Activation of these dehydrogenases increases the NADH/NAD+ ratio and the metabolic rate, which should, in itself lead to a higher rate of ROS production. Mn^{2+} binds to almost every Ca^{2+} binding site and could increase ROS production by simply functioning as a Ca^{2+} analog. However, we also know that Mn^{2+} inhibits the rate of ATP production through inhibition sites in the electron transport chain, the TCA cycle and at other loci [60, 116, 117]. The known sites of mitochondrial production of $O_2^{-\bullet}$ represent loci at which the probability of electron capture by O_2 is relatively high [90]. Any inhibition caused by Mn^{2+} binding to intramitochondrial sites which cause a buildup of occupancy in these sites of $O_2^{-\bullet}$ production could also cause an increase in ROS production [90].

Not all ROS effects are negative; ROS have also been shown to be important in cell signaling [118]. For example, H_2O_2 is important in stabilizing Hif-1a, which is probably the most important factor in inducing the changes that enable a cell to survive hypoxia [119, 120]. H_2O_2 has also been found to activate mitochondrial uncoupler proteins (UCP's), which through uncoupling effects decrease $\Delta\psi$ and $\Delta\mu_H$, and thereby decrease ROS production [121]. ROS signaling has also been implicated in a number of activities which can be beneficial to the cell, including: activation of transcription factors [122] including nuclear factor 2 (erythroid-derived 2)(Nrf2), which protects against ROS damage [123, 124]; cell proliferation and regulation of the cell cycle [125, 126]; regulation of apoptosis [127]; regulation of metalloproteinase [128]; and activation of protein kinases and phosphatases [128, 129].

There are mechanisms present both in the cell cytosol and in mitochondria that protect against ROS-induced damage. One of the most effective protective molecules is the short peptide glutathione (γ-L-glutamyl-L-cysteinyl glycine or GSH) [130]. GSH is made in the cytosol and transported into the mitochondrion. The enzyme catalase, present in both the mitochondrial matrix and the cytosol, can convert H_2O_2 to H_2O and O_2, eliminating the ROS danger. Cytochrome c is not only important as part of the ETC, but can also serve a

protective function. It is present in the intermembrane space at concentrations greater than those needed for its electron transport function, and it can scavenge $O_2^{-\bullet}$ radicals and return the electrons to complex IV of the ETC [131]. Similarly, ubiquinol (reduced co Q_{10}), when present in excess, has been reported to scavenge hydroperoxyl radical [132], which like ubiquinol is lipophilic, and return the electron to the ETC. Finally, while mitochondrial DNA does not have protective histones and elaborate repair mechanisms, as the nucleus does, mitochondria do contain many copies of mitochondrial DNA so that if one is damaged by ROS there are many other copies to take its place.

It has been shown that antioxidants, particularly mitochondrially-targeted antioxidants can ameliorate a range of specific disease conditions associated with increased ROS production and extend the lives of test animals which show these conditions [133–143], however the results of experiments designed to show that treatment with antioxidants leads to longer lifespan generally have not been convincing [144]. Autophagy is a process through which a cell breaks down and reuses components of misfolded proteins and malfunctioning organelles. Many antioxidants inhibit basal and induced autophagy [145]. Since induction of autophagy is associated with increased lifespan, the answer to this apparent inconsistency may lie in the inhibitory effects of antioxidants on autophagy [145]. Essentially, antioxidants only extend lifespan in cases where increased production of ROS contributes to cell death.

