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The Nrf2/SKN-1-dependent Glutathione S-transferase π Homologue GST-1 Inhibits Dopamine Neuron Degeneration in a *Caenorhabditis elegans* Model of Manganism

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Abstract

Exposure to high levels of manganese (Mn) results in a neurological condition termed manganism, which is characterized by oxidative stress, abnormal dopamine (DA) signaling, and cell death. Epidemiological evidence suggests correlations with occupational exposure to Mn and the development of the movement disorder Parkinson's disease (PD), yet the molecular determinants common between the diseases are ill-defined. Glutathione S-transferases (GSTs) of the class pi (GSTπ) are phase II detoxification enzymes that conjugate both endogenous and exogenous compounds to glutathione to reduce cellular oxidative stress, and their decreased expression has recently been implicated in PD progression. In this study we demonstrate that a *Caenorhabditis elegans* GSTπ homologue, GST-1, inhibits Mn-induced DA neuron degeneration. We show that GST-1 is expressed in DA neurons, Mn induces GST-1 gene and protein expression, and GST-1-mediated neuroprotection is dependent on the PD-associated transcription factor Nrf2/SKN-1, as a reduction in SKN-1 gene expression results in a decrease in GST-1 protein expression and an increase in DA neuronal death. Furthermore, decreases in gene expression of the SKN-1 inhibitor WDR-23 or the GSTTπ-binding cell death activator JNK/JNK-1 result in an increase in resistance to the metal. Finally, we show that the Mn-induced DA neuron degeneration is independent of the dopamine transporter DAT, but is largely dependent on the caspases CED-3 and the novel caspase CSP-1. This study identifies a *C. elegans* Nrf2/SKN-1-dependent GSTπ homologue, cell death effectors of GSTTπ-associated xenobiotic-induced pathology, and provides the first *in vivo* evidence that a phase II detoxification enzyme may modulate DA neuron vulnerability in manganism.

Keywords

manganism; neurodegeneration; neurotoxicity; caspase; Nrf2; Parkinson's disease

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1. Introduction

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease, and results in motor deficits, abnormal dopamine (DA) signaling, and DA neuron degeneration (Martin et al., 2011; Nass and Przedborski, 2008). The majority of PD cases are considered to be idiopathic (IPD), with less than 10% due to a known inherited mutation (Nass and Przedborski, 2008). It is likely that the environment and/or multiple genes contribute significantly to the development of the disorder. Exposure to high levels of manganese (Mn) can result in a neurological disorder termed manganism (Burton and Guilarte, 2009). Individuals with manganism present with motor deficits, dysfunctional DA signaling, and DA neuron degeneration. Epidemiological evidence suggests an association between occupational exposure to Mn and the development of PD (Crossgrove and Zheng, 2004; Jiang et al., 2006; Roth, 2009; Martin, 2011; Racette et al., 2012). Welders and Mn miners are particularly susceptible to developing parkinsonism and PD (Jiang et al., 2006; Racette et al., 2001; Sriram et al., 2010). The molecular basis of the increased propensity of individuals exposed to high levels of Mn to develop manganism and PD is ill-defined.

GSTπ (glutathione S-transferase pi) is a member of the phase II class of detoxification enzymes that are responsible for conjugating a broad range of electrophiles with glutathione to prepare them for cellular excretion (Goto et al., 2009). Decreased GSTπ expression has been identified as a significant risk factor for the development and progression of PD (Kelada et al., 2003; Menegon et al., 1998; Wilk et al., 2006; Shi et al., 2009; Vilar et al., 2007). GSTπ is expressed in the substantia nigra and plays a role in DA neuron sensitivity to the PD-associated neurotoxicants 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) and rotenone (Shi et al., 2009; Smeyne et al., 2007). The protein has been shown to inhibit apoptosis by binding to c-Jun N-terminal kinase (JNK)

(Wang et al., 2001; Burg et al., 2006; Thevenin et al., 2011). The role and regulation of GST π in manganism or Mn-induced cell death has not been previously defined. In rat primary hepatocytes, the expression of GSTπ has recently been shown to be regulated by the PD-associated transcription factor Nrf2, a key redox sensitive regulator of antioxidant enzymes, suggesting that Nrf2 may also play an important role in modulating GSTπ-associated DA neuron vulnerability (Lin et al., 2012).

The nematode *Caenorhabditis elegans* (*C. elegans*) is a robust genetic model to dissect the molecular players involved in DA neuron vulnerability (Nass et al., 2008; Nass and Blakely, 2003). The DA neurons contain the full complement of genes involved in DA neurotransmission and signaling in vertebrates. As the animals are transparent, a transgenic strain expressing green fluorescent protein (GFP) in the 8 DA neurons allows for analysis of neuronal integrity *in vivo* (Nass et al., 2002; Nass and Settivari, 2008). *C. elegans* are also sensitive to a number of PD-associated toxicants, including 6-OHDA, rotenone, and heavy metals, as well as to the human PD-associated protein α-synuclein, and contain homologues to genes involved in cell death pathways (Lakso et al., 2003; Nass and Settivari, 2008; Vanduyn et al., 2013; Ved et al., 2005; Vistbakka et al., 2012).

The development of *C. elegans* models for PD and manganism and the recent discovery that the nematode homologue of Nrf2, SKN-1, is expressed in DA neurons, provide an opportunity to identify mechanisms of Mn-associated DA neuron vulnerability (Oliveira et al., 2010; Park et al., 2009; Settivari et al., 2009; Vanduyn et al., 2010). In this study we asked whether the PD-associated GSTp and its molecular modulators contribute to DA neuron vulnerability to Mn. Here we show that the *C. elegans* SKN-1 regulates DA neuron vulnerability to Mn through the GSTπ homologue GST-1. We also identify upstream and

downstream modulators of toxicant-associated cell death, and identify a novel caspase involved in the toxicant-induced DA neurodegeneration.

2. Materials and Methods

2.1 *C. elegans* strains and maintenance

The following strains were obtained from the *Caenorhabditis* Genetics Center: Wild-type Bristol N2 and RNA mediated interference (RNAi) sensitive NL2099 *rrf-3(pk1426)*. BY250 ($P_{dat\text{-}1}$: :GFP) is an integrated, transgenic line expressing GFP from behind the *dat-1* promoter, and has been previously described (Lakso et al., 2003; Nass et al., 2002; Settivari et al., 2009). BY200 vtIs1 [$P_{dat\text{-}1}$: :GFP; *rol-6(su1006)*], BY215 vtIs1 [P *dat-1*: :GFP; *dat-1(ok157)*; *rol-6(su1006)*] and RJ928 ($P_{dat\text{-}1}$: :GFP; *rrf-3(pk1426)*) have also been previously described (Nass et al., 2002; Settivari et al., 2009). *C. elegans* strains were cultured on OP50 or NA22 bacteria on NGM or 8P media, respectively, at 20°C according to standard methods (Hope, 1999; Brenner, 1974).

