CHARACTERIZATION OF NEUROTROPHIC FACTORS IN *C. ELEGANS*

CHARACTERIZING THE EXPRESSION AND FUNCTION OF MESENCEPHALIC ASTROCYTE-DERIVED NEUROTROPHIC FACTOR IN *CAENORHABDITIS ELEGANS*

By

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Abstract

Neurotrophic factors are proteins involved in the maturation, differentiation and survival of neurons. Due to their neuroprotective properties, they have been regarded as potent candidates for the treatment of neurodegenerative diseases. Recently, a novel family of neurotrophic factors was discovered comprising mesencephalic astrocyte-derived neurotrophic factor (MANF) and cerebral dopamine neurotrophic factor (CDNF). These factors have been shown to protect against the degeneration of nigrostriatal dopaminergic neurons in mammalian models of Parkinson's disease, however their neuroprotective mechanisms of action are not yet understood. Although distinct in vertebrates, MANF and CDNF constitute a single homolog in invertebrates. In the present study, we have characterized the *in vivo* expression and function of the *C. elegans* homolog *manf-1*. We have shown that *manf-1* is not essential for neuronal development, however when knocked down, mutants exhibit enhanced age-related dopaminergic neuronal degeneration accompanied by an increase in the endogenous ER stress response. Loss of *manf-1* function also results in enhanced alpha-synuclein expression and aggregation, a pathological hallmark of Parkinson’s disease.

Acknowledgements

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I am extremely appreciative to my colleagues in the Gupta Lab, both past and present, for their help, encouragement and friendships. You helped blur the lines between work and play, and made every day enjoyable…even during the most arduous experiments.

This thesis is dedicated to my family. You always push me to pursue what I love, and my appreciation is beyond words.

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List of Abbreviations

α-syn – Alpha-synuclein
CDNF – Cerebral dopamine neurotrophic factor
ER – Endoplasmic reticulum
ERSE – ER stress response element
GFP – Green fluorescent protein
HSP – Heat shock protein
L1 to L4 – Larval stages
MANF – Mesencephalic astrocyte-derived neurotrophic factor
NTF – Neurotrophic factor
PCR – Polymerase chain reaction
PDI – Protein disulfide isomerase
PD – Parkinson's disease
qRT-PCR – Quantitative real time polymerase chain reaction
ROS – Reactive oxygen species
SAP – SAF-A/B, Acinus, and PIAS
UPR – Unfolded protein response
UTR – Untranslated region
WT – Wild type
YFP – Yellow fluorescent protein

Declaration of Academic Achievement

Chapter 2: *C. elegans* mesencephalic astrocyte-derived neurotrophic factor homolog protects dopaminergic neurons and regulates the ER stress response

Contributions:
I performed most of the experiments for this paper including the genetic confirmation of the *manf-1* deletion strain (*tm3630)* and qRT-PCR, assessment of the dopaminergic, GABAergic and serotonergic neurons, growth delay and brood size assays, dopaminergic age dependent degeneration (replicated by Sabih Rashid), quantification of ER and mitochondrial stress reporter lines, analysis of the *manf-1* transcriptional reporter line and quantification of alpha-synuclein inclusions in the α-syn-YFP chimeric strains. Additionally, I generated all transgenic lines required for this study along with all figures.

Chemotaxis assay was done by Anika Gupta (Figure 3A). Lifespan assay was conducted by Tina Chatterjee (Figure 3C). Sabih Rashid performed the paraquat and valproic acid neurodegenerative assays (Figure 6). Endogenous expression of *manf*-1 in aging wild type animals was performed by Shreya Prashar (Figure 5A).

Chapter 1: Introduction

Neurotrophic Factors: An Overview

Neurotrophic factors (NTFs) are secreted proteins capable of mediating a variety of signaling responses in neurons affecting growth, survival, development, differentiation, neurogenesis, and synaptic plasticity. The guided formation of synaptic contacts is mediated by a variety of cell-intrinsic and extrinsic factors. NTFs constitute an important group of extrinsic signaling peptides that regulate neuronal survival at the time of synapse formation. The target tissues of innervating neurons are the source of these key factors. During embryogenesis, neurons are generated in excess. As such, many are pruned throughout development by undergoing programmed cell death. The selective survival of target-innervating neurons is a well-established concept that arose with the discovery of nerve growth factor (NGF) (Levi-Montalcini & Angeletti, 1968). It is known as the neurotrophic factor hypothesis, which stipulates that the target of innervation of a particular set of neurons secretes a small amount of an essential nutrient (or trophic factor), which is taken up by neurons within close proximity, thus permitting their survival. It is a competitive process whereby only those neurons that are closest to the target tissue and are able to access the NTFs survive. As such, the targets' area of release influences the size of the neuronal populations that innervate them. It has been well established that NTFs can be secreted by multiple sources aside from the target tissue itself (Davies, 2003). NTFs are endocytosed by proximal synaptic terminals and retrogradely transported to the soma to initiate pro-survival signaling pathways that inhibit apoptosis. Such regulation is carried out in both the peripheral and central nervous

systems (Figure 1).

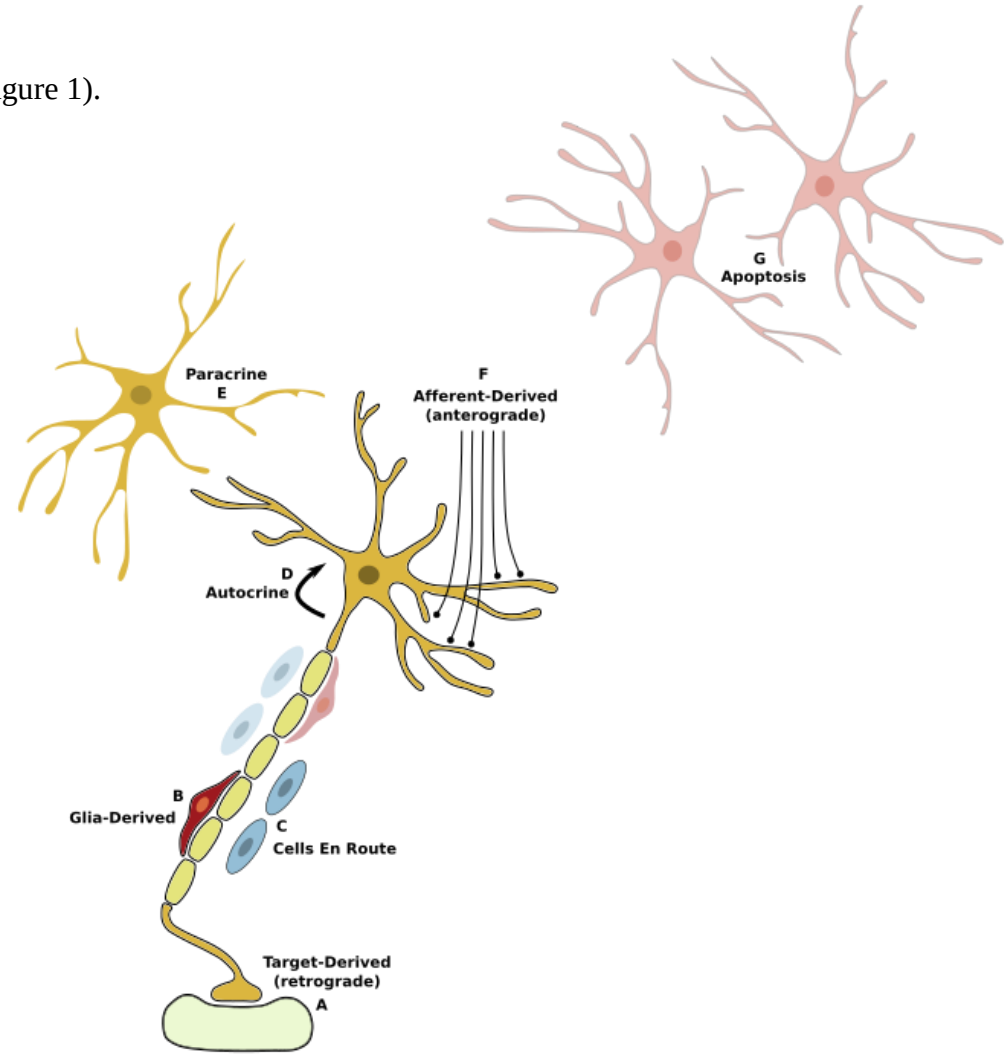


Figure 1 Routes by which neurotrophic factors can influence neuronal survival. Neurons may receive NTFs from a variety of sources including the target tissues/neurons **(A)**, glial cells **(B)**, intermediate target cells en route **(C)**, autocrine **(D)** and paracrine release **(E)**, or presynaptic neurons **(F)**. Distant neurons deprived of NTFs undergo apoptosis **(G)**.

The classification of NTFs has come to include many growth factors with different functional properties, each capable of specifically targeting unique subpopulations of neurons and tissues. The best characterized neurotrophic factors comprise the neurotrophin family of mammalian proteins, initiated with the discovery of nerve growth factor (NGF), including brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) (Lewin & Barde, 1996). The other

families of neurotrophic factors include the glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFL), neuropoietic cytokines and a recently discovered group comprising mesencephalic astrocyte-derived neurotrophic factor (MANF) and cerebral dopamine neurotrophic factor (CDNF) (Barbacid *et al.*, 1995; Lindholm *et al.*, 2007; Petrova *et al.*, 2003). MANF has previously been recognized by the name ARMET, or arginine-rich mutated in early stages of tumour, until it was determined that the protein is translated downstream of the arginine rich sequence upon which it was first named. ARMET was subsequently renamed MANF in accordance with its discovery in a rat ventral mesencephalic cell line.

Using *in vitro* immunofluorescence, MANF has been localized to the endoplasmic reticulum (ER) and Golgi apparatus (Apostolou *et al.*, 2008). It can be found on the lumenal surface of these organelles, and its secretory function is regulated by COPII-mediated transport within the ER-Golgi pathway (Colla *et al.*, 2012; Oh-Hashi *et al.*, 2012). MANF has been shown to co-localize *in vivo* with reticulons, which belong to a family of evolutionarily conserved eukaryotic proteins residing in the ER membrane where they play an important role in shaping the ER membrane, trafficking to the Golgi apparatus, the induction of ER stress and apoptosis (Chen *et al.*, 2014; Yang & Strittmatter, 2007). Its expression *in vivo* has been shown in a developing murine model, where it is widely expressed in the brain as well as non-neuronal tissues. In the brain, relatively high levels of MANF are detected in the cerebral cortex, hippocampus and cerebellar Purkinje cells (Lindholm *et al.*, 2007, 2008). Outside of the central nervous system, high levels of MANF mRNA and protein are especially abundant in mouse

secretory cells and tissues, including the embryonic and adult choroid plexus, salivary gland and embryonic pancreas (Lindholm *et al.*, 2008). Given the neuroprotective qualities of endogenous trophic factors and the novelty of this particular family of proteins, they present as an attractive new therapeutic approach to neurodegenerative disorders.

The Structure of MANF

The function of the MANF/CDNF family of proteins is believed to be tightly regulated by the ER, in large part due to its unique structural composition. The following discussion will mostly focus on the structure of MANF, however that of CDNF is very similar. The first 21 residues contain a signal peptide, directing the protein to the ER during synthesis, allowing access to the secretory pathway (Lindholm *et al.*, 2008). Both *N*- and *C*-terminal domains exhibit globular conformations largely composed of alpha helices, connected by a linker region (Hellman *et al.*, 2011; Hoseki *et al.*, 2010; Latge *et al.*, 2015; Parkash *et al.*, 2009). The *N*-terminal domain of MANF is structurally homologous to saposin-like proteins (SAPLIPs), a membrane and lipid binding protein superfamily (Parkash *et al.*, 2009). Interestingly, a rare π-helix structural element resides within this region, which has been implicated in functional domains that bind to enzyme substrates and ligand molecules (Hoseki *et al.*, 2010; Weaver, 2008). The presence of such a structure might suggest a resident enzymatic or signal-transducing role for MANF within the ER.

The *C*-terminal domain has been shown to share structural homology with the

SAP (SAF-A/B, Acinus, and PIAS) domain of the Ku70 protein, involved in nonhomologous DNA double-strand repair (Hellman *et al.*, 2011). The Ku70 protein is capable of inhibiting apoptosis via the binding and stabilization of the pro-apoptotic Bcl-2-associated X (Bax) protein, maintaining it in its inactive conformation. In line with the activity of this domain, the application of MANF has been shown to be equally efficient in preventing neuronal apoptosis (Hellman *et al.*, 2011). At the *C*-terminus, MANF contains a putative ER retention signal sequence (RTDL), resembling the canonical KDEL ER retention signal. This sequence is necessary and sufficient for the binding of MANF to KDEL receptors (KDELRs) (Henderson *et al.*, 2013). The *C*-terminal also contains a C-X-X-C consensus sequence which exists in proteins of the thiol-protein oxidoreductase superfamily, such as protein disulfide isomerases (PDIs) (Henderson *et al.*, 2013; Ni & Lee, 2007). During periods of ER stress, PDIs can catalyze the formation of intramolecular disulfide bonds to restore proper protein folding. However, despite the presence of this motif, no oxidoreductase activity has been found in MANF (Mizobuchi *et al.*, 2007). MANF contains a type 2 ER stress response element (ERSRE II) within its promoter region in contrast to CDNF (Mizobuchi *et al.*, 2007). It also has two patches of conserved positively charged residues, composed largely of lysines and arginines. In similarly structured membrane-lytic proteins such as granulysin and NK-lysin, the conserved dispersal of positive residues across the domains are believed to aid in anchoring themselves to membranes via the headgroups of anionic phospholipids (Parkash *et al.*, 2009). These findings support the activity of the MANF/CDNF NTFs through the ER.

Involvement in the ER & Therapeutic Potential

The endoplasmic reticulum (ER) is an essential organelle involved in the regulation of calcium homeostasis, lipid biosynthesis, protein folding and transport. Misfolded proteins are normally discarded by ER-associated degradation (ERAD). However, the accumulation of these proteins can result in ER stress triggering the unfolded protein response (UPR). The UPR signaling pathway counteracts stress via the activation of three main ER transmembrane receptor signal transducers: inositol-requiring enzyme 1 (IRE1), protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (Hetz, 2012). Many lines of evidence have implicated ER stress in a wide array of neurodegenerative disorders, resulting in the accumulation of misfolded proteins and disrupted calcium homeostasis (Lindholm *et al.*, 2006). The impaired clearance of protein triggers the ER UPR resulting in the up-regulation of genes (e.g. chaperone proteins) to re-establish homeostasis. MANF has previously been identified as a UPR up-regulated gene directed by ER stress , thereby making it desirable for use in neurodegenerative models exhibiting ER stress, such as Parkinson's, Alzheimer's and Huntington's disease (Lee *et al.*, 2003; D. Lindholm *et al.*, 2006). The presence of protein aggregates, ER and oxidative stress have been implicated in the subsequent neurodegeneration observed (Higo *et al.*, 2010; Lindholm *et al.*, 2006). Protein aggregation irrespective of its localization tends to inhibit proteasomal degradation, further leading to the buildup of misfolded proteins in the ER, triggering ER stress and the UPR. MANF is known to carry a wide array of neuroprotective roles via its endogenous expression, protecting against ER stress induced apoptosis (Apostolou *et al.*,

2008; Glembotski *et al.*, 2012; Mizobuchi *et al.*, 2007; Oh-Hashi *et al.*, 2012; Yu *et al.*, 2010).

Of particular interest to our study is Parkinson's disease (PD), the second most common neurodegenerative disease marked by the death of dopaminergic neurons within the substantia nigra pars compacta (Kalia & Lang, 2015). A known hallmark of PD is the presence of intracellular protein inclusions within the soma and processes of neurons, known as Lewy bodies and neurites respectively. These aggregates are shown to be strongly correlated with dementia and the onset of bradykinesia (Selikhova *et al.*, 2009). Familial forms of PD implicate genes that encode proteins that converge on cellular processes related to the mitochondrion, the ER, protein handling, and the ubiquitin proteasome system. Further, neurotoxins regularly used to mimic disease pathology and dopaminergic cell death (e.g. 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-pyridinium (MPP), and rotenone) have been shown to trigger ER stress and the UPR (Holtz & O'Malley, 2003; Ryu *et al.*, 2002). Gene profiling performed subsequent to exposure of these neurotoxins results in the up-regulation of ER chaperones and other components of the UPR (Holtz & O'Malley, 2003; Ryu *et al.*, 2002).

