ROLE OF DIETARY FAT AND SUPPLEMENTATION IN MODULATING NEURODEGENERATIVE PATHOLOGY IN TWO ANIMAL MODEL SYSTEMS

By

Malabika Maulik, B.S, M.S.

A Dissertation Submitted in Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

in

Biochemistry and Neuroscience

University of Alaska Fairbanks

December 2018

APPROVED:

Abel Bult-Ito, Committee Co-Chair
Barbara E Taylor, Committee Co-Chair
Lawrence Duffy, Committee Member
Thomas Kuhn, Committee Member
Kriya Dunlap, Committee Member
Thomas Green, Chair

Department of Chemistry and Biochemistry
Leah Berman, Interim Dean

College of Natural Sciences and Mathematics
Michael Castellini, Dean of Graduate School
Abstract

Neurodegenerative disorders are progressive conditions that worsen over time and result in death of neurons. Parkinson’s disease (PD) is a prevalent example of one such age-related disease, which is characterized by movement disorder (ataxia) and/or cognitive disability (dementia). Pathologically, PD is characterized by a toxic accumulation of α-synuclein protein in the midbrain leading to degeneration of the dopaminergic neurons. The etiology of PD is intricate, and the cause is attributed to genetic mutations and environmental factors like insecticides or heavy metals. Moreover, treatment options are limited and often aimed at treating the symptoms rather than the actual disease progression. Using the nematode model of Caenorhabditis elegans, I examined the effect of Alaskan bog blueberry (Vaccinium uliginosum) on α-synuclein overexpression and how such indigenous natural treatment can modulate key molecular targets like sirtuins, which are proteins involved in regulating cellular processes including aging, death and their resistance to stress. The impact of extrinsic factors like dietary fat on PD pathology has been sparsely explored and the molecular basis of such changes is not known. Through my thesis research, I also further investigated the influence of fat metabolism on key hallmarks of PD: α-synuclein overexpression and dopaminergic degeneration in the nematode model. Finally, I studied the interaction of dietary fat (normal, low and high fat) and Alaskan blueberry supplementation on metal induced neurotoxicity model of Mus musculus. Our results highlight the beneficial properties of Alaskan blueberries in combating proteotoxic stress and inflammation in both animal models. They also reiterate the benefit of low fat diet, on its own or in combination with supplementation in improving several PD-like molecular features and how consuming high fat can mask such health promoting outcomes. The current thesis work therefore, provides a foundation for further
exploration of neurobiological changes associated with consumption of natural products and different diets and how such alterations can be extrapolated to humans.
Dedication

I would like to dedicate this dissertation to my son, whose relentless sacrifices allowed me to pursue and complete my education at University of Alaska and to my husband, whose enthusiasm and mentoring turned this dream into reality.
Table of Contents

Abstract ............................................................................................................................................ i

Dedication ....................................................................................................................................... iii

Table of Contents .......................................................................................................................... iv

List of Figures ................................................................................................................................... xii

List of Tables ..................................................................................................................................... xv

Acknowledgements ........................................................................................................................ xvi

Chapter 1: General Introduction .................................................................................................. 1

1.1 Caenorhabditis elegans and Mus musculus models of Parkinson’s Disease ...................... 2

1.1.1 Nematode Caenorhabditis elegans ...................................................................................... 2

1.1.2 House Mouse (Mus Musculus) .......................................................................................... 3

1.2 Genetic PD models of C. elegans and M. musculus ............................................................. 4

1.2.1 α-Synuclein .......................................................................................................................... 4

1.2.2 LRRK2 and PINK1 ............................................................................................................... 6

1.2.3 Parkin ..................................................................................................................................... 7

1.2.4 DJ-1 ....................................................................................................................................... 8

1.3 C. elegans and M. musculus Toxin-induced Models of Parkinson’s Disease ................... 8

1.3.1 MPTP and 6-OHDA ............................................................................................................ 8

1.3.2 Insecticides and Herbicides ............................................................................................... 9
1.3.3 Manganese .......................................................................................................................... 10

1.4 Role of Diet in Parkinson’s Disease .......................................................................................... 11