2.2 RNA extraction and cDNA synthesis

Total RNA was isolated from a synchronized *C. elegans* population using Trizol reagent largely as previously described (Novillo et al., 2005; Settivari et al., 2009). Briefly, nematode pellets were resuspended in Trizol after treatment with $MnCl_2$ (1 ml/100 µl compact worm pellet). Impurities were separated from nucleic acids using chloroform, and RNA was precipitated with isopropyl alcohol. The RNA pellet was washed with 75% ethanol and dissolved in RNase-free water, and stored at -80°C. RNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA). One microgram of total RNA was reverse transcribed to cDNA using oligo dT primers (Integrated DNA Technologies, Coralville, IA) and the iScript cDNA synthesis kit (Bio Rad, Hercules, CA) following manufacturer's instructions. The cDNA was purified using Microcon YM30 filters (Millipore, Billerica, MA) and quantified using a NanoDrop ND-1000 spectrophotometer.

2.3 qPCR measurements

Gene specific primers were designed using Primer3 software, and the primers were designed to be exon spanning to avoid amplification of contaminating genomic DNA. Glyceraldehyde-3-dehydrogenase (GAPDH) was selected as the housekeeping gene, as its expression did not change as a result of $MnCl_2$ treatment. The following primers were used to determine changes in gene expression of *gst-1* following $MnCl_2$ exposure: *gst-1* F – CAAGGACGTTCTTCCAGGAG, *gst-1* R - CTGGAACACCATCAAGAGCA GAPDH F - GAAACTGCTTCAACGCATCA, GAPDH R - CCTTGGCGACAAGAAGGTAG. Real-time PCR was performed using 2× SYBR Green PCR master mix and the ABI Prism 7500 sequence detection system (Applied Biosystems, Grand Island, NY). Gene expression studies were performed in triplicate and the formation of a single PCR product was confirmed using dissociation curves. Negative controls with the primers consisted of all of the components of PCR mix except cDNA. Relative fold change in gene expression for each gene was calculated using normalized C_T values (the cycle number at which the fluorescence passes the threshold).

2.4 Toxicant exposures

Synchronized L1 stage worms were obtained by hypochlorite treatment of gravid adults followed by incubation of the embryos in M9 buffer for 18 h, and washed at least three times in dH_2O using standard protocols (Nass et al., 2002; Nass and Hamza, 2007; Settivari et al., 2009). For Mn treatment, L1 stage worms (10 worms/µl) were incubated with dH_2O ± 50 mM manganese chloride ($MnCl_2$, Fisher Scientific, Fair Lawn, NJ), as previously

described for 30 min at room temperature (~22°C) with gentle mixing every 10 min (Nass and Hamza, 2007; Settivari et al., 2009). Following treatment, the worms were placed onto NGM/OP50 plates and allowed to recover for 72 h at 20°C. After exposure and recovery, 50-60 worms were immobilized on 2% agarose pads with 2% sodium azide and were scored for DA neurodegeneration under a fluorescence microscope (Leica MZ 16FA, Switzerland). Worms were scored positive for DA neuron degeneration when GFP in any part of the four cephalic dendrites (CEPs, which run from the nerve ring to tip of the nose) was absent (Nass et al., 2002; Settivari et al., 2009). Each of the experiments was performed at least in triplicate, and the results are reported as mean ± S.E.

2.5 Antibodies and Western blot analysis

Antibodies to amino acids 85-184 from the putative *C. elegans* protein GST-1 (WP:CE00302) were generated using Genomic Antibody Technology at Strategic Diagnostics Inc. (SDI, Newark, DE). Rabbit polyclonal antibodies were further purified at SDI. GAPDH (ab36840 Abcam, Cambridge, MA) was used as a loading control. To prepare protein for Western blot analysis, synchronized L1 stage worms were exposed to $MnCl_2$ for 30 min, washed three times with water and allowed to recover on NGM plates at 20°C for 24 h. Following toxicant exposures and recovery, worms were washed from media plates and pelleted. 150 μl of buffer (20 mM HEPES, pH 7.5, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 μg/ml aprotinin) was added to 300-400 μl of pelleted worms and the tubes were frozen at -20°C until protein purification. Nematode samples stored at -20°C were thawed and homogenized on ice using 50-60 strokes with a 2 ml glass homogenizer. The lysate was centrifuged at 400×g at 4°C for 4 min, the supernatant was collected in a sterile tube, and protein concentration was determined using the Bradford assay with bovine gamma globulin as the standard (Bradford, 1976). The samples were diluted in NuPAGE LDS buffer (Invitrogen, Carlsbad, CA), heated at 95°C for 20 min, and total cell lysates (50 μg protein) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes (Bio-Rad, Hercules, CA). Membranes were blocked with 5% non-fat dry milk dissolved in TBST (tris-buffered saline, 0.1% Tween-20) for 2 h at RT, followed by incubation with the appropriate primary antibody dilution (anti-GST-1 at 1:10,000; anti-GAPDH at 1:10,000) at 4°C overnight. The membranes were washed 3 times at RT for 15 min, and incubated with HRP-conjugated secondary anti-rabbit IgG (611-1302 Rockland, Gilbertsville, PA). The membrane was developed using enhanced chemiluminescence (ECL) (Amersham Biosciences, Pittsburgh, PA), captured using Bio-Rad ChemiDoc XRS, and total-protein intensities were measured using QuantityOne software (Bio-Rad, Hercules, CA).

2.6 Immunohistochemistry

Primary *C. elegans* cultures were prepared as previously described, but with slight modifications (Bianchi and Driscoll, 2006, Settivari et al., 2009). Gravid adult worms were lysed with the synchronization solution, and the egg pellet was washed using egg buffer (118 mM NaCl, 48 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 25 mM HEPES). The eggs were separated from the debris using a 60% sucrose solution. The eggshell was digested using 4 mg/ml chitinase (Sigma, St. Louis, MO), embryonic cells were dissociated using syringe aspiration, and cells were resuspended in L-15 medium (containing 10% FBS and 1% pen/strep) and grown on polylysine-coated slides at 20°C. Following growth for 72 h, the cells were fixed in 4% paraformaldehyde, permeabilized in 0.5% Triton X-100, and blocked using 2% BSA and 20% normal goat serum. The cells were then incubated with GST-1 primary antibodies (1:5,000) at 4°C overnight (14 h), followed by incubation with a Texas Red conjugated goat anti-rabbit secondary antibody (Invitrogen, Eugene, OR) (1:5,000) at

RT for 1 h. Images were captured using a Zeiss LSM 510 confocal microscope (Zeiss, Jena, Germany).

2.7 RNA interference

The RNAi sensitive strain RJ928 has been described previously (Settivari et al., 2009; Vanduyn et al., 2010). RNAi studies were performed on NGM plates containing 1 mM isopropyl β-d-thiogalactoside (IPTG) and 100 μg/ml ampicillin and seeded with HT115 (DE3), an RNase III-deficient *E. coli* strain carrying the L4440 vector with the gene fragment (*gst-1, skn-1, wdr-23, jnk-1, ced-3, or csp-1*) (OpenBiosystems, Thermo Scientific, Waltham, MA) or empty vector (Addgene, Cambridge, MA) (Timmons and Fire, 1998). Synchronized L1 stage RJ928 worms were transferred onto RNAi plates and the feeding protocol was followed with modifications (Kamath and Ahringer, 2003). 50 to 100 second generation gravid adults grown on RNAi bacteria were transferred to fresh RNAi media plates and allowed to lay eggs for 5 h. Adults were then removed from the plate. L1s hatched from the eggs (3^{rd} generation) were exposed to Mn as described above. For *skn-1,* RNAi is embryonic lethal, therefore first generation L1s were exposed to the toxicants (Vanduyn et al., 2010). The animals were allowed to recover on the RNAi plates for 72 h, and evaluated as previously described (Nass et al., 2002; Settivari et al., 2009;Vanduyn et al., 2010).