The use of both MANF and CDNF have been shown to efficiently protect and regenerate dopaminergic neurons in animal models of PD and cerebral ischemia (Lindholm *et al.*, 2007; Voutilainen *et al.*, 2009; Yu *et al.*, 2010). Further, both can be administered concurrently, working in concert to give enhanced neuroprotection (Cordero-Llana *et al.*, 2015). However, such neuroprotective effects have yet to be specifically attributed to an intracellular or secreted extracellular role; a distinction that

will be critical to understanding the therapeutic use of exogenously administered MANF. Given its paracrine and autocrine functions, MANF's extracellular activity could help regulate the intracellular activity of the very cells that secrete it, resulting in a form of auto-regulation in response to insult.

MANF has been shown to play a role in the neurophysiology of cells by potentiating gamma-aminobutyric acid (GABA) receptor-mediated inhibitory postsynaptic currents (IPSCs) in dopaminergic neurons of the substantia nigra (Zhou *et al.*, 2006). When intracellular energy levels are reduced, glutamate can be toxic due to its excessive activation of N-methyl-D-aspartate (NMDA) receptors, resulting in subsequent neuronal damage (Novelli *et al.*, 1988). This inhibitory function may be a means by which MANF imparts neuroprotection, as NMDA antagonists have previously demonstrated neuroprotection in animal models of PD (Sonsalla *et al.*, 1991).

Aside from neurodegenerative disorders, MANF has also been shown to mediate protective activity in several non-neuronal tissues of the periphery. The pathophysiology of ischemia is accompanied by ER stress, and multiple lines of evidence suggest that MANF expression is induced in both neurons and cardiomyocytes subsequent to ischemic conditions (Lindholm *et al.*, 2008; Tadimalla *et al.*, 2008; Yu *et al.*, 2010). Additionally, ER stress causes MANF upregulation in fibroblasts and in pancreatic β cells, exemplifying the breadth of its therapeutic potential (Lee *et al.*, 2003; Mizobuchi *et al.*, 2007).

Caenorhabditis elegans as a Model System

Neurodegenerative diseases can be modeled in *Caenorhabditis elegans*, a simple nematode system comprising only 302 neurons with almost invariant structure allowing for the easy assessment of compounds on the nervous system (Altun & Hall, 2011). The structure and connectivity of the entire nervous system has been determined from reconstructions of serial section electron micrographs. In addition, the complete cell lineage has been mapped revealing the developmental origin of every neuron, and the genome has been fully sequenced and analyzed for conserved gene systems (Altun & Hall, 2011).

Evidence suggests that NTFs play a role in the development of invertebrate nervous systems. Like vertebrates, there is an overproduction of neurons during embryogenesis in *Drosophila melanogaster*, requiring refinement via the programmed cell death of select populations of neurons during development (White *et al.*, 1994). In addition, a neurotrophin homolog known as *Drosophila* Neurotrophin1 (DNT1/spätzle2) was identified along with an orphan homolog for the GDNF transmembrane receptor tyrosine kinase RET (Airaksinen *et al.*, 2006; Zhu *et al.*, 2008). However, in *C. elegans* the only homology indicative of the existence of NTFs has been a putative GDNF family receptor (GFR)-like gene (Airaksinen *et al.*, 2006). This absence of literature might stem from fundamental differences in the development of invertebrate nervous systems, which despite advances in genomics has resulted in a lack of known orthologs (Bargmann, 1998; Ruvkun, 1998). Up until the recent discovery of MANF/CDNF, no conserved NTFs were deemed to exist, nor their commonly corresponding tropomyosin receptor kinase (Trk)

family of tyrosine kinase receptors (Bargmann, 1998; Chao, 2000).

With the lack of conventional trophic factors existing within *C. elegans*, inquiries have previously been made into other signaling pathways to derive a means by which developmental pruning and trophic support might occur. Although there is no predominant model by which neuronal protection and trophic support is conferred, the first evidence for neurite pruning in *C. elegans* implicated a novel transcription factor, Mblk-1, normally expressed in the mushroom bodies of honeybee brains (Kage *et al.*, 2005). Conserved amongst many species, its role in the nervous system had never been explored and it was determined to promote the elimination of neurites. The same group subsequently demonstrated a trophic role for Wnt-Ror kinase signaling, whereby neurite survival was dependent on the recognition of Wnt ligands by Ror Kinase CAM-1, a transmembrane tyrosine kinase with a Frizzled domain (Hayashi *et al.*, 2009). This non-canonical Wnt signaling pathway implicated CAM-1 as an inhibitor of neurite pruning. Another study found that the phosphatidylinositol 3- kinase (PI3K) signaling pathway, fundamental for the correct development of the nervous system, was implicated via the negative regulator DAF-18 (PTEN) (Christensen *et al.*, 2011). It was determined that DAF-18 could activate DAF-16 (FOXO) and promote neurite outgrowth during larval development. They additionally showed that the ability of DAF-16 to regulate neuron morphology is conserved amongst mammalian neurons.

Given the selectivity of MANF and CDNF for dopaminergic neurons, its effect on neuronal and dendritic morphologies can easily be addressed in *C. elegans*. There are only eight dopaminergic neurons in the hermaphroditic system, and an additional six

located in the tail of the male. All fourteen dopaminergic neurons are believed to be mechanosensory, modulating environmental interactions pertaining to locomotion and learning (Chase, 2007). There are two pairs of cephalic neurons (CEP) and a single pair of anterior deirid neurons (ADE) located in the head (Figure 2), along with another posterior deirid (PDE) pair found laterally in the posterior body.

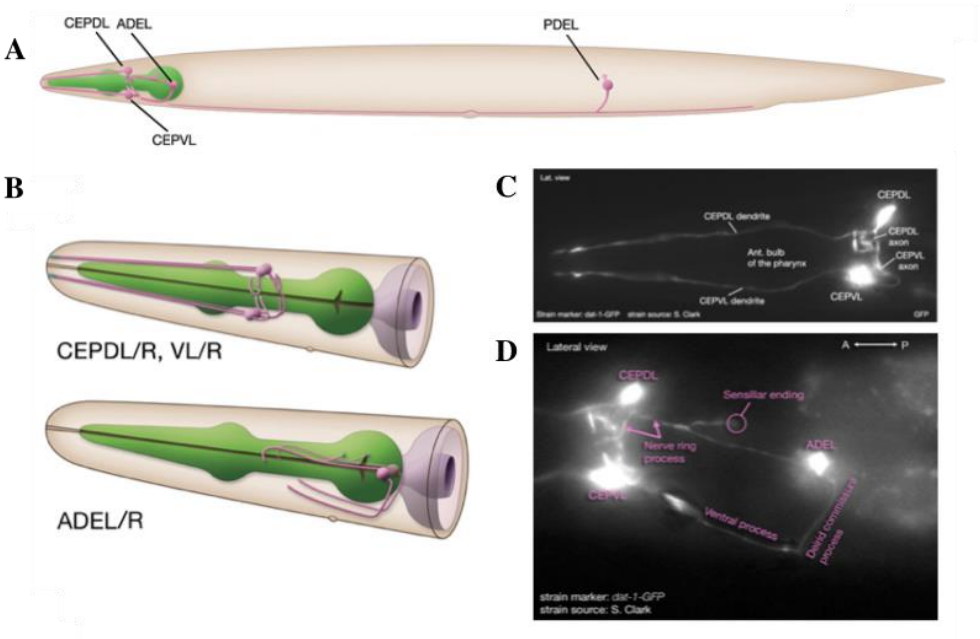


Figure 2 Dopaminergic neurons in *C. elegans*. (A) Schematic depicting dopamine neurons of hermaphroditic nervous system, lateral view. All neurons exist in pairs. **(B)** Within the head, CEPs are depicted in pairs located to the dorsal (D) and ventral (V) regions. Trajectories of the ADE neurons are depicted below. **(C)** Dopaminergic neurons visualized under the expression of P*dat-1*::*gfp* showing a single visible pair of CEPs, with others located below the plane of view. **(D)** Posterior to the CEPs, a single ADE neuron with typical neuritic trajectory can be viewed. Adapted from Altun & Hall, 2011.

These neurons can easily be assessed for degeneration by expressing GFP under a dopamine transporter (*dat-1*) promoter and assessing the integrity of existing cell bodies and neurites. It is important to note that only the neurons located in the head (CEPs and ADEs) are scored given their stable expression and trajectories, whereas the posterior PDEs tend to demonstrate unstable expression.

The neurotrophic factors CDNF and MANF, although distinct in vertebrates comprise a single homolog in invertebrates. Given the ancestral nature of MANF determined from phylogenetic reconstruction (Figure S1), and the greater similarity of the *C. elegans* homolog to mammalian MANF versus CDNF, the gene currently known as Y54G2A.23 will now be referred to as *manf-1*.

Aims and hypothesis of Study

Currently, very little is understood regarding the novel MANF/CDNF family's neuroprotective mechanisms of action, and even less is understood regarding their endogenous expression in *C. elegans*. As a result, I aimed to characterize the expression and functional role of MANF in *C. elegans*.

Based on data from *Drosophila* and mammalian systems, my hypothesis is that *manf-1* plays a role in ER stress mediated neurodegeneration in *C. elegans*. In order to test this hypothesis, neuronal degeneration will be assessed in a *manf-1* knockdown strain, along with ER stress using reporter gene assays. Additionally, the expression profile of *manf-1* will be determined. The results of these experiments will support future investigations to understand whether ER stress plays a causative role in neurodegeneration, assisting in MANF's development as a targeted therapeutic.

Chapter 2: Characterizing the expression and function of *manf-1* in *C. elegans*

Preface:

This chapter consists of a manuscript in preparation for publication entitled "***C. elegans* mesencephalic astrocyte-derived neurotrophic factor homolog protects dopaminergic neurons and regulates the ER stress response"**

The manuscript was written by me, with comments and revisions provided by Bhagwati Gupta. It is currently being edited for submission to a peer-reviewed journal.

C. elegans mesencephalic astrocyte-derived neurotrophic factor homolog protects dopaminergic neurons and regulates the ER stress response

Abstract

Neurotrophic factors (NTFs) are proteins important for the development, function, and survival of neurons. Mesencephalic astrocyte-derived neurotrophic factor (MANF) and cerebral dopamine neurotrophic factor (CDNF) are two members of a novel family of NTFs in vertebrates that share similar domain structure and mediate the protection of dopaminergic neurons. Both these factors have the potential to be developed as new treatments for Parkinson's disease (PD), however their mechanisms of action are poorly understood. Sequence analysis of MANF and CDNF genes has identified a single homolog in invertebrates that is most related to MANF. Here we have characterized the function of the *Caenorhabditis elegans* (nematode) MANF gene, *manf-1*, using a deletion allele and show that *manf-1* is necessary for the protection of dopaminergic neurons in older adults. *manf-1* mutants show a progressive age-dependent increase in the death of dopaminergic neurons. Using a combination of reporter gene assay and stress markers, we find that *manf-1* animals have increased ER stress. Finally, we show that the loss of *manf-1* function causes enhanced aggregation of alpha-synuclein protein, a pathological hallmark of PD, suggesting that MANF plays a role in reducing these inclusions. Overall, our work demonstrates the important role of *manf-1* in dopaminergic neuronal survival and maintaining ER homeostasis in *C. elegans*.

Introduction

Neurotrophic factors (NTFs) are secreted proteins capable of mediating a variety of neuronal signaling responses throughout development including growth, survival, differentiation, neurogenesis and synaptic plasticity. The best characterized neurotrophic factors comprise three major families; the neurotrophins, glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFLs) and neuropoietic cytokines (Barbacid, 1995). More recently a novel family of NTFs has been identified that includes mesencephalic astrocyte derived neurotrophic factor (MANF) and its paralog cerebral dopamine neurotrophic factor (CDNF) (P. Lindholm *et al.*, 2007; Petrova *et al.*, 2003).

The MANF/CDNF family of NTFs have been shown to protect dopaminergic neurons against a variety of insults. When administered intracranially, both compounds are neuroprotective against the degeneration of nigrostriatal dopaminergic neurons in a 6-hydroxydopamine induced rat model of PD and models of ischemia (Airavaara *et al.*, 2009; P. Lindholm *et al.*, 2007; M. H. Voutilainen *et al.*, 2009; Yu *et al.*, 2010). The expression of both NTFs in the presence of ER stress protects from unfolded protein response (UPR)-induced apoptosis, even when MANF is restricted intracellularly (Lee et al 2003, Apostolou *et al.*, 2008 Voutilainen *et al.*, 2015). Such findings have suggested that these proteins can mediate their neuroprotective activity intracellularly in response to ER stress, or as secreted proteins (Petrova *et al.*, 2003; Lindholm *et al.*, 2007; Apostolou *et al.*, 2008; Voutilainen 2015) Little is currently understood regarding this novel family's neuroprotective mechanisms of action. However, given their potent neuroprotective

activity specific to dopaminergic neurons, elucidation of their cellular function holds great therapeutic potential.

The well-defined structures of MANF and CDNF can be characterized as bifunctional, with neurotrophic activity believed to largely reside within the *N*-terminal of the protein and the ER stress response within the *C*-terminal (Hellman *et al.*, 2011; Parkash *et al.*, 2009). They are composed of two globular alpha helical domains connected by a short linker, with eight highly conserved cysteines which all form disulphide bonds in their mature conformations (Figure 1A) (Hoseki *et al.*, 2010; Latge *et al.*, 2015; Mizobuchi *et al.*, 2007; Parkash *et al.*, 2009). The *N*-terminal is homologous to the saposin protein superfamily, involved in the degradation of glycosphingolipids from the plasma membrane (Kolter & Sandhoff, 2005). It is the first saposin-like protein (SAPLIP) structure found to impart neuroprotective properties and act via the ER stress response (Hellman *et al.*, 2011; Hoseki *et al.*, 2010; Parkash *et al.*, 2009).

The *C*-terminus of both MANF and CDNF contain putative ER retention signal sequences, RTDL and KTEL respectively, which closely resemble the canonical KDEL ER retention signal. This sequence is necessary and sufficient for the binding of MANF to KDEL receptors (KDELRs) (Henderson *et al.*, 2013). These proteins also contain a C-X-X-C motif common to protein disulfide isomerases (PDIs). During periods of ER stress, PDIs can catalyze the formation of intramolecular disulfide bonds to restore proper protein folding (Ni & Lee, 2007). The *C*-terminus is also structurally similar to the SAP (SAF-A/B, Acinus, and PIAS) domain of the Ku70 protein, a key subunit of the heterodimeric Ku protein involved in DNA repair, and known inhibitor of apoptosis.

MANF is believed to stabilize the Bcl-2-associated X (Bax) protein, the main pro-apoptotic effector in neurons, conferring its cytoprotective activity. In fact, the *C*-terminal domain of MANF has been shown to be equally efficient to Ku70 at inhibiting Bax-mediated neuronal apoptosis *in vitro*, even after undergoing protein recombination (Hellman *et al.*, 2011).