1.5 Role of Natural Compounds in Parkinson's Disease ................................................................. 13

1.6 Research Objectives .............................................................................................................. 14

1.6.1 Aim I: Study the Role of Alaskan Bog Blueberries on α-synuclein Overexpression in a Transgenic C. elegans Model of PD .......................................................................................................................... 15

1.6.2 Aim II: Silencing Fat Metabolism Genes in Parkinson's like Models of Caenorhabditis elegans .................................................................................................................................................................................. 16

1.6.3 Aim III: Complex Interaction of Dietary Fat and Alaskan Bog Blueberry Supplementation Influences Manganese Mediated Neurotoxicity and Behavioral Impairments ................................................................. 16

1.7 References ............................................................................................................................... 18

Chapter 2: Sir-2.1 Mediated Attenuation of α-synuclein Expression by Alaskan Bog Blueberry Polyphenols in a Transgenic Model of Caenorhabditis elegans ................................................................. 39

2.1 Abstract .................................................................................................................................. 39

2.2 Introduction ................................................................................................................................ 40

2.3 Materials and Methods ........................................................................................................... 42

2.3.1 Strains, Maintenance and Synchronization ........................................................................ 42

2.3.2 Berry Extract Preparation .................................................................................................. 42

2.3.3 Treatment Administration .................................................................................................. 43

2.3.4 Lifespan Analysis ................................................................................................................. 44

2.3.5 Motility .................................................................................................................................. 44
2.3.6 Fecundity............................................................................................................................44
2.3.7 RNA Interference.................................................................................................................44
2.3.8 Lipid Staining.......................................................................................................................45
2.3.9 DCF-DA Assay ....................................................................................................................45
2.3.10 qPCR Analysis .................................................................................................................45
2.3.11 Protein Extraction and Quantification ..................................................................................46
2.3.12 Western Blots .....................................................................................................................46
2.3.13 Microscopy and Image Quantification ..................................................................................47
2.3.14 Statistical Analysis .............................................................................................................47
2.4 Results ...................................................................................................................................48
2.4.1 Crude extract of Alaskan bog blueberry Attenuated Expression of Human α-synuclein in C. elegans ...........................................................................................................................................48
2.4.2 Alaskan Bog Blueberry Polyphenolic Fraction Reduced Expression of Human α-synuclein in C. elegans ................................................................................................................................................49
2.4.3 Alaskan Bog Blueberry Polyphenols Reduced Human α-synuclein Expression in C. elegans through RNA Interference Mediating sir-2.1 .................................................................................................................................49
2.4.4 Alaskan Bog Blueberry Extract Improved Motility Mediated by sir-2.1 in C. elegans Expressing Human α-synuclein .................................................................................................................................50
2.4.5 Alaskan Bog Blueberry Treatments did not Affect Lifespan or Total Progeny in C. elegans Expressing Human α-synuclein .................................................................................................................................50
2.4.6 Alaskan Bog Blueberry Restored Lipid Content in C. elegans Expressing Human α-
synuclein

2.4.7 Alaskan Bog Blueberry Treatment Reduced Gene Expression Levels of sir-2.1 in C. elegans Expressing Human α-synuclein

2.5 Discussion

2.6 Conclusion

2.7 Acknowledgements

2.8 Figures and Tables

2.9 References

Chapter 3: Silencing Fat Metabolism Genes in Parkinson's like Models of Caenorhabditis elegans