2.8 Statistical analysis

Data in all graphs is presented as the mean ± SEM. GraphPad Prism software (GraphPad Software, San Diego, CA) was used for statistical analysis. A *t-test* was used to compare the difference between two groups. One-way ANOVA followed by a Bonferroni post hoc test was used for the comparison of multiple groups. For experiments involving RNAi and toxicant exposure, two-way ANOVA analysis with a Bonferroni post hoc test was performed. Differences were considered significant when $p < 0.05$, or lower where indicated.

3. Results

3.1 Manganese induces GST-1 gene and protein expression

Manganese is an environmental toxicant that has been positively correlated with the development of IPD (Hudnell, 1999; Gorell et al., 1999; Racette et al., 2001; Roth, 2009; Tanner et al., 2011; Martin, 2011). Epidemiological and neuropathological studies implicate differential expression of the inducible phase II detoxification enzyme GSTπ as a possible contributor to the development of IPD (Shi et al., 2009; Vilar et al., 2007). A BLAST search with human GSTπ (accession number: AAH10915.1) and the *C. elegans* genome indicates that the nematode GST-1 is the most homologous protein. A sequence alignment of the results using ClustalW2 indicates that the *C. elegans* GST-1 is highly conserved with its human counterpart (58% similar, 39% identical), and contains the 11 residues that are involved in the formation of the glutathione binding site as well as the 8 conserved C-terminal amino acids that are characteristic of the pi class GSTs (data not shown). The alignment suggests that GST-1 may be a functional GSTπ homologue, and its expression may be induced upon xenobiotic exposure.

Mn exposures have been used to model manganism and PD in vertebrates, *C. elegans,* and cell culture (Settivari et al., 2009; Shi et al., 2009, Sanchez-Betancourt et al., 2012; Ved et al., 2005). In order to determine if GST-1 gene expression is induced upon exposure to the toxicants, we utilized RT-PCR to determine levels of gene expression in controls and in worms exposed to sub-lethal concentrations of Mn as previously described (Nass and Hamza, 2007; Settivari et al., 2009). Young nematodes exposed to $MnCl_2$ for 30 min show a

3-fold increase in GST-1 mRNA levels (Fig. 1A). These results suggest that GST-1 protein levels may also be induced following exposure to Mn. In order to determine if Mn can induce GST-1 protein expression, we generated antibodies to the putative GST. The antigenic sequence is unique to GST-1 and spans from amino acid 85 to 184. As can be seen in Fig. 1B, exposure to Mn results in a 3-4 fold increase in GST-1 protein levels. These results indicate that GST-1 protein levels are sensitive to Mn, and suggest that the enzyme may play a role in modulating DA neuron sensitivity to the metal.

3.2 GST-1 is expressed in DA neurons

Vertebrate studies indicate that GSTπ is expressed in DA neurons and can affect DA neuron sensitivity to toxicants (Smeyne et al., 2007). To determine whether GST-1 is expressed in *C. elegans* DA neurons, we generated primary cultures from RJ928 animals fed RNAi bacteria containing the empty vector (WT) or RNAi bacteria targeting *gst-1* to decrease its expression (*gst-1*$_{\mathrm{RNAi}}$) (Settivari et al., 2009). The DA neurons are easily identified under a fluorescent dissecting scope, as GFP is expressed at high levels in the neurons both *in vivo* and *in vitro* (Settivari et al., 2009; Vanduyn et al., 2010). The morphology of the DA neurons in animals in which GST-1 expression is reduced appears identical to WT, suggesting that GST-1 does not play a significant role in maintaining DA neuron integrity (data not shown). We used affinity-purified anti-GST-1 to evaluate the GST-1 expression levels. GST-1 immunoreactivity is observed in all DA neurons, as well as in other cell types (Fig. 2A-D; data not shown). There is no apparent staining in DA primary neurons or other cells exposed to *gst-1* RNAi, suggesting that the RNAi significantly reduces GST-1 protein expression (Fig. 2E-H). These results indicate that GST-1 is expressed in DA neurons, and suggest that the antibody is likely specific for this GST.

3.3 GST-1 inhibits toxicant-induced DA neuron degeneration

Compounds that generate reactive oxygen species (ROS) and confer oxidative stress have been shown to contribute to DA neuron vulnerability (Bove et al., 2005; Sherer et al., 2003). GSTπ has been shown to protect against oxidative stress (Goto et al., 2009). We have previously demonstrated that a brief exposure of *C. elegans* to Mn can increase oxidative stress and lead to DA neuronal death (Settivari et al., 2009). In order to determine if GST-1 may inhibit Mn-associated DA neuropathology, we exposed WT or *gst-1*$_{\mathrm{RNAi}}$ transgenic worms expressing GFP in the eight DA neurons to the toxicant (Nass et al., 2002; Settivari et al., 2009). An acute low concentration exposure to Mn results in approximately 15% of the WT animals displaying significant DA neurodegeneration (Fig. 3A, B). The Mn-induced DA neurodegeneration that we observe is similar to our prior studies in which we characterized Mn- and 6-OHDA-induced DA neurodegeneration by loss of dendritic GFP and loss of neuronal integrity by electron microscopy (Nass et al., 2002; Settivari et al., 2009). As described earlier, only the CEP processes that cannot be visually followed from the cell body to the tip of the nose are considered to have DA neuron degeneration (Nass et al., 2002). Following genetic knockdown of GST-1, we found that Mn exposure resulted in degeneration in an additional 10% of nematodes (Fig. 3C). These results show that Mn exposures cause DA neuronal death, and that expression of GST-1 inhibits the toxicant-associated pathology.

3.4 SKN-1 modulates GST-1 protein expression

SKN-1, the homologue to the vertebrate PD-associated transcription factor Nrf2, can regulate antioxidant and detoxification enzymes including PD-associated proteins and GSTs. We have recently shown that SKN-1 is expressed in *C. elegans* DA neurons (Vanduyn et al., 2010). Analysis of the sequence 5′ of the GST-1 start codon suggests that there is a SKN-1 binding site approximately 230 bases upstream from the ATG in the promoter region that may be involved in GST-1 regulation (data not shown). In order to determine if GST-1

protein expression may be dependent on SKN-1, we knocked down SKN-1 gene expression by RNAi and examined GST-1 protein levels by Western blot analysis. As can be seen in Fig. 4A, a reduction in SKN-1 gene expression resulted in approximately 2-fold reduction of GST-1 protein levels. These results indicate that SKN-1 can regulate GST-1, and suggest that the transcription factor could play a role in GST-1-associated neuroprotection following toxicant exposure.