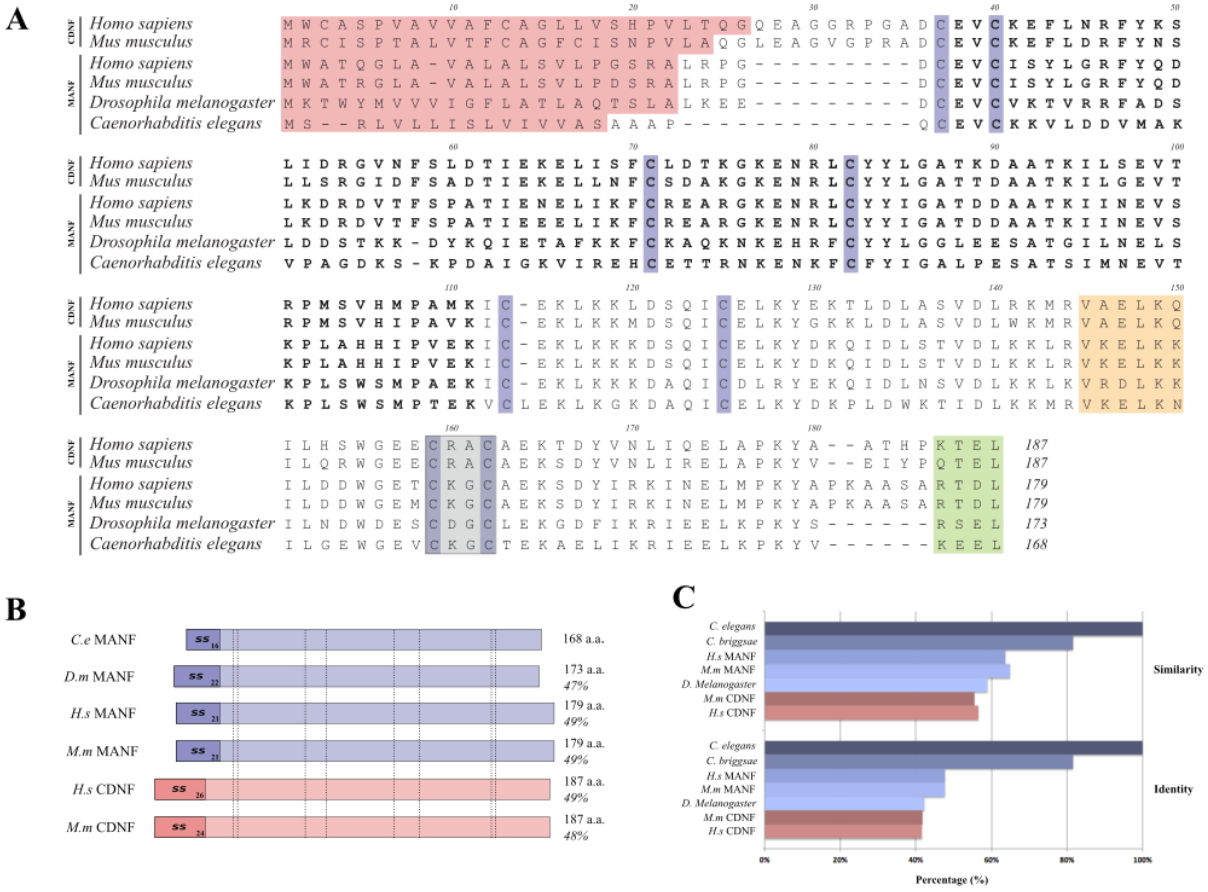


Figure 1 *C. elegans* Y54G2A.23 protein shares conserved domains with vertebrate MANF. (A) Alignment of CDNF and MANF amino acid sequences. The signal sequence is depicted in red, eight conserved cysteines in blue, *N'*-terminal saponin-like domain in bold, *C'*-terminal SAP-domain in orange, C-X-X-C motif in grey and putative ER retention signal in green. **(B)** Schematic representation of MANF and CDNF proteins from *Caenorhabditis elegans* (*C.e*), *Drosophila melanogaster* (*D.m*), Homo sapiens (*H.s*) and *Mus musculus* (*M.m*) with percent identity indicated relative to *C. elegans.* Conserved domains are aligned and signal sequences (ss) are indicated with sizes, all presented to scale. **(C)** Percent amino acid identity and similarity amongst select species as compared to *C. elegans.* Y54G2A.23/*manf-1* is most similar to MANF.

While much work has been done on the roles of MANF and CDNF in vertebrates, very little is known about their invertebrate homologs. Previously, sequence searches in two leading invertebrate model species, *Drosophila melanogaster* and *C. elegans*, revealed the presence of a single MANF/CDNF homolog (P. Lindholm *et al.*, 2007; Petrova *et al.*, 2003). This suggests that the ancestral gene duplicated in the vertebrate lineage, leading to the specialization of MANF and CDNF roles in the nervous system. *DmManf* has been shown to be necessary for the maintenance of dopaminergic neurites in *Drosophila* (Palgi *et al.*, 2009). It is expressed within both glia and dopaminergic neurons, however it is not required cell autonomously for the survival or differentiation of either cell type (Stratoulias & Heino, 2015). *DmManf* is upregulated in response to ER stress, where it interacts *in vivo* with *Drosophila* homologs of key components of the mammalian UPR like GRP78 (BiP), PERK and XBP1 (Lindström *et al.*, 2016). Interestingly, only the full length protein is able to rescue the larval lethality associated with a knockout *DmManf* mutant, however its *C*-terminal is sufficient to protect against the drug-induced apoptosis of mammalian neurons *in vitro* (Lindström *et al.*, 2013). Downstream, the absence of *DmManf* most dramatically perturbs the expression of genes involved in metabolism and membrane transport, particularly in exocytosis and the endosomal recycling pathways (Palgi *et al.*, 2012). Despite the many similarities of MANF and CDNF, only human MANF is capable of rescuing the larval lethality associated with a *Drosophila* knockout (Palgi *et al.*, 2009).

Although the nematode *Caenorhabditis elegans* (*C. elegans*) contains a MANF homolog, Y54G2A.23, its biological function has not been reported. It is currently unclear whether *C. elegans* MANF has a role in the dopaminergic system. As a simple animal model that carries just 302 neurons, of which 8 are dopaminergic in the hermaphrodite, *C. elegans* offers many advantages to model neurodegenerative diseases and investigate the function of associated genes and pathways. The dopaminergic neurons in *C. elegans* are believed to be mechanosensory, modulating environmental interactions pertaining to locomotion and learning (Chase, 2007). These consist of two pairs of cephalic neurons (CEPs) and a single pair of anterior deirid neurons (ADEs) located in the head (Figure 2D), along with another posterior deirid (PDE) pair found laterally in the posterior body. These neurons can easily be assessed for degeneration in live animals by examining the integrity of existing cell bodies and neurites when visualized using a fluorescent reporter. Given the neuroprotective qualities of endogenous trophic factors and the novelty of this particular family of proteins, we aimed to characterize the expression and function of MANF in *C. elegans*.

Here we use a *manf-1* loss of function mutation to characterize dopaminergic and behavioral phenotypes. We also use reporter gene assays to localize the expression of *manf-1* throughout development and assess the induction of the ER and mitochondrial stress pathways. The effects of drug-induced oxidative stress on neurodegeneration, and subsequent rescue with VPA was determined in the *manf-1* knockdown mutant, as was MANF's effect on the expression and aggregation of alpha-synuclein (α-syn).

Results

MANF/CDNF homolog in C. elegans

As reported earlier, the Y54G2A.23 gene product in *C. elegans* represents the closest homolog of vertebrate MANF and CDNF, sharing conserved structural features including eight cysteines located at characteristic positions and a *C*-terminus ER-retention signal (P. Lindholm *et al.*, 2007; Petrova *et al.*, 2003). (Figure 1A,B). The amino acid sequence alignments of Y54G2A.23 shows that it most closely resembles the MANF genes in *Drosophila* and vertebrate homologs (Supplementary Figure S1). We found that the *C. elegans* protein is roughly 65% similar to mouse and human MANF (Figure 1C). In addition, other nematode species also contain a MANF-like gene (www.wormbase.org). Based on these results, the gene currently known as Y54G2A.23 will be regarded as *manf-1*.

A deletion allele of *manf-1*, *tm3603,* was generated earlier by the Japanese Knock-out Consortium (National BioResource Project, https://shigen.nig.ac.jp/c.elegans). This allele causes a deletion of the third exon (www.wormbase.org), which we confirmed by sequencing. The allele lacks 204bp at the genomic location 2,927,897 and inserts a random 21 bp in place of the missing sequence. The mutant allele lacks three conserved cysteines, as well as nearly half of the highly folded *N*'-terminal saponin-like domain. Furthermore, the majority of surface residues believed to impart the functional neuroprotective activity unique to human MANF (in contrast to CDNF) in a *Drosophila manf-1* knockout mutant are lost within this deletion site (Parkash *et al.*, 2009). Interestingly, the remaining *manf-1* (*tm3603*) transcript maintains the original reading

frame, and by cDNA analysis we confirmed that mutant animals carry a truncated product (Figure 2A,B). We do not know whether the *tm3603* allele is translated, although any such product is unlikely to be biologically functional.

The finding that *manf-1*(*tm3603*) animals carry a transcript led us to determine its expression level by a quantitative real time PCR (qRT-PCR) approach. We observed a four-fold decrease in *manf-1* gene expression (Figure 2C), which is expected due to the action of the nonsense mediated decay pathway (Lykke-Andersen & Jensen, 2015). More recently we have acquired an additional allele of the gene (*gk3677*) that lacks the entire coding sequence (see Methods and Figure 2B).

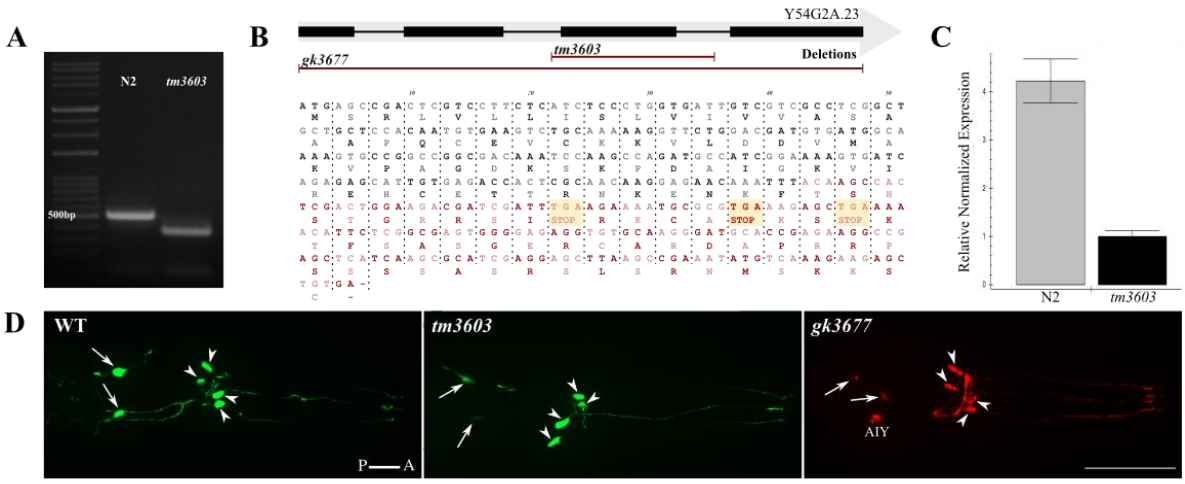


Figure 2 Characterization of *manf-1* alleles. (A) Agarose gel of PCR fragments obtained from amplification of *manf-1* wild type (N2) and mutant (*tm3603*) RNA. **(B)** Location and size of deletions in two *manf-1* mutant alleles (*tm3603* and *gk3677*). Sequence of truncated transcript generated from (A) of *tm3603* animals indicating the presence of three nonsense mutations (highlighted). **(C)** Expression of *manf-1* gene measured by qRT-PCR of day one adult animals. Results are means of experiments performed using three biological replicates ± SEMs **(D)** Visualization of dopaminergic neurons in the head of wild type, *tm3603* and *gk3677* mutant alleles. The posterior pair of ADEs is indicated by arrows and four anterior CEPs are indicated by arrowheads. Scale bar = 50 μm.

Phenotypic characterization of *manf-1* animals

Dopaminergic neurons in *tm3603* and *gk3677* animals were examined at day one of adulthood using a *dat-1* (dopamine transporter) promoter-driven fluorescent reporter in order to determine whether the loss of trophic support would impact their development. Both mutants displayed normal dopaminergic neuritic trajectories and number of soma (Figure 2D). Two other neuronal subtypes, GABAergic and serotonergic, were also inspected using *unc-47*::*gfp* and *tph-1*::*gfp* markers, respectively, but no obvious defect was observed (Supplementary Figure S2). These results suggest that *C. elegans manf-1* does not play a major role in the formation and guidance of neurons.

Because the ability to recognize environmental olfactory and gustatory cues is mediated by a significant portion of the nervous system, we then tested the chemosensory responses of *manf-1* mutants (Bargmann, 2006). The *tm3603* animals were able to mount a normal chemotactic response to an attractant (NaCl) and a repellant ($CuSO_4$) (Figure 3A).

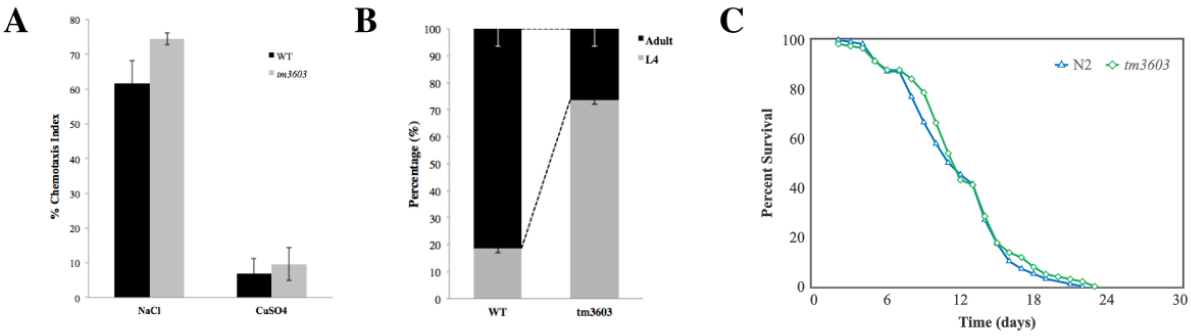


Figure 3 Chemosensory and life traits of *tm3603* animals (A) Results of chemotaxis assays for NaCl (chemo-attractant) and $CuSO_4$ (chemo-repellant). Results are means of four batches ± SEMs. **(B)** Growth delay represented by percentage of adults reached by 54 hours after plating L1 arrested animals. Results are means of two biological replicates ± SEMs. **(C)** Survival curve of wild type and *tm3603* animals. Data is plotted as the mean values of experiments carried out using two biological replicates, n=120, generated using OASIS 2 software (Han *et al.*, 2016).

Visual inspection of the *manf-1*(*tm3603*) strain revealed no apparent morphological or movement defects, although we did observe a slight growth delay as evidenced by a lower proportion of mutants reaching adulthood by 54 hours of growth (post L1-arrest) compared to controls (Figure 3B). However, adults appeared healthy and had a normal lifespan (Figure 3C).

Neurodegeneration is enhanced in *manf-1* animals

The results described above show that the loss of *manf-1* has no impact on the development of neurons in one-day old adults. However, *manf-1* may still play a neuroprotective role later in life. To investigate this possibility, we proceeded to assess age-dependent changes in dopaminergic neurons of *manf-1*(*tm3603)* animals. The cell bodies of ADEs and CEPs were counted every couple days for up to nine days. The results revealed significantly enhanced degeneration in *manf-1* mutants by day 3, a phenotype that became progressively worse with age such that by day nine there were roughly one-third less dopaminergic neurons in *manf-1*(*tm3603*) compared to controls (Figure 4A). These findings allow us to conclude that *manf-1* has a neuroprotective role in *C. elegans*.