3.1 Abstract

3.2 Introduction

3.3 Materials and Methods

3.3.1 C. elegans Strains and Maintenance

3.3.2 RNA Interference

3.3.3 Rotenone Administration

3.3.4 Lifespan Analysis

3.3.5 Body Bends

3.3.6 Fecundity

3.3.7 Pharyngeal Pump Rate
3.3.8 Stress Resistance Assays: Heat Stress and Osmotic Stress ........................................... 99
3.3.9 Dopamine Dependent Behaviors .................................................................................... 99
  3.3.9.1 Basal Response to Food ......................................................................................... 99
  3.3.9.2 Ethanol Avoidance Assay .................................................................................... 99
3.3.10 Microscopy and Image Quantification ......................................................................... 100
3.3.11 Statistical Analysis .................................................................................................... 100
3.4 Results .......................................................................................................................... 100
  3.4.2 Genetic Silencing of fat-5 and fat-7 Genes Improved Resistance to Heat in a Human Wild-type α-synuclein Overexpression Model of C. elegans ........................................................... 101
  3.4.3 Genetic Silencing of fat-5 and fat-7 Genes Improved Dopaminergic Neuron Degeneration and Related Behaviors in a Model of C. elegans Expressing Human Mutated α-synuclein (A53T Mutation) .................................................................................................................. 102
  3.4.4 Defects in Stress Resistance was Improved by Silencing of fat-5 and fat-7 Genes in C. elegans Expressing Human Mutated α-synuclein (A53T Mutation) .......................................................... 102
  3.4.5 Silencing of fat-5 and fat-7 Genes Rescued Dopaminergic Degeneration and Associated Behaviors in a Rotenone-induced Model of C. elegans ......................................................... 103
  3.4.6 Lifespan or Healthspan Measures were Unaltered by Silencing of Fat genes in the Rotenone-induced Model of C. elegans ......................................................................................... 104
3.5 Discussion .................................................................................................................... 104
3.6 Conclusion .................................................................................................................... 107
3.7 Acknowledgements ...................................................................................................... 107
Chapter 4: Complex Interaction of Dietary Fat and Alaskan Bog Blueberry Supplementation Influences Manganese Mediated Neurotoxicity and Behavioral Impairments ........................................ 125

4.1 Abstract ................................................................................................................................... 125

4.2 Introduction ............................................................................................................................... 126

4.3 Materials and Methods ............................................................................................................ 128

4.3.1 Experimental Animals ........................................................................................................... 128

4.3.2 Experimental Design ............................................................................................................. 128

4.3.3 Drugs ...................................................................................................................................... 129

4.3.4 Behavioral Tests ..................................................................................................................... 129

4.3.4.1 Open Field ........................................................................................................................... 129

4.3.4.2 Novel Object Recognition ................................................................................................ 130

4.3.5 Brain Tissue Collection and Molecular Analysis ............................................................... 131

4.3.5.1 Perfusion and Brain Sectioning .......................................................................................... 131

4.3.5.2 Nissl Staining ..................................................................................................................... 132

4.3.5.3 Immunohistochemistry ...................................................................................................... 132