3.5 SKN-1 inhibits Mn-induced DA neuron degeneration and contributes to GST-1-associated neuroprotection

We have previously shown that the PD-associated transcription factor SKN-1 can inhibit MeHg-associated DA neuron degeneration (Vanduyn et al., 2010). It has previously been demonstrated that SKN-1 lowers the incidence of *C. elegans* death in presence of high Mn concentrations, but to date SKN-1 has not been shown to play a role in Mn-induced DA neuroprotection (Benedetto et al., 2010). We therefore asked whether SKN-1 may inhibit Mn-associated DA neuronal death following toxicant exposure. As can be seen in Fig. 4B, genetic knockdown of SKN-1 alone significantly increases DA neuronal death following exposure to Mn, indicating that the loss of SKN-1 increases DA neuron vulnerability to this metal. Since GST-1 protein expression is partially dependent on SKN-1, we asked whether SKN-1 and GST-1 could function in the same molecular pathway to inhibit toxicant-associated DA neuron degeneration. The double knockdown of *skn-1/gst-1* demonstrates similar levels of DA neuronal death, approximately 30%, as the RNAi of each of the single genes following Mn exposure (Fig. 4B). These results are consistent with SKN-1 and GST-1 functioning via a similar molecular pathway. The WD40 repeat protein WDR-23 negatively regulates SKN-1-associated transcription, and can also play a significant role in the response of cells to stress (Choe et al., 2009). To determine if WDR-23 may contribute to the vulnerability of DA neurons to Mn, we knocked down WDR-23 gene expression using RNAi. Consistent with WDR-23 negatively regulating SKN-1, we find a significant increase in DA neuron viability following WDR-23 genetic knockdown and Mn exposure (Fig. 4C). Furthermore, there is very little difference in DA neuron degeneration between WT and *wdr-23/gst-1* double knockdown, suggesting that the neuroprotection conferred by WDR-23 may also largely function through a SKN-1-associated pathway. Taken together, these results indicate that DA neuron vulnerability to Mn is dependent on SKN-1 and GST-1 expression that may function in overlapping molecular pathways.

3.6 Mn-induced DA neurodegeneration is not dependent on DAT-1

Mn-induced neurotoxicity is largely dependent on the transport of Mn into cells through the divalent metal transporter, DMT-1 (Liu and Culotta, 1999; Culotta et al., 2005; Supek et al., 1996). The Mn-induced DA neurotoxicity in vertebrates has been demonstrated to be independent of the expression of the dopamine transporter DAT (Hirata et al., 2008; Roth et al., 2013). Studies in *C. elegans* also indicate that the primary mechanism of Mn-induced DA neurotoxicity is dependent on the *C. elegans* orthologue of DMT-1, SMF-1 (Settivari et al., 2009), although a recent nematode study suggests Mn-induced DA neurodegeneration is dependent on the nematode orthologue of DAT, DAT-1 (Benedetto et al., 2010). In order to determine if DAT-1 contributes to Mn-induced DA neuron degeneration in the nematode, we exposed WT animals and animals containing a deletion of *dat-1* to 50 mM Mn, and examined DA neuron integrity 72 hours later. We also exposed both animals to the DA neurotoxin 6-OHDA as a control, as DA neurons lacking DAT-1 are resistant to 6-OHDA induced DA neurodegeneration (Nass et al., 2002; Nass and Blakely, 2003). As can be seen in Fig. 5A, there is no difference in the extent of Mn-induced DA neurodegeneration between WT and *dat-1* knockout animals. Importantly, approximately 60% of WT animals exposed to 6-OHDA display DA neuron degeneration, while none of the *dat-1* knockout animals display neurodegeneration, consistent with the complete loss of DAT-1. These

results indicate that *dat-1* knockout DA neurons are not more resistant to Mn-induced DA neuron degeneration relative to WT animals. In order to further elucidate whether DAT may play a role in Mn-induced DA neuron pathology, we utilized RNAi to knockdown *dat-1* and/or *smf-1* expression in animals sensitive to RNAi. As can be seen in Fig. 5B, there is no difference in Mn-induced DA neurodegeneration between WT and *dat-1*$_{RNAi}$ animals, or between animals in which the primary DA neuron Mn transporter, SMF-1, is knocked down and *smf-1*/*dat-1* double knockdown. Furthermore as reported earlier, the loss of SMF-1 results in a significant reduction of Mn-induced DA neuronal death, indicating that Mn-induced DA neuron degeneration is dependent on SMF-1 expression (Settivari et al., 2009). Exposure to 6-OHDA as a control in the *dat-1* RNAi experiment suggests that the vast majority of DAT-1 expression is reduced. These studies strongly indicate that Mn-induced DA neuron degeneration is independent of DAT-1 expression.

3.7 JNK-1 contributes to toxicant-associated DA neuron degeneration

Under non-stress conditions in vertebrates, GSTπ inhibits the activation of JNK by binding JNK in a protein complex with c-Jun (Wang et al., 2001). Under oxidative stress, GSTπ dissociates from JNK allowing JNK to participate in downstream events that include apoptotic signaling and cell death (Ruscoe et al., 2001). To determine if the *C. elegans* JNK homologue, JNK-1, may play a role in cell death following Mn exposure, we knocked down *jnk-1* gene expression using RNAi and exposed the animals to Mn as described above. As can be seen in Fig. 6A, genetic knockdown of *jnk-1* results in significant inhibition of DA neuronal death following exposure to Mn, consistent with JNK's role in apoptotic signaling. The double knockdown of *gst-1* and *jnk-1* shows a significant yet small difference in the percentage of animals demonstrating DA neuron pathology relative to the *jnk-1*$_{RNAi}$ exposed animals, suggesting that JNK-1 and GST-1 may largely function in the same signaling pathway. Taken together, these results indicate that JNK-1 contributes to Mn-induced DA neuron pathology, and suggest that the toxicants may be precipitating cell death through apoptosis.

3.8 Mn-induced DA neuron degeneration is dependent on the caspases CED-3 and CSP-1

The mechanisms of cell death in PD and manganism remain elusive. Apoptosis has been implicated in both disorders, although the role of classical apoptosis is controversial and not well defined, as recent studies suggest that several types of programmed cell death may contribute to DA neuron degeneration (Venderova and Park, 2012). In order to determine if the canonical programmed cell death/apoptotic pathway in *C. elegans* contributes to Mn-induced DA neuron toxicity, we examined DA neurodegeneration in *ced-3* deficient backgrounds (Nass et al., 2002). Animals in which CED-3 expression is reduced have 50% less DA neuron degeneration following Mn exposure relative to WT (Fig. 6B). These results suggest that Mn activates the classical apoptotic pathway to confer DA neuron degeneration, and that another cell death pathway may also be contributing to the toxicant-associated DA neurodegeneration.

C. elegans contains several other putative caspase genes that may be involved in the activation of *ced-3*, or in an independent cell death pathway (Shaham, 1998). Three caspase-like genes, *csp-1, csp-2,* and *csp-3*, have been shown to encode a total of at least 7 RNA transcripts, although only the CSP-1 splice variant CSP-1B has been shown to be an active protease (Shaham, 1998). Recently CSP-1 has been shown to be a pro-apotic caspase involved in programmed cell death in embryogenesis (Denning et al., 2013). In order to determine if CSP-1 may contribute to Mn-associated DA neuron degeneration, we evaluated the DA neuronal death following genetic knockdown of *csp-1* and exposure to Mn. Mn-induced DA neuron death is inhibited by approximately 50% in *csp-1* genetic knockdown

animals (Fig. 6B). Taken together, these results indicate that both caspase CED-3 and CSP-1 play a role in Mn-associated DA neuron vulnerability.