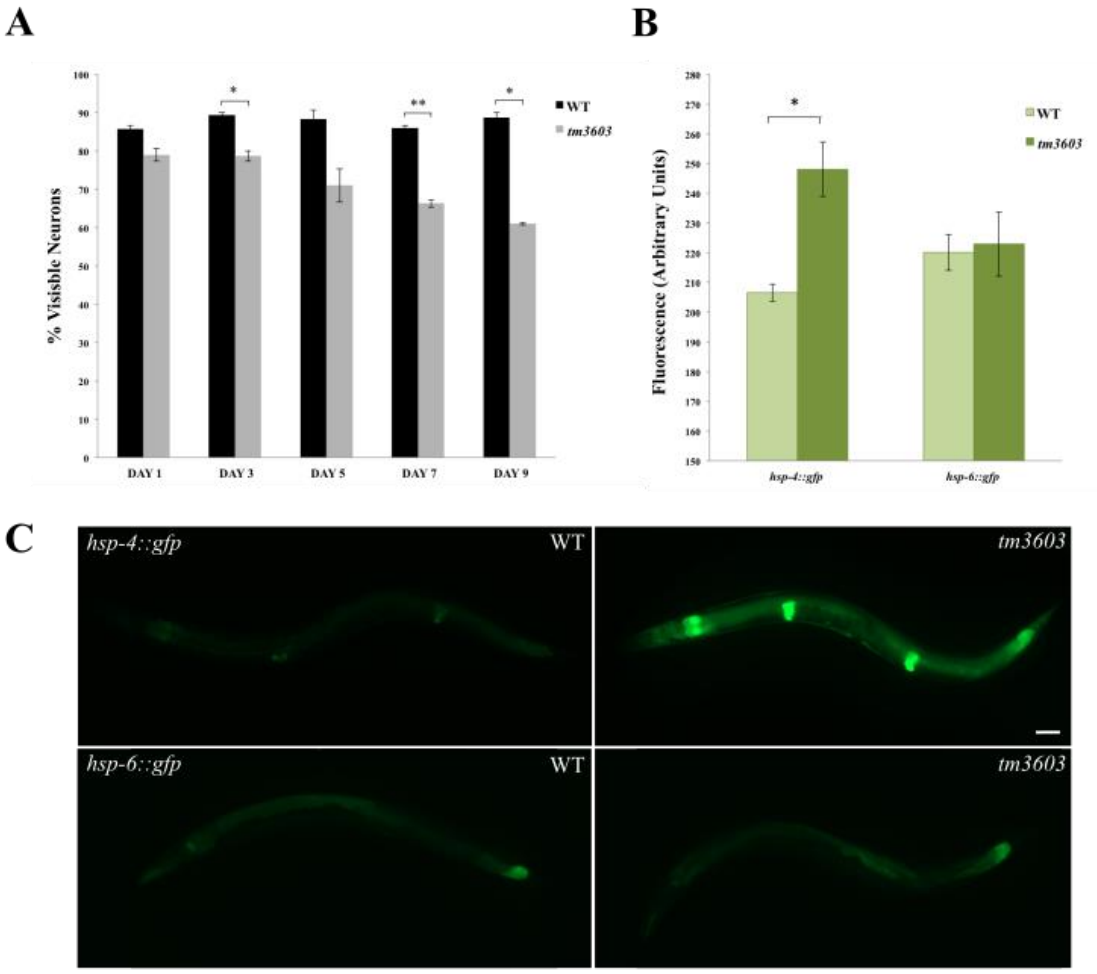


Figure 4 *manf-1* serves a neuroprotective role and is involved in ER stress homeostasis
(A) Neurodegeneration of dopaminergic neurons with age in day 1-9 adults of wild type and *tm3603* mutant animals. Performed using two biological replicates, n=50 ± SEMs, **P*<0.05, **<0.01. **(B)** The average GFP pixel intensity values quantified via whole animal analysis. Performed using three biological replicates, n=120 ± SEMs, *p<0.05. **(C)** Differential induction of ER and mitochondrial stress. Whole animal ER (*hsp-4*::*gfp*) and mitochondrial (*hsp-6*::*gfp*) reporters visualized in wild type and *tm3603* animals; worms with average fluorescence shown. Scale bar = 50 μm.

manf-1 animals show an increase in ER stress response marker expression

Studies in other animal models have shown that the cytoprotective roles of MANF and CDNF are mediated by the ER stress-induced UPR (Merja H. Voutilainen, Arumäe, Airavaara, & Saarma, 2015). Although both trophic factors are secreted, they localize largely in the ER via the ER retention signal sequence (Figure 1) and may facilitate

protein folding. As such, we assessed its role in regulating ER stress that may mediate its neuroprotective action. For this an ER stress reporter line, *hsp-4*::*gfp*, was used. The *hsp-4* gene is a *C. elegans* ortholog of the vertebrate binding immunoglobulin protein (BiP), also known as glucose-regulated protein 78 (GRP78), a molecular chaperone involved in the ER stress response (Calfon *et al.*, 2002). Analysis of the *hsp-4*::*gfp* pattern in *manf-1* animals revealed a significant increase in GFP fluorescence, particularly in the intestinal, hypodermal and spermathecal cells (17% higher relative to controls) (Figure 4B,C). Thus, ER stress was elevated in worms in the absence of *manf-1* function. We also examined mitochondrial stress using an *hsp-6*::*gfp* marker, an ortholog of the vertebrate HSP70 mitochondrial matrix specific chaperone, but saw no change in reporter expression (Yoneda *et al.*, 2004) (Figure 4B,C).

manf-1 is widely expressed throughout development

We next determined whether *manf-1* expression levels were maintained throughout adulthood. Gene expression data was obtained from wild type worms at three and nine days of adulthood via qRT-PCR and normalized to day one. There was a notable decline in *manf-1* expression with age, decreasing to ~55% by day three and ~15% by day nine (Figure 5A).

The embryonic expression profiles of all genes in *C. elegans* has previously been determined, providing a means to determine the gene regulatory states underlying developmental processes (Levin, Hashimshony, Wagner, & Yanai, 2012). This dataset

has provided us with an embryological gene profile for *manf-1* in *C. elegans* (Supplementary Figure S3).

In order to visualize the expression pattern of *manf-1* throughout development, a transcriptional reporter plasmid was constructed by placing GFP under the control of a 2.7kb 5'UTR sequence of *manf-1*. Bright fluorescence was detected in post gastrulating embryos in regions corresponding to intestinal cells and the dorsal body wall muscle quadrant (Figure 5B). Throughout the early larval stages, expression was fairly ubiquitous with the exception of gonadal regions. By mid-L4, worms appeared to express GFP throughout the intestinal and muscle cells, with concentrated regions within the pharyngeal and vulval muscles. There was a reduction in expression by day one of adulthood with expression maintained within the intestine and pharynx, and high concentration within the spermatheca (Figure 5C). Such findings are in line with the protein's secretory function and high expression within analogous structures (i.e. the testes) in mammals (P. Lindholm *et al.*, 2008). Interestingly, no expression was observed in the male gonad (Figure 5D), suggesting sex-specific differences in the mechanism of *manf-1* expression. To determine whether MANF accumulation in the spermatheca has a role in reproduction, we examined the brood size of hermaphrodites. The reproductive span of *tm3603* animals was found to be normal however there was a reduction in the number of fertilized eggs laid each day, leading to a smaller cumulative brood size (Figure 5E). We also observed expression within the excretory system in structures resembling the excretory gland cells as well as the intestinal-rectal valve and anus (Figure

5F-H). Reporter expression appears to decline with age, which is not surprising given our findings indicating a substantial decrease in *manf-1* expression during adulthood.

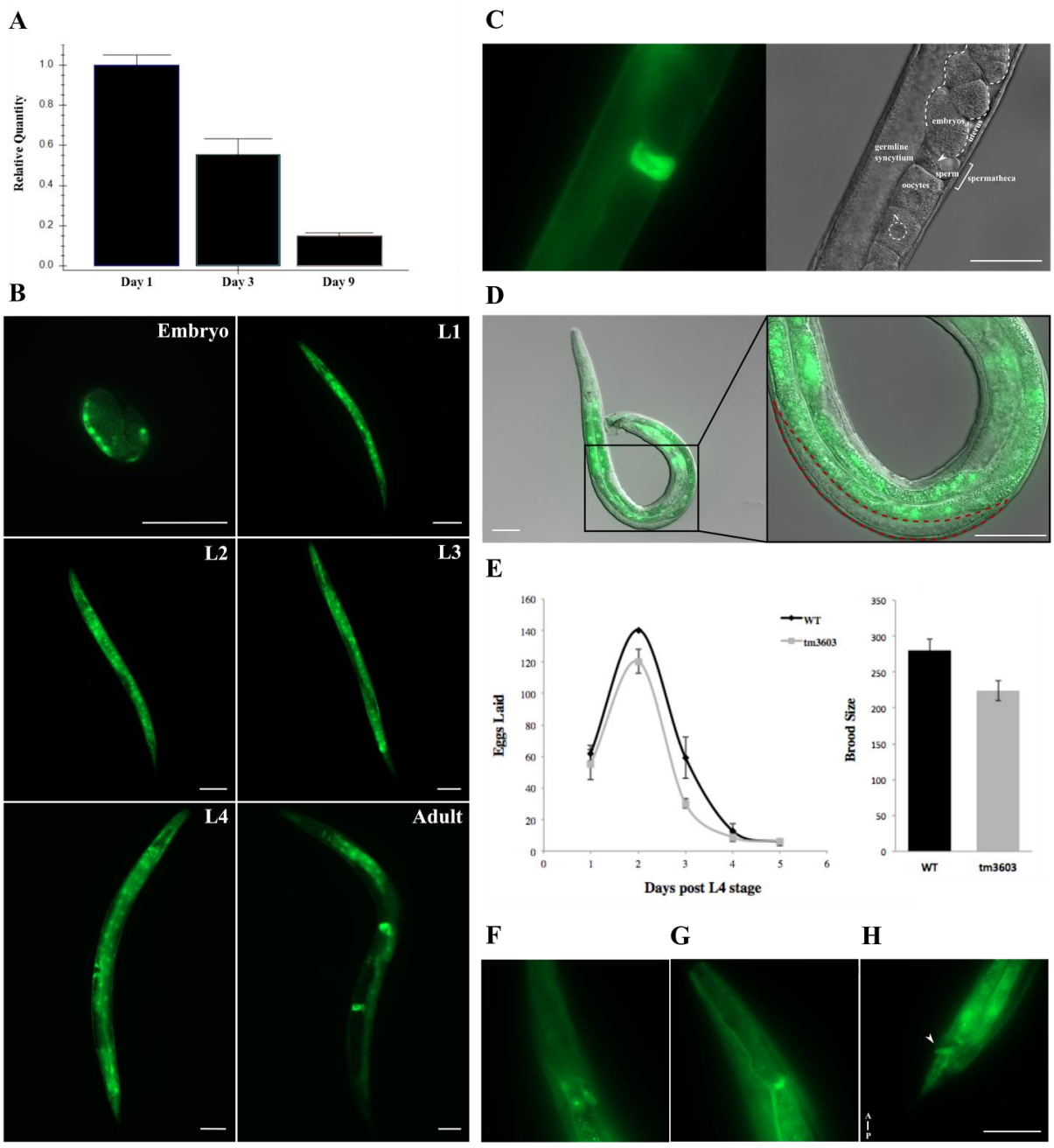


Figure 5 Expression of *manf-1* throughout development and effects on fecundity. (A) qRT-PCR data showing decline in endogenous *manf-1* expression with age in wild type animals. Batches are normalized to the first day. Results are means of experiments performed using two biological replicates ± SEMs **(B)** Expression throughout larval stages. **(C)** Concentrated expression within the spermatheca of adults. **(D)** Gonad of adult male lacking fluorescence within sperm (indicated by red dashed line). **(E)** Progression of egg laying and brood size for wild type and *tm3603* animals. Done using two biological replicates ± SEMs. **(F-H)** Expression within the excretory system; including excretory gland cells **(F)**, canal **(G)** and anus **(H)**. Scale bar = 50 μm.

Chronic exposure to Paraquat and VPA do not alter neurodegeneration in *manf-1* animals

To determine whether *manf-1* mutants were more sensitive to oxidative insult as they demonstrate a significant induction of ER stress (Figure 4B,C), wild type and *tm3603* worms were exposed to 0.25 mM paraquat, a herbicide known to cause oxidative stress and activation of JNK and caspase-3 mediated dopaminergic cell death (Peng *et al.*, 2004). Wild type animals exposed to paraquat displayed significantly enhanced neurodegeneration by day three of adulthood, however defects were less severe than loss of *manf-1* alone. Given the severity of degeneration in the untreated *tm3603* allele, loss of *manf-1* does not seem to have a synergistic effect with paraquat treatment, as there is no significant further reduction in number of dopaminergic neurons (Figure 6A).

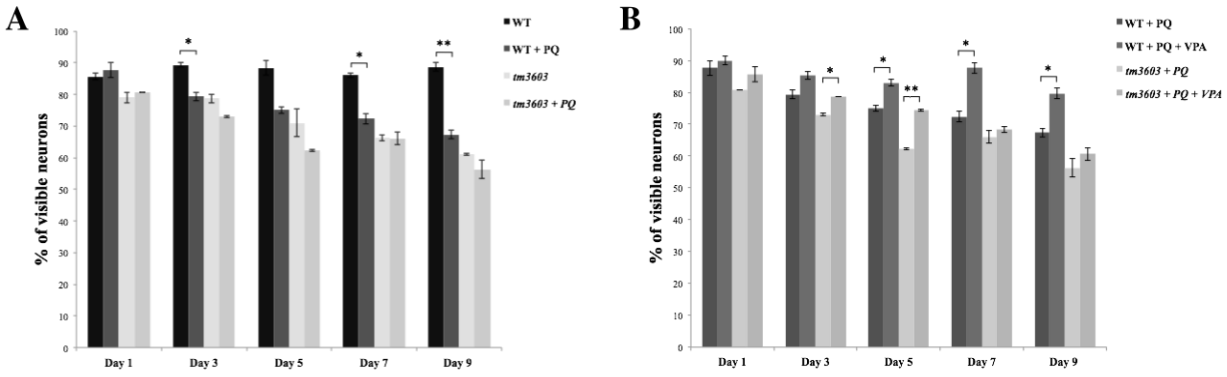


Figure 6 Sensitivity of the *tm3603* allele to paraquat exposure and rescue with valproic acid. (A) Neurodegeneration of dopaminergic neurons upon the administration of paraquat (0.25mM) in wild type and *tm3603* mutant animals. **(B)** Application of VPA has significant neuroprotection of neurons in wild type exhibited from day 5, but not *tm3603* animals. Both assays performed using two biological replicates, n=50 ± SEMs. **P*<0.05, **<0.01.

We next asked whether the application of VPA could rescue the degenerative phenotype exhibited by paraquat. This compound was selected as it functions using the same pathway as NTFs (i.e. ERK-MAPK), possibly through their direct regulation (Hao *et al.*, 2004; Kautu *et al.*, 2013; Niles *et al.*, 2012). Since VPA is capable of upregulating MANF, it is possible that VPA exerts it's neuroprotection via its overexpression. If this is in fact the case, we would anticipate no effect in the *manf-1* background. In wild type animals, there was a significant percentage of neuroprotection beginning at day five of adulthood. However, *tm3603* failed to exhibit this trend, with fairly equal degrees of degeneration amongst most days (Figure 6B). This finding supports the involvement of trophic factors in mediating VPA's mechanism of neuroprotection.

Loss of *manf-1* enhances the expression and aggregation of α-synuclein

Accumulating evidence has implicated ER stress in a wide array of neurodegenerative disorders which result in the accumulation of misfolded proteins and disrupted calcium homeostasis. The impaired clearance of these protein aggregates triggers the ER UPR (Lindholm *et al.*, 2006). A known hallmark of PD is the presence of intracellular protein inclusions within the soma and processes of neurons, known as Lewy bodies and neurites respectively. We seeked to determine whether *manf-1* could play a role in the clearance of alpha-synuclein (α-syn), the main constituent of these inclusions. To assess the presence of aggregates, we made use of a strain containing human α-syn fused to a yellow fluorescent protein (YFP) under the control of a muscle promoter (van Ham *et al.*, 2008). The chimeric protein can be visualized with age by the clear presence

of fluorescent puncta within the body wall muscle cells, the largest and most easily visualized cells in the organism. There was a significant increase in the level of α-syn-YFP expression in day one *tm3603* adults (Figure 7A), as well as number of inclusions within the head region (Figure 7B-C). This was quantified manually by counting the number of aggregates of a minimum diameter (2μm) and by automated analyses of total pixel area after thresholding to eliminate background (Figure 7C).

