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Examining Potential Gene-Environment Interactions between the Parkinson's Disease-Associated Gene *parkin*, Manganese, and its Transporter DMT1

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Abstract
Parkinson's disease (PD) is a neurodegenerative brain disorder that is marked by the loss of dopaminergic (DAergic) cells in the substantia nigra (SN). It is characterized by overall motor dysfunction, along with cognitive and emotional disturbances. Various treatment options currently exist to combat PD symptomatology, but are not able to directly target its pathogenesis due to a lack of knowledge concerning its etiology. In hopes of leading to therapy, many genes have been linked to PD, including *parkin*, which encodes for an E3 ubiquitin ligase involved in targeting proteins for proteasomal degradation. However, as the majority of PD is idiopathic[A], environmental factors must be taken into consideration as contributing to the disease as components of gene-environment interactions. One such environmental factor is manganese (Mn), an essential nutrient in our diet that can become toxic at high levels, leading to a condition known as "manganism" that resembles PD. Mn intoxication from excessive Mn exposure could arise from impairments in proper Mn transport mediated by the divalent metal transporter 1 (DMT1), or problems associated with intracellular Mn storage capabilities. Parallel findings (both clinical and pathological) between manganism and PD suggest common mechanisms that demand further examination. This review aims to shed light on the potentially interactive roles of parkin, Mn and DMT1 that may be involved in the pathogenesis of both PD and manganism.

Keywords: *Parkinson's disease, neurodegeneration, basal ganglia, parkin, ubiquitin-proteasome system, gene-environment interaction, manganese, DMT1, mitochondria, autophagy*

Parkin's Functional Connections to PD Pathophysiology

PD is one of the most common neurodegenerative disorders in the U.S. population, with a median age of onset around 60 years[1]. This disease affects more than 1% of the population over the age of 60 by causing preferential damage to the nigrostriatal[B] circuit of the brain. More specifically, distinct degeneration of DAergic neurons in the substantia nigra pars compacta (SNpc) is the prominent pathological hallmark of the disease, along with the presence of α-synuclein-rich[C] Lewy body inclusions. These features ultimately lead to motor dysfunction, with cardinal symptoms including bradykinesia (slowness in movement), tremors, rigidity, and postural instability[2]. Cognitive deficits and emotional and behavioral problems are also seen in diseased individuals. Later stages of the disease are often marked by appearance of a masked face, along with a forward-flexed posture, gait freezing, shuffling steps, and gastrointestinal issues[1]. Despite the myriad of known symptoms, the mechanisms behind this neurodegenerative disease are still unclear.

Although the majority of PD cases are sporadic in nature, about 10-20% of PD cases have a genetic basis. Many PD-associated genes have been identified in the literature, including *dj-1, pink1, parkin, NURR1, lrrk2, UCH-L1*, and *α-synuclein*[2]. This review will specifically focus on one of these genes, *parkin*, and its protein product, also called "parkin." This protein functions as an E3 ubiquitin ligase, a component of the ubiquitin-proteasome system (UPS) to target substrate proteins, along with itself, for proteasomal degradation[3]. It consists of 465 amino acids, and contains a ubiquitin-like domain that is responsible for substrate recognition, along with RING finger domains that interact with other components of the UPS[4]. Parkin expression in the brain is distributed within basal ganglia structures, including the SN and caudate-putamen, but also with some expression in the cerebellum as well[5]. Beside itself, parkin has many substrates, including the synaptic vesicle-associated protein CDCrel-1[3], α-synuclein[6], the α-synuclein-interacting protein synphilin-17, and the membrane receptor Pael-R[8]. Parkin has also recently been shown to form an E3 ligase complex with DJ-1 and PINK1, two other proteins implicated in PD[9]. Homozygous mutations found in parkin have been linked to an early-onset familial form of PD, with no presence of Lewy bodies[4]. Homozygous mutations in *parkin* result in altered intracellular localization, impaired substrate binding and enzymatic activity, both *in vitro* and *in vivo*. Consequently, a functional effect of mutations in this gene is an inability to degrade substrate proteins[10]. Parkin-/- mice show an increase in extracellular striatal dopamine (DA) concentration[11], while wildtype parkin seems to increase cell surface expression of the dopamine transporter (DAT) for increased DA reuptake *in vitro*[12]. *Parkin*-/- mice also show impairments in synaptic plasticity[13], as parkin seems to negatively regulate the strength and number of excitatory synapses[14]. Moreover, animal models expressing mutant *parkin* exhibit selective DAergic degeneration as well as hypokinetic deficits[15], as seen in PD cases. However, the mechanism behind these effects is still unclear and needs further investigation.