4.3.5.4 Brain Tissue Harvesting .................................................................................................... 133
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.6 Acknowledgments</td>
<td>145</td>
</tr>
<tr>
<td>4.7 Figures and Tables</td>
<td>146</td>
</tr>
<tr>
<td>4.8 References</td>
<td>157</td>
</tr>
<tr>
<td>Chapter 5: General Conclusions</td>
<td>164</td>
</tr>
<tr>
<td>5.1 General Overview</td>
<td>164</td>
</tr>
<tr>
<td>5.2 Effect of Alaskan Bog Blueberry on α-synuclein Overexpression in a Transgenic Model of <em>Caenorhabditis elegans</em></td>
<td>165</td>
</tr>
<tr>
<td>5.3 Silencing Fat Metabolism Genes in Parkinson's like Models of <em>Caenorhabditis elegans</em></td>
<td>165</td>
</tr>
<tr>
<td>5.4 Complex Interaction of Dietary Fat and Alaskan Bog Blueberry Supplementation Influences Manganese Mediated Neurotoxicity and Behavioral Impairments</td>
<td>166</td>
</tr>
<tr>
<td>5.5 Overall conclusion</td>
<td>167</td>
</tr>
<tr>
<td>5.6 References</td>
<td>169</td>
</tr>
<tr>
<td>Appendix</td>
<td>172</td>
</tr>
</tbody>
</table>
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2.1</td>
<td>Crude Extract of Alaskan Blueberry Reduced Expression of α-synuclein in the OW13 Strain of <em>C. elegans</em></td>
<td>58</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>Alaskan Blueberry Polyphenols Reduced Expression of α-synuclein in the OW13 Strain of <em>C. elegans</em></td>
<td>60</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>Alaskan Blueberry Polyphenols Reduced Expression of α-synuclein in the OW13 Strain of <em>C. elegans</em> Through a Sirtuin Mediated Pathway</td>
<td>61</td>
</tr>
<tr>
<td>Figure 2.4</td>
<td>Alaskan Blueberry Polyphenols Improved Motility in Aged (Day 12) OW13 Strain of <em>C. elegans</em></td>
<td>62</td>
</tr>
<tr>
<td>Figure 2.5</td>
<td>Reduction of sir-2.1 Expression Reverses the Motility Improving Effects of Alaskan Bog Blueberry Polyphenols in Aged (Day 12) OW13 Strain of <em>C. elegans</em></td>
<td>63</td>
</tr>
<tr>
<td>Figure 2.6</td>
<td>Alaskan Bog Blueberry did not Alter Lifespan in OW13 Strain of <em>C. elegans</em></td>
<td>64</td>
</tr>
<tr>
<td>Figure 2.7</td>
<td>Alaskan Bog Blueberry and/or RNA Interference Treatments did not Influence Total Fecundity in OW13 Strain of <em>C. elegans</em></td>
<td>65</td>
</tr>
<tr>
<td>Figure 2.8</td>
<td>Alaskan Bog Blueberry Increased Lipid Content by a Marginal Increase in Reactive Oxygen Species in OW13 Strain of <em>C. elegans</em></td>
<td>66</td>
</tr>
<tr>
<td>Figure 2.9</td>
<td>Alaskan Bog Blueberry Extract Reduced the Gene Expression of sir-2.1 in OW13 Strain of <em>C. elegans</em></td>
<td>68</td>
</tr>
<tr>
<td>Supplemental Figure 2.1</td>
<td>Graphical Representation of Fluorescence Intensity of the OW13 animals Fed on Different RNA Interference Treatments: <em>L4440</em> and <em>GFP</em></td>
<td>69</td>
</tr>
</tbody>
</table>
Supplemental Figure 2.2 Graphical Representation of Fluorescence Intensity of the OW13 Animals Fed on Different Concentrations of Alaskan Bog Blueberry Crude Extract (0, 100, 200 and 400 μg/ml) and Heat-killed OP-50-1 as a Food Source ...................................................... 70

Supplemental Figure 2.3 qPCR Analysis of mRNA Levels of sir-2.1 between Wild-type N2 and α-synuclein Overexpressing OW13 Strain ............................................................ 71

Figure 3.1 Silencing of fat-5 and fat-7 Genes Reduced Human wild-type α-synuclein Overexpression and Improved Dopamine Related Behaviors in Models of C. elegans ................................................................. 108

Figure 3.2 Genetic Silencing of fat-5 and fat-7 Genes Conferred Heat Resistance in Human wild-type α-synuclein Overexpression Model of C. elegans .............................................. 109

Figure 3.3 Silencing of fat-5 and fat-7 Genes Rescued Dopaminergic Degeneration and Related Behaviors in Model of C. elegans Expressing Human Mutated α-synuclein (A53T mutation) ......................................................................................................... 110

Figure 3.4 Defects in Stress Resistance is Ameliorated by Silencing of fat-5 and fat-7 Genes in C. elegans Expressing Human Mutated α-synuclein (A53T mutation) .................. 112

Figure 3.5 Silencing of fat-5 and fat-7 Genes Rescued Dopaminergic Degeneration and Associated Behaviors in a Rotenone Induced Model of C. elegans .............................. 113

Figure 3.6 Lifespan or Healthspan Measures are Unaltered by Silencing of fat-5 and fat-7 genes in Rotenone-induced Model of C. elegans ................................................................. 115

Supplemental Figure 3.1 Graphical Representation of the Touch Response as a Measure of Batch Control for the RNAi Plates ................................................................. 116