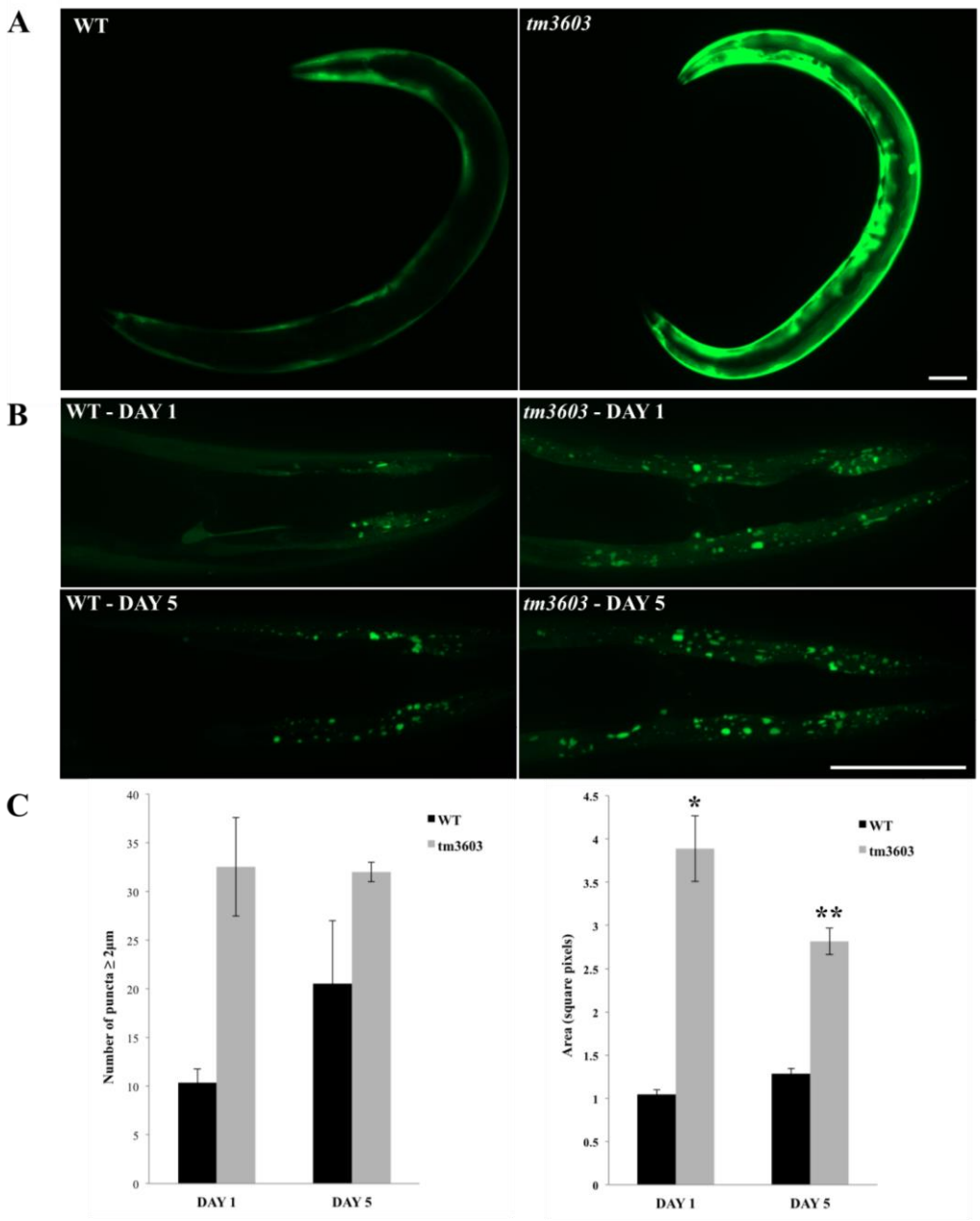


Figure 7 Effect of *manf-1* knockdown on α-synuclein aggregation. (A) Expression of human α-syn::YFP chimeric protein within the body wall muscle cells of wild type and *tm3603* strains. **(B)** Maximum projection of Z-stack images at days 1 and 5 of adulthood. **(C)** Number of inclusions via manual counting of a 2µm minimum diameter and automated analysis of total pixel area after thresholding. Experiments performed using two (manual) and three (automated) biological replicates, n=10 per batch, ± SEMs, **P*<0.05, **<0.01. Scale bar = 50 μm.

Discussion

Our findings present the first evidence of *manf-1* expression and phenotypic characterization in *C. elegans,* illustrating a functional role in maintaining dopaminergic health and ER homeostasis. Utilizing a *manf-1* knockdown (*tm3603*) and knockout (*gk3677*) strain, we found that MANF does not serve as a necessary developmental cue for neuronal guidance in the dopaminergic, GABAergic or serotonergic nervous systems.

Mutants were screened for pleiotropies that may accompany the protein's role in the nervous system. The *tm3603* allele was not found to exhibit chemotactic defects, inferring the normal health of the animal's complex chemosensory system. Mutants appeared to have a slight growth delay (~ 2-3 hours), which was exemplified by the decreased proportion of worms reaching adulthood by 55 hours post L1 synchronization. Despite this developmental delay, mutants display a normal lifespan comparable to that of wild type animals, indicating that *manf-1* does not influence longevity.

The endogenous expression of *manf-1* decreases throughout adulthood in wild type animals, yet their dopamine neurons appear to be fairly robust, resisting degeneration until beyond the timespan of our analyses. Therefore MANF may be required in very minute quantities in order to mediate its neuroprotective effects, meaning only the loss of function or complete depletion of MANF has a causative role in the enhanced susceptibility of dopaminergic neurons to degenerate with age. Dopaminergic degeneration as a result of a loss of MANF has been demonstrated in *Drosophila*, whereby it is necessary for the maintenance of dopaminergic neurites (Palgi *et al.*, 2009).

It is feasible that MANF plays a role in sustaining dopaminergic health, likely by mediating the ER stress response. In support to this notion, we have demonstrated a significant induction of ER stress when *manf-1* is knocked down, indicating that MANF may help regulate the resting state of the ER's UPR. Further, MANF is believe to be retained within the ER via the presence of KDEL receptors and a calcium dependent interaction with GRP78/BiP (Glembotski *et al.*, 2012; Henderson *et al.*, 2013). Aside from GRP78's role as an ER resident chaperone with anti-apoptotic activity, it also serves as a master regulator of the UPR by maintaining ER transmembrane signalling proteins in an inactive state (A. S. Lee, 2005). MANF may form a complex with GRP78 to stabilize and facilitate its association with signal transducers.

In accordance, the expression pattern of *manf-1* in adulthood resembles that of the ER stress reporter line (*hsp-4*::*gfp*); ortholog of the GRP78 chaperone. This similarity in patterning is not surprising given the presence of a putative ER stress response element (ERSEII) site conserved within *C. elegans*, 480bp upstream of the *manf-1* promoter (Mizobuchi *et al.*, 2007). Upon induction of the UPR, transcription factors bind to the ERSEs of ER chaperone genes to trigger their upregulation. The presence of this ER stress response element within both MANF and GRP78 proteins might explain their similarity in patterning, indicating a shared manner of regulation. Further support comes from the similarity of MANF and GRP78 expression patterns within murine tissues, as well as their time course of induction in response to ER stress (Mizobuchi *et al.*, 2007).

The expression of *manf-1* within the spermatheca and excretory cells of adult animals is in line with the protein's secretion-based neuroprotective role, as well as ER stress-induced cytoprotection. It is interesting to note that *manf-1* did not appear to be expressed within the sperm of adult male gonads, whereas it is detected via immunohistochemistry within the spermatocytes of mice (Mizobuchi *et al.*, 2007). The cause of this discrepancy is unclear, however the generation of a translational fusion reporter would help elucidate the protein's active location, possibly resolving this difference in patterning. Given the high expression of protein within the reproductive system, we assessed the role of *manf-1* within the context of fertility. By performing a brood size assay, *manf-1* knockdown exhibited a slightly decreased, although insignificant difference in the progression of laying and summed number of fertilized eggs compared to controls.

Paraquat administration in *C. elegans* has previously been shown to cause reactive oxygen species (ROS)-dependent toxicity and dopaminergic neurodegeneration (González-Hunt *et al.*, 2014). By exacerbating the oxidative stress already active in the *manf-1* mutant, animals did not display further loss of dopaminergic cell bodies. Given this lack of a synergistic effect, it is possible that the loss of *manf-1* results in a saturated oxidative stress response that cannot be disrupted further, or that paraquat toxicity is mediated through the regulation of MANF. The existence of a shared mechanism could explain why a loss of *manf-1* would preclude further degeneration with the application of paraquat.

To assess whether this ROS-dependent toxicity could be inhibited, we tested the use of valproic acid (VPA) on paraquat-exposed worms. VPA is a drug commonly used for the treatment of epilepsy and bipolar disorder that protects dopamine neurons via the ERK-MAPK signalling pathway (Kautu *et al.,* 2013). This signalling cascade is known to be utilized by NTFs in order to mediate their neuroprotective properties including growth, regeneration and neurogenesis (Hao *et al.,* 2004). VPA has also been shown to upregulate the expression of MANF and CDNF, as well as heat shock proteins (e.g. HSP70) in neurons conferring protection against paraquat-induced oxidative stress and dopaminergic cell death (Marinova *et al.*, 2009; Niles *et al.*, 2012; Shukla *et al.*, 2014). As such, it was of interest to see whether VPA could mediate its neuroprotective ability in the *manf-1* knockdown background. The chronic application of VPA was shown to significantly rescue the paraquat induced neurotoxicity by day five of adulthood in wild type animals but not *tm3603* mutants. Assuming that VPA activity relies upon the upregulation of NTFs and heat shock proteins, it is possible that *manf-1* knockdown prevents the induction of a functional stress response (via the ER) and resulting neuroprotection.

The ER is a major site for protein synthesis and homeostasis. Perturbations in ER function, e.g. oxidative stress, genetic mutations, viral infection and pathology can result in the accumulation of aberrant peptides that require immediate mitigation. A prime example is that of alpha-synuclein (α-syn), the main constituent of aggregates found within the dopaminergic neurons of patients with PD. Oxidatively modified α-syn is more prone to aggregation than the native protein, and the accumulation of α-syn within the ER is linked to pathogenic chronic ER stress contributing to neurodegeneration (Giasson *et*

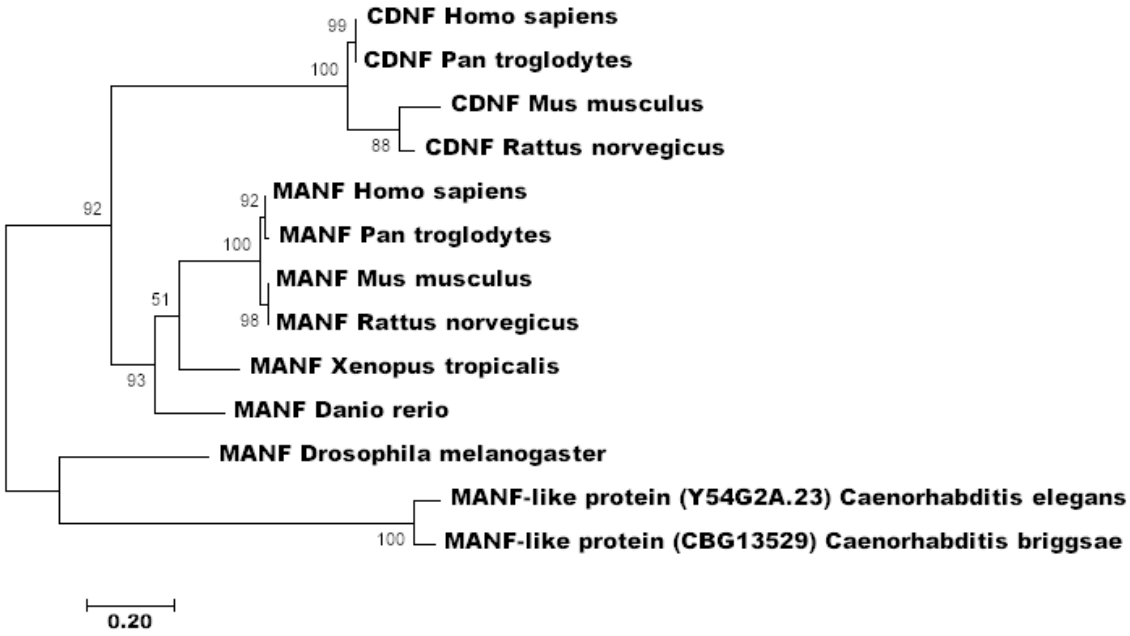
al., 2000; Higo *et al.*, 2010; D. Lindholm *et al.*, 2006; Souza, 2000). α-syn within microsomes has previously been shown to co-immunoprecipitate with GRP78 and GRP94 luminal chaperones in both symptomatic and asymptomatic transgenic lines, indicating a normal endogenous interaction (Colla, Coune, *et al.*, 2012). Given the putative association of MANF with HSP-4 in *C. elegans*, we were intrigued by the trophic factors role in mitigating the pathological aggregation of α-syn given its proximity and possible direct association with inclusions. Further, both MANF and CDNF demonstrate neuroprotection against the toxicity induced by α-syn oligomers, whereby MANF is believed to mediate its protection via the upregulation of GRP78 (Huang *et al.*, 2016; Latge *et al.*, 2015). We noted a dramatic increase in α-syn reporter expression in the *tm3603* mutant, accompanied by enhanced aggregation. Further study will be required to determine whether this enhanced aggregation is a result of increased expression, or whether this correlation is independent. Regardless, MANF seems to be capable of attenuating the level of these neurotoxic protein inclusions *in vivo*. This presents a novel platform for the assessment of MANF as a therapeutic in alleviating the ER stress implicated in neurodegenerative diseases, and provides a simple system to elucidate its molecular mechanisms of neuroprotection.

Given the activity of *manf-1* in the ER pathway, the suitability of this trophic factor as a therapeutic in other ER-associated neurodegenerative models warrants investigation. Since many diseases currently rely on the use symptomatic treatments, management strategies are quite poor. The potential for NTFs to reduce

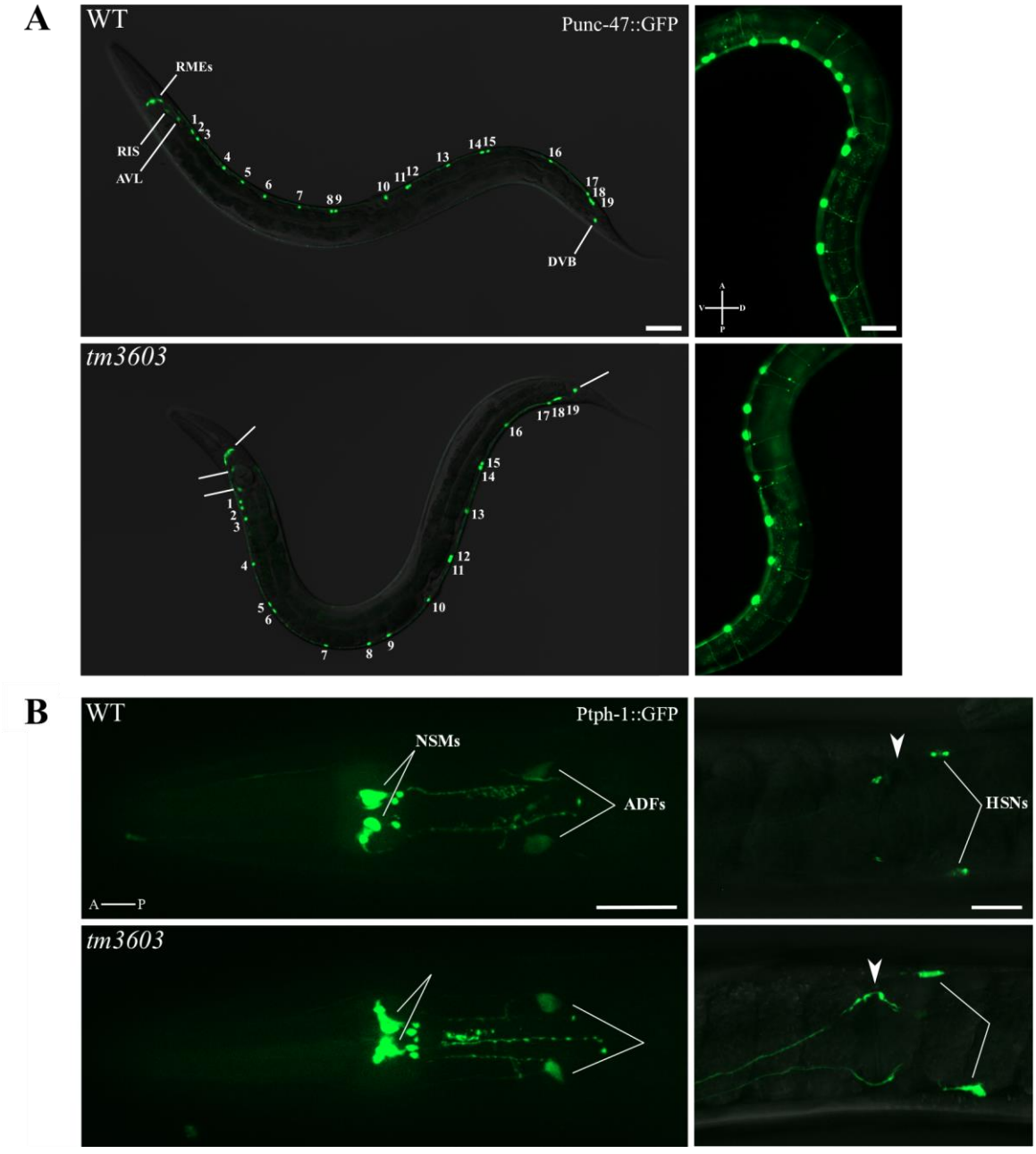
neurodegeneration, and perhaps even halt progression and stimulate neurogenesis represent the apex of hope for these therapeutic compounds.

