

THE ROLE OF SMF 1, SMF-2, SMF-3 IN METAL-INDUCED WHOLE ANIMAL  
VULNERABILITY AND DOPAMINE NEURON DEGENERATION IN  
*CAENORHABDITIS ELEGANS*

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## LIST OF ABBREVIATIONS

6-OHDA	6-hydroxydopamine
AAAD	aromatic L-amino acid decarboxylase
AD	Alzheimer's Disease
ADE	anterior deirid
Al <sup>3+</sup>	aluminum
ALS	Amyotrophic lateral sclerosis
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CEP	cephalic neuron
COMT	catechol-O-methyl transferase
Cu <sup>2+</sup>	copper
DA	dopamine
DAT	dopamine transporter
DMT1	divalent metal transporter- also known as Nramp2, DCT1, or SLC11A2
DMSO	dimethyl sulfoxide
DOPAC	3,4-dihydroxyphenylacetic acid
Fe <sup>2+</sup>	iron
GFP	green fluorescent protein
GSH	glutathione
GSH-Px	glutathione peroxidase
GST	glutathione s-transferase
GTPCH	GTP cyclohydrolase
H2-DCF-DA	2,7-dichlorodihydrofluorescein diacetate
H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
HVA	homovanillic acid
L1	larval 1 stage
L-DOPA	L-3,4-dihydroxyphenylalanine
Mn <sup>2+</sup>	manganese



MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NGM	Nematode Growth Medium
$\cdot\text{O}_2^-$	super oxide radical
$\cdot\text{OH}$	hydroxyl radical
PCR	polymerase chain reaction
PD	Parkinson's Disease
PDE	posterior deirid
qPCR	quantitative PCR
RNAi	RNA-mediated interference
ROS	reactive oxygen species
SN	substantia nigra
TMRE	tetramethyl rhodamine ethyl ester
TH	tyrosine hydroxylase
VMAT	vesicular monoamine transporter
WT	wild type

## Chapter One: Introduction and Background

The etiology of many neurodegenerative diseases is unknown, but a number of studies indicate that a combination of both genetic and environmental factors contribute to the progression of disease. Metals are important in numerous biological processes in the brain. Metal homeostasis is regulated through multiple mechanisms of transport, storage, and secretion, and breakdown of these processes have been implicated in the development of a number of neurodegenerative diseases including the loss of dopamine (DA) neurons in Parkinson's disease (PD) [1,2]. The focus of these studies are to determine whether the genetically tractable nematode *Caenorhabditis elegans* (*C. elegans*) is sensitive to PD-associated metals  $Mn^{2+}$ ,  $Fe^{2+}$ ,  $Cu^{2+}$ , and  $Al^{3+}$ , whether these metals can induce dopamine neuron degeneration, and whether the *C. elegans* homologues to the human divalent metal transporter (DMT1) may contribute to metal induced neuropathology.

### Metals and Neurodegenerative Diseases

Neurodegenerative diseases such as PD, manganism, Alzheimer's disease (AD), and Wilson's disease display an age-related loss of specific neurons, increased oxidative stress, and metal accumulation in surviving cells. Exposures to high concentrations of metals such as  $Mn^{2+}$ ,  $Cu^{2+}$ , and  $Al^{3+}$  can confer overlapping pathologies and exposures have been correlated with an increased propensity to develop these disorders.

For example,  $Mn^{2+}$  and  $Cu^{2+}$  have been implicated in the development of PD, while  $Al^{3+}$  has been implicated in PD as well as AD, ALS, and Friedreich's ataxia [7 - 9]. It has been suggested that the long half-life of  $Al^{3+}$  in numerous brain tissues, along with the long life of neurons contribute to its accumulation and elevated levels in neurodegenerative diseases [10]. A study of the hippocampus from patients with ALS and Parkinsonism-dementia of Guam supports  $Al^{3+}$  as a potential environmental factor contributing to increased accumulation of  $Al^{3+}$  in the neurofibrillary tangles [10, 11].

## Parkinson's Disease

PD is the second most prevalent neurodegenerative disease after AD. Early descriptions of PD were detailed by James Parkinson in 1817, which now is estimated to affect over 2% of the population over the age of 65 [22, 23]. The most common form of PD is called idiopathic or sporadic PD (~95% of the PD population), with the remaining portion of patients inheriting the disease directly due to genetic contributions [22, 24 - 25]. Identification of genes involved in PD has provided insights into the molecular mechanisms involved in sporadic PD [25]. PD is generally considered a disease that slowly progresses over time, and there is no cure for the disease. PD is characterized by the selective loss of dopamine (DA) neurons in the *substantia nigra* (SN) and often the presence of Lewy bodies in surviving cells. Lewy bodies are cytoplasmic inclusions of protein aggregates [26]. Clinical symptoms include resting tremor, bradykinesia, stiffness of limbs, and gait or balance problems [24]. Animal studies suggest that idiopathic PD results from a complex combination of genetic and environmental interactions in combination with the factors involved in aging [22, 23]. Central to PD research is the involvement of oxidative stress, and mitochondrial and proteasomal dysfunction [24, 25]. Postmortem studies of PD brains show increased oxidative stress, lipid peroxidation, as well as decreased mitochondrial complex I activity and glutathione levels in the SN [27, 28].

Epidemiological studies suggest that metal exposure contributes to the development of PD. A study by Zayed J. *et al.* 1990 suggests that occupational exposure to  $Mn^{2+}$ ,  $Fe^{2+}$ , and  $Al^{3+}$  for a time period greater than 30 years increases the risk for PD. Postmortem analysis of brain tissues from Parkinsonism patients has shown an increase in total  $Fe^{2+}$ ,  $Zn^{2+}$ , and  $Al^{3+}$  in the SN compared to control tissues [29 - 31]. In addition, a recent study suggests that  $Al^{3+}$  is one of the metals that significantly increase in early PD patient serum and may be a good indicator of disease progression [32]. Epidemiological studies have also suggested the potential involvement of occupational exposure to copper in the etiology of PD. A study of Michigan counties by Rybicki B. *et al.* 1993 suggests a statistically significant increase in PD death rates in areas with industries

using heavy metals. These industries included paper and chemical production in addition to iron and copper mining. Gorell J. *et al.* 1999 showed that chronic occupational exposure (>20 years) to  $\text{Cu}^{2+}$  or to dual combinations of lead,  $\text{Fe}^{2+}$ , and  $\text{Cu}^{2+}$  was associated with PD.

Environmental agents were first recognized as potential risk factors in the development of PD with the identification of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), in a preparation of an illegally manufactured heroin-like drug. MPTP-exposed individuals exhibited signs and symptoms of PD [39]. High exposures to the transition metal  $\text{Mn}^{2+}$  can cause a parkinsonism-like syndrome called manganism. Manganism patients present with a similar loss of DA neurons in the SN, increased indicators of oxidative stress, motor disturbances, and often psychological disturbances [40, 41 - 45].  $\text{Mn}^{2+}$  may also reduce DA release, and inhibit complex-I of the mitochondrial electron transport chain and increase the generation of reactive oxygen species (ROS) [46 - 50].

PD does not naturally occur in species other than humans, therefore animal models are utilized to recapitulate various aspects of the human disorder [22, 34]. A model of PD is to expose DA neurons is 6-hydroxydopamine (6-OHDA), a hydroxylated analogue of DA. This model of PD was first proposed over 40 years ago, and is a well-studied mammalian toxicant model due to its ability to induce specific DA neuron degeneration both *in vitro* and *in vivo* [35, 36]. The specificity of 6-OHDA for causing degeneration in DA neurons is attributed to its ability to be transported into the cells by the DA transporter (DAT). Studies have shown that DAT antagonists like imipramine or nisoxetine or mutations that render DAT non-functional are able to inhibit 6-OHDA effects [37, 38].

### Alzheimer's Disease

AD is the most common neurodegenerative disorder, characterized by the development of extracellular plaques consisting of aggregated insoluble amyloid- $\beta$  polymer, intracellular neurofibrillary tangles, and a selective loss of neurons in the

hippocampal and cerebral cortical regions [7, 51]. The role of  $\text{Al}^{3+}$  in AD is still controversial and there are many studies supporting as well as refuting the involvement of the metal. In the 1960's the "aluminum hypothesis" was first proposed based on studies that found  $\text{Al}^{3+}$  injections induce neurofibrillary tangle-like lesions, increased  $\text{Al}^{3+}$  levels in AD patients, and increased prevalence of AD in regions with high  $\text{Al}^{3+}$  in the drinking water [52 - 54]. It has been suggested that many of these initial studies were inaccurate due to morphological differences in neurofibrillary changes, potentially contaminated brain samples, or inconclusive data sets [55, 56]. Another hypothesis suggests metals such as  $\text{Al}^{3+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Fe}^{2+}$  can alter oligomerization and induce conformational changes that result in amyloid- $\beta$  polymer-induced neurotoxicity [7, 51]. Epidemiological data suggest  $\text{Al}^{3+}$  exposure increases memory deficits in AD patients [7].  $\text{Fe}^{2+}$  and  $\text{Cu}^{2+}$  are consistently found at high levels in the brain regions most prone to AD-associated neurodegeneration [14, 15]. Some studies suggest that the source of oxidative stress in the AD brain may come from the generation of ROS via molecular oxygen and  $\text{Cu}^{2+}$  [58]. Amyloid precursor protein has also been shown to reduce  $\text{Cu}^{2+}$  to  $\text{Cu}^+$  generating hydrogen peroxide that can form hydroxyl radicals via the Fenton reaction [16]. In *C. elegans*, aggregation of amyloid- $\beta$  protein is accelerated by exposure to  $\text{Cu}^{2+}$ , and the aggregation is inhibited by the  $\text{Cu}^{2+}$  chelators, histidine or clioquinol [59]. Hyperphosphorylated tau, a significant component of neurofibrillary tangles, has been shown to bind  $\text{Cu}^{2+}$  causing protein aggregation that may increase neuronal oxidative stress [60].

#### Menkes and Wilson's Diseases

Menkes disease and Wilson's disease are neurodegenerative diseases in which mutations in  $\text{Cu}^{2+}$  transport can cause altered brain development, defective synthesis of collagen, increased ROS and neurological problems [20, 40, 61, 62]. In Wilson's disease a mutation in a  $\text{Cu}^{2+}$  transporter results in abnormal  $\text{Cu}^{2+}$  accumulation and parkinsonian like symptoms [63, 64]. Unbound  $\text{Cu}^{2+}$  can catalyze the Fenton and/or Haber-Weiss reactions, which can result in the generation of the highly reactive and damaging

superoxide and hydroxyl radicals [16, 20].  $\text{Cu}^{2+}$  can also be toxic due to its binding to protein sulfhydryl groups, resulting in enzyme inactivation or altered protein folding which contributes to inclusion body formation. Exposure to toxic levels of  $\text{Zn}^{2+}$  has been shown to alter copper homeostasis by upregulating metallothioneins which can increase  $\text{Cu}^{2+}$  elimination. Imaging studies have shown some Wilson's disease patients to have severe loss of striatal DAT and significantly reduced integrity of both pre- and post-synaptic DA neuronal regions [66, 67]. In addition, Long-Evans Cinnamon rats, which are used as a rodent model of Wilson's disease, have shown increased indicators of oxidative stress in the brain regions that typically accumulate  $\text{Cu}^{2+}$  due to the disease [67, 69].

## Aluminum

Aluminum is the most abundant metal in the earth's crust and the third most abundant element in the environment [70, 71]. Humans are primarily exposed to  $\text{Al}^{3+}$  through food or environmental contamination.  $\text{Al}^{3+}$  also comes in direct contact with humans as it is used in pharmacological products like antacids and antiperspirants [74].  $\text{Al}^{3+}$  is a non-essential metal ion, and exposure to high concentrations of  $\text{Al}^{3+}$  has been shown to contribute to toxicity across many organisms [31, 70, 75]. The proximal intestine appears to be the primary site of  $\text{Al}^{3+}$  absorption by calcium channels and sodium transporters, while transferrin and urine are potentially the primary routes for excretion [71].  $\text{Al}^{3+}$  has a high propensity to form hydroxyl complexes in an acidic environment [154].  $\text{Al}^{3+}$  can cross the blood brain barrier, but the mechanism of transport has been controversial. Studies have suggested  $\text{Al}^{3+}$  may be transported by transferrin receptor-mediated endocytosis or through a glutamate transporter [7, 71].  $\text{Al}^{3+}$  has also been shown to alter mitochondrial function by interfering with ATP production and reducing antioxidant defenses [175, 190]. In addition,  $\text{Al}^{3+}$  has been suggested to alter DA neurotransmission by altering membrane integrity and dopamine receptor density, as well as decreasing DA levels [72, 75, 76].

## Copper

In biological systems  $\text{Cu}^{2+}$  is an essential cofactor for many enzymes and is utilized in electron transport [1, 77]. The primary source of  $\text{Cu}^{2+}$  exposure is through food or environmental contamination [77, 78]. After being absorbed through the GI tract,  $\text{Cu}^{2+}$  is transported by albumin.  $\text{Cu}^{2+}$  in the liver is bound by metallothionein, and secreted into plasma or excreted in the bile [79, 80]. Some of the critical enzymes requiring  $\text{Cu}^{2+}$  are involved in mitochondrial respiration, peptide hormone production, pigmentation, neurotransmitter metabolism, and  $\text{Fe}^{2+}$  transport. These enzymes include cytochrome c oxidase, Cu/Zn superoxide dismutase, tyrosinase, dopamine  $\beta$ -hydroxylase, and metallothionein [20, 79].  $\text{Cu}^{2+}$  is also one of the few metals within the cell that causes increased oxidative stress with either excess accumulation or deficiency [81]. Some studies have suggested that DMT1 and the high affinity  $\text{Cu}^{2+}$  transporter, CTR1, may play a significant role in  $\text{Cu}^{2+}$  transport across the plasma membrane [91].

## Oxidative Stress

The nervous system is particularly sensitive to oxidative damage due to the high rate of oxygen consumption, relatively high  $\text{Fe}^{2+}$  levels, and concentration of oxidizable polyunsaturated fatty acids in neuronal membranes [29]. Understanding how oxidative imbalance contributes to DA neuron sensitivity in diseases such as PD could provide insight into the etiology of the disease [83, 84]. Studies have shown factors which contribute to DA neuron specific increased oxidative stress include large stores of  $\text{Fe}^{2+}$ ,  $\alpha$ -synuclein accumulation, and easily oxidized DA [85 - 87]. PD patients present with increased oxidative stress and lipid peroxidation, decreased levels of glutathione (GSH) and mitochondrial complex I activity, and the presence of dopamine quinones [27, 30, 88].

Glutathione synthesis is one of the cell's most important antioxidant responses to scavenge free radicals [46, 92]. Post mortem studies of PD brains have shown a decrease in GSH levels in the SN, and a correlation between PD severity and GSH deficit [29, 93]. Loss of GSH has also been associated with impaired mitochondrial electron

transport chain function [94]. As a major antioxidant in the brain GSH has multiple functions including scavenging of superoxide and hydroxyl radicals [95]. GSH serves as an electron donor for glutathione peroxidase, and as a cofactor for glutathione s-transferases (GSTs). Toxic exposure of  $\text{Al}^{3+}$  is suggested to inhibit mitochondrial NADP-isocitrate dehydrogenase, decrease GSH, and decrease GSH peroxidase and catalase activities effecting cellular oxidative stress levels [96 - 98]. Exposure to high  $\text{Cu}^{2+}$  levels significantly decreases total glutathione levels [1, 101]. In addition, *in vitro* studies suggest that exposure to  $\text{Cu}^{2+}$  inhibit GST and alter glutathione peroxidase activity [102].