4. Discussion

Mn-induced neurotoxicity resembles a number of aspects of IPD, including tremors, bradykinesia, and rigidity (Burton and Guilarte, 2009). As in IPD, high Mn exposures result in mitochondrial dysfunction, increases in ROS levels and oxidative stress, and DA neurodegeneration. Epidemiological evidence also suggests that there may be a correlation between Mn exposure and the development of PD (Gorelle, et al., 1999; Verina et al., 2013). The similarity between manganism, and PD suggests that there may be common modalities in the underlying pathophysiology, although the molecular determinants involved in both disorders have largely remained elusive. (Sherer et al., 2003, Tanner et al., 2011). A contributing factor to the sensitivity of DA neurons to PD-associated neurotoxicants may be the expression or activity of GSTπ. GSTπ enzymes conjugate endogenous and exogenous electrophiles, including pesticides, with glutathione to reduce ROS, oxidative stress, and cellular toxicity. GSTπ polymorphisms have been shown to be a risk factor for the development of PD, and the PD-associated risk can be exacerbated by exposure to herbicides (Menegon et al., 1998; Wilk et al., 2006; Sherer et al., 2003; Tanner et al., 2011). In this study, we show that the *C. elegans* GSTπ homologue GST-1 is expressed in DA neurons, Mn exposures induce GST-1 gene and protein expression, and the loss of SKN-1 or GST-1 gene expression results in an increase in DA neurodegeneration. This study also shows for the first time that Mn-induced DA neuron degeneration is associated with GSTπ gene expression, and further supports a possible common genetic linkage between manganism and IPD.

In vertebrates, the basic leucine zipper class transcription factor Nrf2 regulates the expression of antioxidant genes, and is negatively regulated by Keap1 (kelch-like ECH-associated protein 1) (Nguyen et al., 2009). Under non-oxidative stress conditions, Keap1 physically interacts with Nrf2 and promotes Nrf2 ubiquitination that targets the transcription factor for proteosomal degradation (Itoh et al., 1999; Kobayashi et al., 2004; Nguyen et al., 2009). Under conditions of oxidative stress, the interaction of Keap1 with Nrf2 is decreased, resulting in decreased ubiquitination and an increase in Nrf2 binding to antioxidant response elements (AREs) in the promoters of cytoprotective genes. Nrf2 regulates a number of phase II detoxification enzymes including GSTs, and Nrf2 deficient vertebrate cells are sensitive to PD-associated toxicants (Kobayashi et al., 2009; Lee et al., 2003; Toyama et al., 2007). SKN-1 is the *C. elegans* homologue to Nrf2, and although there does not appear to be a nematode homologue to Keap1 based on genetic sequence, the WD40 repeat containing protein WDR-23 appears to be a functional homologue, as it physically interacts with and negatively regulates SKN-1, likely through a ubiquitination-associated pathway, and plays a role in stress resistance and longevity (Choe et al., 2009). We have recently shown that SKN-1 is expressed in the *C. elegans* DA neurons and can modulate expression of at least two other GSTs (Vanduyn et al., 2010). Chromatin immunoprecipitation binding studies indicate that SKN-1 binds in the putative promoter of GST-1, consistent with the binding site predicted by the consensus sequence (An and Blackwell, 2003). Our current studies show that constitutive expression of GST-1 is partially dependent on SKN-1 expression, and the loss of SKN-1 expression results in increase sensitivity of DA neurons to Mn. This study shows for the first time that Mn-associated

DA neuron pathology is associated with SKN-1/Nrf-2 expression. Consistent with the role of SKN-1 in DA neuroprotection, the loss of the SKN-1 inhibitor WDR-23 renders the neurons significantly more resistant to Mn. Furthermore, genetic repression of both GST-1 and WDR-23 renders the DA neurons in the double knockdown similarly vulnerable to

exposures as WT, suggesting that the role of WDR-23 in the stress response may be at least partially through a GST-1-associated pathway. The small but significant increase in resistance relative to WT following Mn exposure in the double knockdown suggests that WDR-23 may contribute to the DA neuroprotection also through a GST-1-independent pathway, possibly through a reduction in target protein ubiquitination (Kobayashi et al., 2009; Nguyen et al., 2009).

Our studies show that Mn-induced DA neurodegeneration is not dependent on DAT-1. This is in contrast to Aschner and colleagues, whose studies indicate that DAT-1 is required for Mn-induced DA neuronal death. The reasons for the different results from the studies are not clear. It is possible that the genetic background of the *dat-1* knockout strain used in Benedetto et al. 2010 was different from the WT strain; the full genotype of the *dat-1* knockout strain used in their experiments was not reported (Benedetto et al., 2010). Also, the genotype of the WT strain (BY200) used in their study was reported incorrectly (see Nass et al., PNAS, 2002, for details on the genotype), therefore it is not clear which WT strain may have been used in discerning the role of DAT in Mn-induced toxicity. In the current study, we compare Mn-induced DA neurodegeneration in a WT strain (BY200) and a strain with a deletion in *dat-1* (BY215) from identical genetic backgrounds (each contain the selection marker *rol-6*), and find that DAT-1 does not contribute to Mn-induced DA neuron vulnerability (Nass et al., 2002). We also show that a genetic knockdown of *dat-1* does not alter Mn-induced DA neuron sensitivity relative to WT animals in a similar genetic background. Taken together, these results are consistent with vertebrate studies that demonstrate that DAT does not contribute to Mn-induced DA neuron toxicity.

JNKs are a class of MAPKs that are activated by endogenous or environmental stressors and play a role in proapoptotic signaling (Kanda and Miura, 2004; Liu and Lin, 2005; Lu et al., 2007). GSTπ enzymes have been shown to negatively regulate kinase pathways by directly binding to the C-terminus of JNK and inhibiting JNK activation and the subsequent apoptosis (Wang et al., 2001; Thevenin et al., 2011). Our findings are consistent with JNK playing a role in apoptosis, as genetic knockdown of JNK results in increase in DA neuron viability following Mn exposure. Conversely, genetic knockdown of *gst-1* alone results in an increase in DA neuronal death, consistent with a loss of JNK inhibition and an increase in apoptosis.