Supplemental Figure 3.1 Lipid Staining to Validate the Effect of Silencing of Fat Metabolism Genes ......................................................................................................... 117
Figure 4.1 Study Design .............................................................................................................. 146
Figure 4.2 Alaskan Blueberry Supplementation Enhanced Locomotor, Anxiety and Memory Functions in Mice on a LFD ........................................................................................................ 147
Figure 4.3 Alaskan Blueberry Supplementation Ameliorates Mn-induced Sensory and Motor coordination Deficits in Mice Consuming NFD and LFD ................................................................. 149
Figure 4.4 Alaskan Blueberry Improves Hippocampal BDNF and Attenuates Levels of Inflammatory Markers in the SN Region ....................................................................................... 151
Figure 4.5 HFD Consumption Decreases Total Cell Count and Tyrosine Hydroxylase Immunoreactive Neurons in the SN region ....................................................................................... 152
List of Tables

Supplemental Table 2.1 Total Phenolic Content (Quantified By Folin–Ciocalteu Assay with Gallic Acid Equivalent), and Anthocyanin Content (Quantified by pH-differential with Cyanidin-3-glucoside) of Alaskan Bog Blueberry.................................................................72
Supplemental Table 2.2 Sequencing Results of the RNA Interference Clones .................................................................................................................................................................73
Supplemental Table 2.3 Total Number of Animals Used for Lifespan Experiments.............81
Table 4.1 Summary of Statistical Effects of Individual Factors (Diet, BB and MnCl₂ treatment) and their Interactions on Behavioral and Molecular Measures used in the Study........................................................................................................................................153
Table 4.2 Diet Composition................................................................................................156
Acknowledgements

I would like to express my sincere gratefulness to my advisors Dr. Barbara Taylor and Dr. Abel Bult-Ito for all the support they have provided me during the course of this PhD. Without their constant understanding, patience and guidance this thesis would have never been completed. Besides my advisors, I would like to thank my committee members Dr. Thomas Kuhn, Dr. Larry Duffy and Dr. Kriya Dunlap for their constant support, encouragement and critique of my research. I sincerely thank my undergraduate mentor Dr. Hillol Jyoti Singha for introducing me to the word ‘science’ and Dr. Kelly Drew for teaching me the subject of neuroscience.

I would like to thank my husband, Swarup Mitra for his constant help, enthusiasm and guidance for completion of this degree. This PhD wouldn’t be a reality without his mentoring. I am grateful to Shailesh Narayan Khatri, Lalu Murali Krishna, Uday Turlapati, Courtney Scerbak, Skyler Hunter, Heather Currey, Cristiane Bastos and Mitchell Reed who trained me, worked with me and motivated me for pursuing this degree for endless hours. I would also like to thank all the undergraduate members of the Taylor / Bult-Ito lab for giving me the opportunity to train them in various research projects. A special thanks to Justin and Savanna Olnes, and Soumik and Debarati Basu for their friendship, help and the mental support they have provided me throughout, especially in the last year of my PhD.

A big thank you to all the funding bodies and offices that supported me throughout my academic endeavors: Alaska IDeA Network of Biomedical Research Excellence (INBRE), University of Alaska Fairbanks Biomedical Learning and Student Training (BLaST), UAF Department of Chemistry and Biochemistry, College of Natural Science and Mathematics (CNSM), UAF International Office and Office of the Vice Chancellor for Research. Last but not the least, I would like to dedicate this PhD to my son for his relentless sacrifice to foster my passion for science.
Chapter 1: General Introduction