DA can be degraded by monoamine oxidase to generate hydrogen peroxide, or can contribute to the formation of superoxide and quinones that can cause denaturation of proteins, lipid peroxidation, and DNA damage as depicted in Figure 1 [83]. A number of studies indicate that DA in the presence of the transition metals  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Mn}^{2+}$  can catalyze production of free radicals [20, 40, 92]. While  $\text{Al}^{3+}$  is not a redox-active metal, it has been shown to facilitate pro-oxidant activities like  $\text{Fe}^{2+}$ -induced lipid peroxidation, oxidation of NADH, and formation of hydroxyl radical [99]. Neuromelanin is a redox-active brain pigment associated with neurodegeneration in the DA neurons in the SN of PD patients [73, 103]. *In vitro* studies have also shown that  $\text{Al}^{3+}$  can facilitate  $\text{Fe}^{2+}/\text{Cu}^{2+}$  oxidation of DA to neuromelanin. Once formed, neuromelanin is capable of binding metals such as  $\text{Al}^{3+}$  resulting in increased lipid oxidation due to the formation of an  $\text{Al}\cdot\text{O}_2^-$  complex with increased oxidant capacity [73, 99]. Studies have also shown  $\text{Al}^{3+}$  co-exposure with the PD toxicants 6-OHDA or MPTP increases free radical generation, DA neuron degeneration, lipid peroxidation, and DA turnover [96, 104-107].

#### Divalent metal transporter

The divalent metal transporter (DMT1), or natural resistance-associated macrophage protein 2 (Nramp2), is a proton-coupled membrane transporter with 12 transmembrane domains that is expressed in most tissues [108]. The transporter has been shown to transport divalent cations such as  $\text{Fe}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ , and lead [14,



109, 110]. DMT1 family members share a consensus transport sequence located between transmembrane domain 8 and 9 that is involved in metal translocation across the membrane [46]. Homologues of human DMT1 have been identified across multiple species including plants, yeast, *C. elegans*, and many vertebrates [46, 111]. In mammalian systems, DMT1 has been identified to function primarily in Fe<sup>2+</sup> uptake and distribution [84]. In yeast and *C. elegans*, three homologues to DMT1 have been identified and are called SMF-1, SMF-2, and SMF-3 [46, 111]. In yeast the cellular localization of the three SMF proteins may depend on the extracellular environment [111]. For example, Portnoy M.E. *et al.* 2000 found when Mn<sup>2+</sup> was added to growth media, both Smf1p and Smf2p were targeted to the vacuole for degradation. In contrast, Mn<sup>2+</sup> starvation caused Smf1p expression within the plasma membrane, while Smf2p localized to intracellular vesicles. Salazar J. *et al.* 2008 showed protein extracts from the SN of PD patients had an increase in the Fe-responsive isoform of DMT1 and a decrease in non-Fe responsive DMT1 isoform, compared to SN tissues from patients without PD. In addition, a mutation in DMT1 which impairs Fe<sup>2+</sup> transport was able to protect against both MPTP and 6-OHDA-induced DA neuron degeneration in mice [84]. PARK2, an E3-ligase contributing to protein proteasomal degradation, has been identified as one of the genetic factors resulting in early-onset PD. Mutation in the PARK2 gene results in the selective loss of DA neurons in the SN [5]. There is additional evidence which suggests mutation may also alter DA homeostasis and contribute to late onset of PD [6, 12]. A recent *in vitro* study has shown that PARK2 can also selectively regulate DMT1 expression [113]. This study suggested that overexpression of PARK2 not only reduces transport of Mn<sup>2+</sup>, but also decreases expression of DMT1. This suggests an additional regulatory mechanism for DMT1 and further implicates DMT1's potential role in DA neuron degeneration.

### *C. elegans* as a model system

The nematode *C. elegans* is a powerful model system to explore the cellular and molecular basis of PD-associated DA neuronal death [46, 116, 117]. *C. elegans* have

been used as an important model system for biological research in the fields of genomics, cell biology, neuroscience and aging [118]. Its small size, rapid development, large brood size, and a food source primarily of bacteria lend to the ease and low cost of laboratory culture. The adult hermaphrodite is approximately 1mm in length and can progress from embryo to adult within 3 days [118]. Other advantages include its transparent body, along with a well-defined cell pattern and nervous system [119]. There are two sexes of *C. elegans*, a self-fertilizing hermaphrodite and a male. Each adult hermaphrodite typically produces 300-350 progeny, but this is only limited by the number of sperm produced. Male fertilization can increase the number of oocytes fertilized to closer to 1000 [120]. Males naturally arise infrequently (0.1%), but this frequency can be increased by environmental stresses or through genetic mutations [121].

The self-fertilization of the hermaphrodite allows homozygous animals to generate genetically identical progeny, and male mating allows for the isolation and maintenance of mutant strains along with the addition of new mutations [119]. Another advantage of *C. elegans* as a model system is the relatively short period of time to generate mutant strains. Confirmation of new mutants and genetic crosses can be quickly performed through whole animal polymerase chain reaction (PCR), termed single worm PCR [122]. An alternative to generating genetic knockout lines is incorporating genetic knockdown through RNA-mediated interference (RNAi) [115]. The first evidence that double stranded RNA could cause gene silencing came from *C. elegans* studies by Andy Fire's and Craig Mello's laboratory [124]. Although neurons are largely insensitive to RNAi, a mutation in a RNA polymerase gene, *rff-3*, increases the efficacy of RNAi-mediated genetic knockdown in most neurons including the DA neurons [127, 128].

In humans, DA, in addition to being essential for coordinating body movements, is involved in addiction, motivation, reward, and reinforcement [129]. Loss of DA neurons and depletion of DA in the basal ganglia results in the movement abnormalities associated with PD [130]. DA synthesis is dependent on the essential amino acid

tyrosine. Tyrosine is metabolized by tyrosine hydroxylase (TH) to L-3,4-dihydroxyphenylalanine (L-DOPA), which is then decarboxylated to DA by DOPA decarboxylase [130, 132]. Following neurotransmission, DA inactivation involves reuptake of DA by the presynaptic DAT where it is then metabolized or repackaged for storage. DA is metabolized by catechol-O-methyl transferase and monoamine oxidase [130]. Dopamine synthesis and metabolism are very tightly regulated as DA alone is very reactive, and degradation results in the generation of free radicals [92]. *C. elegans* have similar molecular components as mammalian systems that are involved in DA synthesis, packaging, signaling, transport, and degradation [(Figure 1), 38, 115].

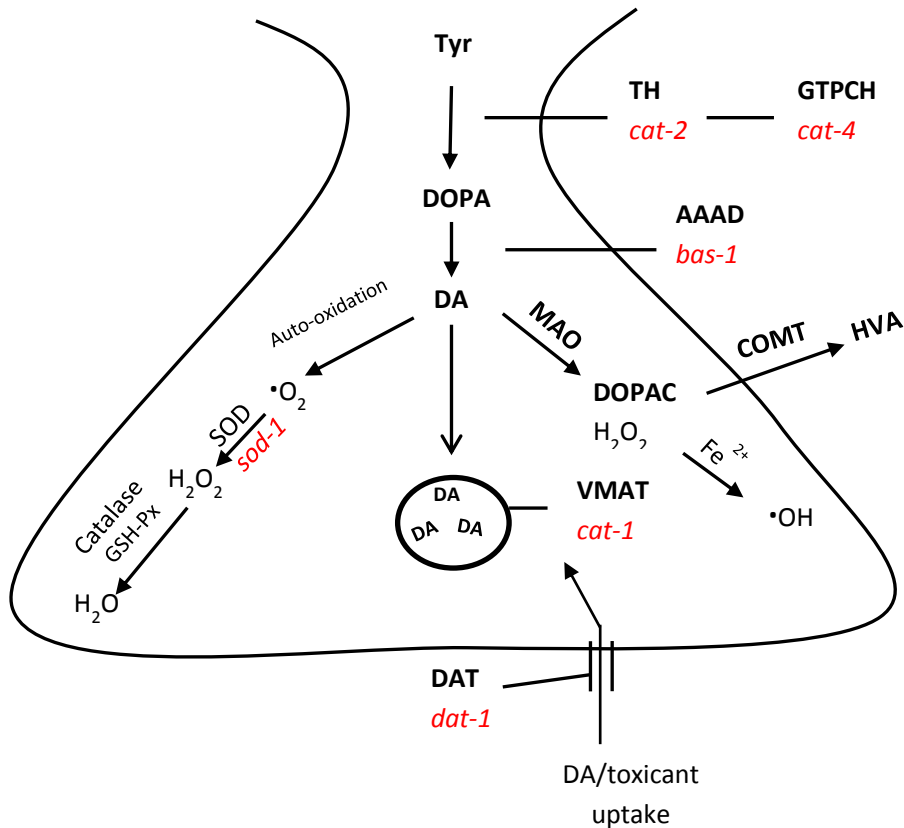


Figure 1: Dopamine synthesis, metabolism, and DA free radical production in mammals and *C. elegans*. *C. elegans* genes in red. DA, dopamine; TH, tyrosine hydroxylase; GTPCH, GTP cyclohydrolase; AAAD, aromatic L-amino acid decarboxylase; MAO, monoamine oxidase; DOPAC, 3,4-dihydroxyphenylacetic acid; COMT, catechol-O-methyl transferase; HVA, homovanillic acid; VMAT, vesicular monoamine transporter; DAT, dopamine transporter; GSH-Px, glutathione peroxidase; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; •O<sub>2</sub><sup>-</sup>, super oxide radical; •OH, hydroxyl radical [38, 92].

While DA is not required for *C. elegans* viability, DA signaling is involved in the mechanosensation of food, egg-laying, defecation, movement, and mating [133 - 136].

*C. elegans* have 302 neurons in the adult hermaphrodite and 383 neurons in the adult male [118, 121]. Hermaphrodites have eight DA neurons and the males have three additional pairs of DA neurons in the tail. These neurons can be easily seen *in vivo* under a fluorescent dissecting scope when the green fluorescent protein is expressed behind the DAT promoter (Figure 2) [38]. The nematode head contains four cephalic cells (CEPs) and two anterior deirids (ADEs) (Figure 2). The CEP dendrites extend from the cell body, which is near the nerve ring, to the tip of the nose [115]. The tail also contains two posterior deirids (PDEs) near the vulva (refer to [115] for image).

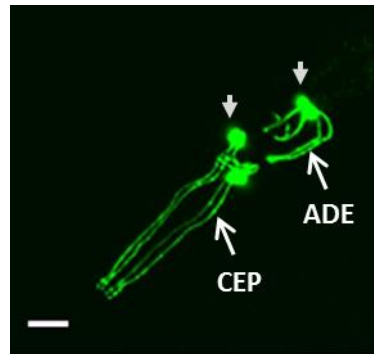


Figure 2: Dopamine neurons in the head region of an adult hermaphrodite *C. elegans*. Confocal images of BY250 strain which expresses GFP behind the *dat-1* promoter showing the 6 DA neurons in the head region, four CEPs and two ADEs. The thick arrow points to cell bodies, while the thin arrow points at the appropriate dendritic processes CEP and ADE from left to right. Photos taken 72 hours after L1 stage and were captured using confocal microscopy (Zeiss LSM 510 microscope). Scale bar indicates 20  $\mu\text{m}$ .

## Chapter Two: Experimental Data

The molecular mechanisms involved in PD and manganese is largely unknown, but numerous studies indicate increased DA neuron cell death is a common factor [22, 24]. As previously introduced excess exposure to environmental metals such as  $Mn^{2+}$ ,  $Fe^{2+}$ ,  $Al^{3+}$ , or  $Cu^{2+}$  have been shown to induce cell-death and increase oxidative stress which has been implicated in PD. In addition, vertebrate evidence suggests DMT1 transports ions such as  $Mn^{2+}$ ,  $Fe^{2+}$ , or  $Cu^{2+}$  into DA neurons [84, 112]. Therefore I propose that exposure to  $Mn^{2+}$ ,  $Fe^{2+}$ ,  $Al^{3+}$ , or  $Cu^{2+}$  can induce DA neurodegeneration and DMT1 contributes to increased DA neuron sensitivity. To explore this hypothesis, I use the powerful animal model *C. elegans* to evaluate animal vulnerability, changes in oxidative stress, DA neuron degeneration, and contribution of DMT1 homologues SMF-1, SMF-2, and SMF-3 after metal toxicant exposure.

### Materials and Methods

***C. elegans* strains and maintenance** - Nematode Growth Medium (NGM) or 8P plates containing bacterial lawns of either OP-50 or NA-22, respectively, were used to grow *C. elegans* strains at 20°C consistent with standard methods [137, 140]. OP-50 is a uracil auxotroph *E. coli* whose growth is limited on NGM plates to enable easier observation [141], while NA-22 *E. coli* grow in very thick layers and are an optimal food source for large quantities of worms when fed on 8P plates [13]. The *C. elegans* strains used in these experiments are listed in Table 1. Homozygotes from genetic crosses were verified using single worm PCR [122]. Frozen stocks (-80°C freezer) were generated by mixing a largely L1 population of worms as previously described [141].

Table 1: *C. elegans* experimental strains used

Strain name	Genotype	Strain name	Genotype
N2	Wild type Bristol	RJ907	$P_{dat-1}::GFP;smf-1(eh5)X$
IG6	$smf-1(eh5) X$	RJ905	$P_{dat-1}::GFP;smf-2(gk133)X$
VC171	$smf-2(gk133) X$	RJ906	$P_{dat-1}::GFP;smf-3(ok1035)IV$
RB1074	$smf-3(ok1035) IV$	RJ928	$P_{dat-1}::GFP;rrf-3(pk1426) II$
BY250	$P_{dat-1}::GFP$	BY200	$P_{dat-1}::GFP; rol-6$
		BY215	$P_{dat-1}::GFP;rol-6;dat-1(ok157)III$

**Whole-animal vulnerability assay** - Gravid adults were collected and rinsed 3 times with dH<sub>2</sub>O, and eggs were harvested after hypochlorite treatment following standard protocols [38, 117]. Eggs were then incubated in M9 buffer, a standard *C. elegans* laboratory solution containing essential salts necessary for eggs to continue development to the L1 stage, on a rocker for 18 hours at room temperature. After which the synchronized population of L1 animals were rinsed again 3 times with dH<sub>2</sub>O and counted to determine the approximate number of worms per  $\mu$ l using a Zeiss dissecting microscope. L1 animals (30 - 50) were placed on NGM plates with OP50 bacteria +/- various concentrations of metal solutions. These solutions included MnCl<sub>2</sub> [10 - 30 mM] (Fisher Scientific, Fair Lawn, NJ), CuCl<sub>2</sub> [50 - 500  $\mu$ M] (Alfa Aesar, MA), AlCl<sub>3</sub> [.1 - 1 mM] (Fisher Scientific, UK), and FeCl<sub>2</sub> [100 - 500  $\mu$ M] (Sigma Aldrich, St. Louis, MO), and were added to autoclaved NGM agar prior to pouring the 60 mm plates. L1 animals were grown for 72h at 20°C and assayed for viability. Animals were considered to be alive when moving or when responding to a gentle touch with a metal pick on the nose [142]. All experiments were performed at least in triplicate, the results were reported as mean +/- S.E.M., and statistical analysis was performed using GraphPad Prism5.