Supplemental Data

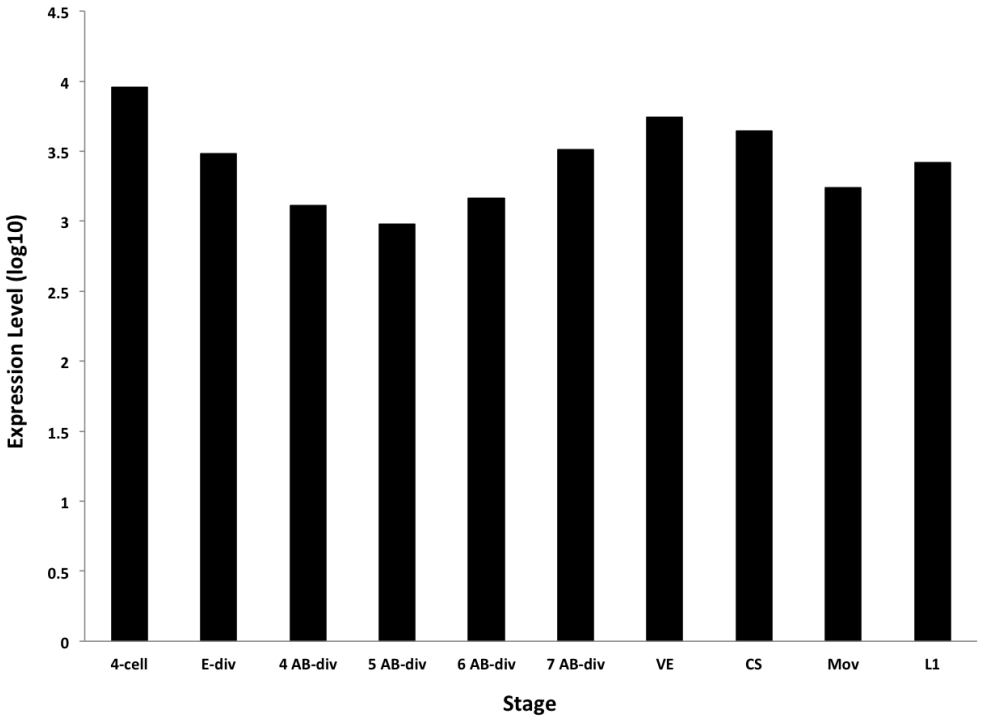
Supplementary Figure S1. Phylogenetic analysis for selected MANF and CDNF full-length amino acid sequences. Evolutionary analyses were conducted using *MEGA7* (Kumar *et al.*, 2016). Peptide sequences were aligned using ClustalW and evolutionary history was inferred using the maximum likelihood method based on the Le_Gascuel_2008 model with substitution rates determined using the invariant and discrete gamma distribution models. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and bootstrap values indicated at branch points. GenBank accession numbers for each of the amino acid sequences used in the alignment (from top to bottom, with the species names preceding in italics) include: **CDNF** *H.s* NP_001025125.2; *P.t* XP_507666.3; *M.m* NP_808315.1; *R.n* NP_001032632.1; **MANF** *H.s* NP_006001.4; *P.t* JAA43993.1; *M.m* NP_083379.2; *R.n* P0C5H9.1; *X.t* NP_001016425.1; *D.r* NP_001070097.1; *D.m* NP_477445.1; *C.e* Q9N3B0.2; *C.b* CAP32313.1.



Supplementary Figure S2. Examination of GABAergic (A) and serotonergic (B) neuronal marker expression in day one adult wild type and *tm3603* animals. Number of cell bodies and neurites exhibiting stereotyped trajectories are the same between strains. Arrowhead indicates vulva structure. Scale bar = 50 μm.



Supplementary Figure S3. Y54G2A.23 (*manf-1)* gene expression throughout embryogenesis using a species-specific microarray. Levels of transcript are shown from the 4-cell stage to the first juvenile (L1) stage. Description of developmental stages and raw data can be derived from Levin *et al.*, 2012.



Materials and Methods

Strains and Handling

C. elegans strains were cultured on nematode growth medium (NGM) plates using *Escherichia coli* strain OP50, maintained by standard protocols (Brenner, 1974). The wild type N2 Bristol strain and following transgenic lines and mutant strains were used: SJ4005 (zcIs4 [*hsp-4*::*gfp*]), SJ4100 (zcIs13 [*hsp-6*::*gfp*]), GF1366 (mgIs42 [*tph-1*::*gfp* + *rol-6*(*su1006*)]), EG1285 (oxIs12 [*unc-47*p::*gfp* + lin-15(+)]), NL5901 (pkIs2386 [*unc-54*p::α-syn::*yfp* + unc-119(+)]) were obtained from the Caenorhabditis Genetics Center (University of Minnesota, St. Paul, MN, USA). VC3705 (*manf-1*(*gk3677*)) was provided as a gift from Donald Moerman's lab.

Strains generated in house include: DY353 (bhEx138[pGLC72(*Cel-dat-1* 5'UTR::*yfp*)]), DY482 (*unc-119*(*tm4063*); bhIs7 [*unc-119*(+), *dat-1*::α-syn(A53T), *dat-1*::*yfp*]), DY487 (*manf-1*(*tm3603*)) was generated by out-crossing FX03603 obtained from Shohei Mitani's laboratory (Tokyo Women's Medical University, Tokyo, Japan), DY581 (*manf-1*(*tm3603*); zcIs4 (*hsp-4*::*gfp*), DY597 (*manf-1*(*tm3603*); bhEx247 [pGLC72(*Cel-dat-1* 5'UTR::*yfp*)]), DY598 (*manf-1*(*tm3603*); zcIs13 (*hsp-6*::*gfp*), DY612 (bhEx259 [pGLC135 (*Cel-manf-1*-5'UTR::*gfp*) + pRF4(*rol-6*(*su1006*)], DY613 (*manf-1*(*tm3603*); mgIs42 [*tph-1*::*gfp* + pRF4(*rol-6*(*su1006*)]), DY623 *(manf-1*(*tm3603*); oxIs12 [*unc-47*p::*gfp* + lin-15(+)]), DY627 *(manf-1*(*gk3677*); otIs181 [*dat-1*::*mcherry* + *tax-3*::*mcherry*]), DY644 (*manf-1*(*tm3603*); pkIs2386 [unc-54p::α-syn::*yfp* + unc-119(+)]), DY645 (*manf-1*(*tm3603*); bhIs7 [*unc-119*(+), *dat-1*::α-syn(A53T), *dat-1*::*yfp*]).

Molecular Biology and Transgenics

All primers used are listed in **Table 1.**

The *manf-1::gfp* reporter plasmid (pGL135) was generated by subcloning a *Pst*I/*BamH*I-digested 2755bp DNA fragment containing the 5'UTR of *manf-1* from *C. elegans* into the Fire lab vector pPD95.69 (using primers GL1123/GL1126). This vector contains an SV40 nuclear localization signal, allowing for concentrated expression within the nuclei of cells to assist in identification (Figure A1). Transgenic strains carrying extrachromosomal plasmids were generated using a standard microinjection technique (Mello *et al.*, 1991). DY597 was generated by injecting the *tm3603* background with 50ng/µl pGLC72 (*Cel-dat-1* 5'UTR::*yfp*). In order to generate DY612, *C. elegans rol-6*(*su1006*) was used as a marker, injected at 100ng/µl, along with 30ng/µl of pGLC135 (*Cel-manf-1*-5'UTR::*gfp*) (Kramer *et al.*, 1990).

Microscopy

Strains were mounted on 2% agar pads and anesthetized using 30mM sodium azide. Fluorescent reporter expression and neuronal imaging were visualized using a Zeiss Observer.Z1 microscope equipped with an Apotome.2 structured illumination attachment and X-Cite® 120LED fluorescence illuminator. Neurodegeneration was manually scored for number of cell bodies and neuritic morphologies using a Zeiss Axio Imager.D1 microscope equipped with the same fluorescence illuminator (Figure A2). Neurites were determined to be normal when exhibiting smooth dendritic projections from the CEPs anteriorly directed towards the tip of the head. The presence of breaks or blebs/puncta

along their lengths is indicative of degeneration. Whole animal fluorescence was quantified from stress reporter lines using a Nikon Eclipse 80i microscope equipped with a Nikon mercury lamp power supply (C-SHG1) and Hamamatsu ORCA-ER digital camera (C4742-80). Images were processed using NIH ImageJ (rsbweb.nih.gov/ij) software. Experiments were conducted using at least two biological replicates, with n = 25-30 worms per genotype per experiment.

Brood Size and Growth Delay

Progression of egg laying and overall brood size were determined by selecting synchronized L4 nematodes and placing them individually on NGM plates seeded with OP50 bacteria. Ten cloned worms from two biological replicates per strain were used, and worms were transferred to fresh plates daily. Outliers indicative of sick animals or contamination were excluded. Nematode brood size was determined based on the sum of total eggs laid by individual hermaphrodites, which were counted from plates once offspring reached adulthood. Growth delay between strains was determined by assessing the percent of worms that reached adulthood by 55 hours post L1 arrest grown at 20°C via random sampling.

Analysis of α-synuclein Aggregation

To quantify the number of α-syn inclusions, images were captured using a Zeiss Observer.Z1 microscope equipped with an Apotome.2 structured illumination attachment. The optimal exposure rate for each animal was individually determined from the

brightest/most densely aggregated plane. Acquisitions parameters were fixed to 15 Z-stacks of varied slice intervals, accounting for variations in animal size and ensuring unbiased imaging of planes. The maximum projection of each Z-stack set was generated using Zen2 blue edition software (zeiss.com/microscopy/int/software-cameras.html), with the first and last image being determined by the furthest plane in each respective direction containing clear inclusions. Foci with a minimum diameter of 2um from the nose to posterior pharyngeal bulb were analyzed and manually counted. Automated quantification of inclusions was performed by calculating total pixel area after thresholding images using ImageJ software [26]. Statistical significance was determined using t-tests.

RNA Isolation

Worms were synchronized by hypochlorite treatment (3:2 ratio of NaClO:NaOH) and grown for 60 hours at 20°C to gravid adults. After incubation, worms were collected and thoroughly washed in M9 buffer, removing any contaminating OP50 bacteria and debris. Approximately 100µl of pelleted worms were collected in eppendorf tubes and used for a TRIzol-based method of total RNA extraction. Four times the volume of TRIzol (Invitrogen, cat. #15596026) was added to the initial volume of harvested worms and vortexed until re-suspended. Samples were then flash frozen in liquid nitrogen, thawed at 37°C and vortexed. This was repeated three times. An additional two to three times the volume of TRIzol was added and vortexed, followed by two times chloroform. The solutions were shaken by hand for fifteen seconds and left at room temperature for three minutes. Each sample was then spun at 13.2k rpm for fifteen minutes at 4°C. The

resulting top aqueous layer containing RNA was then removed and pipetted into a new sterile eppendorf tube, where the volume of supernatant was estimated. An equal volume of isopropanol was added to the aqueous layer, mixed gently by hand and left at room temperature for ten minutes. The samples were then spun at 13.2k rpm for ten minutes at 4°C, after which the supernatant was discarded whilst avoiding the disruption of the formed pellet. The pellet was washed in 500µl of 75% ethanol and once again spun at full speed for 5 minutes at 4°C, after which the supernatant was discarded. The pellets were air dried at room temperature for three to five minutes and dissolved in RNase-free water preheated to 55°C. RNA samples were DNase treated (Thermo Scientific DNase I, cat. #EN0521) and the quantity of RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, cat. #ND-2000). The purity of each sample was determined using their 260/280 absorbance ratios. Additionally, RNA integrity was assessed by the visual inspection of discrete 18S and 28S risbosomal RNA bands using a 0.8% agarose gel stained with ethidium bromide. RNA preparations were reverse transcribed to generate cDNA using an AMV First Strand cDNA Synthesis Kit (NEB, cat #E6550S) in preparation for qRT-PCR.

Quantitative RT-PCR

Reaction mixes for qRT-PCR were prepared using a SensiFAST SYBR Hi-ROX Kit (Bioline, cat #BIO-92005) on a Bio-Rad CFX96 Touch Real-Time PCR Detection System. *manf-1* was amplified using the primer pair GL916/GL1232. Generated data was analyzed using BioRad CFX manager software 3.1.

Primers for quantitative real-time PCR (qRT-PCR) were designed to span an intron in order to differentiate between genomic DNA contamination and cDNA. They were designed to amplify 50-150bp amplicons, the sequences of which were entered into the mfold web server in order to select primers with minimal secondary structure for efficiency (Zuker, 2003). Primer pairs were then optimized for annealing temperature and product specificity based on melt curve analysis and amplicon specificity via gel electrophoresis (Figure A3).

Three biological replicates were carried out for both wild type (N2) and *tm3603* strains, each containing three technical replicates. Experiments were normalized to the reference gene *pmp*-3, a gene that exhibits little variation between larval stages (Hoogewijs *et al.*, 2008). Two–step cycling was carried out as follows: an initial denaturation step at 95°C for 2 minutes, followed by 40 cycles of: denaturation for 5 seconds at 95°C and a combined annealing/extension step for 30 seconds at 58°C.

Assays for endogenous *manf-1* expression throughout aging in wild type animals were carried out using a custom RT^2 qPCR Primer Assay (Qiagen, cat #330001-PPL05030A) which includes pre-designed, optimized primers.

Table 1 - List of oligonucleotide primers used in this study

Locus	Oligo Name	Sequence (5' to 3')	
Cel-manf-1	**GL915-RP**	CTCGGTGCATCCCTTGCACA	**Genotyping**
	GL916-FP	AGCCGACTCGTCCTTCTCAT	
	GL1174-FP	ACGGTTCGTTTGGCGCATAATCTCG	**Confirmation (Internal to *tm3603* deletion)**
	GL1041-FP	GAGGATCCATGAGCCGACTCGTCCTTCTC	**Insert for *hs::manf-1* & Genotyping**
	GL1042-RP	GACCATGGTCACAGCTCTTCTTTGACATATTTCGGC	
	GL1123-FP	CGCTGCAGTCGGAGCGATTTATATGCGGAG	**Translational Reporter Insert**
	GL1110-RP	CCGGATCCCACAGCTCTTCTTTGACATATTTCGGC	
	GL1126-RP	ACGGATCCGACGACAATCACCAGGGAGATGAG	**Transcriptional Reporter Insert (+ GL1123)**
	GL1232-RP	GCATCTGGCTTGGATTTGTCG	***manf-1* qRT-PCR (+ GL916)**
	GL1283-FP	GATGCACCATGACCCGATCTC	**Genotyping *gk3677* allele**
	GL1284-RP	GTTGCATCACCTTCACCCTCTC	
Cel-pmp-3	**GL747-FP**	CTTAGAGTCAAGGGTCGCAGTGGAG	**qRT-PCR**
	GL748-RP	ACTGTATCGGCACCAAGGAAACTGG	
Human-SNCA	**GL1134-RP**	CGAGGCATTTCATTGATCTGG	**Sequencing *α*-synuclein**
	GL860-FP	AAACTGCAGCTATGGATGTATTCATGAAAGGACTTTCAAAGGCCAAGG	**Genotyping**
	GL861-RP	AAAGTCGACTTAGGCTTCAGGTTCGTAGTCTTGATACCC	

Chapter 3: Summary and Future Directions

In this thesis, we have characterized the expression and functional role of the *C. elegans* MANF homolog, *manf-1*. We have validated a deletion strain (*tm3603*) by assessing its level of transcript (reduced four-fold) and determining a mutant phenotype. Dopaminergic, GABAergic and serotonergic neurons appear morphologically wild type at day one of adulthood, indicating that *manf-1* does not have a necessary function in neuronal development, or may simply be redundant. However, there is an enhanced propensity for dopaminergic cell bodies to degenerate with age. The hypersensitivity of the dopaminergic system with age is accompanied by a significant increase in ER stress, but not mitochondrial stress. This is in line with previous evidence suggesting that MANF carries out its function via the ER UPR.

We further characterized life and behavioural traits, determining that mutants are able to mount a normal chemotactic response, indicative of its fully functioning chemosensory nervous system. There is a growth delay in the *tm3603* mutant along with a smaller number of fertilized eggs laid daily and final brood size.

Wild type animals exhibit a significant decrease in *manf-1* expression throughout adulthood, however this decline in expression does not present a degenerative phenotype, suggesting the normal maintenance of dopaminergic health even in the presence of reduced levels of MANF. The expression pattern of *manf-1* was determined by generating a transcriptional reporter, displaying expression at all larval stages. In early development expression is fairly ubiquitous, with no detection within the gonadal regions. It is later visible in the intestine and highly concentrated in the spermatheca by adulthood. Reporter

expression declines with age, in line with our previous finding indicating a substantial decrease in *manf-1* expression throughout adulthood.

The induction of oxidative stress from chronic paraquat exposure did not enhance the degeneration exhibited by the *tm3603* mutant alone, and cannot be rescued using VPA. This may suggest the involvement of MANF in paraquat's mode of neurotoxicity and rescue from VPA. In an effort to assess the therapeutic potential of *manf-1*, we determined the protein's ability to attenuate ER-associated α-syn inclusion pathology, exhibited in PD. There was a significant increase in α-syn expression and oligomerization in the *tm3603* mutant, indicating that MANF can help mitigate α-syn expression and aggregation.