The two types of vertebrate caspases involved in apoptosis are initiator caspases and effector caspases. Initiator caspases (caspase-8, caspase-9, and caspase-10) cleave inactive pro-forms of effector caspases (caspase-3, caspase-6, and caspase-7), resulting in activation of the effector caspase and initiation of the apoptotic cascade leading to cell death (Bratton and Salvesen, 2010; Venderova and Park, 2012). In *C. elegans*, one caspase has been shown to function as a positive regulator of apoptosis *in vivo*. CED-3 is the core cell-death effector (homologous to caspase-3) that acts both in programmed cell death and as recently shown, in neuronal regeneration (Pinan-Lucarre et al., 2012; Yuan et al., 1993). Our studies show that CED-3 contributes to Mn-induced DA neuronal death. The nematode also contains three proteins that have some homology to vertebrate caspases CSP-1-3 (Shaham, 1998). Considering that CSP-2 has a highly divergent consensus caspase sequence and CSP-3 lacks a middle caspase domain, these proteins are unlikely to function as active caspases. In addition, both proteins have been shown to be negative regulators of CED-3 (Brady and Duckett, 2009; Geng et al., 2008; Geng et al., 2009). CSP-1 is highly conserved with vertebrate caspase proproteins, and *in vitro* studies show that activated CSP-1 can cleave the CED-3 protein, and activated CED-3 and CSP-1 have different substrate specificities (Shaham, 1998). Furthermore, CSP-1 has recently been shown to promote programmed cell death during *C. elegans* embryogenesis (Denning et al., 2013). Here we provide the first evidence that CSP-1 contributes to neuronal vulnerability to a toxicant. CSP-1 may act

upstream of CED-3 as an initiator caspase *in vivo*, or cleave other substrates downstream to initiate apoptosis and cell death (Shaham, 1998). Considering that apoptosis plays a significant role in PD-associated DA neurodegeneration, yet the precise molecular pathways in the neuropathology are ill-defined, the identification of CSP-1's role in Mn-associated DA neuron pathology may provide new insights into the molecular basis of DA neuron vulnerability.

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Abbreviations used

DA	dopamine
ECL	enhanced chemiluminescence

GFP	green fluorescent protein
PAGE	polyacrylamide gel electrophoresis
PD	Parkinson's disease
ROS	reactive oxygen species
SN	substantia nigra
TH	tyrosine hydroxylase
WT	wild type

Highlights

1. GST-pi expression in *C. elegans* DA neurons inhibits Mn-associated DA neuron pathology
2. A PD-associated transcription factor and molecular modulators affect DA neuron vulnerability to Mn
3. Dopamine transporter does not play a role in Mn-associated DA neurodegeneration
4. Identifies apoptosis-associated caspases involved in Mn-induced DA neurodegeneration

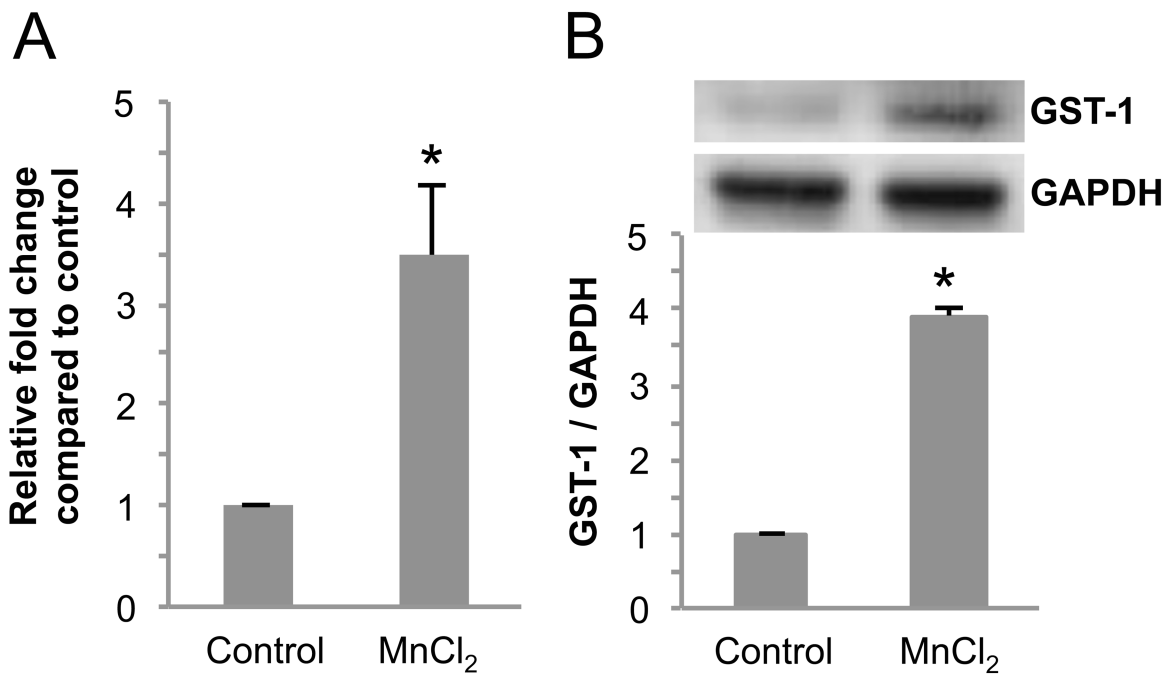

Fig 1. Exposure to Mn induces GST-1 gene and protein expression

(A) Synchronized L1 stage WT *C. elegans* were exposed to 50 mM $MnCl_2$ for 30 mins, mRNA was extracted and reverse transcribed to cDNA. Relative gene expression changes of *gst-1* were quantitated using real-time PCR. The fold change in gene expression relative to GAPDH was calculated following the ΔΔCt method. Shown are mean ± S.E. of three individual replicates. *p* value was calculated using t-test analysis. Asterisk indicates $p \leq 0.04$ between control and toxicant-exposed group ΔCt values. (B) Synchronized L1 stage WT nematodes were exposed to 50 mM $MnCl_2$ for 30 mins and allowed to recover for 24 h on NGM plates at 20°C. Following recovery/exposure, the worms were collected, homogenized and protein was quantified following standard a Bradford assay. For Western blot analysis, protein samples were separated by electrophoresis, transferred to a membrane, and probed with anti-GST-1 or GAPDH primary antibodies. Shown are mean ± SE of at least three individual replicates. *p* values were calculated using t-test analysis. Asterisks indicate $p \leq 0.03$ between controls and Mn-exposed groups.