**RNA extraction and cDNA synthesis** - After treatment with AlCl<sub>3</sub> for 30 min and recovery on NGM plates for 48 hrs., the worms were washed from the plates and rinsed with dH<sub>2</sub>O at least 3 times or until the dH<sub>2</sub>O appeared clear. Worm pellets were then

resuspended in Trizol (1 ml worms/100 µl Trizol). Total RNA was extracted as previously described, but with minor modifications [143]. Chloroform was used to denature and separate proteins and lipids from the sample, and isopropyl alcohol was added to remove the DNA and allow RNA precipitation. The RNA pellet was washed with 75% ethanol, allowed to dry, and then resuspended in RNase-free water. A small volume from each sample was then diluted to measure the RNA concentration, while the remaining portion of the sample was stored at -80°C. RNA concentrations were determined using a ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, DE). cDNA synthesis was followed according to manufacturer's instructions (Bio Rad, CA). The cDNA was purified using dH<sub>2</sub>O and Microcon YM30 filters (Millipore corp., Bedford, MA), and the concentration determined using the ND-1000 spectrophotometer. cDNA sample were stored at -20°C.

**Real-time PCR measurements** - Primers were designed with Primer3 software. All qRT-PCR measurements were determined relative to glyceraldehyde-3-dehydrogenase (GAPDH) as its expression does not change as a result of exposures (unpublished lab results). The primers in Table 2 were used to determine changes in gene expression of the following genes:

Table 2: Primers used to determine changes in gene expression

Gene	Forward primer 5' - 3'	Reverse primer 5' - 3'
<i>Gapdh</i>	GAAACTGCTTCAACGCATCA	CCTTGCGACAAGAAGGTAG
<i>smf-1</i>	GTGGGTTTTGCTCTCAGCTC	TGGCAATTGCTGTTCCAATA
<i>smf-2</i>	GCACTGGTTGGCTGATTTTT	GGAGCATCCAGTTCCAGTGT
<i>smf-3</i>	GGAGTGCGAAAGTTTGAAGC	TTGACAAGTGCCGAGTGAAG

The real-time reaction was performed using a 2X SYBR Green PCR master mix which included SYBR Green, cDNA at 25 ng/µl, and dH<sub>2</sub>O. The PCR master mix with the cDNA of interest and primers were added to the appropriate wells within a 96-well plate. Negative controls were added to wells that contained the entire PCR master mix minus the cDNA. The ABI Prism 7500 sequence detection system (Applied Biosystems,

Warrington, UK) was used to detect the quantity of PCR product. A  $C_T$  value indicates the cycle number that the fluorescence passes the threshold determined for SYBR Green fluorescence. Studies were performed in triplicate and the  $\Delta C_T$  was calculated for each exposure condition based on the difference in  $C_T$  value for control and GAPDH. The  $\Delta\Delta C_T$  was calculated by subtracting the control  $\Delta C_T$  from the  $AlCl_3$   $\Delta C_T$ . Fold change was then calculated based on 100% efficiency ( $2^{(\Delta\Delta C_T)}$ ).

**Toxicant exposures** - My initial studies determined DA neurons were more sensitive to 30 min exposure to  $MnCl_2$  when exposed in the first larval stage (L1) as compared to animals exposed at the fourth larval (L4) stage or adult stage. The life cycle of *C. elegans* begins with the embryonic stage, progresses through four larval stages, and then reaches adulthood [118]. To eliminate additional potential variability in results due to the developmental stage of animal exposed, I began each of my experiments with a population of animals all at the L1 stage by using a common *C. elegans* laboratory procedure called synchronization [117]. To determine the appropriate recovery time to evaluate DA neuron degeneration after  $MnCl_2$  exposure, I evaluated the number of animals that displayed DA neuron cell death at multiple time points. I found that after 30 minute exposure to 50 mM  $MnCl_2$ , the number of animals that displayed DA neuron degeneration was greater 72 hours post exposure compared to 48 hours (Figure 3). I therefore decided to use the 72 hour time point to examine DA neurons in all future experiments.



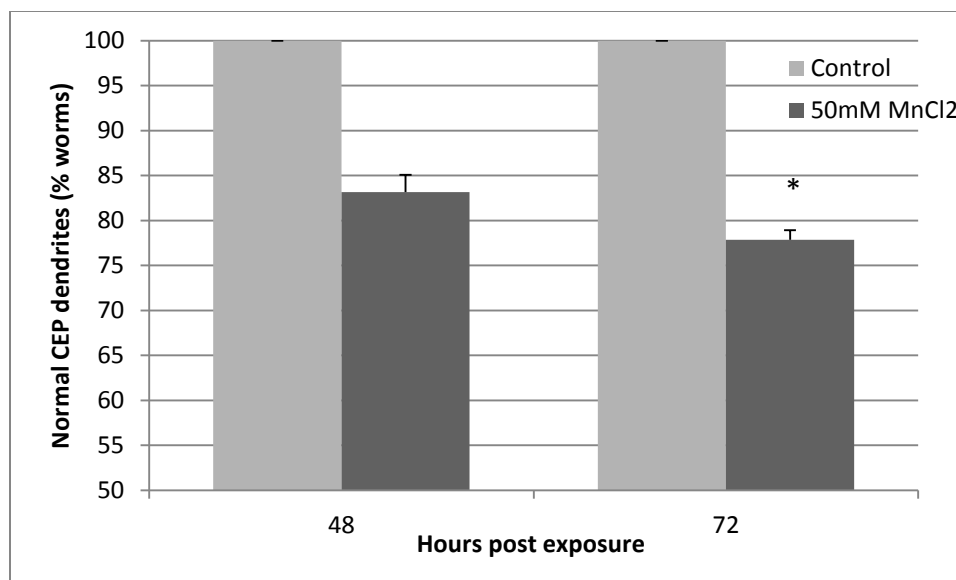


Figure 3: *C. elegans* DA neuron degeneration increases 72 hours post-MnCl<sub>2</sub> exposure. A synchronized population of L1 BY250 animals was exposed for 30 min to dH<sub>2</sub>O ± 50 mM MnCl<sub>2</sub>, and then recovered on NGM plates for 48 or 72 hours prior to evaluation of DA neuron degeneration. \*p ≥ .05 as determined by two-tailed t-test comparing MnCl<sub>2</sub> exposure groups at each time point.

Samples of gravid adults were collected from plates by washing with dH<sub>2</sub>O, spinning at 2500 rpm for 2 min, and removing the supernatant and repeating the washes until all bacteria was removed from the sample using standard protocols [38, 116]. Then animals were synchronized using hypochlorite treatment and incubated on a rotating plate at room temperature for 18 hours in M9. L1 stage worms were then washed at least 3 times with dH<sub>2</sub>O and counted using a dissecting microscope.

Acute exposures of L1 animals were set up with 10 worms/μl and incubated with varying concentrations of MnCl<sub>2</sub>, CuCl<sub>2</sub>, AlCl<sub>3</sub>, or FeCl<sub>2</sub>, added to dH<sub>2</sub>O in 1.5 ml centrifuge tubes [116]. Samples were incubated for 30 min at room temperature with gentle mixing every 10 min throughout exposure. After exposure, each sample was spun down at 2500 rpm for 2 min, supernatant removed, and the rinse repeated 3x with dH<sub>2</sub>O. Animals were then placed onto NGM plates seeded with OP50 bacteria and allowed to recover for 72 hours at 20°C. Between 50 - 60 worms were then picked onto microscope slides containing 2% agarose pads and immobilized using 2% sodium azide. Animals were scored for DA neuron degeneration under the fluorescent microscope

(Leica MZ 16FA, Switzerland). DA neuron degeneration was considered positive if there was not a continuous GFP signal along the CEP dendrite [38]. Figure 4 A & C shows an example of animals without and with degeneration respectively. For exposures with more than one toxicant, the first 30 min exposure remained the same as described above, animals were then washed at least 3x in dH<sub>2</sub>O using standard protocols and resuspended in the second solution +/- toxicant at 10 worms/ $\mu$ l and exposed for 30 minutes. Animals were allowed to recover, prepared for imaging, and then scored as described above.

For chronic exposures, synchronized populations of L1 animals were plated on NGM containing varying concentrations of MnCl<sub>2</sub>, FeCl<sub>2</sub>, AlCl<sub>3</sub>, or CuCl<sub>2</sub>. The toxicants were added to the NGM agar prior to pouring the plates per standard protocols [116]. OP-50 bacteria was spread on each of the metal toxicant plates and allowed to grow as previously described [116]. After 72 hours of toxicant exposure, DA neuron integrity was evaluated as described above. Each of the experiments was performed at least in triplicate, the results are reported as means  $\pm$  S.E.M., and statistical analysis was performed using GraphPad Prism5.

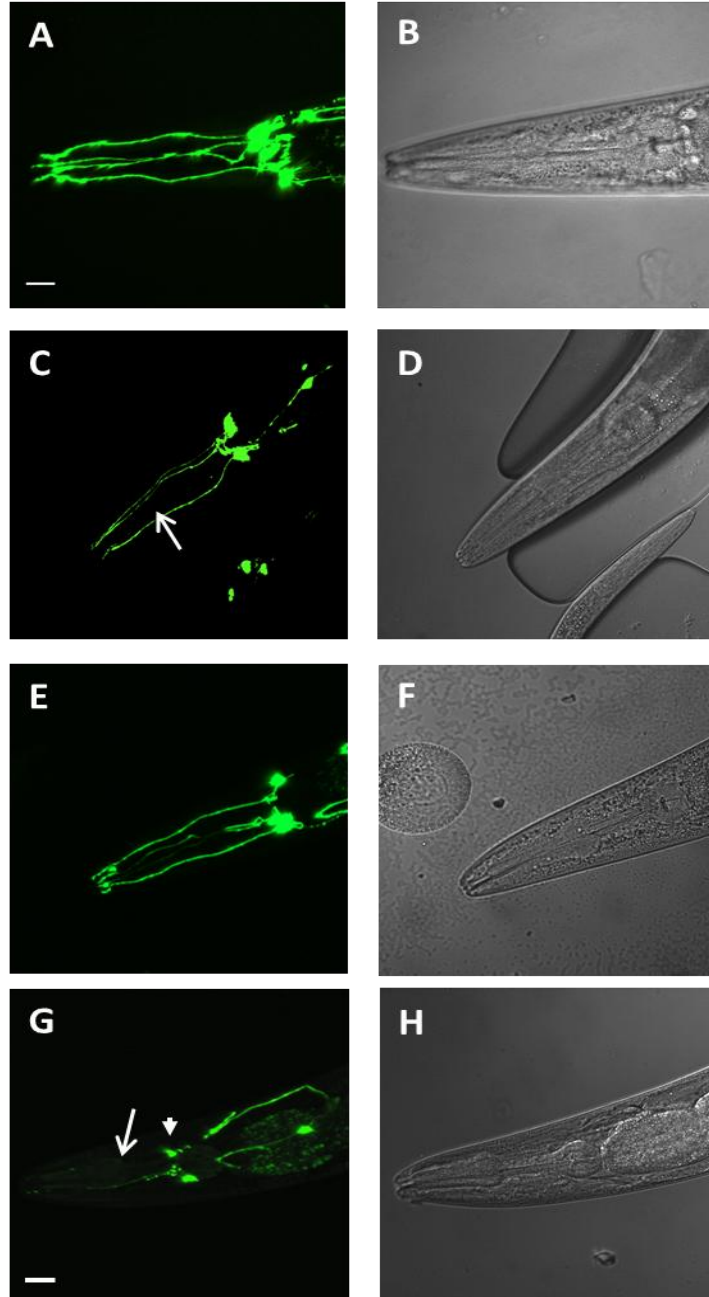


Figure 4. PD-associated toxicants MnCl<sub>2</sub> or 6-OHDA induces DA neuron degeneration in *C. elegans*. L1 BY250 animals were exposed dH<sub>2</sub>O +/- 50 mM MnCl<sub>2</sub> or 1% dimethyl sulfoxide (DMSO) +/- 5 mM 6-OHDA for 30 min and then images were taken 72 hrs. after exposure. Images are displayed as fluorescent and corresponding DIC image of animals exposed to A & B) dH<sub>2</sub>O C & D) 50 mM MnCl<sub>2</sub> E & F) 1% DMSO in dH<sub>2</sub>O G & H) 5 mM 6-OHDA. Long arrows point to representative CEP process damage, short arrow points to representative CEP cell body damage. Images are representative of toxicant-induced DA neuron degeneration and were captured using confocal microscopy (Zeiss LSM 510 microscope). Scale bar indicates 20  $\mu$ m.

**Mitochondrial Membrane Potential Analysis** - Tetramethylrhodamine ethyl ester perchlorate (TMRE, Sigma Aldrich, St. Louis, MO) is a voltage-sensitive fluorescent indicator that accumulates in active mitochondria and was used to determine changes in mitochondrial membrane potential [144]. Acute exposure of L1 stage worms for 30 min to 100  $\mu\text{M}$   $\text{AlCl}_3$  was carried out similar to described above, then animals were placed on NGM plates containing 0.1  $\mu\text{M}$  TMRE and incubated in the dark [144, 145]. After 48 hours, 20 - 30 animals were picked onto slides with 2% agarose pads and immobilized with 2% levamisole. Levamisole has been identified to cause muscle paralysis in *C. elegans* due to the prolonged activation of nicotinic acetylcholine receptors in the body wall muscles [18]. Pictures of the head region of each animal were captured using a Leica MZ 16FA fluorescent microscope with a Texas Red filter. The change in membrane potential was calculated based on changes in average pixel intensity using Image Pro Plus v6.2 software (Media Cybernetics, MA). Each exposure condition was evaluated in triplicate.

**ROS Analysis** - ROS was determined using 2,7-dichlorodihydrofluorescein diacetate (H<sub>2</sub>-DCF-DA)-associated fluorescence [46, 146, 147]. A synchronized population of worms was exposed to 1  $\mu\text{M}$   $\text{CuCl}_2$  for 30 min and rinsed 3x with dH<sub>2</sub>O, followed by resuspension in M9 at a concentration of 50,000 worms/ml. A 96-well plate was used to set up four replicates in different wells of each control and experimental condition. Experimental conditions included 50  $\mu\text{l}$  of resuspended worms from either the dH<sub>2</sub>O or 1  $\mu\text{M}$   $\text{CuCl}_2$  groups in addition to 50  $\mu\text{l}$  of 100  $\mu\text{M}$  H<sub>2</sub>-DCF-DA. Control wells were set up on each plate in triplicate and included 50  $\mu\text{l}$  of resuspended worms from each experimental condition with 50  $\mu\text{l}$  of M9 instead of H<sub>2</sub>-DCF-DA, and 50  $\mu\text{l}$  of H<sub>2</sub>-DCF-DA with 50  $\mu\text{l}$  of M9 instead of resuspended worms. Total ROS was determined based on emitted light at 520 nm on a Tecan Spectrafluor Plus spectrophotometer [46, 147, 148]. The 96-well plate was covered and placed on a shaker for 60 min and then read again. The final values were normalized with the appropriate control wells and compared between experimental conditions. This assay was performed in triplicate.