Gene-Environment Interaction: Manganese Neurotoxicity as a Cue

Despite the wealth of knowledge already gathered about the disease, mutations in parkin and other PD-associated genes still only partially explain a minority of PD cases. The possibility exists that a convergence between the individual effects of a genetic predisposition and exposure to

[A] **Idiopathic**: used to describe a disease in which the etiology is unknown.
[B] **Nigrostriatal:** major dopamine pathway that connects the substantia nigra with the striatum; involved in movement generation.
[C] **α-synuclein**: a soluble protein that can form abnormal aggregates (Lewy bodies) in neurons.

an environment factor on the same neural pathways ultimately leads to PD. A twin study conducted in 1999 was one of the first to suggest that gene defects may not be the only causes of PD[16]. Furthermore, the recent Geoparkinson study found a significant association between pesticide use and PD[17]. Others have shown that exposure to the pesticide rotenone, a mitochondrial complex I inhibitor, causes selective degeneration of nigrostriatal DAergic cells[18]. Investigation into the effects of genetic risk factors on the ability of environmental factors to reproduce PD neuropathology has become more prevalent, as evidence for gene-environment interactions begins to expand. For example, in addition to causing selective DAergic cell loss, rotenone also induces oxidative modification of DJ-1, α-synuclein accumulation, and proteasomal impairment[19] (*DJ-1* and *α-synuclein* are also genetic risk factors for PD). However, support of a gene-environment interaction involving *parkin* in PD has been marginally examined. A recent study in Drosophila shows that *parkin* mutants exhibit a significantly shortened lifespan compared to control flies upon exposure to environmental pesticides[20]. With *parkin*'s prevalence as a major genetic risk factor for PD, further work must be done to look at its interactions with environmental factors that may be causing the pathophysiology seen in patients harboring a *parkin* mutation. Temporal parallels between PD and manganism further support a gene-environment hypothesis, where environmental exposure to manganese (Mn) and a juvenile, familial form of PD due to *parkin* mutations can both be found in younger individuals[21]. Thus, further work is necessary to assess whether Mn exposure is an added risk for individuals harboring polymorphisms in the *parkin* gene.

Consequently, environmental PD risk factors now include heavy metals. One such metal is Mn, a vital trace element that is necessary for proper metabolic function and detoxification of superoxide free radicals. Mn ions are important cofactors for several enzymes, including but not limited to glutamine synthetase, arginase, and superoxide dismutase (SOD)[22]. Mn also has a significant role in proper immune function, bone growth, digestion, reproduction, and defense against free radicals[23]. Despite being an abundant and essential micronutrient, rare Mn deficiency can result in abnormal bone growth, birth defects, impaired macromolecular metabolism, and seizures[23,24]. The essentiality of Mn, however, is mirrored by neurotoxicity that can develop from excess Mn exposure. Outside of dietary Mn intake, environmental sources of exposure can include drinking water (groundwater)[25], pesticides[26], and airborne exposure upon combustion of the fuel additive methylcyclopentadienyl manganese tricarbonyl (MMT)[27]. However, excessive environmental exposure to Mn can also occur through a variety of occupations, including welders, steel miners, smelters, and other industrial workers[28]. Excessive exposure to Mn can result in an irreversible condition known as "manganism," which involves neurological disturbances that highly resemble PD. This disorder is marked by progressive bradykinesia, gait disturbances, fixed facial expression, and slurred speech[29]. Its pathology includes increased Mn concentrations in the basal ganglia, including the caudate-putamen, SN, subthalamic nuclei, and the globus pallidus[30,31]. Unlike PD, damage from Mn intoxication primarily occurs downstream of the nigrostriatal pathway, focusing on the globus pallidus output target. Interestingly, similar to *parkin* mutants, it is rare to see Lewy bodies in the SN of individuals suffering from manganism[24]. Mn has been shown to promote cytotoxicity in dopamine- producing cells[32], along with affecting neurite length and integrity within the basal ganglia[33]. Mitochondrial dysfunction is also generated upon Mn exposure in a concentration-dependent manner, with depletion of ATP and an increase in reactive oxygen species[34]. Furthermore, Mn also impairs proper astrocytic function, consequently hindering their buffering roles in protecting the more sensitive neurons from Mn toxicity[35]. Aside from affecting the same neural substrates damaged by PD[36], Mn has also been identified as a potential component of a gene-environment interaction. Few studies have begun to look at this connection[37], with one study finding that α-synuclein may interact with Mn to result in increased Mn toxicity[38]. Moreover, in the only study thus far to specifically examine a *parkin*-Mn interaction, Mn was found to increase parkin protein levels specifically in DAergic cells. Parkin also conferred protection from Mn-induced DAergic cell death *in vitro*, and was selectively redistributed to the perinuclear region in DAergic cells upon Mn exposure[39]. However, it still remains unknown how parkin is able to selectively protect DAergic cells, and how Mn is able to alter this ability.