Given the multiple lines of evidence implicating MANF's function in the ER along with the aforementioned findings, an early model can be proposed for the endogenous cellular regulation and function of *manf-1* (Figure 1).

The transcriptional regulation of *manf-1* is not currently known, however there are several transcription factor binding sites determined by ChIP data from the modENCODE project that merit analysis and *in vivo* testing (Araya *et al.*, 2014). Those transcription factors implicated in nervous system development are indicated aside (Figure 1A). Other relevant transcription factors of particular interest include SKN-1, a transcription factor orthologous to the mammalian Nuclear factor-erythroid-related factor (Nrf) involved in the regulation of the oxidative stress response, PQM-1 encoding a protein that is upregulated in response to oxidative stress induced by paraquat, and NHR-6, orthologous to mammalian orphan nerve growth factor-induced clone B (NGFI-B/NR4 subgroup)

receptors required for spermatheca development (An & Blackwell, 2003; Gissendanner *et al.*, 2008; Tawe *et al.*, 1998).

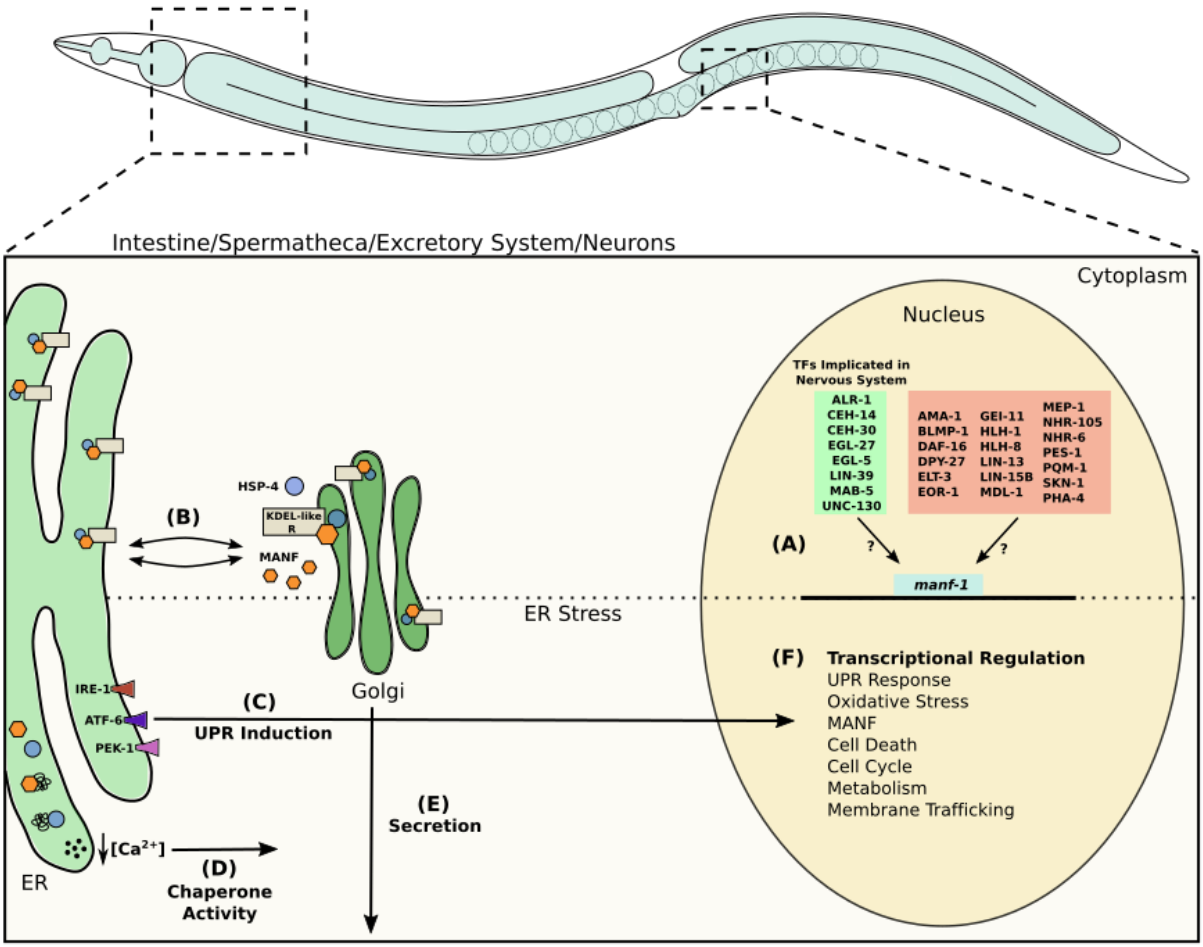


Figure 1 Putative model for *manf-1* regulation and function.

Because MANF is implicated in the ER stress response and secretory pathway which inherently involve transport between the ER and Golgi, MANF may serve a role in mediating trafficking or translocation between organelles in response to insult (Figure 1B). As MANF and GRP78 (homolog of HSP-4) both share KDEL motifs and have a calcium dependent interaction, it is possible that MANF forms a complex with the HSP-4 luminal chaperone to help stabilize the main ER UPR activators, maintaining them in

their inactive state (Figure 1C) (Mizobuchi *et al.*, 2007). The induction of ER stress using thapsigargin depletes the ER (and sarcoplasmic reticulum) of calcium, increasing the levels of MANF and yielding less MANF/GRP78 complexes (Glembotski *et al.*, 2012). The dissociation of MANF from HSP-4 upon ER stress may allow either/both proteins to carry out chaperone activity (Figure 1D). The combination of MANF's secretion and intracellular role could indicate its own regulation via autocrine signaling (Figure 1E). Thus it may also play a role in self-regulating its own expression. The result of these summed intra- and extracellular effects is the induction of a large transcriptional program (Figure 1F), counteracting stress and maintaining ER homeostasis under normal conditions.

Future Directions

The novelty of this class of NTFs presents many unexplored avenues for research to proceed. First and foremost should be the determination of a respective receptor and primary downstream signaling pathway of MANF, which has yet to be reported. The MANF protein could be retrieved using a pull down assay (e.g. Co-IP) followed by mass spectrometry to determine the corresponding binding partner. Given the presence of the putative ER retention signal sequence in *C. elegans*, the receptor mediated retention of MANF-1 within the ER could be determined via the generation of a translational fusion reporter line (the plasmid of which was generated in this study - Figure A1) and localization of protein subsequent to mutating KDELR homologs in *C. elegans* (C28H8.4 and *erd-2*). Removal of the RTDL sequence has been shown to increase MANF levels in

cell supernatants, possibly due to reduced ER/Golgi retention by the KDELRs (Henderson *et al.*, 2013). However, the secretion of MANF can also be attenuated by the overexpression of GRP78. Interestingly, the GRP78 interaction is not fully dependent on the presence of the RTDL sequence, as even with the loss of this element, MANF can be retained within the ER via its interaction with GRP78 (Oh-Hashi *et al.*, 2012). As such, complex formation seems to prevent secretion. It would be of interest to determine whether these binding interactions mediate differences in intracellular activities, or whether they simply function as a means of retention to the ER and Golgi apparatus.

Study of the structural differences between MANF and CDNF will help determine the cause of differences in function. Since human MANF (and not CDNF) could rescue the lethality of a *Drosophila* knockout mutant, individual differences in residues were identified to account for this functional difference (Palgi *et al.*, 2009; Parkash *et al.*, 2009). Genetic dissection of the identified residues should be carried out by generating a variety of deletion mutations to attribute importance to specific changes in structure.

It would also be interesting to assess whether MANF's neuroprotective properties are functionally conserved by using heterologous promoters/proteins (e.g. human/murine) to determine the level of conservation in *C. elegans*.

MANF-1 appears to regulate components of the ER UPR; it would be of interest to elucidate which cellular components are implicated in this regulation. Of particular interest would be the main UPR signal transduction protein homologs IRE-1, ATF-6 and PEK-1. Components of these specific signalling pathways could be targeted by RNAi to determine suppressor mutations in our *tm3603* stress reporter background.

MANF is a neuroprotective compound capable of inhibiting apoptosis, where its application reduces the activation of caspase 3 following oxidative insult (Huang *et al.*, 2016). Previous attempts to verify the direct MANF-Bax interactions have failed (Voutilainen *et al.*, 2015). As such, how MANF prevents apoptosis upstream of Bax needs to be determined. MANF's regulation of the apoptotic signalling cascade would help determine its particular method of neuroprotection.

To complement our analysis of *manf-1* deletion mutants, rescue experiments in response to different neurodegenerative insults could be conducted by overexpressing MANF under the control of a heat shock promoter. This was attempted however the generated transgenic could not be validated due to a lack of detectable overexpression (Figure A4).

The levels of neurotransmitter within the *manf-1* mutant, particularly dopamine, should be determined to assess whether MANF plays a role in synthesis. Dopamine levels have been shown to be reduced in *Drosophila* knockout and zebrafish knockdown mutants (Chen *et al.*, 2012; Palgi *et al.*, 2009) We aimed to quantify the dopamine levels within *C. elegans*, but unfortunately could not achieve levels above the limit of detection (Figure A5). Sample enrichment (e.g. derivatization) can be done in the future to facilitate detection.

MANF's action is intimately tied to the oxidative state of cells. To test its therapeutic potential in response to oxidative stress directed to the dopamine neurons, we treated animals with the neurotoxic herbicide paraquat which increases ROS levels,

activates the JNK pathway and caspase-3 leading to neurotoxicity (Peng *et al.*, 2004). The application of this neurotoxin to the *tm3603* deletion background did not enhance the degeneration exhibited by the mutant alone. It is important to note that the cellular mechanisms underlying the degenerative process induced by paraquat are still unclear, and it is possible that this pathway implicates MANF in its function. This could be assessed using the MANF overexpression line previously described in a *manf-1* mutant background. Other lines of evidence have suggested that paraquat's toxicity may result from the generation of superoxides by microglia, or in the acetylation of core histones (Song *et al.*, 2011; Wu *et al.*, 2005). Paraquat is unique from other environmental toxins as it has been implicated in the etiopathogenesis of PD. Given its many mechanisms of toxicity, determining how MANF may regulate paraquat's role in neurodegeneration can help develop novel targets for the treatment of resulting exposure or the affiliated disease itself.

Differences have been found in the transcriptional profiles of cortical cells from PD patients (Gebremedhin & Rademacher, 2016). A reduction in histone deacetylase (HDAC) enzymes has been detected in the midbrain of PD patients and is believed to be degraded in part by the induction of autophagy (Park *et al.*, 2016). The emergence of therapeutics targeting epigenetic processes is not very recent, however its re-appropriation to the treatment of neurodegenerative diseases has presented as a novel field of research. This was fueled by the ability of many histone deacetylase inhibitors (HDACIs) to cross the blood brain barrier and by findings indicating that the inhibition of

the 4 classic families of HDAC enzymes exhibit neuroprotective properties both *in vitro* and *in vivo* in various models of neurodegenerative disease (Hahnen *et al.*, 2008). By altering levels of histone acetylation, we can modify the transcriptional program of diseased cells in order to re-establish homeostasis.

Valproic acid (VPA) is one of the most commonly used HDACIs for the treatment of neurological disorders, and is primarily used as an antiepileptic and mood stabilizing drug. It functions as a pan-inhibitor of HDAC classes I and IIa, and has been shown to impart neuroprotection in several models of PD (Harrison & Dexter, 2013). VPA is capable of inducing the expression of several genes, including neurotrophic factors (e.g. BDNF, GDNF, MANF & CDNF) and heat shock proteins (e.g. HSP70) (Chen *et al.*, 2006; Marinova *et al.*, 2009; Niles *et al.*, 2012). VPA is also capable of mediating a variety of signaling pathways, including the ERK/MAPK pathway utilized by neurotrophic factors, possibly through their up-regulation (Di *et al.*, 2005). We have shown that the loss of MANF function prevents the neuroprotection imparted by VPA in animals treated with paraquat. Given the shared signalling mechanism between VPA and NTFs, determining how they converge and modulate one another's signalling is important, especially if both can be adapted for concurrently administered treatments.

MANF plays a role in preventing the aggregation of α-syn *in vivo* (demonstrated here) and is able to counter apoptosis via caspases-3 in response to the overexpression of α-syn *in vitro* (Huang *et al.*, 2016). However its neuroprotective action countering this pathology requires further study. α-syn has been shown to sensitize cells to oxidative

insult and elicit pathogenic chronic ER stress, upregulating a variety of stress markers like GRP78 and p-EIFa (an indicator of PERK activation) (Jiang *et al.*, 2010; Smith *et al.*, 2005). It also mediates direct effects on the ER, having been found within the lumen of microsomes, supporting a direct association with the organelle. In PD mouse models and tissues derived from patients, these microsome fractions are immunoreactive for toxic soluble oligomers and fibrils (Colla, Jensen, *et al.*, 2012). Further support for an ER stress mediated form of toxicity comes from the array of protective properties imparted by the ER stress inhibitor salubrinal which includes a reduction in α-syn accumulation, extension of lifespan, decreased Golgi apparatus fragmentation (considered an early event preceding neuronal death in response to ER stress) and enhanced cell survival (Colla *et al.*, 2012; Jiang *et al.*, 2010; Nakagomi *et al.*, 2008; Smith *et al.*, 2005) Additionally, treatment with tunicamycin exacerbates the oligomerization of α-syn (Jiang *et al.*, 2010). As such, α-syn's method of toxicity is well suited to MANF's intracellular protective activity within the ER.

The association of α-syn with GRP78 proteins which can also immunoprecipitate MANF, presents a proximity that questions whether there is a direct interaction between MANF and α-syn themselves, perhaps supporting endogenous chaperone or clearance activities (Colla, *et al.*, 2012). Because MANF can help reduce endogenous oxidative stress, this effect alone could influence pathology since oxidatively modified α-syn is more prone to aggregation than the native protein (Giasson *et al.*, 2000; Souza, 2000). Currently, the exact mechanism of neuroprotection is not known.

Two common familial point mutations in PD are frequently replicated in disease

models, namely A53T and A30P, for their increased tendency to aggregate (Krüger *et al.*, 1998; Polymeropoulos, 1997). Inducing the expression of A53T α-syn results in decreased chymotryptic proteasome activity, increased intracellular ROS and increased apoptosis, indicated by higher levels of caspase 3, 9 and 12 activities (a hallmark of ER stress induced apoptosis) (Smith *et al.*, 2005). We recently generated a strain containing A53T α-syn expressed within the dopamine neurons of the *tm3603* deletion background (Figure A6). This strain should be utilized in the future to model the α-syn associated degeneration associated with PD. It would be of interest to know whether a loss of *manf-1* function exacerbates the pathology of α-syn specifically within the dopamine neurons in this model system. High-throughput testing of the efficacy of different toxins and therapeutics could also be performed by assessing neurodegeneration in this strain and/or the expression of the α-syn-YFP chimeric protein described earlier.

A large field of inquiry moving forward will include MANF's role in non-neuronal tissues. For example, glial support cells (e.g. astrocytes, oligodendrocytes, microglia) show an up-regulation of MANF following middle cerebral artery occlusion (MCAO), a model of stroke (Shen *et al.*, 2012). Other tissues demonstrating protection from MANF include cardiac myocytes, fibroblasts, pancreatic b, and the hematocytes of *Drosophila* amongst others (Lee *et al.*, 2003; Mizobuchi *et al.*, 2007; Neves *et al.*, 2016; Tadimalla *et al.*, 2008). Given the many cell types that are capable of utilizing MANF for cell protection, its therapeutic potential is system wide.

Finally, the application of MANF as a general therapeutic against ER stress is promising. For example, ER stress can be a significant contributing factor to tumour pathogenesis (Feldman *et al.*, 2005). MANF has been shown to inhibit cell proliferation and reduce cell size *in vitro* (Apostolou *et al.*, 2008). Therefore, it is possible that MANF plays a role in mediating tumour responses via its induction under hypoxic conditions to inhibit cell proliferation and protect cells from ER stress-induced death. With the ultimate aim of therapeutically modulating trophic levels, we require a much better understanding of the regulation, biological activity and transcriptional profile of this novel NTF family.

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Appendix

A1- In order to assess the localization of MANF protein, both transcriptional and translational fusion reporter constructs were generated. Transcriptional reporter expression has been described, however we were not able to obtain a stable translational line. This can be revisited in the future.