**RNA Interference** - RNAi-sensitive NL2099 *rrf-3(pk1426)* nematode strain was crossed with BY250, and homozygosity was determined by single worm PCR [46, 127]. The new strain was named RJ928. RNAi experiments were carried out on NGM plates seeded with HT115 (DE3), an RNase III-deficient *Escherichia coli* strain carrying L4440 vector with the gene fragment (*aco-1*, *gst-1*, *ftn-1*, or *ftn-2*) (GeneService, Source BioScience, PLC, Nottingham, UK) or empty vector (Addgene, Cambridge, MA) with the addition of 1 mM isopropyl 1-thio- $\beta$ -D-galactopyranoside [127]. RJ928 L1 stage worms from a synchronized population were transferred onto the RNAi plates and allowed to grow until gravid. Eggs were harvested and synchronized as above and the second generation L1s were then placed back onto the RNAi plates. After approximately 46 hours, between 50 - 100 gravid adults were transferred to fresh RNAi plates and allowed to lay eggs for 5 hrs. Adults were then removed from the plate and approximately 9 hours later, the recently hatched L1s were exposed to 100  $\mu$ M  $\text{AlCl}_3$  for 30 min. After the exposure, L1 animals were washed and allowed to recover on the appropriate RNAi plates for 72 h and then evaluated for DA neuron degeneration as previously described [46].

## Results

### Chronic metal exposure

Prior vertebrate studies have shown that exposure to concentrations, as high as 2.5 mM and 6 mg/kg/day, of  $Mn^{2+}$  result in metal accumulation in the striatum in addition to animal and cell death [19, 57]. *C. elegans* relatively impermeable cuticle may block entry of compounds, thereby requiring higher exposure concentrations. Our lab previously showed 30 min exposure to 50 mM  $MnCl_2$  results in *C. elegans* accumulated  $Mn^{2+}$  tissue concentrations similar to vertebrate systems [46]. In order to determine if  $Mn^{2+}$  exposure affects the viability of animals after long term exposure, I exposed worms to varying concentrations of  $Mn^{2+}$ . Synchronized BY250 L1 animals were added to NGM plates with varying levels of  $MnCl_2$ . Animal viability was determined after 72 hrs. As can be seen in Figure 5,  $Mn^{2+}$  exposure concentration affects animal viability in a concentration-dependent manner, with the  $IC_{50} = 12.97$  mM  $MnCl_2$ .  $IC_{50}$  was calculated using ReaderFit software (Hitachi Solutions America) using a 4 parameter logistic nonlinear regression model.

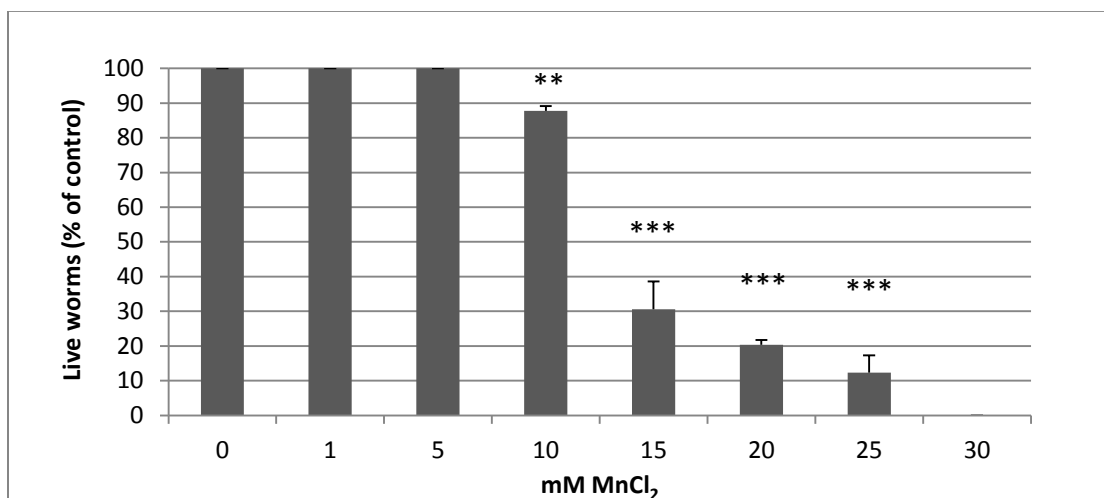


Figure 5: 72 hour exposure to  $Mn^{2+}$  exhibit increased animal death. Graph shows animal viability after 72 hour exposure to mM +/-  $MnCl_2$ . Data was analyzed using one-way ANOVA followed by Dunnett's multiple comparison test (\*\*  $\geq p .01$ , \*\*\*  $\geq p .001$ ).

Epidemiological studies have suggested occupational exposure of greater than 20 years to metals can increase the onset of PD [10, 29], and a pathological hallmark of both PD and manganism is the loss of DA neurons in the SN. To test the hypothesis that

chronic exposure to metals associated with PD may contribute to DA neuron degeneration, synchronized BY250 L1 animals were placed on NGM plates with varying concentrations of  $MnCl_2$ ,  $FeCl_2$ ,  $AlCl_3$ , or  $CuCl_2$  and DA neuron degeneration was evaluated after 72 hr. Figure 6 shows that chronic exposure to  $MnCl_2$ ,  $FeCl_2$ ,  $AlCl_3$ , or  $CuCl_2$  confers DA neuron degeneration. Animals exposed to  $FeCl_2$ ,  $AlCl_3$ , or  $CuCl_2$  did not exhibit any changes in animal viability at the concentrations tested.

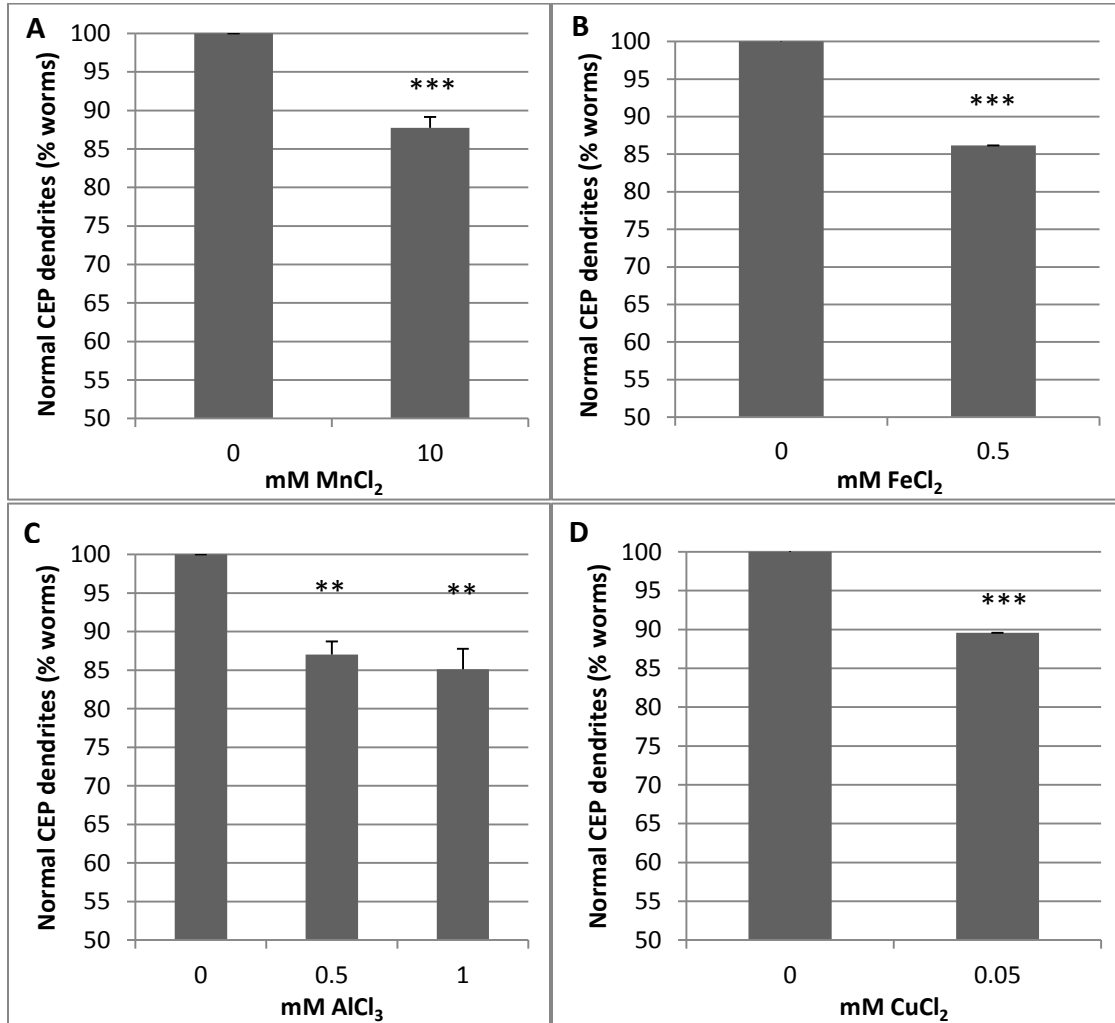


Figure 6:  $MnCl_2$ ,  $FeCl_2$ ,  $AlCl_3$ , and  $CuCl_2$  induce significant DA neuron degeneration after 72 hour exposure. Shown are mM exposures in mean values  $\pm$  S.E.M. of at least three individual replicates. p values (\*\*\*  $p \leq .001$  and \*\*  $p \leq .01$ ) were calculated using t-test analysis for panels A,B, D and using an one-way ANOVA followed by Dunnett's multiple comparison test for panel C.

DMT1 transports  $\text{Fe}^{2+}$  into vertebrate DA neurons and confers  $\text{Fe}^{2+}$ -induced cell death, and our studies have recently shown that the DMT1 homologue, SMF-1, localizes to *C. elegans* DA neurons [14, 46, 109, 110]. I utilized strains created by our lab for previous work with  $\text{Mn}^{2+}$  [46] which were verified to express GFP fusion behind the DAT-1 promoter and have a mutation in one of the SMFs. The strain RJ907 (listed as  $\Delta smf-1$  in figures) contains a 2063-bp deletion in *smf-1* which results in a truncated protein containing only the first 6 transmembrane domains. This mutation is predicted to be a non-functional protein, based on the consensus transport sequence being deleted [46, 65]. The strain RJ905 (listed as  $\Delta smf-2$  in figures) contains a 448-bp deletion in *smf-2* which spans the start codon and the first 3 transmembrane domains, and likely results in a non-functional protein [46, 65]. The strain RJ906 (listed as  $\Delta smf-3$  in figures) contains an 1800-bp deletion spanning transmembrane domains 1-8. This mutation likely results in a non-functional protein due to lack of transmembrane domain 8, in addition loss of transmembrane domain 6, which has been shown to impair symporter activity [65]. In order to determine if  $\text{Fe}^{2+}$ -induced DA neuron degeneration is dependent on any of the SMFs, I evaluated DA neuron integrity following acute and chronic exposures to  $\text{Fe}^{2+}$  in animals with mutations in the transporters. Figure 7 shows that after both 72 hour and 30 min exposures to 500  $\mu\text{M}$   $\text{FeCl}_2$ , SMF-1 significantly contributes to  $\text{Fe}^{2+}$ -induced DA neuron degeneration in *C. elegans*. All strains exposed to  $\text{dH}_2\text{O}$  (wild type (WT),  $\Delta smf-1$ ,  $\Delta smf-2$ , and  $\Delta smf-3$ ) did not exhibit DA neuron degeneration and therefore were not included in Figure 7.



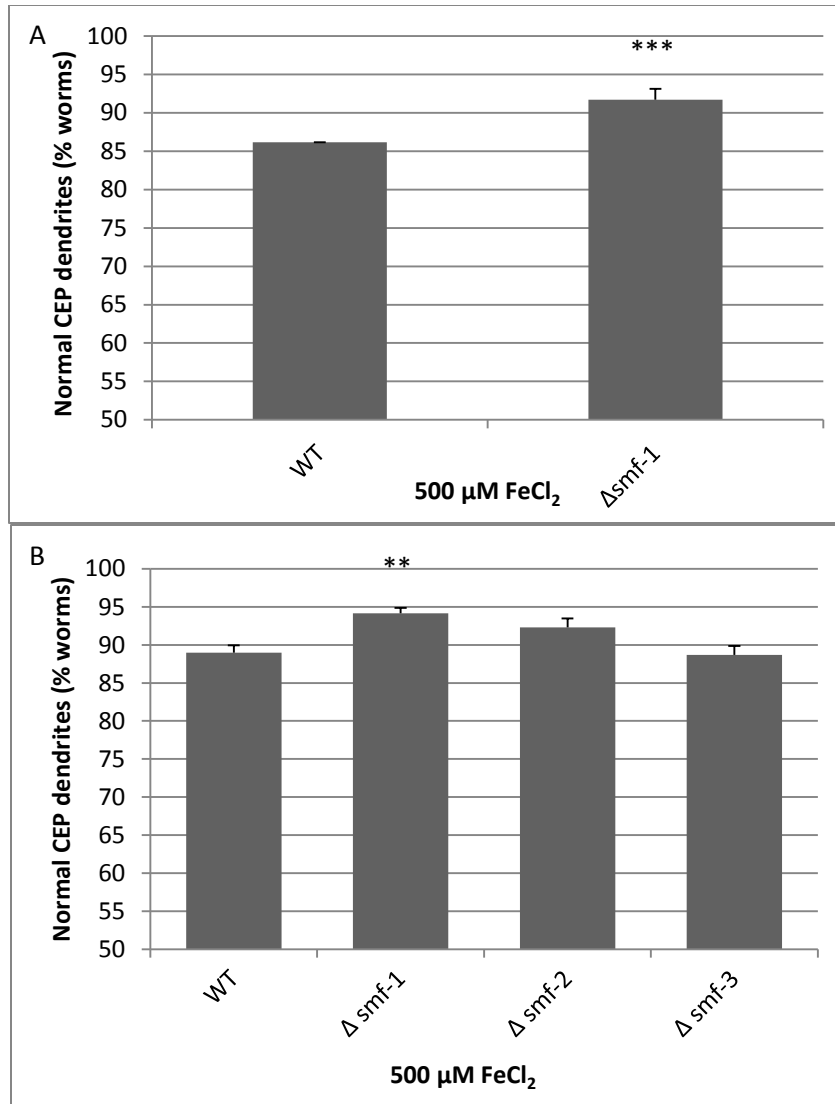


Figure 7: SMF-1 significantly contributes to FeCl<sub>2</sub>-induced DA neuron degeneration after both 72 hour and 30 min exposure. L1 animals were exposed to 500 μM FeCl<sub>2</sub> and then evaluated at the end of the 72 hrs. for degeneration. WT (BY250) and Δ *smf-1* (RJ907) exposed for 72 hr. (panel A) or WT, Δ *smf-1* , Δ *smf-2* (RJ905), and Δ *smf-3* (RJ906), were exposed for 30 min (panel B). Data was analyzed using t-test compared to WT (\*\*\*)  $p \leq .001$  in panel A and using one-way ANOVA followed by Dunnett's multiple comparison test (\* $p \leq .05$ ) in panel B.