Mitochondria, the mitochondrial permeability transition, and apoptosis

Mitochondria in higher eukaryotes carry out many important cellular functions including production of over 90% of the cell's ATP by oxidative phosphorylation [146], cytosolic [Ca^{2+}] buffering [111, 147], partial control of apoptosis [148–150], β oxidation of fatty acids [151], a role in the urea cycle [151], and a role in the synthesis and metabolism of iron-containing proteins [152, 153]; however, oxidative phosphorylation was undoubtedly their initial and is now their most important function. Since intramitochondrial [Ca^{2+}] is understood to be a major factor in controlling the rate of ATP production by oxidative phosphorylation [113–115, 154], and since it also plays a major role in inducing the mitochondrial permeability transition (MPT) (described below), mitochondrial Ca^{2+} transport takes on added importance. Mitochondria contain an elaborate system for Ca^{2+} sequestration and control consisting of two mechanisms of influx, the Ca^{2+} uniporter and the rapid mode or RaM, and two mechanisms of efflux, the Na^+-dependent and the Na^+-independent efflux mechanisms [147]. An additional mechanism, a mitochondrial ryanodine receptor, has also been reported in heart mitochondria [155–157]. In addition, a very unusual mitochondrial mechanism called the MPT, which will be discussed in more detail below, also affects and is affected by intramitochondrial [Ca^{2+}]. Because Mn^{2+} almost always binds to Ca^{2+} binding and transport sites, the mitochondrial Ca^{2+} transport mechanisms are important to Mn toxicity as well. The role of [Ca^{2+}] in activating ATP production by oxidative phosphorylation and the mechanisms of mitochondrial Ca^{2+} transport is important in Mn toxicity for several reasons. First, the mitochondrial Ca^{2+} uniporter has been shown to transport Mn^{2+} as well as Ca^{2+} [158–161]. Second, many studies on Mn toxicity have indicated Mn interference with energy metabolism [60, 76, 117, 162–167] and inhibition of oxidative phosphorylation [60, 116, 117, 167]. Finally, Mn^{2+} has been found not to be transported out of mitochondria via the Na^+-dependent efflux mechanism, which is by far the dominant mechanism in brain mitochondria, but only to be transported outward by the Na^+-independent efflux mechanism, which is much less active in brain mitochondria [168]. This means that once Mn^{2+} is sequestered within brain mitochondria, the target tissue for Mn toxicity by the rapid uniporter mechanism, it is very difficult for the Mn^{2+} to be transported out again. This also explains the relatively long half-life of Mn within the brain.

Mitochondria can undergo a dramatic change in permeability of the inner membrane caused by the opening of a very large pore called the permeability transition pore (PTP). The process is referred to as inducing the MPT [108, 110, 111, 169, 170]. Opening of the PTP rapidly dissipates $\Delta\mu_H$ and blocks oxidative phosphorylation. It is quite remarkable that a process, which dissipates $\Delta\mu_H$ and turns off production of over 90% of the cell's ATP is highly conserved within eukaryotes. This must be because the function of the MPT is very important to the organism. While still speculative, it is likely that the importance of the MPT lies in its relationship to apoptosis and the mitochondrion's role in control of apoptosis, which is discussed further below [147].

By the middle 1970's, the MPT had been described in mitochondria as a process which causes rapid swelling and ultrastructural changes, and also allows NADH and NADPH to directly enter the mitochondrial matrix, something which they cannot otherwise do [171–173]. This transition was originally referred to as the Ca^{2+}-induced MPT, because intramitochondrial Ca^{2+} was always required for its induction. Hunter and Haworth noted that what was known about the MPT could be explained by the opening of a proteinaceous pore, later called the PTP [174, 175]. The opening of the PTP was found to have the following characteristics: 1) it could be induced by Ca^{2+} alone or by smaller amounts of Ca^{2+} in the presence of other agents called "inducing agents" [111]; 2) common endogenous inducing agents include inorganic phosphate, oxaloacetate and acetoacetate, while Mg^{2+}, ADP and ATP act as endogenous inhibitors of PTP opening [108, 111, 170]; 3) molecules with a molecular weight less than 1,500 Daltons penetrate the PTP rapidly [175], while even larger molecules can slowly penetrate the PTP [176]; 4) PTP opening is greatly aided by production of ROS [108, 110, 111, 169, 170], whereas, acidic pH and a high mitochondrial $\Delta\psi$ are potent inhibitors of PTP opening [177, 178]. Commonly used non-endogenous inducing agents include phenylarsine oxide, tert butyl hydroperoxide, atractyloside and carboxyatractyloside, while commonly used inhibitors are cyclosporin A, bongkrekate, adenine nucleotides and chelation of Ca^{2+} [111, 170].