Neurodegeneration Parkinson's disease (PD) is one of the most commonly occurring age-related neurodegenerative disorders. Seven to 10 million individuals are affected by PD worldwide [1]. The mean age of onset is around 60 years, where 1% of all individuals over the age of 60 and 4% of those over 80 exhibit PD symptomology. Pathophysiologically, PD is characterized by the continuous and aggravated loss of dopaminergic neurons in the substantia nigra pars compacta (nigrostriatal pathway) area of the brain [2]. At the cellular level, intra-cytoplasmic inclusions of misfolded α-synuclein protein result in formation of Lewy bodies and dystrophic Lewy neurites in neurons [3, 4]. This results in loss of dopaminergic neurons causing motor impairments, including tremors, hypokinesia, bradykinesia, rigidity, and postural instability [5-7]. Festination, speech and swallowing disorders and handwriting changes constitute the other identifiable motor deficits [6, 8]. Due to the neuronal loss in both central and peripheral nervous systems, patients often experience several non-motor symptoms such as anxiety, depression, memory loss, and olfactory deficits [9, 10]. Genetic (familial) and environmental (sporadic) triggers have emerged as two major factors in PD pathophysiology, with environmental exposure accounting for over two-thirds of all reported cases [11-14]. Susceptibility to both sporadic and familial types of PD is linked to polygenic factors. Some of the implicated genes include α-synuclein, Leucine rich repeat kinase 2 (LRRK2), pink-1, parkin 2 (PARK2), protein deglycase DJ-1, and others. For example, a mutation in the glucocerebrosidase (GBA) gene, which codes for an enzyme essential for metabolism of lysosomal substrates, is linked to the pathogenesis of sporadic PD [15]. Similarly, a mutation in the ubiquitin carboxyl-terminal hydrolase L1 (UCHL1), an enzyme involved in the removal and recycling of ubiquitin molecules from degraded proteins, and ligation of ubiquitin to proteins to mark them for degradation, has been linked to the early-onset of familial PD [2]. The identification
of these and other genes, and the discovery that certain toxins such as MPTP (1-methyl-1, 2, 3, 6-tetrahydropyridin) [16], 6-OHDA (6-hydroxy dopamine) [16], and rotenone [17] lead to PD symptoms, has informed the development of genetic and toxin-induced PD models [13, 16, 18, 19] and resulted in a better understanding of disease etiology, pathology, and molecular mechanisms [20, 21].

1.1 Caenorhabditis elegans and Mus musculus models of Parkinson's Disease

1.1.1 Nematode Caenorhabditis elegans

Invertebrate models such as Caenorhabditis elegans are useful in investigating the molecular mechanisms of PD pathology [22]. These models promote the studies of PD-associated molecular signaling pathways and first-round screening that can be further examined in mammalian models [23]. The nematode C. elegans is a useful model organism for studying healthy and abnormal neuronal aging, including cellular pathologies of PD. C. elegans share many conserved cellular pathways and mechanisms with mammals, including humans [24-26]. These cellular pathways can be genetically manipulated using RNA interference (RNAi) by gene-specific feeding bacteria that deliver RNA for gene specific interference [27], which enables rapid screening of target genes [28, 29]. RNAi screening is an important tool for predicting pathogenic mechanisms before moving to complex organisms for further investigation [28, 30, 31]. Despite major anatomical differences from humans, the C. elegans nervous system consists of a circumpharyngeal nerve ring and contains key cellular and molecular features of mammalian neurons, including conserved neurotransmitter systems (dopamine, GABA, acetylcholine, serotonin, etc.), receptors, axon guidance molecules, ion channels, and synaptic features [32]. Although α-synuclein is not endogenous to C. elegans, expression of this human PD-associated protein in C. elegans dopaminergic (DA) neurons results in age-dependent neurodegeneration [33-
Moreover, most familial PD genes such as PINK1, PARK, DJ-1, and LRRK2 have at least one *C. elegans* homolog [36-38]. Hermaphroditic *C. elegans* have 302 neurons, of which eight (ADEL, ADER, CEPDL, CEPDR, CEPVL, CEPVR, PDEL, and PDER) are dopaminergic such as those implicated in PD in humans [39]. This has led to creation of transgenic models with fluorescently labelled dopaminergic neurons to examine the effect of environmental factors on neuronal health [40]. In addition, four dopamine receptors (DOP-1, DOP-2, DOP-3, and DOP-4) have been identified in *C. elegans*, including homologs of each of the two classes of mammalian dopamine receptors (D1- and D2-like) [41]. *C. elegans* have low maintenance costs, and their shorter lifespan (2–3 weeks) reduces the time needed for each experiment. These advantages make *C. elegans* a valuable model system for genetic and chemical screening, and pre-clinical research.

In contrast, the limitations of a *C. elegans* PD model include a lack of defined organs, including the complex brain structure seen in humans and, therefore, the inability to recapitulate the same set of complex interactions involving various brain cells and tissues seen in human PD patients [42]. In addition, impermeability of the cuticle and inability of intestinal cells to absorb certain chemicals may require high exposure doses to affect the animal's physiology [30, 42]. Despite these limitations, *C. elegans* have proven useful in aging research [42] and numerous studies have used nematodes to investigate the cellular mechanisms associated with PD [22].