### Aluminum

It has not been established that Al<sup>3+</sup> is an essential metal ion, but exposure to high concentrations has been shown to contribute to toxicity and cell death in many species [154 - 156]. *In vitro* studies have shown that even though it is not a redox-active

metal,  $\text{Al}^{3+}$  can facilitate increased reactive oxygen species by interacting with other metals to increase oxidation of DA or lipids [73, 99]. Therefore to test the hypothesis that  $\text{AlCl}_3$  exposure increases oxidative stress in *C. elegans*, a synchronized population of L1 animals was exposed for 30 min to 100  $\mu\text{M}$   $\text{AlCl}_3$ , similar to previous studies [46]. Worms exposed to  $\text{dH}_2\text{O}$  + 100  $\mu\text{M}$   $\text{AlCl}_3$  did not result in any changes in animal vulnerability (data not shown). Animals were placed on TMRE plates for 48 hr. and mitochondrial membrane potential in the head region was evaluated. A brief exposure to a low  $\text{Al}^{3+}$  concentration significantly reduces mitochondrial membrane potential in the head relative to control animals. This supports previous vertebrate data and suggests  $\text{Al}^{3+}$  may impair mitochondria function in *C. elegans* (Figure 8).

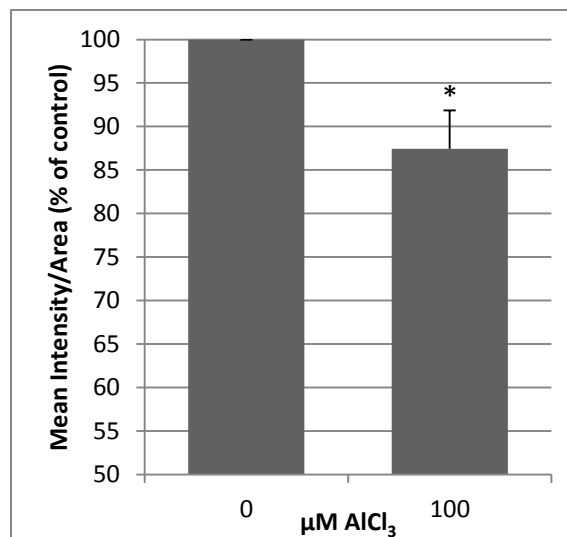


Figure 8: Acute  $\text{Al}^{3+}$  exposure significantly decreases mitochondrial membrane potential in the head region of worms. Animals were allowed to recover on TMRE plates for 48 hours after 30 min exposure  $\pm$  100  $\mu\text{M}$   $\text{AlCl}_3$ . \*  $p \leq .05$  as determined by t-test of toxicant exposure to control.

Environmental exposure to low levels of metals including  $\text{Fe}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Al}^{3+}$  have been shown to induce oxidative stress, which is a contributing factor of neurodegenerative diseases. In order to determine whether a brief acute exposure to  $\text{Al}^{3+}$  causes DA neurodegeneration, I evaluated DA neuron integrity following a 30 min exposure to low concentrations of  $\text{Al}^{3+}$ . As seen in Figure 9, acute sub lethal exposure to  $\text{Al}^{3+}$  induces significant DA neuronal death.

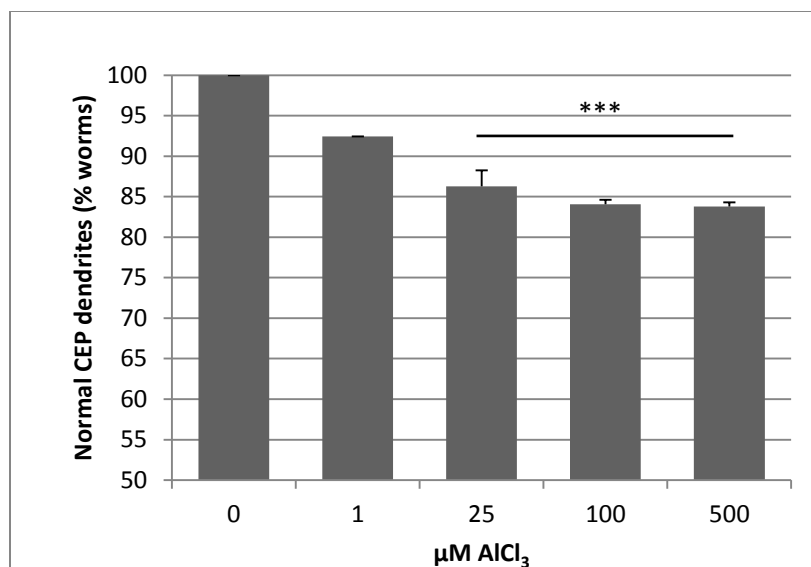


Figure 9: DA neuron degeneration significantly increases in WT animals with exposure to  $\geq 25 \mu\text{M AlCl}_3$ . BY250 L1 worms were exposed for 30 min dH<sub>2</sub>O +/-  $\mu\text{M AlCl}_3$  and evaluated for DA neuron degeneration after 72 hr. Shown are mean values  $\pm$  S.E.M. Data was analyzed using one-way ANOVA followed by Dunnett's multiple comparison test \*\*\* $p \leq .001$ .

In the mammalian brain the molecular basis of  $\text{Al}^{3+}$  transport is still unknown. Previous studies have suggested that  $\text{Al}^{3+}$  can interfere with iron homeostasis, and DMT1 has been shown to transport  $\text{Fe}^{2+}$  into cells [99, 158, 159]. Therefore I hypothesized that one of the *C. elegans* homologues SMF-1, SMF-2, or SMF-3 may play a role in regulating  $\text{Al}^{3+}$  transport, and exposure may cause a significant change in gene or protein expression. To determine whether  $\text{Al}^{3+}$  may affect *smf-1*, *smf-2*, or *smf-3* gene expression, I exposed worms to  $100 \mu\text{M AlCl}_3$  for 30 min and allowed them to recover on NGM plates for 48 hr. A reduction of mRNA levels after exposure suggests that *C. elegans* DMT homologues, specifically SMF-2 and SMF-3 are highly sensitive to excess  $\text{AlCl}_3$  exposure and may be part of cellular response to limit  $\text{Al}^{3+}$  accumulation (Figure 10).

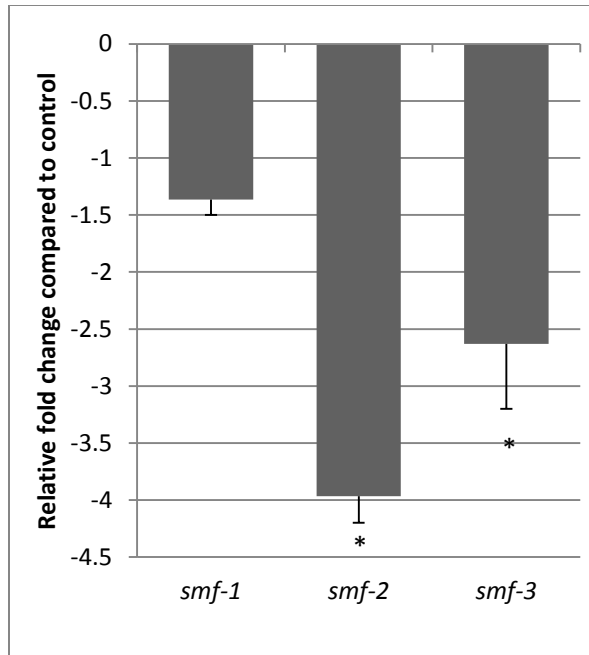


Figure 10: Transcript levels of *smf-2* and *smf-3* decrease following acute  $Al^{3+}$  exposure. L1 animals were exposed to 100  $\mu M$   $AlCl_3$  for 30 min and then allowed to recover for 48h on NGM plates then mRNA was extracted, reverse-transcribed to cDNA, and relative gene expression changes of *smf-1*, *smf-2*, *smf-3* were quantitated using qPCR. Fold change was calculated relative to GAPDH following the  $\Delta\Delta C_T$  method. Shown are mean values for fold change  $\pm$  S.E.M. of three individual replicates. \*  $p \leq 0.05$  between control and  $Al^{3+}$  treated group  $\Delta C_T$  values.

Since SMF-1, SMF-2 [46] and SMF-3 are expressed in DA neurons (lab results, unpublished), acute exposure to  $Al^{3+}$  induces DA neuron degeneration, and  $Al^{3+}$  exposure elicits gene DMT1 homologue response, I tested the hypothesis that *C. elegans* SMF's contribute to  $Al^{3+}$ -induced DA neuron degeneration. As mentioned before an assumed non-functional mutation in *smf-3* significantly protects against  $Al^{3+}$ -induced DA neuron cell death (Figure 11). Since there was no change in DA neuron degeneration after  $Al^{3+}$  exposure in strains containing mutations in either *smf-1* or *smf-2*, the data suggests that these genes do not play a role in  $Al^{3+}$ -induced DA neurodegeneration (Figure 11 A). Similar results were seen with both WT and SMF-3 mutant animals exposed to 500  $\mu M$   $AlCl_3$  for 72 hrs. (data not shown). All strains exposed to  $dH_2O$  (WT,  $\Delta smf-1$ ,  $\Delta smf-2$ , and  $\Delta smf-3$ ) did not exhibit DA CEP dendrite degeneration and therefore were not included in Figure 11 A.

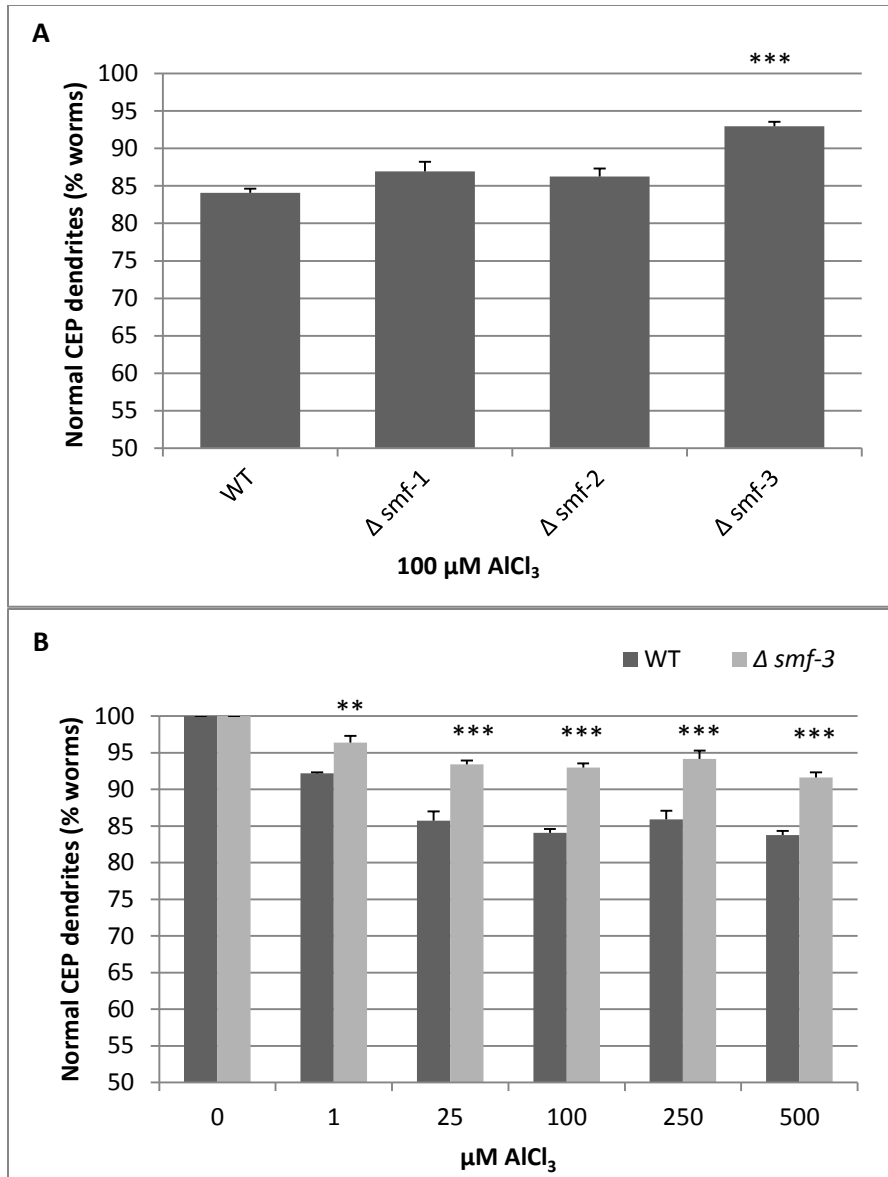


Figure 11: SMF-3 contributes to  $AlCl_3$ -induced DA neuron degeneration in *C. elegans*. L1 animals were exposed to  $dH_2O + AlCl_3$  for 30 min and placed on NGM for evaluation after 72hr A) WT (BY250),  $\Delta smf-1$  (RJ907),  $\Delta smf-2$  (RJ905), and  $\Delta smf-3$  (RJ906) respectively exposed to  $100 \mu M AlCl_3$  for 30 min, B) WT (BY250) and  $\Delta smf-3$  (RJ906) exposed  $dH_2O \pm AlCl_3$  for 30 min. Data was analyzed using one-way ANOVA with a Dunnet post-test (\*\* $p \leq .001$ ) compared to WT at  $100 \mu M AlCl_3$  (panel A) or using two-way ANOVA with Bonferroni post-test (\*\* $p \leq .01$  or \*\*\* $p \leq .001$ ) compared to WT for each concentration tested (panel B).

$Al^{3+}$  exposure has been suggested to increase  $Fe^{2+}$  accumulation, as well as  $Fe^{2+}$ -induced oxidative injury [7, 159 - 161]. Iron-sulfur clusters are cofactors for aconitase

enzymes which are important for the isomerization of citrate to isocitrate in the Krebs cycle. *C. elegans* has two aconitase genes, *aco-1* which is cytosolic and a homolog to human iron regulatory protein-1. While the cytosolic aconitase function is not known in *C. elegans*, *aco-1* has been suggested to exhibit aconitase activity and be post-translationally regulated by  $\text{Fe}^{2+}$  [162]. To determine if an aconitase linked  $\text{Fe}^{2+}$ -responsive pathway contributed to  $\text{Al}^{3+}$  neurotoxicity, I asked if *aco-1* altered  $\text{Al}^{3+}$ -induced DA neuron degeneration. A synchronized population of RNAi sensitive L1 animals were exposed for 30 min  $\text{dH}_2\text{O}$  +/- 100  $\mu\text{M}$   $\text{AlCl}_3$  and then allowed to recover on RNAi plates with bacteria expressing either empty vector or *aco-1* dsRNA. After a 72 hour recovery, animals were evaluated for DA neuron degeneration. Figure 12 suggests knock down of *aco-1* increases  $\text{Al}^{3+}$ -induced DA neuron degeneration, but verification of *aco-1* knock down needs to be completed to confirm initial findings.