The discovery that the immunosuppressant cyclosporin A (cys A) was a potent inhibitor of PTP opening [179, 180] was followed by studies establishing that this inhibition occurs by its binding to a mitochondrial matrix protein, cyclophilin D (cyp D). This led to efforts to determine the makeup of the pore protein and the development of the hypothesis that the crucial step in pore opening was the Ca^{2+}-induced binding of cyp D to the adenine nucleotide translocase (ANT). Evidence showed that cys A inhibited this process by binding to cyp D, therefore keeping it from binding to ANT [112, 181, 182]. Later Wallace's laboratory demonstrated the MPT in mitochondria from the livers of newborn mice that had been genetically modified to lack the ANT, thereby establishing that the ANT was not required for the MPT [183]. Many still support the hypothesis that the Ca^{2+}-induced binding of cyp D to the pore protein is the crucial step in PTP opening with the modification that a set of mitochondrial membrane proteins and not just the ANT can form the basis for the pore [184].

The MPT is viewed as an important component of the mechanisms which give mitochondria a significant role in control of apoptosis or programmed cell death, and ROS are viewed as playing a significant role in induction of the MPT and therefore in apoptosis. Unlike necrosis, apoptosis preserves components of cells for reuse by other cells, usually macrophages [150]. Characteristics that are commonly observed in apoptosis include contraction of cell volume rather than the swelling seen in necrosis, chromatin condensation, nuclear fragmentation, blebbing of the plasma membrane, and the formation of apoptotic vesicles containing cellular material that can be taken up and reused by the surrounding cells [150]. In recent years the relatively clear distinction between apoptotic and necrotic cell

death has been blurred somewhat. It is presently believed that many types of cell death include both apoptotic and necrotic characteristics [95].

Mn, ROS, the Mitochondria and Apoptosis

In terms of electron structure, Mn^{2+} ion has a half filled d shell, giving it spherical symmetry similar to that of Ca^{2+} and Mg^{2+}, and its ionic radius is intermediate between that of Ca^{2+} and Mg^{2+} [160, 161]. For these reasons, it binds to almost every Ca^{2+} or Mg^{2+} binding site, and can often either substitute for Ca^{2+} or Mg^{2+} in biological processes or act as an inhibitor of these processes. Therefore, it comes as no surprise that Mn^{2+} can interfere with Ca^{2+}'s role in activating ATP production by oxidative phosphorylation.

Using a *C. elegans* model, Benedetto and colleagues demonstrated extracellular Mn-induced oxidation of synaptic DA, generating ROS and leading to lipid peroxidation [69]. In their study, SKN-1 (mammalian NRF2 homolog) and BLI-3, proteins important for antioxidant/oxidant homeostasis, were linked to the Mn-induced toxicity. Loss of function of SKN-1 increased sensitivity to Mn, and Mn was able to induce expression of antioxidant proteins through SKN-1 [69]. BLI-3 is a dual oxidase involved in di-tyrosine bond formation in the worm cuticle; pathogen-induced ROS production and loss of this gene resulted in no increase in ROS production by Mn [69].

Gavin and colleagues, using isolated mitochondria and concentrations of Mn^{2+} greater than 1μM, showed evidence that Mn^{2+} causes impairment of energy metabolism [60, 168]. In another study of Mn inhibition of energy metabolism, Brouillet and colleagues measured ATP and lactate levels following injection of 2 μM Mn in the rat striatum. They reported N-methyl-D-aspartate (NMDA) excitotoxic lesions as well as a 51% reduction of ATP and a 97% increase in lactate compared to control. Dose-dependent decreases in DA, GABA, and substance P were also noted. These data suggest that oxidative metabolism was impaired following exposure to Mn [76].