1.1.2 House Mouse (*Mus Musculus*)

PD models of house mouse (*Mus musculus*) models have been established to critically understand PD pathology and to investigate new therapeutic strategies [43]. These models display many of the clinical features of PD such as the Lewy body formation, loss of dopaminergic neurons in substantia nigra [44-46], disease progression associated with age, motor dysfunction, and non-motor symptoms including cognitive decline, autonomic dysfunction, depression, and hyposmia...
Over the last decade, several mouse models have been generated with genetic mutations that have been associated with familial PD [48]. These models have provided valuable insights in understanding the impaired cellular mechanisms that contribute to the molecular pathology of the disease. However, the available models do not exhibit the considerable extent of neurodegeneration and related behavioral phenotypes [49]. This includes failure to reproduce the dopaminergic cell loss in the substantia nigra or locus coeruleus [50-52]. This limitation of genetic models can be addressed by models with toxin-induced dopaminergic neuron loss in these regions. Several pharmacological and neurotoxic rodent models have been developed that feature the major symptoms of human PD including rigidity, tremor, postural instability, and slowness of movement [48, 53, 54]. PD mouse models closely mimic human PD pathology but none present all the pathological features, and therefore, only partial knowledge can be acquired from studies using these models. Despite these shortcomings, rodent models are still considered to be very useful in understanding the underlying mechanisms and development of therapies to treat PD.

1.2 Genetic PD models of *C. elegans* and *M. musculus*

1.2.1. α-Synuclein

α-synuclein is a small, presynaptic cytoplasmic protein, which is highly conserved in vertebrates and has been implicated in PD and other synucleinopathies [55]. Studies suggest that α-synuclein may be important for synaptic transmissions [56]. The α-synuclein gene, SNCA, is causatively related to PD and its mutation was the first gene to be linked to the disease [18]. Mutations in SNCA, including rare point mutations as well as duplications and triplications of wild-type α-synuclein in the form of A30P, A53T, and E46K cause familial forms of PD in humans [11, 57, 58].
C. elegans do not have an α-synuclein homolog. Thus, to study the pathogenicity of α-synuclein overexpression and aggregation in PD, several transgenic C. elegans strains with human α-synuclein have been created. These strains are particularly useful for studying the toxicity of protein aggregates, and cellular and behavioral abnormalities [35, 59]. Strains OW13 ([unc-54p::α-synuclein::YFP + unc-119(+)]), NL5901 ([unc-54p::α-synuclein::YFP+unc-119(+)]), and DDP1 (uonEx1[unc-54p::α-synuclein::CFP + unc-54::α-synuclein::YFP(Venus)]) express α-synuclein in body wall muscle cells [59, 60]. In these strains, the human α-synuclein gene is fused to yellow fluorescent protein (YFP) and expressed under the control of the unc-54 promoter [35, 59, 60]. These strains have been used to study α-synuclein aggregation, changes in movement, animal behavior and genes that modulate these and other PD-related hallmarks. In addition to strains that overexpress α-synuclein in body wall muscle cells, strains that overexpress wild-type or mutant (A53T) human α-synuclein in dopaminergic neurons have been generated by multiple research groups [33, 34, 61, 62]. In these models, the dopamine transporter promoter dat-1 is fused to green fluorescent protein (GFP), following co-expression of wild-type or mutant (A53T) α-synuclein and GFP. In C. elegans expressing α-synuclein (wild-type or mutant) in dopaminergic neurons, neuronal abnormalities, accumulation of aggregates and cell loss were observed, typically in an age-dependent manner [33, 34, 61, 62].