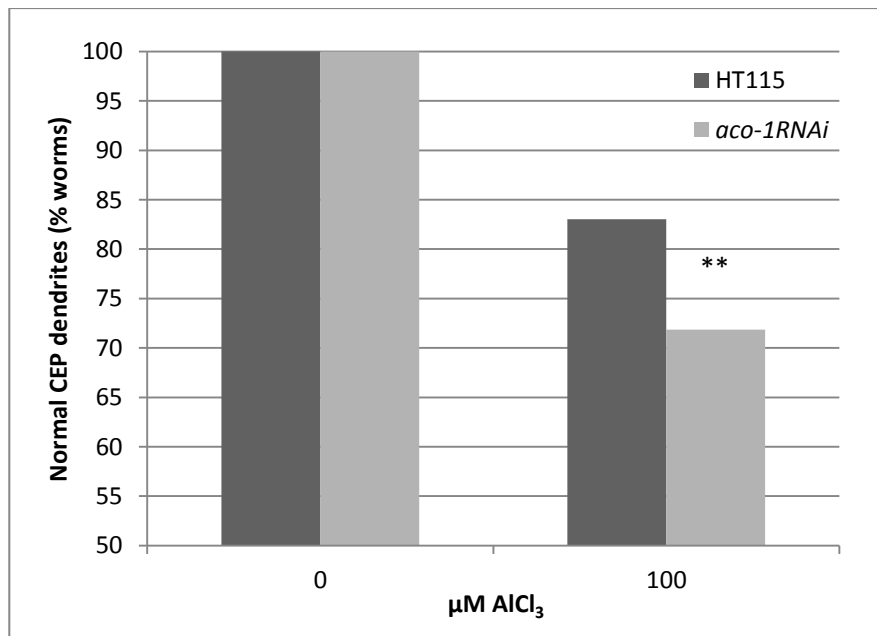


Figure 12: Knockdown of cellular aconitase *aco-1* increases DA neuron degeneration after  $\text{Al}^{3+}$  exposure. RJ928 L1 animals were exposed for 30 min  $\pm$  100  $\mu\text{M}$   $\text{AlCl}_3$  then allowed to recover on RNAi plates expressing HT115 or *aco-1* dsRNA bacteria respectively for 72 hrs. and then degeneration determined. \*\* $p \leq .01$  determined by t-test comparing the two  $\text{Al}^{3+}$  exposed groups.

Brain glutathione has multiple functions including scavenging of highly reactive superoxide and hydroxyl radicals, and serving as a cofactor for a number of radical

scavenging enzymes such as glutathione peroxidase and glutathione s-transferases (GSTs) [95]. GSTs are part of a family of phase II detoxifying enzymes responsible for catalyzing the conjugation of the thiol group of reduced glutathione to electrophilic centers on a wide variety of substrates [163, 164]. An increase in ROS levels can induce GST expression that is involved in detoxification of exogenously and endogenously derived toxic compounds [142, 143]. In mammals, three of the seven classes of GSTs have been identified in the central nervous system and only GST pi class, specifically GSTP1, has been identified in dopaminergic neurons of the SN [88]. Prior studies have suggested toxic  $Al^{3+}$  exposure effects GSH enzyme activity [96, 97]. Since brief  $Al^{3+}$  exposure increases ROS through mitochondrial inhibition similar to results seen with  $Mn^{2+}$  exposure [46], I tested the hypothesis that *gst-1* is also important in cellular response to  $Al^{3+}$  neurotoxicity. To test this, RNAi sensitive animals were grown on either empty vector or *gst-1* dsRNA bacteria. A synchronized population of L1 animals were exposed to 100 $\mu$ M  $AlCl_3$  and then placed on the appropriate RNAi plates for 72hr to recover. While knock down of *gst-1* increased  $Al^{3+}$  induced DA neuron degeneration, it was not significant compared to control (Figure 13). *gst-1* knock down was not verified, so one potential explanation for the results not producing further increased DA neuron sensitivity to  $Al^{3+}$  exposure as originally expected could be a result of incomplete penetrance of RNAi knock down. Assuming verification of knock down of *gst-1* produced statistically significant increased DA neuron degeneration, further experiments to determine *gst-1* mRNA and/or protein response after  $AlCl_3$  exposure would help to determine if DA neuron degeneration correlates with *gst-1* involvement in cellular response to  $AlCl_3$  induced DA neuron degeneration.

Overall these studies suggest that  $Al^{3+}$  toxicity in *C. elegans* increases oxidative stress through mitochondrial inhibition, and  $Al^{3+}$  exposure confers DA neuron degeneration. These studies also show that  $Al^{3+}$ -induced DA neurodegeneration is dependent on SMF-3 and suggests *C. elegans* aconitase *aco-1* may contribute to the neuropathology.

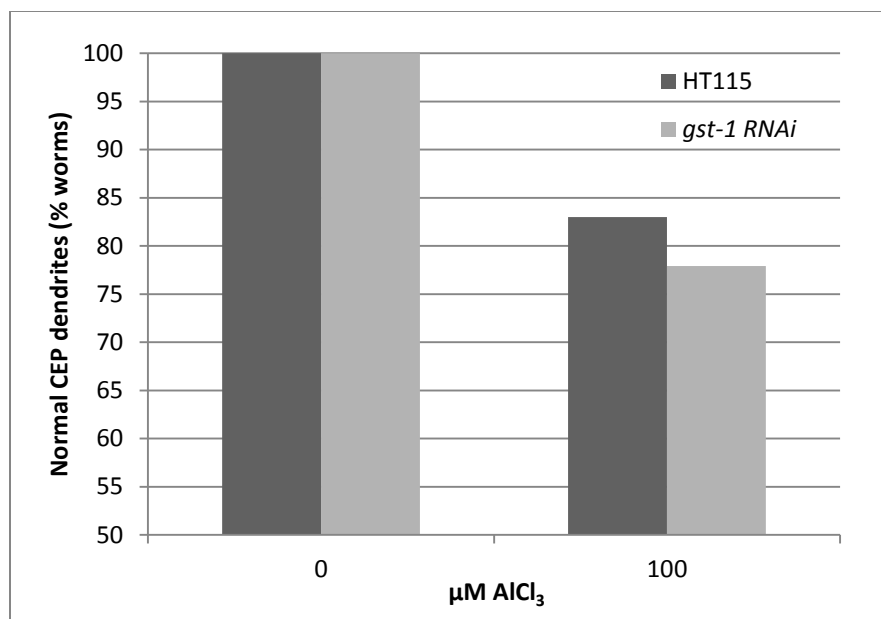


Figure 13: RNAi knockdown of *gst-1* does not significantly increase DA neuron degeneration after Al<sup>3+</sup> exposure. RJ928 L1 animals were exposed for 30 min dH<sub>2</sub>O ± 100 μM AlCl<sub>3</sub> then recovered on RNAi plates expressing HT115 or *gst-1* dsRNA bacteria respectively for 72 hours prior to determination of degeneration. p=.18 as determined by t-test comparing the two Al<sup>3+</sup> exposed groups.

## Copper

While the molecular mechanisms for copper toxicity are largely unknown, free Cu<sup>2+</sup> may be able to react with ROS and catalyze the production of highly toxic hydroxyl radical via Fenton reactions [20, 122]. Utilizing previous techniques [46], an hour after 1 μM CuCl<sub>2</sub> exposure whole animal ROS increased ~1.9 fold (Figure 14).

Considering that an acute Cu<sup>2+</sup> exposure increases whole animal ROS, I hypothesized that a brief Cu<sup>2+</sup> exposure may also induce DA neuron degeneration. As can be seen in Figure 15, a 30 min exposure in liquid culture to 1 - 50 μM CuCl<sub>2</sub> significantly increased the number of animals counted with DA neuron degeneration.



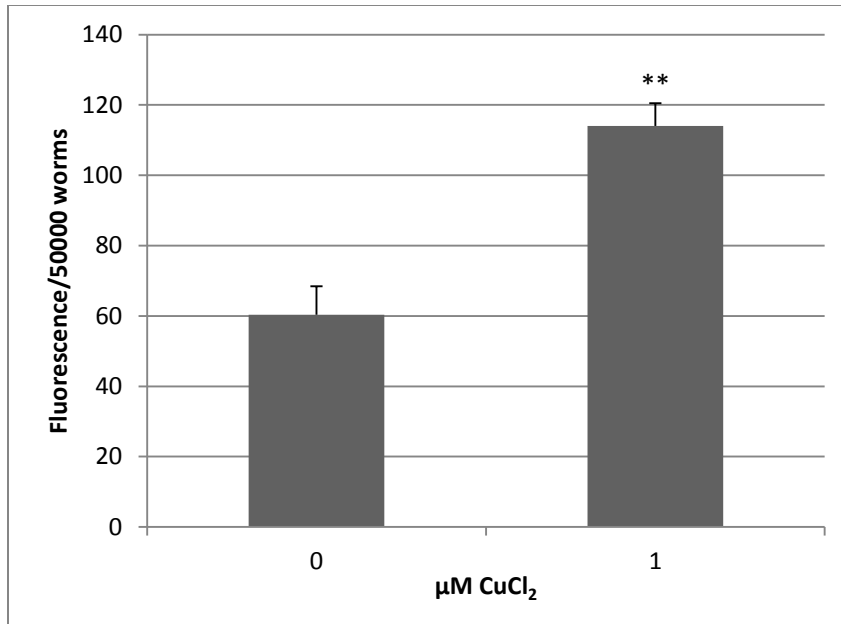


Figure 14: 30 min CuCl<sub>2</sub> exposure significantly increases ROS levels in whole animals. Whole animal ROS levels were measured following dH<sub>2</sub>O +/- 1 μM CuCl<sub>2</sub> treatment for 30 min and incubation with DCF-DA for 60 min. \*\* p<sub>≤</sub> .01 as determined by t-test compared to control.

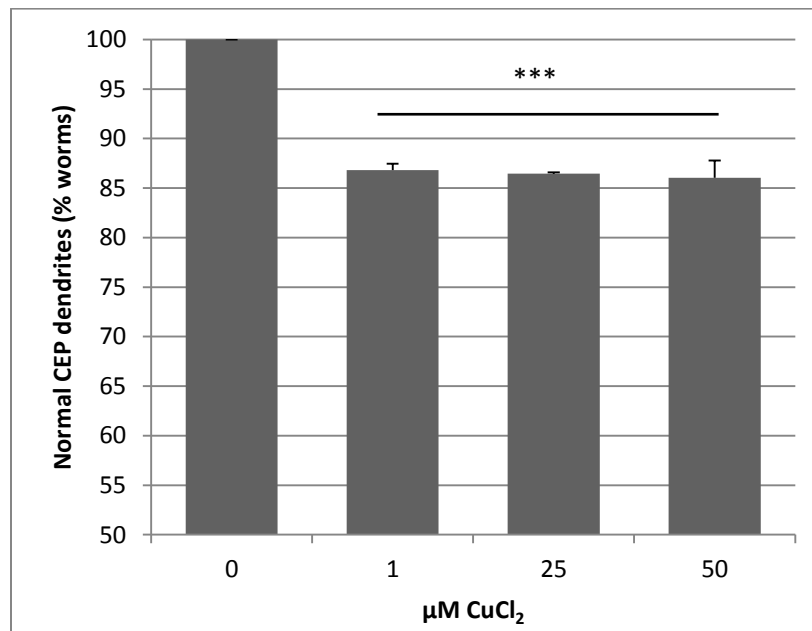


Figure 15: Sub-lethal CuCl<sub>2</sub> exposure significantly increases DA neuron degeneration. BY250 L1 animals were exposed for 30 min to dH<sub>2</sub>O ± 1 - 50 μM CuCl<sub>2</sub> then examined 72 hr. later. Concentrations 1-50 μM CuCl<sub>2</sub> were determined significant (\*\*\*) p<sub>≤</sub> .001 by a Dunnett test following a one-way ANOVA .

Since  $\text{Cu}^{2+}$  transport is mediated by DMT1 or SMFs in mammals or yeast respectively, I asked if any of the SMF's are able to mediate  $\text{Cu}^{2+}$ -induced DA neuron degeneration. A synchronized population of L1s for WT along with each of the non-functional SMF mutant strains was exposed for 30 min to 1  $\mu\text{M}$   $\text{CuCl}_2$  and degeneration determined 72 hrs. later. I found that *C. elegans smf-2*, but not *smf-1* or *smf-3* is involved in  $\text{Cu}^{2+}$ -induced DA neuron degeneration (Figure 16). All strains exposed to  $\text{dH}_2\text{O}$  (WT,  $\Delta smf-1$ ,  $\Delta smf-2$ , and  $\Delta smf-3$ ) did not exhibit DA CEP dendrite degeneration and therefore were not included in Figure 16 A.

Nass *et al.* 2002 previously established 6-OHDA as a PD model for inducing DA neuron degeneration in *C. elegans* and showed that mutations in *dat-1* was able to protect against 6-OHDA induced DA neuron degeneration. Additional studies have shown that  $\text{Cu}^{2+}$  can accelerate autoxidation of DA in addition to increasing 6-OHDA induced oxidative stress such as DNA damage and mitochondrial dysfunction [105 - 107, 167]. Also, *in vitro* studies have suggested that copper and dopamine can form a complex which is specifically transported by DAT [167 - 169]. To test the hypothesis that  $\text{Cu}^{2+}$ -induced DA neuron degeneration may be dependent on DAT, I utilized the BY215 strain (referred to as  $\Delta dat-1$  in the figure) that has a functional knockout of DAT and expresses GFP in the DA neurons  $P_{dat-1}::\text{GFP}; rol-6; dat-1(ok157)III$  [38]. BY215 was generated from the background strain BY200,  $P_{dat-1}::\text{GFP}; rol-6$ , and therefore BY215 was used as the control strain. A synchronized population of L1 animals were exposed for 30min to  $\text{dH}_2\text{O} \pm 1$  or 100  $\mu\text{M}$   $\text{CuCl}_2$  and placed on plates to recover for 72hrs. As can be seen in Figure 17,  $\text{Cu}^{2+}$ -induced DA neuron cell death appears to be dependent on DAT.

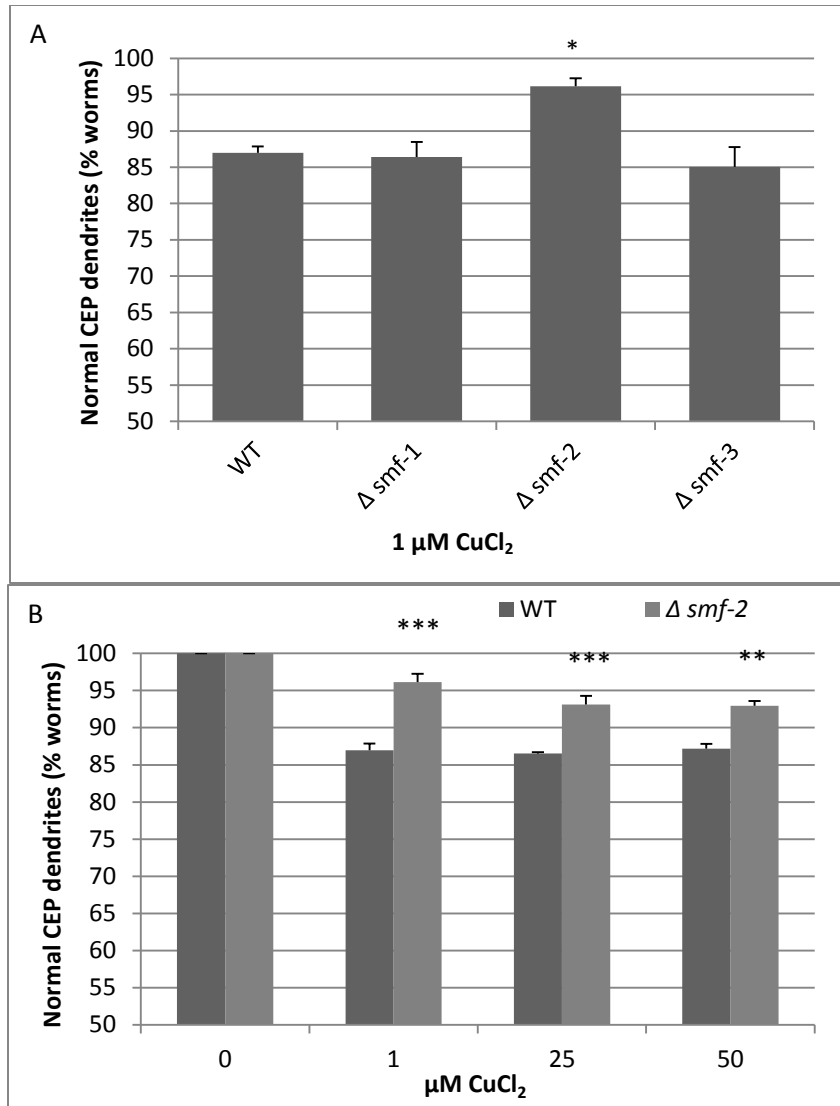


Figure 16: SMF-2 significantly contributes to  $\text{CuCl}_2$ -induced DA neuron degeneration in *C. elegans*. L1 animals were exposed to  $\text{dH}_2\text{O} \pm \text{CuCl}_2$  for 30 min and placed on NGM for evaluation after 72hr A) WT (BY250),  $\Delta smf-1$  (RJ907),  $\Delta smf-2$  (RJ905), and  $\Delta smf-3$  (RJ906) respectively exposed to  $1 \mu\text{M}$   $\text{CuCl}_2$ , B) WT (BY250) and  $\Delta smf-2$  (RJ905) exposed  $\text{dH}_2\text{O} \pm \text{CuCl}_2$ . Data was analyzed using one-way ANOVA with Dunnett post-test ( $*p \leq .05$ ) compared to WT (panel A) or two-way ANOVA with Bonferroni post-test ( $***p \leq .001$ ) compared to WT for each concentration tested (panel B).