Additional support for oxidative stress as a mechanism of toxicity comes from studies on the use of antioxidants to combat Mn-induced ROS. Hazell and colleagues (2006) co-treated rats with N-acetylcysteine (an antioxidant) and 50mg/kg $MnCl_2$ for 4 days. Addition of N-acetylcysteine prevented the pathological changes observed in Mn exposed animals [185]. In another study using catecholaminergic cells, Stredrick and colleagues found that supplementing culture media with glutathione or N-acetylcysteine protected against Mn cytotoxicity [61]. Brenneman et al (1999) measured ROS in the brains of neonatal rats exposed to 22mgMn/kg/day and reported no increase in ROS in the striatum, hippocampus or hindbrain at postnatal day (PND) 21 but a significant increase in ROS levels in the cerebellum. Interestingly, Mn levels were not significantly increased in the cerebellum but were increased in the striatum when measured at PND 49 [186]. Klockgether and Turski reported that the pathophysiology of PD is due to dis-inhibition of glutamatergic inputs to the globus pallidus regulated by differential activation of glutamate receptor subtypes in the basal ganglia [51].

The death of cultured cells and cells in the brains of non-human primates [187] exposed to Mn has largely been described as apoptotic and is often associated with increased ROS production. The primary tools used to measure the increase in ROS are 2′,7′-dichlorofluoroscein (DCF) and its acetate form, DCF-DA. DCF fluorescence was used to show an increase in ROS induced by Mn addition to a primary culture of astrocytes [99] and to a culture of neural stem cells [102], with cell death showing apoptotic characteristics. DCF-DA was used with primary mesencephalic cells prepared from ventral mesencephalic tissues of fetal rat brain to show an ROS increase and cell death with apoptotic characteristics [101]. Latchoumycandane et al reported strong evidence for apoptotic cell

death in a rat mesencephalic DAergic N27 cell line. Cytochrome c release, caspase 3 activation, DNA fragmentation and the effects of the apoptosis inhibitors Z-DEVD-FMK were all studied, and showed apoptotic characteristics [188]. Guilarte's laboratory found signs of apoptosis in the brains of cynomolgus macaques after treatment with 3.3 – 5.0 mg Mn/kg body weight weekly for 10 months [187, 189].

In an effort to study the contribution of Mn to L-DOPA toxicity, Migheli and colleagues used the 3-(4,5-demethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, flow cytometry, fluorescence microscopy and TUNEL staining to measure apoptosis in PC12 (catecholaminergic) cells. They showed that neither exposure to a subtoxic dose (0.2mM) of $MnCl_2$ or 10–20 μM L-DOPA alone affected any of their experimental parameters but apoptotic cell death occurred following $MnCl_2$ and L-DOPA co-exposure [190]. Use of N-acetylcysteine (NAC) inhibited apoptosis and decreases in cell viability [190]. Additionally, Ramesh and colleagues (2002) measured the DNA binding activity of nuclear factor kappa B (NF-κB) after exposure to Mn in PC12 cells [191]. They reported a five-fold induction of NF-κB following Mn exposure for just 30–60 minutes and activation of mitogen activated protein kinase kinase (MAPKK); the authors suggest that this activation could lead downstream to excessive transcription of proteins that contribute to cell death [191]. Kitazawa and colleagues, using the same cell line, highlighted the importance of proteolytic activation of protein kinase Cδ (PKCδ) by caspase-3 in mediating apoptosis following Mn exposure [192]. Mitochondrial membrane depolarization, cytochrome c release, caspase-3 activation and DNA fragmentation were all reported in Mn exposed cells. These events, as well as the proteolytic cleavage of PKCδ, were blocked by overexpression of Bcl-2. Expression of the catalytically inactive mutant PKCδ attenuated apoptosis and administration of active recombinant PKCδ induced DNA fragmentation [192]. Taken together, this evidence points to apoptosis as a mechanism of toxicity and the involvement of PKCδ and caspase-3 in mediating apoptosis following Mn exposure.

A case of the use of mitochondrially-targeted antioxidants

Untargeted antioxidants have been shown to ameliorate Mn-induced damage. Vitamin E was assessed as an antioxidant treatment option and it was found that pretreatment attenuated the Mn-induced increase in cerebral isoprostanes and protected medium spiny neurons from dendritic atrophy and dendritic spine loss [193].