The translational fusion was generated by placing a *Pst*I/*BamH*I-digested 3420bp fragment of the *manf-1* 5' UTR along with the unspliced coding sequence in frame with a GFP reporter using the pPD95.75 Fire Lab vector (primers - GL1123/GL1110); this backbone contains no localization sequence permitting the endogenous localization of protein, indicating where the protein is stored, expressed, and/or secreted.

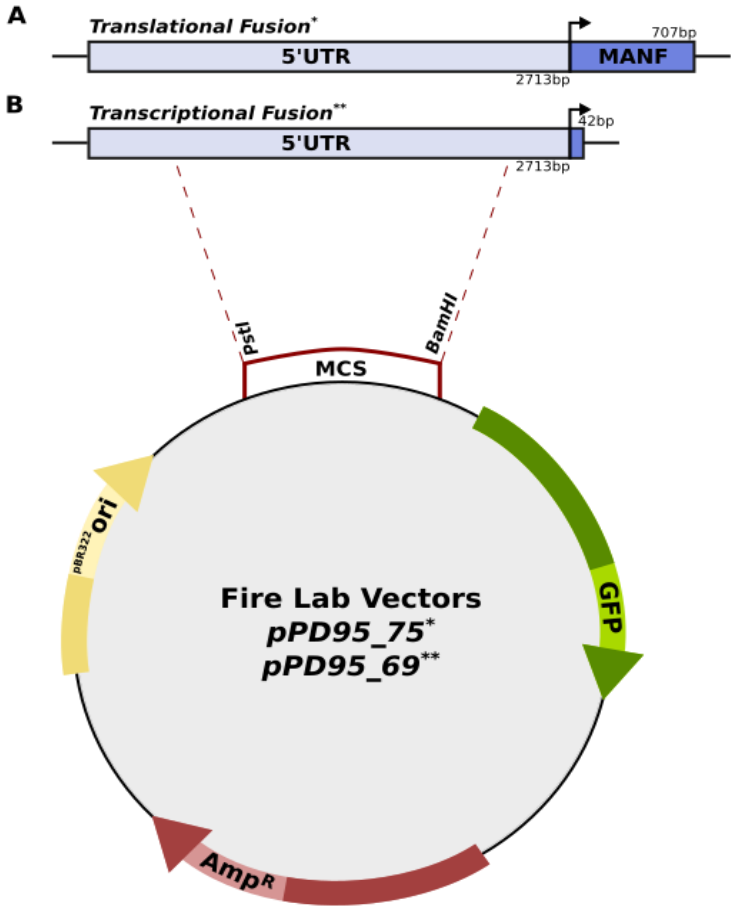


Figure A1 Transcriptional and translational reporter fusion constructs. (A) Translational fusion containing MANF 5' UTR along with the coding sequence in frame with GFP using the pPD95.75 Fire Lab vector. **(B)** Transcriptional fusion contains the same 5'UTR sequence as well as the start codon of the gene in frame with GFP in the pPD95.69 Fire Lab vector.

A2 – Quantification of neuronal degeneration phenotype in aging *manf-1* mutants. The manuscript in chapter 2 (Figure 4A, p.24) has a similar graph generated by Sabih Rashid which is a replication of the data presented here. The results in this figure show a similar trend although exact numbers vary from Figure 4A possibly due to differences in scoring criteria and/or batches.

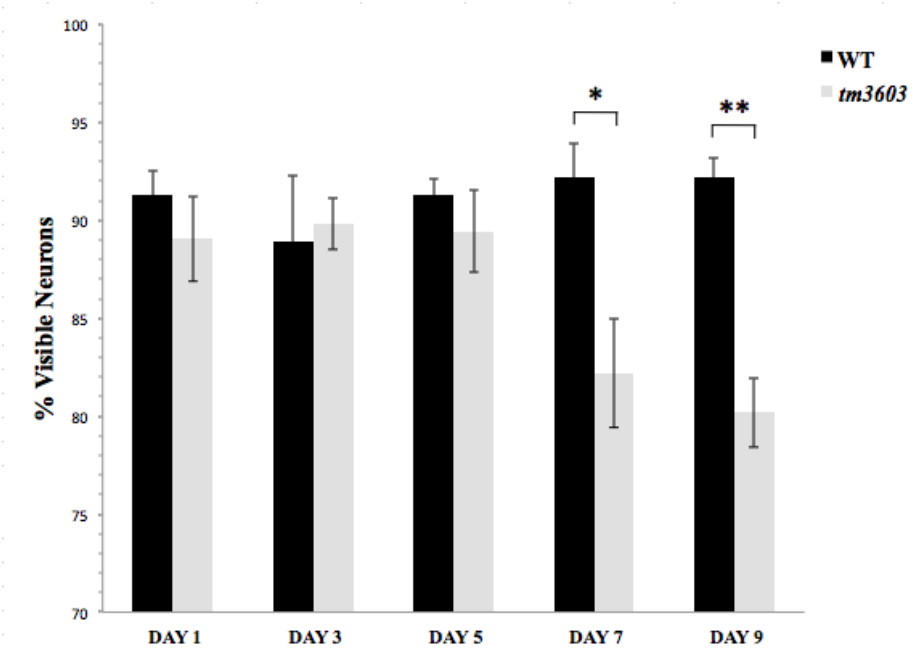


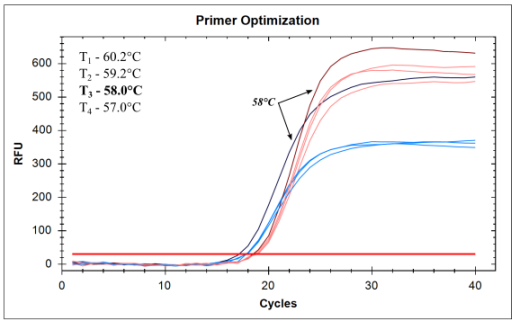
Figure A2 Age-dependant dopaminergic neurodegeneration in wild type and *tm3603* animals. Presence of dopaminergic neurons with age in day 1-9 adults of wild type and *tm3603* mutant animals. Performed using three biological replicates, n=90 ± SEMs, **P*<0.05, ***P*<0.01.

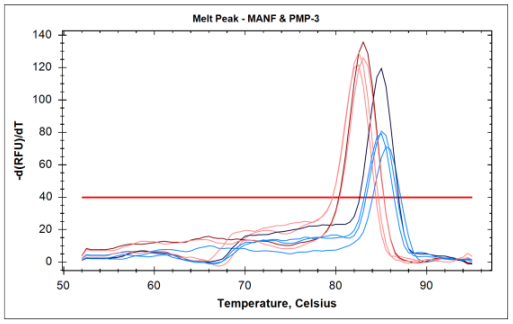
A3 - Primer optimization for qRT-PCR

A

Gene	Primer	T_m	Size		Sequence (5' – 3')
manf	GL916	58°C	134bp	Fwd	AGCCGACTCGTCCTTCTCAT
	GL1232	56.8°C		Rvs	GCATCTGGCTTGGATTTGTCG
pmp-3	GL747	60.6°C	98bp	Fwd	CTTAGAGTCAAGGGTCGCAGTGGAG
	GL748	61°C		Rvs	ACTGTATCGGCACCAAGGAAACTGG

B





C

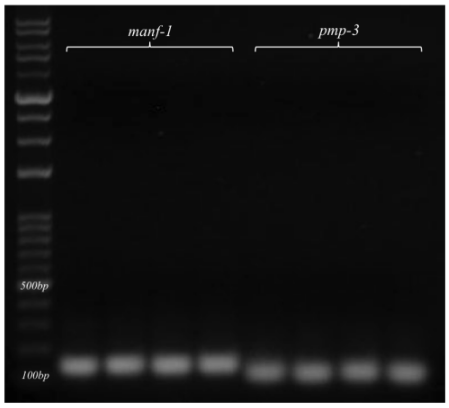


Figure A3 Primer Optimization for qRT-PCR amplification of *manf-1*.
(A) Primers along with corresponding melting temperatures and fragment lengths of amplicons. **(B)** Melting curves depict optimal temperature (58°C) for *manf-1* and *pmp-3*. No additional peaks observed, indicative of nonspecific amplification **(C)** Agarose gel of qRT-PCR fragments obtained from amplification of *manf-1* and *pmp-3* in wild type background as an additional means of confirming primer specificity.

A4 - The *manf-1* gene was placed under the control of the heat shock *hsp16.2* gene promoter previously designed by undergraduate student Komal Prajapati. This was injected into the *tm3603* background to conduct rescue experiments. In order to validate the strain, we attempted to quantify protein levels after heat shocking by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining. A difference in band intensity could not be detecting. Since no commercial antibody is available, this was left aside. Future studies could utilize the existing plasmid to incorporate a common epitope tags (e.g. His-tag, Myc-tag) to validate the use of this transgenic.

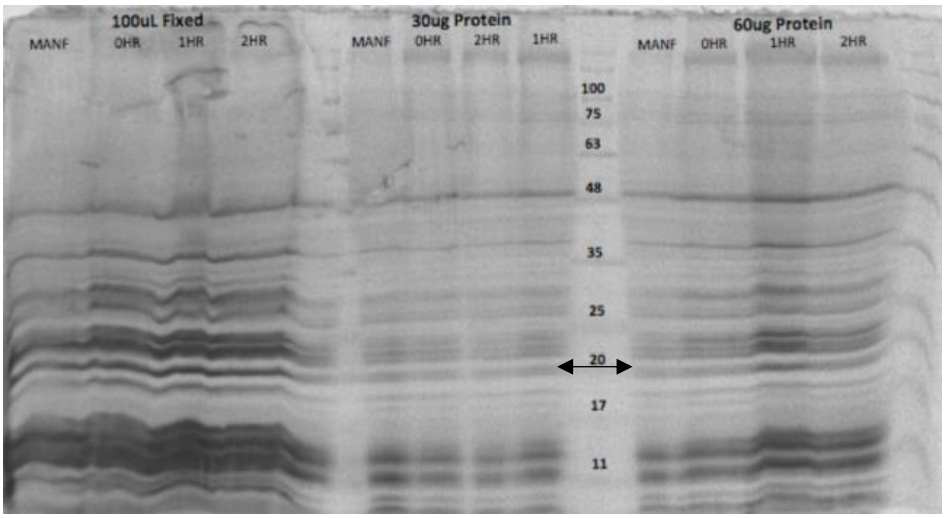


Figure A4 Validation of m*anf-1* overexpression using a heat shock vector. SDS-PAGE Coomassie stained gel, P*hsp16.2*::*manf-1* heat shock strain after 0, 1 and 2 hour exposures to 33°C, with *tm3603* mutant as a control. Anticipated location of increased band intensity indicated by arrows (~19kDa). None detected.

A5 - Dopamine measurements via LC-MS in *C. elegans*

Measurement of dopamine was standardized using a Quadrupole Time-of-flight mass spectrometer (Q-TOF). Despite successful measurements in standards, we could not get dopamine measurements above the limit of detection when testing *C. elegans* samples. Future attempts should use electrochemical detection or derivatization of the samples.

A PFP column was used with Buffers A and B, composed of standard water in 0.1% FA and acetonitrile in 0.1% FA respectively.

Q-TOF Method
MS scan rate=3
MS/MS scan rate = 2
Isolation width MSMS = Narrow
Collision Energy = 10
Excluded masses: 922.091, 121.0507
Preferred mass = 154.0867
Max precursor/cycle = 5
Threshold = 10000
Target (counts/spectra) = 50000
Use MSMS accumulation time = yes
Purity Stringency = 100%
Purity cut off = 30%
Isotope model = common
Active exclusion enabled = yes
Sort precursors = by charge state and then abundance

Source Parameters:
Gas Temp = 175°C
Fragmentor Voltage = 175
Scan rate (spectra/sec) = 3
Gas Flow = 16 L/min
Nebulizer = 35 psig
Sheath Gas temp = 350°C
Sheath gas Flow = 12 L/min
VCap = 3500
Nozzle Voltage = 500
Skimmer 1 = 0
Octopole RF Peak = 750

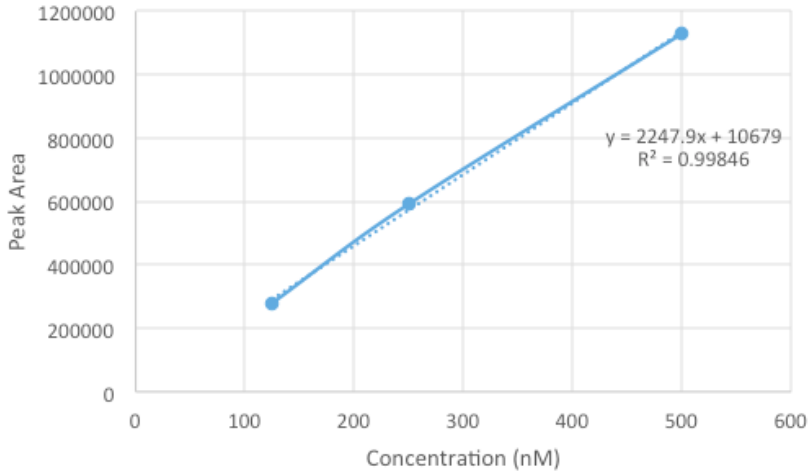


Figure A5 Dopamine measurement via LC-MS in *C. elegans*.
Standard curve for using 125, 250 and 500 nM of dopamine.

A6 - In an effort to create a PD model in *C. elegans*, p*dat-1*::αSyn(A53T) plasmid was obtained as a gift from Takeshi Iwatsubo's laboratory (University of Tokyo, Japan). Unfortunately, the fragment of human α-synuclein cDNA present in the plasmid was never described in literature and was obtained from another researcher in Japan, Dr. Shigeo Nakajo, who never disclosed the sequence. Therefore, in order to determine the identity of the insert, the backbone was digested using the same restriction enzymes as previously described (Kuwahara *et al.*, 2006), yielding a fragment ~420bp . Upon sequencing, the segment of α-synuclein was confirmed and shown to have the desired A53T mutation. This plasmid was previously injected and integrated (DY482) and has been crossed to the tm3603 background (DY645) for future use.

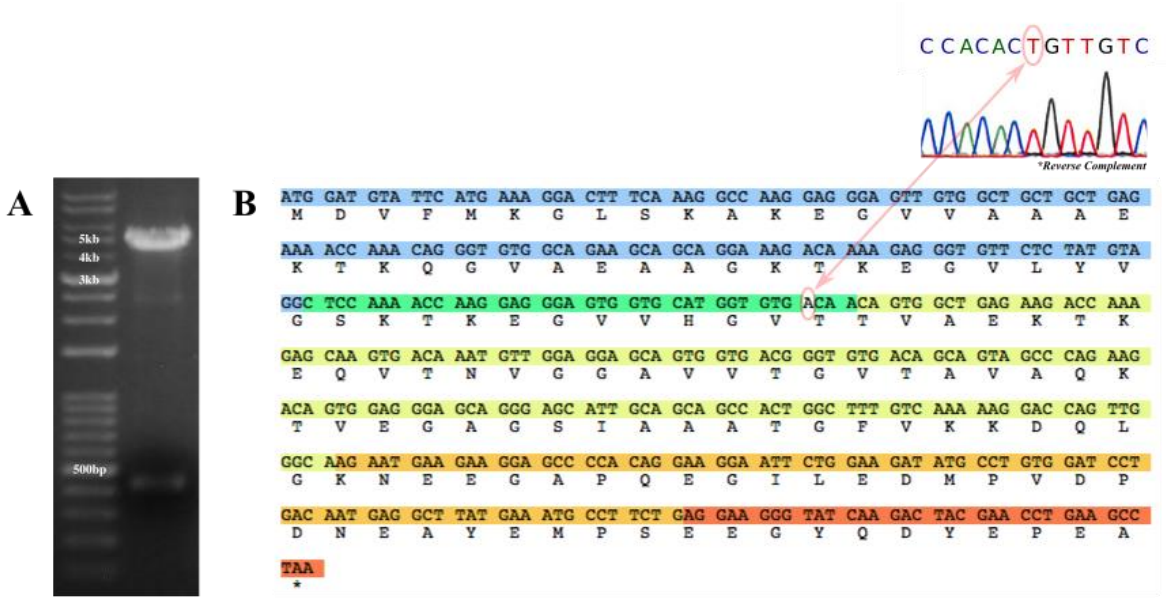


Figure A6 Validation of A53T mutation in alpha-synuclein. (a) Digestion of p*dat-1*::αSyn(A53T) plasmid with NotI and BglII to determine approximate size of α-syn cDNA insert. (b) Sequencing results verified the presence of the A53T mutation, indicated by the circled nucleotide.