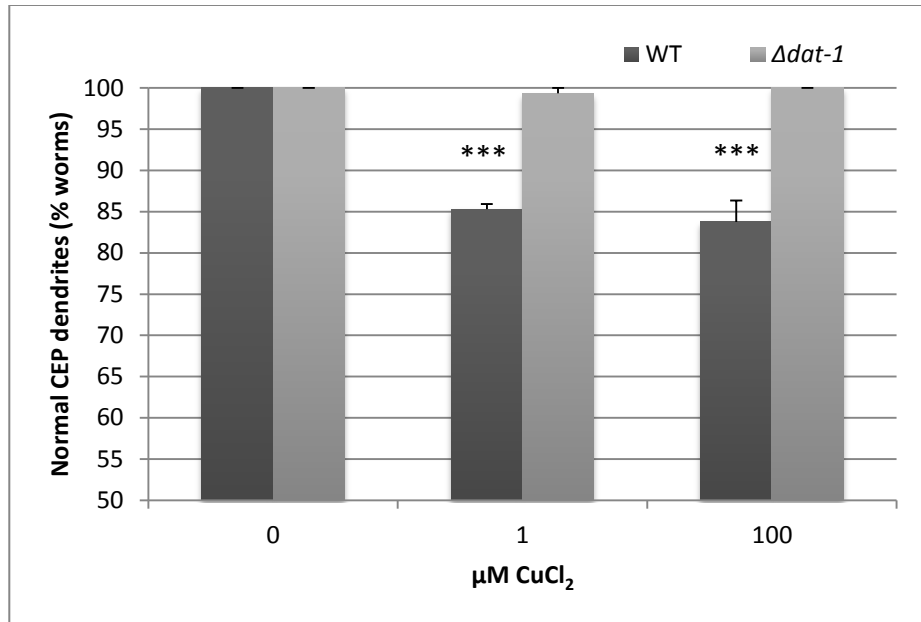


Figure 17: DAT-1 contributes to Cu<sup>2+</sup>-induced DA neuron degeneration. Strains BY200 (WT) and BY215 (*Δdat-1*) respectively were exposed for 30min and degeneration evaluated after 72h. Data was analyzed using two-way ANOVA with Bonferroni post-test (\*\*\*)  $p \leq .001$  compared to WT at each concentration.

In summary low level CuCl<sub>2</sub> exposure increases both whole animal ROS and DA neuron cell death. My data also indicates that Cu<sup>2+</sup>-induced DA neuron degeneration is dependent both on DAT-1 and SMF-2.

## Discussion and Future Direction:

While the etiologies of many neurodegenerative diseases are still unknown, epidemiological data has suggested that most of the pathologies develop from a combination of both genetic and environmental risk factors. A number of studies have shown environmental metal exposure increases cell death that is characteristic of neurodegenerative disorders such as AD, PD, Wilson's disease and Menkes disease. Mounting evidence collected from post mortem patients suggests metal exposure can decrease neurodegenerative disease age of onset [29, 30, 33, 148, 149]. High oxygen consumption rate, membranes enriched in oxidizable polyunsaturated fatty acids, lower antioxidant enzyme activity, DA catabolism, and large stores of  $\text{Fe}^{2+}$  have been suggested as factors contributing to DA neuron-associated disorders [29, 85 - 87]. For example, loss of dopamine neurons in the *substantia nigra* is a hallmark of PD and  $\text{Mn}^{2+}$ -induced neurotoxicity, and mitochondrial dysfunction has been implicated in disease progression. In these studies, I utilized *C. elegans* translational GFP fusions to view DA neurons *in vivo* and identify metals that contribute to DA neuron cell death and oxidative stress. These studies suggest that *C. elegans* DA neurons are sensitive to  $\text{Mn}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Al}^{3+}$ , and  $\text{Cu}^{2+}$ . The loss of CEP dendritic GFP seen in my experiments is similar to prior studies from our lab after exposure to  $\text{Mn}^{2+}$  or 6-OHDA, which correlated loss of GFP in toxicant exposed animals with a loss of total animal DA concentration and loss of neuronal integrity by electron microscopy [38, 46]. High performance liquid chromatography coupled to an electrochemical detection system determined approximately 60% loss of DA in animals exposed to  $\text{Mn}^{2+}$  as compared with control animals, which is consistent with DA neuronal loss [46]. Also my experiments show that the *C. elegans* transporters SMF-1 SMF-2, and SMF-3 contribute significantly to  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Al}^{3+}$ -induced DA neuron degeneration respectively. Furthermore, I show that DAT plays a role in  $\text{Cu}^{2+}$ -induced DA neuronal death. These studies provide insight into potential molecular pathways involved in neurodegenerative disease progression.

## Aluminum

Exposure to high concentrations of  $\text{Al}^{3+}$  confers cell death in many organisms [155, 156].  $\text{Al}^{3+}$  is not a redox-active metal, yet has been shown to facilitate  $\text{Fe}^{2+}$ -induced oxidative stress and inhibit multiple antioxidant enzymes [73, 96 - 100, 174]. Our laboratory previously determined  $\text{Mn}^{2+}$  exposed animals exhibit an approximate 15% reduction in TMRE intensity in the head region of the animals and also showed a greater than 2 fold reduction of cellular oxygen consumption, which has been correlated with dysfunctional mitochondria in both vertebrates and *C. elegans* [46]. I found that a 30 min exposure of *C. elegans* to sub-lethal concentrations of  $\text{AlCl}_3$  significantly decreased TMRE intensity in the head region of animals (Figure 8), consistent with prior studies, and suggests  $\text{Al}^{3+}$  impairs mitochondria function [76, 175]. Elevated cellular levels of  $\text{Al}^{3+}$  have been reported in multiple neurodegenerative diseases including AD, parkinsonism-dementia of Guam, and ALS [7 - 10, 31, 146].  $\text{Al}^{3+}$  has also been shown to alter neurotransmission by binding negatively charged lipids to alter membrane integrity, alter dopamine receptor density, and decrease dopamine levels [73, 75, 76]. In addition,  $\text{Al}^{3+}$  has been shown to exacerbate both MPTP and 6-OHDA induced DA neuron degeneration in animal models of PD [96, 104, 176]. My studies show that animals subjected to either chronic (72 hr.) or brief (30 min) exposure of sub-lethal levels of  $\text{AlCl}_3$  exhibit significantly increased DA neuron degeneration (Figures 6 C & 9 respectively). Additional studies from our lab have verified that this  $\text{Al}^{3+}$ -induced neuron degeneration is specific to DA neurons (manuscript in progress). Taken together, this data recapitulates vertebrate studies suggesting  $\text{Al}^{3+}$  exposure increases oxidative stress and DA neuron vulnerability in *C. elegans*.

$\text{Al}^{3+}$  has a small ionic radius and high charge and has been shown to have similar inorganic chemistry to  $\text{Fe}^{3+}$  [177]. The molecular basis for  $\text{Al}^{3+}$  transport across cell membranes in mammals is unknown, but it has been suggested that transferrin receptor mediated endocytosis or glutamate transporter uptake of an  $\text{Al}^{3+}$ -citrate complex may play a role [7]. A few studies have suggested  $\text{Al}^{3+}$  can interfere with iron homeostasis [99, 158, 159]. The vertebrate  $\text{Fe}^{2+}$  transporter DMT1 has been shown to play a role in

iron uptake system [84, 161]. To determine whether *C. elegans* DMT1 homologues may contribute to  $\text{AlCl}_3$  toxicity, I determined changes in gene expression following a 30 min exposure to sub-lethal  $\text{AlCl}_3$ . I found that *smf-2* and *smf-3* are significantly down regulated following the exposure (Figure 10), suggesting they may be down-regulated in order to limit cellular  $\text{Al}^{3+}$  accumulation. While there are limited studies available, SMF expression patterns in *C. elegans* suggest that SMF-1 is localized to DA neurons [46] and is primarily expressed in the intestine and associated gland cells with faint expression observed in a subset of anterior sensory neurons, ring neurons, and posterior-head neurons [150,151]. Expression of SMF-2 has been identified in DA neurons [46], epithelial cells of pharynx, and pharyngeal-intestinal valve cells [150]. Expression of SMF-3 has been identified in DA neurons (our lab unpublished data), along the intestine, and a weak expression in head and tail neurons [150, 151]. I then evaluated strains with mutations in SMF, as they have been shown to be localized to DA neurons, to determine whether they played a role in  $\text{Al}^{3+}$ -induced DA neuron degeneration. I found that SMF-3 contributes to  $\text{Al}^{3+}$ -induced DA neuron degeneration (Figure 11). Since my original experiments, other experiments from our lab using RNAi to knock down gene expression of both *smf-1* and *smf-2* simultaneously, also confirmed that expression of *smf-3* contributes to  $\text{Al}^{3+}$ -induced DA neuronal death. SMF-3 expression seems to be important specifically in  $\text{Al}^{3+}$ -induced DA neuron degeneration, as there was no significant change in the number of animals observed with DA neuron degeneration in *smf-3* mutant animals after exposure to  $\text{MnCl}_2$ ,  $\text{FeCl}_2$ , and  $\text{CuCl}_2$  ([46] and Figures 7B & 16A). To the best of my knowledge there was not any priore data suggesting that a trivalent ion such as  $\text{Al}^{3+}$  can be transported by DMT 1. It had been proposed that  $\text{Al}^{3+}$  might interact with an unknown protein to alter DMT 1 functionality as an explanation for the altered  $\text{Fe}^{2+}$  accumulation [161]. A recent study by Xia J. *et al.* 2010 identified an  $\text{Al}^{3+}$  specific rice plasma membrane transporter, *Nrat1*, which is highly homologous to DMT1. To determine if *Nrat1* is homologous to SMF-3, a BLAST search with the SMF-3 and rice transporter and a sequence alignment of the results using ClustalW2 indicates that SMF-3 has approximately a 36% amino acid identity and a 55% similarity to the rice





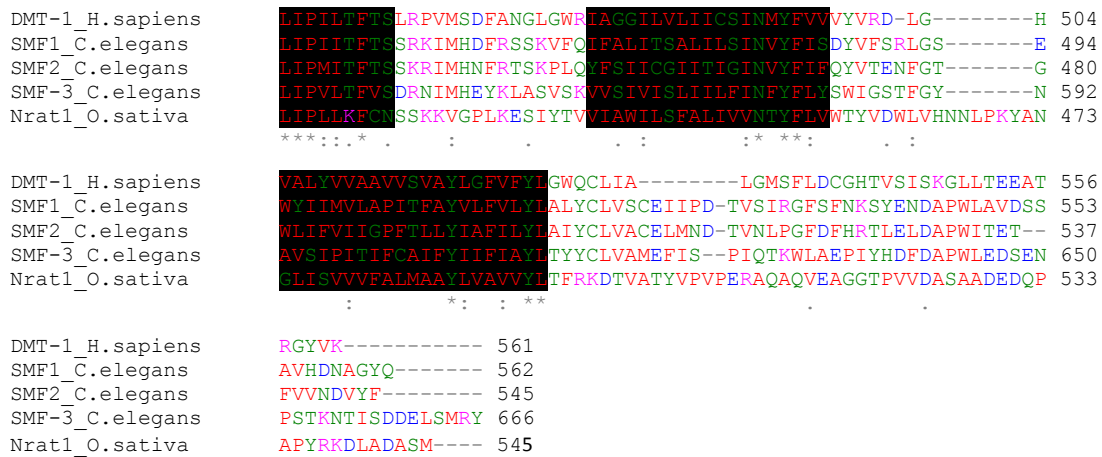


Figure 18: Sequence alignment of rice Nrat1 Al<sup>3+</sup> specific transporter with *C. elegans* and human DMTs. The sequences of human DMT-1 (GenBank accession number NP\_000608), *C. elegans* SMF-1 (GenBank accession number AAC465690), *C. elegans* SMF-2 (GenBank accession number AAC46568), *C. elegans* SMF-3 (GenBank accession number AAL27264), and *O. sativa* Nrat1 (GenBank accession number NP\_001045794) were aligned using the ClustalW2 program following an NCBI Blast search. The characteristic 12 transmembrane domains of DMT-1 are highlighted in *black*, and the consensus transport sequence is located between transmembrane domains 8 and 9 is highlighted in *yellow*. The *red font* in the sequence represents hydrophobic amino acids, whereas *blue* represents acidic, *magenta* represents basic, and *green* represents hydroxyl. The *asterisk* indicates amino acids in that column are identical, *colons* represent conserved substitutions (same color group), and *periods* represent semi-conserved substitutions (similar shapes).

Iron-sulfur clusters are cofactors for aconitase enzymes that are important for the isomerization of citrate to isocitrate in the Krebs Cycle. Aconitases are found in the cytosol, mitochondria, and glyoxysomes and have been shown to alter activity based on changes in iron availability [162]. I found that *C. elegans* homologue to cytosolic aconitase/iron regulatory protein-1, *aco-1*, plays a role in Al<sup>3+</sup> induced DA neuron degeneration. RNAi knock down of *aco-1*, increases DA neuron degeneration after a 30 min exposure to AlCl<sub>3</sub> (Figure 12). These results are consistent with prior studies suggesting that Al<sup>3+</sup> exposure may increase free Fe<sup>2+</sup> due to the lack of cytosolic aconitase and may be a contributing factor to DA neuron cell death. As only two experiments were performed, it would be important to determine degeneration from at least one more group to ensure statistical significance. It would also be important to

determine if the mitochondrial aconitase, identified in *C. elegans* as *aco-2*, is also playing a role in the neuropathology particularly since  $Al^{3+}$  has been shown to be a potent mitochondrial toxicant. Ferritin's main function is to provide bioavailable, but non-toxic storage of iron [178]. A few studies have suggested that after  $Al^{3+}$  exposure  $Fe^{2+}$  incorporation into ferritin changes, potentially suggesting ferritin may act as an aluminum detoxicant [161, 179]. My preliminary studies using RNAi to knock down either ferritin gene *ftn-1* or *ftn-2* do not show significant changes in DA neuron degeneration after exposure to 100  $\mu M$   $AlCl_3$ , but RNAi knock down was not confirmed, these studies were only performed in duplicate, and both ferritin genes were not knocked down in combination. Knock down of both genes simultaneously would eliminate potential protective compensatory effects.