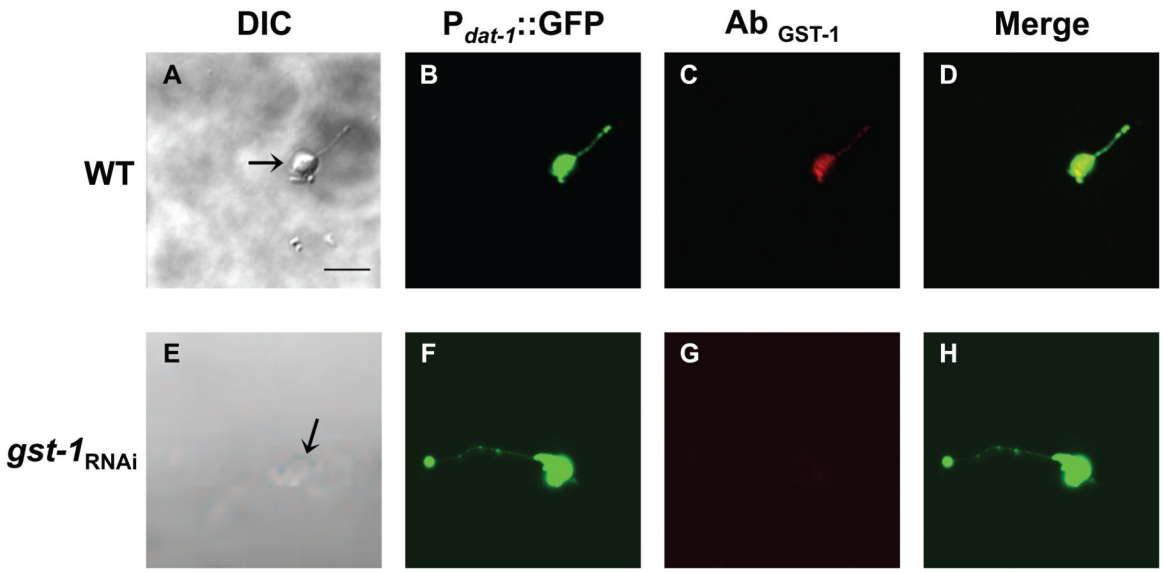

Fig 2. GST-1 is expressed in DA neurons

Primary *C. elegans* cultures expressing GFP in the dopamine neurons were generated with WT (A-D) or *gst-1* knockdown animals (E-H). Primary cultures were incubated with GST-1 primary antibody followed by incubation with Texas Red conjugated goat-anti-rabbit secondary antibody. DIC images (A) and (E) of WT and *gst-1*$_{\text{RNAi}}$ cultures, respectively. DA neurons from WT and *gst-1*$_{\text{RNAi}}$ animals expressing GFP driven by the *dat-1* promoter (B) and (F), respectively. GST-1 is expressed in DA neurons in WT animals (C), but not in *gst-1*$_{\text{RNAi}}$ (G). (D) Overlay of (B-C) and (H) overlay of (F-G). Images were observed under a Zeiss confocal microscope (Zeiss LSM 510). Scale bar represents 5 μm.

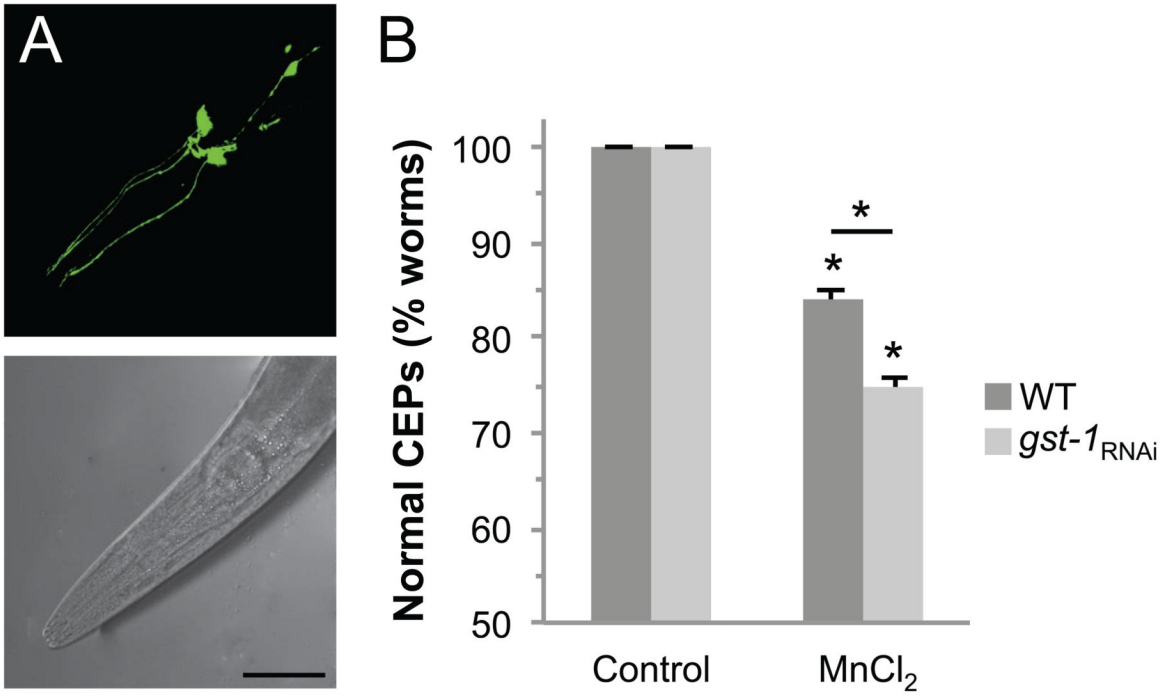

Fig 3. GST-1 inhibits dopamine neuron degeneration

Third generation L1 stage RJ928 animals were exposed to 50 mM $MnCl_2$ for 30 mins and the dopamine neurons were visualized after a 72 h recovery on control or *gst-1* RNAi plates at 20°C. DA neurons were considered degenerated when a break in or complete loss of GFP expression was observed in the CEPs (dendritic processes ending at the tip of the nose). An unexposed (normal) nematode has 4 complete CEP processes (A). Mn induces DA neuron degeneration (B) (arrows indicate the location of missing process), scale bar is 50 μm. Quantification of DA neuron degeneration is expressed as the percentage of worms with normal CEPs after exposure to $MnCl_2$ in WT and *gst-1*$_{RNAi}$ animals (C). Shown are mean values ± SE of at least three individual replicates. Two-way ANOVA analysis was used and an asterisks indicates $p \leq 0.001$. For both genotypes, Mn exposure significantly decreased the % normal CEPs, and the difference between WT and *gst-1*$_{RNAi}$ Mn exposed groups is significant.

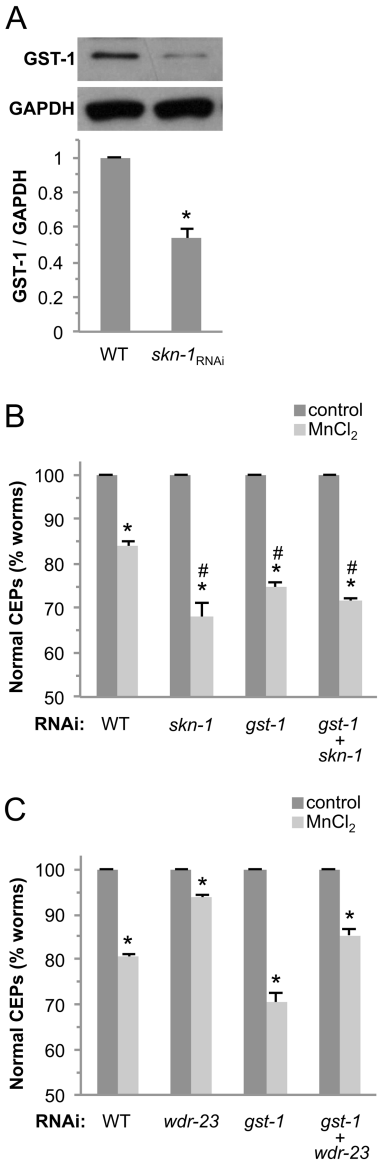

Fig 4. GST-1 requires SKN-1 for inhibition of toxicant-associated DA neurodegeneration