Consequences Of An Impaired Manganese Transport Profile

DMT1-Mediated Import

A potential mechanism for the neurodegeneration evident in *parkin*-linked PD cases could involve disrupted Mn homeostasis, implicating problems with Mn transport and/or storage capabilities within the cell. The delicate balancing act between Mn's necessity for proper physiological processes and its paralleled toxicity calls for tight regulation to maintain proper homeostasis. Mn can cross the blood brain barrier through a variety of processes[40], but the identity of a single and definite Mn transporter remains a mystery. A well-researched transporter prominently involved in Mn regulation is the divalent metal transporter 1 (DMT1). DMT1 is a member of the natural resistance-associated macrophage protein (NRAMP) family and was formerly known as the divalent cation transporter (DCT)[41], in which a missense mutation in Belgrade rats or microcytic mice was found to impair proper iron (Fe) uptake[42,43]. At the cellular level, DMT1 seems to localize to the membrane. In tissue, it is ubiquitously expressed, but shows most prominent expression in the proximal intestine, kidney and brain[41]. Light and electron microscopy has also found DMT1 expression in glial cell bodies of the neocortex, subcortical white matter, and the hippocampus[44], while immunocytochemistry has found dense DMT1 staining in the caudate, putamen, and substantia nigra pars reticulata (SNpr) of the monkey basal ganglia[45]. The commonality in neural substrates and transporter expression between manganism and PD requires further examination of potential mechanisms surrounding DMT1's interactions.

DMT1 was given its name due to its ability to non-specifically transport various divalent metal ions into the cell. The transporter's specific connection to Mn has come from studies that have looked at the effects of Mn exposure on the expression of DMT1. For example, Mn-exposed rat pups are found to have increased levels of Mn *in vivo*, as well as increases in the protein expression of DMT1 throughout the brain[46]. Similarly, increased Mn uptake is evident upon impaired cellular iron status in astrocytic cultures, corresponding with enhanced DMT1 protein expression in these cultures[47]. These data provide evidence that DMT1 is likely a Mn transporter.

DMT1's possible role in a gene-environment interaction in PD emerges from its functional transport of an environmental toxin like Mn into cells. The transporter's connection to PD genetics has only recently been investigated. The motivation behind this investigation comes from the existence of an alternatively spliced DMT1B isoform that could be affected by post-translational modifications through the ubiquitin-proteasome pathway. Keeping in mind that parkin is a known E3 ligase in this pathway, overexpression of parkin in a neuroblastoma cell line lead to decreased DMT1B isoform levels and increased Mn toxicity. Additionally, immunoprecipitation and immunofluorescent studies revealed co-localization between this isoform and parkin in cells transfected with wild-type *parkin*[48]. This study serves as just an initial *in vitro* examination of parkin's modulation of DMT1

via ubiquitination (see Figure 1A). Furthermore, recent evidence has shown that in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, expression of DMT1 increases significantly in treated animals compared to untreated animals. Treated animals also exhibit increased Fe accumulation, increased oxidative stress, and overall DAergic neurodegeneration in the SN. Moreover, the mutation in the DMT1 gene previously shown to impair Fe transport actually confers protection against MPTP and 6-hydroxydopamine-induced toxicity[49]. Thus, higher expression of DMT1 in the basal ganglia could potentially explain this area's particular sensitivity to Mn toxicity, as well as its vulnerability to the specific neuronal damage done in PD. Further work is necessary to assess if and how this mechanism is affected *in vivo* upon exposure to high levels of Mn. In addition, investigating how high exposure affects Mn transport kinetics in *parkin*-linked PD models could help explain the variations seen among these cases.

Intracellular Mn Storage

Outside of its import process, another component of proper Mn homeostasis is non-toxic storage of Mn within the cell. Despite its necessity for the actions of mitochondrial antioxidant enzymes (Mn-SOD)50, a possible mechanism for the neuronal death seen in *parkin*¬-associated PD could involve improper storage issues that promote toxic Mn accumulation within mitochondria[51]. Chronic Mn treatment leads to Mn increase specifically in the mitochondria of neurons and astrocytes[52]. Mn-induced neurotoxicity notoriously shows signs of increased intramitochondrial oxidative stress, increased release of cytochrome c, and overall mitochondrial dysfunction, similar to PD cases. Striatal neurons show dose-dependent decreases in the mitochondrial membrane potential and Complex II activity upon Mn exposure[53]. This decline in proper mitochondrial function is similar to what is seen in PD, or in PD models induced by poisons like MPTP and rotenone that both inhibit mitochondrial complexes[54].