Overall my results are consistent with prior studies suggesting that  $Al^{3+}$  increases oxidative stress *in vivo*. My studies also show that SMF-3 contributes to  $Al^{3+}$ -induced DA neuron degeneration, and knock down of *C. elegans* aconitase *aco-1* increases DA neuron sensitivity to  $Al^{3+}$ . Further experiments to measure SMF transport of  $Al^{3+}$  and dissection of the  $Fe^{2+}$  regulatory pathways may lead to an understanding of how SMF-3, whether direct or indirect, plays a role in  $Al^{3+}$  induced DA neuron degeneration.

## Copper

$Cu^{2+}$  is an essential metal ion and its dysregulation has been implicated in the promotion of oxidative stress and the development of neurodegenerative diseases.  $Cu^{2+}$ 's ability to donate or accept electrons facilitates the Fenton or Haber-Weiss reaction and produces highly reactive superoxide and hydroxyl radicals that may play a role in  $Cu^{2+}$ -induced DA neuron degeneration [16, 20, 106, 166]. Consistent with vertebrate studies, I found that exposure of *C. elegans* to sub-lethal  $CuCl_2$  concentrations increases whole animal ROS (Figure 13). I also found that both chronic (72 hr.) and short term (30 min) exposure to  $CuCl_2$  increases DA neuron degeneration (Figure 6 D & 15 respectively). Similarly after 30 min exposure in liquid, concentrations above 50  $\mu M$   $Cu^{2+}$  resulted in large numbers of animal death in addition to delayed

development. One potential hypothesis for this narrow range of toxicant concentration could be explained by the fact that excess copper is toxic, and while DA neurons are clearly sensitive to copper exposure, the exposure of the whole worm overrides other essential copper regulatory mechanisms at concentrations higher than 50  $\mu\text{M}$  in my experimental paradigm. In a preliminary experiment I looked at GABAergic neurons after brief 1  $\mu\text{M}$   $\text{CuCl}_2$  and did not find any significant change in GFP expression as I expected, but to verify  $\text{Cu}^{2+}$ -induced degeneration was DA neuron specific further experiments would need to be conducted.

DMT1 is known to transport  $\text{Cu}^{2+}$  in vertebrates, and may play a role in increased cellular free  $\text{Cu}^{2+}$ . I found that *C. elegans* DMT1 homologue SMF-2 plays a role in  $\text{CuCl}_2$ -induced DA neurodegeneration (Figure 16). SMF-2 localization to DA neurons in *C. elegans* [46] suggests that SMF-2 may transport  $\text{Cu}^{2+}$  into the DA neurons or into a  $\text{Cu}^{2+}$  sensitive intracellular compartment.

The neurotoxicant 6-OHDA has been shown to confer DA neuron degeneration, and rapidly oxidize to produce hydrogen peroxide, hydroxyl radicals and quinones [185, 186]. Vertebrate studies incorporating 6-OHDA have shown increased  $\text{Cu}^{2+}$  accumulation in dopaminergic pathways, and co-exposure with  $\text{Cu}^{2+}$  accelerates autoxidation of DA and production of its metabolites [105 - 107]. Prior studies have found DMT1 isoforms upregulated in the SN of PD patients, and DMT1 has been implicated in transport of the excess  $\text{Fe}^{2+}$  capable of catalyzing deleterious Fenton reactions after 6-OHDA or MPTP exposure [84, 112, 114]. Previous studies have also implicated DAT in  $\text{Cu}^{2+}$ -induced cell death [167]. Here I show that DAT also contributes to DA neuron vulnerability in *C. elegans* as a functional knockout of DAT protects the DA neurons from  $\text{Cu}^{2+}$ -induced pathology (Figure 17). Previous studies by Nass et al. 2002 show that a DAT deletion or inhibition with imipramine inhibits 6-OHDA-induced DA neurodegeneration. Taken as a whole my studies suggest that 6-OHDA and  $\text{Cu}^{2+}$  may share a common pathway to increased DA neuron pathology.

*In vitro* studies have suggested that  $\text{Cu}^{2+}$  and DA are able to form a complex that is transported by DAT [167 - 169]. Considering that both 6-OHDA and DA are easily

oxidized in the presence of  $\text{Cu}^{2+}$ , it may be possible that SMF-1 or SMF-2 may indirectly influence DA-associated proteins or DA metabolism to alter DA neuron degeneration. 6-OHDA treated cells have shown increased levels of DMT-1 along with increased iron influx. Also changes in free intracellular  $\text{Fe}^{2+}$  have been shown to affect DAT expression levels [84, 187]. Furthermore a reduction of DAT expression or function in the SMF mutant animals could result in lower accumulation of neurotoxicants attributing to neuron resistance [46]. Therefore it would be important to determine whether functional knockdown of any of the SMFs may modulate DAT-1 expression or function.

A  $\text{Cu}^+$ -GSH<sub>2</sub> complex has been previously reported to accumulate in the cell after copper exposure [102]. This complex has been shown to reduce cellular  $\text{Fe}^{3+}$  levels as well as releasing it from ferritin [102, 188]. This reaction would cause an increase in the intracellular free  $\text{Fe}^{2+}$  pool that in turn may increase the cell's ROS levels through Fenton chemistry or modulate SMF or DAT expression (Figure 19). If  $\text{Fe}^{2+}$  is playing a role in regulation of DAT-1, SMF-1, SMF-2, or both, it would be important to determine changes in ferritin (FTN-1/FTN-2) expression and  $\text{Fe}^{2+}$  concentrations after  $\text{Cu}^{2+}$  exposure.

In summary, excess  $\text{Cu}^{2+}$  may indirectly regulate DAT through a SMF pathway responsible for maintaining divalent metal ion homeostasis or directly based on changes in oxidative stress. Further studies examining DAT expression changes in association with SMF,  $\text{Fe}^{2+}$ , and oxidative stress pathways will assist in elucidating the mechanisms involved in  $\text{Cu}^{2+}$ -induced DA neuron degeneration.

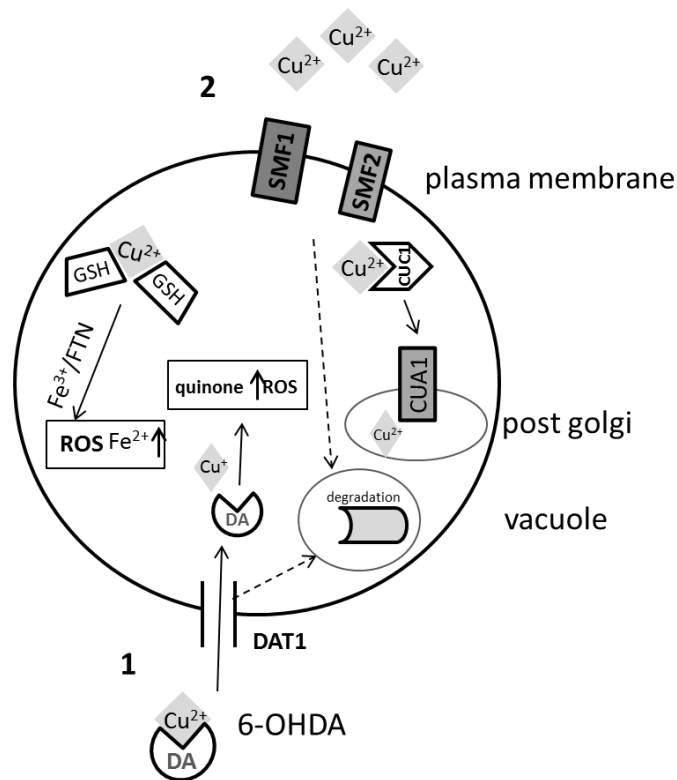


Figure 19: Potential mechanism of  $\text{Cu}^{2+}$ -induced DA neuron degeneration. SMF-2 plays a role in  $\text{Cu}^{2+}$  and 6-OHDA induced DA neuron cell death, SMF-1 plays a role in 6-OHDA induced DA neuron cell death, and DAT-1 inhibits  $\text{Cu}^{2+}$ -induced cell death. Therefore potential mechanisms are: 1) Cu-DA complex transported by DAT-1 introduces neurotoxic quinone radicals which facilitate increased ROS and degradation of SMF-1 and SMF-2 to prevent further accumulation of  $\text{Cu}^{2+}$  or other divalent metal ions 2) SMF-2 plays a role in regulation of DAT-1 expression or function due to changes in free  $\text{Fe}^{2+}$ , similar to *in vitro* data showing decreased intracellular free  $\text{Fe}^{2+}$  decreases DAT-1 expression, thereby limiting  $\text{Cu}^{2+}$  induced DA neuron cell death. GSH, glutathione; ROS, reactive oxygen species; FTN, ferritin; DA, dopamine; CUC-1, copper chaperone; CUA-1, copper ATPase; DAT-1, dopamine transporter.

## Conclusion

DMT1 and its homologues have been shown to transport divalent cations in a number of eukaryotes [14, 109, 110]. DMT-1 has also been implicated in the development of PD, and it has been suggested that its regulation may contribute to dysfunctional metal homeostasis leading to increased neuronal stress and cell death. Our lab previously identified three putative *C. elegans* homologues to human DMT1, SMF-1, SMF-2, and SMF-3 and determined that they are expressed in *C. elegans* DA

neurons [46, unpublished data]. Here I demonstrate that SMF-1, SMF-2, and SMF-3 play specific roles in divalent metal ion-induced DA neurodegeneration. I show that SMF-1 contributes to  $\text{Fe}^{2+}$ -induced DA neuron death, and SMF-2 and DAT-1 contribute to  $\text{Cu}^{2+}$ -induced DA neuron vulnerability. In addition my studies show that SMF-3 contributes to trivalent  $\text{Al}^{3+}$ -induced cell death.

These studies suggest that inhibition of  $\text{Mn}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Al}^{3+}$  or  $\text{Cu}^{2+}$  dysregulation may limit oxidative stress and decrease DA neuron degeneration. *C. elegans* provides a unique and powerful model to evaluate and characterize molecules and pathways involved in metal toxicity, and may identify novel therapeutic targets that are involved in metal-induced DA neuron degeneration and PD.

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## CURRICULUM VITAE

**Jennifer K LeVora**

### **Education**

**Medical Neuroscience**, Master of Science 2007 - May 2012  
Indiana University, Indianapolis, IN

**Computer Engineering**, Bachelor of Science 1997 - 2001  
Rose-Hulman Institute of Technology, Terre Haute, IN

### **Employment/Research Experience**

Graduate Research, Dr. Richard Nass Lab, **Indiana University School of Medicine**, Mar 2008 - May 2012, Department of Pharmacology/Toxicology

Graduate Rotation Research, Dr. Nickolay Brustovetsky, **Indiana University School of Medicine**, Jan - Mar 2008, Department of Pharmacology/Toxicology

Graduate Rotation Research, Dr. Eri Hashino, **Indiana University School of Medicine**, Sept - Dec 2007, Department of Otolaryngology and of Anatomy & Cell Biology

Technical Sales Engineer, **Texas Instruments**, San Jose CA 2001 - 2007

### **Honors and Awards**

Nominee for Student representative graduate student on Society for Neuroscience Membership and Chapters Committee - 2009

Recipient Educational Enhancement Travel Grant from Indiana University Graduate Student Organization - Spring 2009

Third Place Award for poster presentation at IUPUI Research Day - April 2009

Third Place Award for poster presentation in Pathogenesis Division of International C. *Elegans* meeting - June 2009

## Abstracts

**J. LeVora**, R. Settivari, R. Nass. Biochemical and genetic analysis of Parkinson's disease associated proteins, molecular transporters, and stress response proteins in *C. elegans* models of manganism. Society for Neuroscience, Washington DC, 2008.

R. Settivari, **J. LeVora**, R. Nass. Pharmacogenetic and biochemical analysis of Parkinson's disease, manganism, and dopamine neuron-associated proteins in *C. elegans*: effects on mitochondria function and dopamine neuron vulnerability. Society of Toxicology, Baltimore, MD, 2009.

**J. LeVora**, R. Settivari, R. Nass. Biochemical and genetic analysis of Parkinson's disease associated proteins, molecular transporters, and stress response proteins in *C. elegans* models of manganism. IUPUI Research Day, Indianapolis, IN, 2009.

**J. LeVora**, R. Settivari, R. Nass. Biochemical and Genetic Analysis of Parkinson's Disease-associated and Stress Response Proteins in *C. elegans* Models of Manganism. International *C. Elegans* Meeting, Los Angeles, CA, 2009.

R. Settivari, **J. LeVora**, R. Nass. Pharmacogenetic and biochemical analysis of Parkinson's disease-associated proteins in *C. elegans*: effects on mitochondrial function and dopamine neuron vulnerability. International *C. Elegans* Meeting, Los Angeles, CA, 2009.

R. Settivari, **J. LeVora**, R. Nass. Role of the divalent metal transporter, glutathione s-transferase pi, and ER stress response proteins in *C. elegans* models of Parkinson's disease and Manganism. Society of Toxicology, Salt Lake City, UT, 2010.

R. Nass, R. Settivari, **J. LeVora**. Parkinson's Disease and Manganism. Second World Parkinson Congress, Glasgow, UK, 2010.

R. Settivari, G. Sinclair, **J. LeVora**, N. VanDuyn, S. Zhou, R. Nass. SKN-1-dependent GST-Pi inhibits dopamine neuron degeneration in *C. elegans* models of Parkinson's Disease and Manganism. Aging, Metabolism, Stress, Pathogenesis, and Small RNAs, Madison, WI, 2010.

R. Settivari, **J. LeVora**, N. VanDuyn, S. Zhou, G. Sinclair, R. Nass. Gst-pi inhibits dopamine neuron degeneration in *C. elegans* models of Parkinson's disease and manganism. Society of Toxicology, Washington, DC, 2011.

R. Settivari, S. Zhou, N. VanDuyn, **J. LeVora**, G. Sinclair, R. Nass. Role of Nrf2/SKN-1 dependent and ER stress proteins in *C. elegans* models of Parkinson's disease and manganism. International Worm Meeting, Los Angeles, CA, 2011.

## **Publications**

R. S. Settivari, **J. LeVora**, and R. Nass. The divalent metal transporter homologues SMF-1/2 mediate dopamine neuron sensitivity in *Caenorhabditis elegans* models of manganism and Parkinson's disease. *J Biol Chem*. 2009 Dec 18;284(51):35758-68.

## **Presentations**

Pharmacogenetic analysis in novel models of Parkinson's disease, manganism, and methylmercury toxicity. Poster presented at the IUSM Pharmacology and Toxicology Research Retreat, Sept. 2008.

Biochemical and genetic analysis of Parkinson's disease-associated proteins, molecular transporters, and stress response proteins in *C. elegans* models of manganism. Poster presented at the IUSM Biomedical Gateway Program Student Recruitment Weekend, Feb. 2009.

## **Professional Organizations**

Society for Neuroscience, 2008 - 2011

Society for Women in Engineering, 2000 - 2006

Member of Chi Omega Alumni Chapter, multiple locations, 2002 - 2008

## **Activities**

Indiana University School of Medicine Graduate Mentor, 2008 - 2010

Indiana University School of Medicine New Student Recruiter, 2008 - 2010