L-DOPA has been tested for Mn-toxicity due to the similarities between manganism and PD. Some of the motor symptoms associated with manganism were reversed by L-DOPA treatment albeit with serious side effects [52]. Treatment with anti-Parkinsonian drugs may be less effective due to the preferential accumulation of Mn in the globus pallidus, rather than the cell death in the substantia nigra seen in PD.

Treatment of cells and isolated mitochondria with Mn^{2+} has also been found to cause inhibition of ATP production by oxidative phosphorylation [60, 116, 117, 167]. Inhibition of the ETC could cause the proximal part of the ETC to become even more reduced and therefore more likely to produce $O_2^{-\bullet}$, and increases of $O_2^{-\bullet}$, production of this type have been reported [194–197]. These observations as well as those reported above showing an increase in ROS production following Mn treatment [99, 101, 102] suggest that Mn-induced ROS production and probably opening of the PTP are involved with induction of the form of apoptosis leading to cell death in the globus pallidus. If untargeted antioxidants can ameliorate damage in Mn toxicity, how much more clinically effective could drugs that protect against ROS damage, MPT induction and apoptosis at the mitochondrial level be in treating Mn toxicity [198]. A number of different drugs are currently being developed to be used to treat conditions like apoptotic cell death. These drugs are sequestered into

mitochondria where they act as reducing agents and detoxify mitochondrial ROS [137–139, 141, 142].

MitoTEMPO, mitoNAC, and MitoQ are part of a series of antioxidants under development and study containing nitroxide, tocopherol and ubiquinone moieties. They are made membrane permeable and mitochondrially-targeted by attaching a positively charged triphenol phosphonium (TPP) moiety. For example, mitoQ is made up of a combination of ubiquinone or coenzyme Q_{10} (co Q_{10}) and TPP. Analogous to the other members in this series, it can move rapidly across the cell and mitochondrial membranes into mitochondria. Inside the mitochondrion, mito Q can be oxidized by reaction with ROS and then reduced by complex II and reused as an antioxidant. It cannot pass electrons on to complex III as co Q does and so it functions to protect against oxidative damage to the mitochondria [138–140]. Hyperglycemia in diabetes [105], excessive ethanol use [134], and some forms of hypertension [135] are accompanied by an increase in mitochondrial ROS production in some organs. Mito Q was orally administered to mice showing the Akita J model of Type I diabetes over a 12-week period. Interstitial fibrosis and glomerular damage were significantly reduced in the kidneys of treated vs. control animals, showing in this case that mitochondrially-targeted therapies were beneficial in the treatment of diabetic nephropathy [133]. In a four week oral treatment of Mito Q to Sprague-Dawley rats consuming ethanol using the Lieber-Decarli diet vs. controls, it was found that the Mito Q treatment decreased hepatic steatosis in ethanol-treated animals, prevented ethanol-induced formation of 3-NT and 4-HNE, and stabilized HIF1alpha [134]. Stroke-prone, spontaneously hypertensive rats were treated orally with Mito Q for 8 weeks, systolic blood pressure in the treated animals was reduced by approximately 25 mm Hg over the 8-week treatment period [135].

The Szeto-Schiller or SS peptides are a set of mitochondrially-targeted, antioxidant peptides, which have been shown to penetrate the blood-brain barrier and protect brain mitochondria against ROS damage while showing minimal toxicity [141, 142]. The Szeto-Schiller peptide SS-31 has recently been shown to protect against induction of the MPT following a burst of ROS during reperfusion of ischemic kidney tissue reducing apoptosis and necrosis of tubular cells [143].

SkQ1, an antioxidant in the SkQ family, combines plastoquinone, a very effective electron carrier and antioxidant of chloroplasts with decyltriphenolphosphonium to form a cation that easily penetrates membranes [137]. In a number of tests SkQ1 was found to save the lives of young animals after treatments resulting in kidney ischemia, rhabdomyolysis, heart attack, arrhythmia, and stroke and to suppress the appearance of many traits related to senescence [136].