A direct connection between *parkin* and mitochondrial dysfunction that could be relevant to this toxicity has been recently illuminated. In addition to its role as an E3 ligase involved in the proteasomal degradation pathway, recent evidence has identified *parkin*'s involvement in the autophagy of damaged mitochondria. Initial findings show that *parkin* and PINK1 (a mitochondrial-targeted kinase) interact to maintain mitochondrial integrity within DAergic neurons, and that functions downstream of PINK1 in this pathway[55]. Subsequent studies found that the PINK1/*parkin* pathway in Drosophila actually promotes fission and inhibits fusion of mitochondria[56]. More recent evidence has found that *parkin* translocation to damaged mitochondria with lowered mitochondrial membrane potential is dependent on PINK1 expression, followed by the aggregation of these mitochondria into the perinuclear region for autophagic elimination[57,58]. These studies have expanded on *parkin*'s sole duty as an E3 ligase, now adding regulation of mitochondrial trafficking to its resume. Such remarkable results signify that PD pathophysiology could arise from ineffective clearance of defective mitochondria due to mutations in *parkin* or *pink1*, ultimately resulting in neurodegeneration. Furthermore, this neurodegeneration could also be linked to increased DMT1-mediated Mn import in the basal ganglia due to higher transporter expression, with increased levels of Mn entering DAergic neurons and increasing ROS production within accumulated, damaged mitochondria (see Figure 1B).

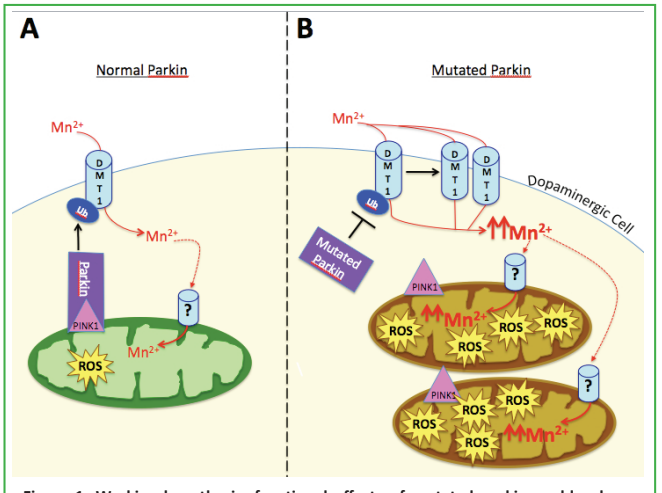

Figure 1. Working hypothesis: functional effects of mutated parkin could enhance Mn(II) toxicity to induce DAergic cell death in the basal ganglia. (A) Normal function of parkin could involve ubiquitination of DMT1 to regulate surface expression levels, controlling how much Mn(II) can enter the cell and, subsequently, the mitochondria to produce ROS. Additionally, interaction with mitochondrial PINK1 can promote mitophagy to reduce the number of damaged mitochondria. (B) A mutation in parkin could result in a protein that can no longer properly ubiquitinate DMT1, allowing for increased Mn(II) entry into the cell, and increased intramitochondrial Mn levels to produce a higher amount of ROS. Moreover, the mutated protein can no longer participate in mitophagy to remove Mn-intoxicated, damaged mitochondria.

Conclusion

Increased human life expectancy will soon designate the elderly as a growing proportion of the population, and a concordant rise in the number of PD cases will become an even more pressing public health concern. However, the signature selectivity of cell loss in the SN, marked by mitochondrial dysfunction and increased oxidative stress at the cellular level, remains an enigma. Nevertheless, the commonality in pathology, the neural substrates affected, and symptomatology shared by both PD and manganism suggests an interaction between genetics and environmental factors to precipitate these disorders. Based on the evidence presented in this review, future studies could focus on gene-environment interactions between Mn, parkin and DMT1. Increased oxidative stress and cell death in parkin mutants exposed to Mn could result from a higher degree of net intracellular Mn levels within aggregated, damaged mitochondria. Subsequently, increased intracellular Mn levels can react with dopamine via Fenton's reaction to create additional oxidative stress[59-61], further enhancing DAergic neurodegeneration, as seen in PD. It is also possible that the mitochondria themselves in DAergic neurons could be selectively vulnerable to environmental toxins like Mn. Thus, a multi-faceted, complex story emerges that could potentially facilitate the study of mechanisms to help point us towards novel therapeutic strategies.

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