(A) GST- 1 expression is partially dependent on SKN-1. Synchronized RJ928 animals were placed on RNAi plates spread with *skn-1* RNAi bacteria. After 48 h, the worms were collected, homogenized and total protein was quantified following the standard Bradford assay. Protein samples were separated by electrophoresis, transferred to a membrane, and probed with anti-GST-1 or GAPDH primary antibodies. Band intensity was quantified relative to GAPDH expression and normalized to WT. *p* value was calculated with a t-test and the asterisk indicates $p \leq 0.0001$ between WT and *skn-1*$_{RNAi}$. *skn-1*$_{RNAi}$ results in increased DA neuron sensitivity to Mn (B), and *wdr-23*$_{RNAi}$ decreases sensitivity (C). L1 stage RJ928 worms were exposed to 50 mM $MnCl_2$ for 30 mins with a 72 h recovery (B and C) on RNAi plates as indicated, and DA neurons were visualized for degeneration. Shown are mean values ± SE of at least three individual replicates. *p* values were calculated using two-way ANOVA analysis. Asterisks indicate $p \leq 0.01$ between control and toxicant exposed groups within each genotype. Number sign indicates $p \leq 0.01$ between toxicant-

exposed WT and gene knockdown groups (B). In C, comparisons between all toxicant-exposed groups are statistically different with $p \leq 0.01$.

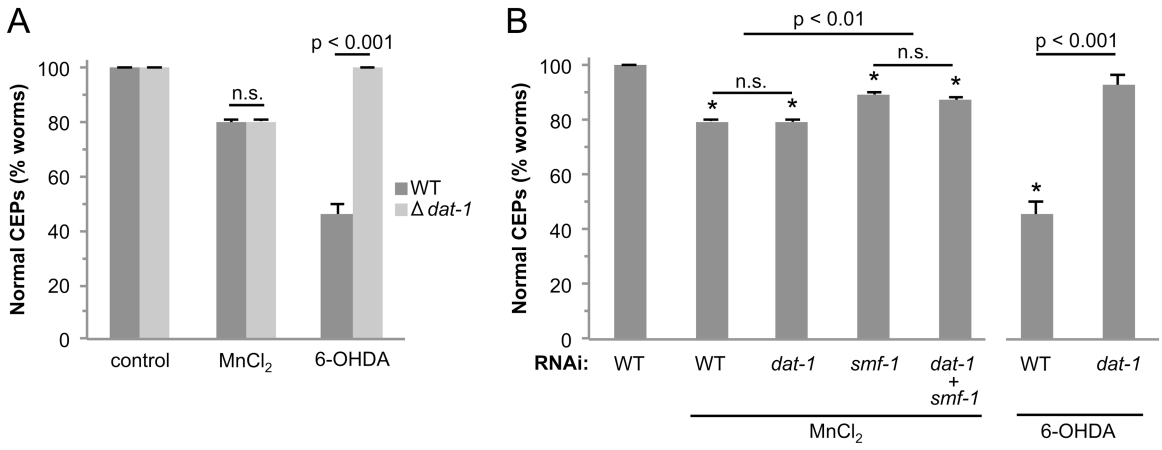

Fig 5. Mn-induced cell death is not dependent on *dat-1*

WT (BY200) and *dat-1* knockout (BY215) synchronized L1 worms (A) or third generation RJ928 nematodes grown on *dat-1* or *smf-1* RNAi or the combination (B) were exposed to 50 mM $MnCl_2$ or 1 mM 6-OHDA for 30 mins and allowed to recover on NGM plates (A) or plates with RNAi bacteria (B) for 72 h. DA neurons expressing GFP were visualized under a fluorescent microscope following the exposure or recovery (> 50 worms/condition). Shown are mean ± SE of at least three individual replicates. Two-way ANOVA analysis indicates a significant difference between WT and dat-1 genetic knockout animals exposed to 6-OHDA (A). Oneway ANOVA was performed in (B). Asterisks indicate $p \leq 0.01$ for all Mn-exposed groups compared to untreated. Within the Mn-treated groups, WT and *dat-1*$_{\text{RNAi}}$ are significantly different from *smf-1*$_{\text{RNAi}}$ and *dat-1/smf-1*$_{\text{RNAi}}$. *A* t-test was used to compare 6-OHDA-treated WT animals vs *dat-1*$_{\text{RNAi}}$.

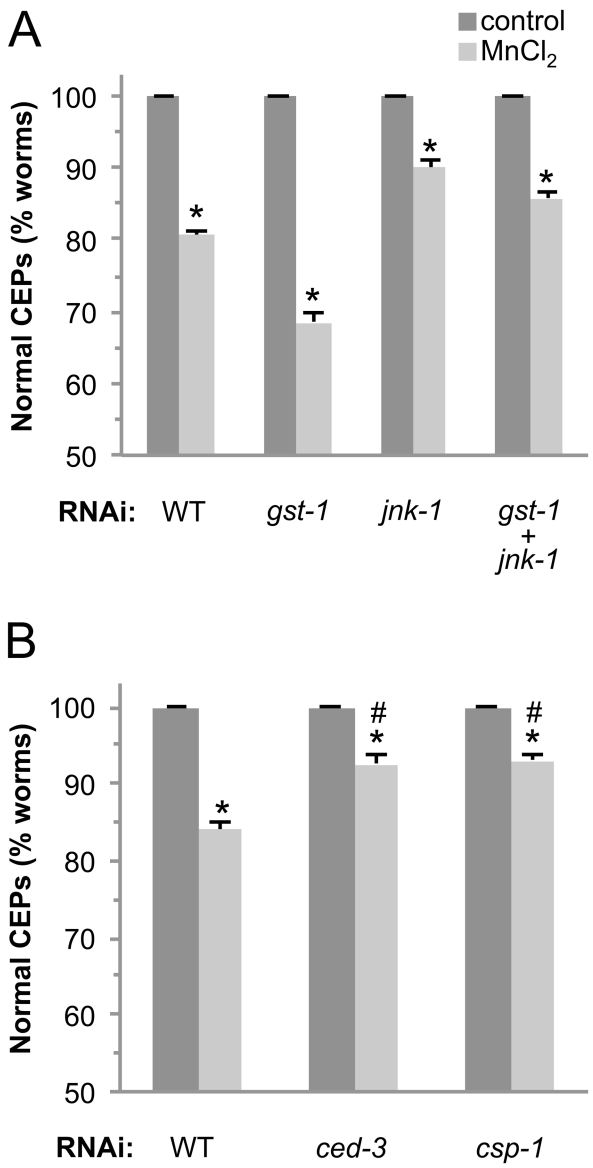

Fig 6. Mn-induced cell death is dependent on apoptosis

Third generation RJ928 nematodes grown on *gst-1* or *jnk-1* RNAi or the combination (A) or *ced-3* or *csp-1* RNAi (B) were exposed to 50 mM $MnCl_2$ for 30 mins and allowed to recover on plates with RNAi bacteria for 72 h. DA neurons expressing GFP were visualized under a fluorescent microscope following exposure and recovery (> 50 worms/condition). Shown are mean ± SE of at least three individual replicates. *p* values were calculated using two-way ANOVA. Asterisks indicate $p \leq 0.01$ between control and toxicant-exposed groups. Comparisons between all Mn-treated groups are significant with $p \leq 0.001$ (A) or $p \leq 0.01$ (B).