Conclusion

Manganese is an essential nutrient, which can be harmful at higher doses or accumulations. Because of its essentiality, stable tissue levels are maintained making it difficult to determine if overexposure has occurred. As Mn toxicity can cause a number of functional problems, such as manganism, it is imperative that we understand the mechanism(s) of damage to attempt to prevent or counter them. Such mechanisms include the autoxidation of DA, production of free radicals, ROS and toxic metabolites, depletion of cellular antioxidant defense mechanisms and alterations of mitochondrial function and ATP production. Decades of research have also shown that the energy requirements of certain brain regions (i.e. globus pallidus) make them more vulnerable. Future research endeavors should be aimed at not only pinpointing the mechanism(s) underlying the damage but also identifying biomarkers for exposure and treatment strategies.

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Highlights

- Mn is a key component of the diet, but in excess can be toxic
- Mn is a component of enzymes and forms complexes in the body
- Mn toxicity is the result of reactive oxygen species, and altered energy metabolism
- Therapeutics that target the mitochondria may be useful in combating toxicity

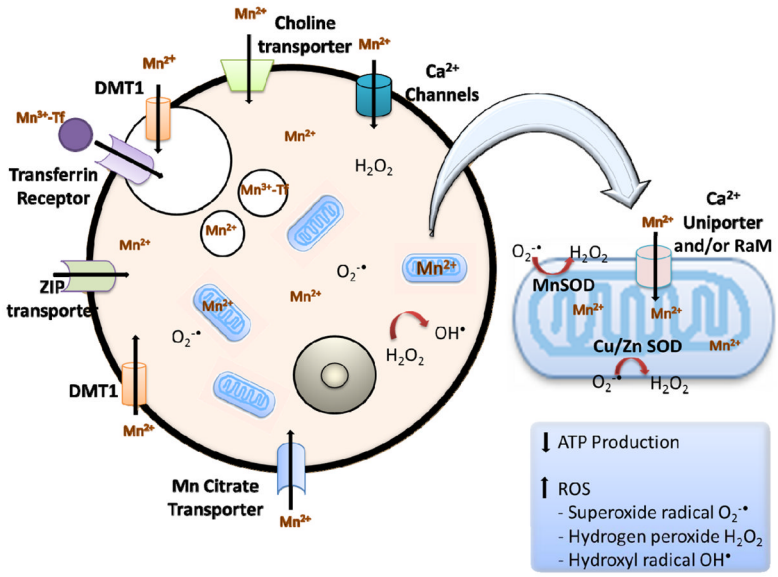

Figure 1.
Mn Transport

Table 1

Similarities and Differences between PD and Manganese Toxicity.

	Parkinson's Disease	Manganese Toxicity	Reference
Genetic Determination	Yes, can be autosomal dominant or autosomal recessive. Approximately 10–20% of cases linked to genetic causes	No	[199]
Selective DA sensitivity	Yes	Yes- however several studies have pointed to the selectivity of glutamate and possibly GABA	[57–59]
Brain regions affected	Substancia nigra pars compacta (SNpc) primarily and striatum	Globus pallidus primarily but striatum, and substancia nigra may also be involved	[6, 19, 199]
Mitochondrial dysfunction/ oxidative stress	Yes	Yes	[85, 199]
L-DOPA responsive	Yes	Generally unresponsive to L-DOPA however, some motor symptoms reversed but with major side effects	[52]
Symptoms	Emotional and cognitive decline, bradykinesia, rigidity, tremors and postural instability.	Extrapyramidal syndrome	[19, 49, 200, 201]
		Slowed hand movements, irritability, aggressiveness and hallucinations.	
	Later stages: masked-face, forward-flexed posture, gait freezing, shuffling steps, GI issues	Later stages: Rigidity, tremor, gait disturbances (slow and clumsy movements), hypokinesia, facial muscle spasms, propensity to fall backward when pushed, less frequent resting tremor, more frequent dystonia, a "cock-walk" collectively referred to as 'manganism'	