In mice, the transgenic models of α-synuclein knockout or expression of the wild-type [63] and mutant (A53T and A30P) forms [64, 65] have been generated to study the normal physiological function of the protein. Apart from that, α-synuclein overexpression models of mice exhibits neurodegeneration which is dependent on the promoter used to express the transgene. The accumulation of α-synuclein in these models leads to disruption in the functioning of dopaminergic neurons [65, 66]. Behavioral deficits have been observed in both the A30P and A53T mice [67-
However, there was no cell loss in the substantia nigra when the transgene was expressed with both the mouse and hamster prion protein promoter [70-73]. Similarly, mice with the PDGF-β promoter showed loss of terminals and dopamine (DA) levels in the striatum but no cell loss [66]. Transgenic mice that overexpressed α-synuclein A53T in the dopaminergic neurons featured motor impairment, degeneration of neurons in the midbrain and decreased levels of DA [74, 75]. In wild-type mice, a single striatal or hind limb intramuscular injection of α-synuclein led to typical Lewy-body pathology, progressive loss of dopaminergic neurons in the midbrain and reduced DA levels in the striatum accompanied by motor deficits [76-78].

1.2.2 LRRK2 and PINK1

In PD patients, mutations in the multi-domain protein leucine-rich repeat kinase 2 (LRRK2) are the most common genetic risk factors for both familial and sporadic PD, accounting for 4% of familial and 1% of sporadic PD across all populations [79]. Mutations are prevalent within the GTPase (R1441C/G) and kinase (G2019S) domains of LRRK2 [79]. Mutations in the gene α-PTEN-induced kinase 1 (PINK1) leads to another form of PD called PARK6 [80].

In C. elegans, the lrk-1 gene is homologous to mammalian LRRK1 and LRRK2, human and mouse leucine-rich repeat kinases, respectively. lrk-1 mutants have been used to study pink-1, homolog of the human PINK1 suggesting that LRK-1 and PINK-1 act antagonistically in stress response and neurite outgrowth [37]. PINK-1 mutation in worms resulted in reduced mitochondrial cristae length and heightened paraquat sensitivity. These losses of function were suppressed upon deleting the lrk-1 gene [37]. In other studies, human wild-type and mutant G2019S and R1441C LRRK2 have been overexpressed in dopaminergic neurons of C. elegans under the control of the dopamine transporter dat-1 promoter co-injected with dat-1p::GFP to generate transgenic lines [62, 81]. Overexpression of these LRRK2 proteins caused age-dependent degeneration of
dopaminergic neurons, behavioral deficits, locomotory dysfunction, and reduced DA levels in transgenic *C. elegans* [62, 81].

LRRK2 knock-out models of mice exhibited accumulation of α-synuclein but no functional loss of dopaminergic neurons in the midbrain [48, 52]. However, over expression of G2019S LRRK2 in mice resulted in mild degeneration of dopaminergic neurons in the substantia nigra [82]. Additionally, some studies also reported viral-vector based models of mutant LRRK2 [83], which demonstrated considerable neuronal loss compared to wild-type LRRK2. Similarly, PINK-1 knockout in mice on its own does not present any of the typical features of PD-like movement deficit and reduced DA levels [72, 84]. However, in PINK1 knock-out mice, overexpression of α-synuclein in the substantia nigra resulted in enhanced degeneration of dopaminergic neurons and higher levels of α-synuclein [85].

1.2.3 Parkin

Some autosomal recessive forms of familial PD are associated with mutations in Parkin (PARK2), an E3 ubiquitin ligase that is important for neuronal protein homeostasis [19, 86-88]. In *C. elegans*, the PARK2 homolog pdr-1 is an essential component in the degradation machinery during the response to proteotoxic stressors [89]. Specifically, pdr-1 was shown to play a role in the unfolded protein response (UPR) pathway, and co-expression of mutant α-synuclein A53T and truncated pdr-1 exacerbated mutant α-synuclein-induced toxicity in a UPR-independent way [89].

Several Parkin knockout mice have been generated by deleting different exons in the PARK2 gene [51, 90-92]. Such mice exhibited motor deficits, age-dependent neuronal degeneration and reduced levels of DA in the striatum [93].
1.2.4 DJ-1

In humans, the DJ-1 gene is mutated in 1-2 % of early onset PD [94]. Its functions include transcriptional regulation, antioxidant activity (particularly after toxic insults), chaperone activity, protease cleavage, and mitochondrial regulation [95].

*C. elegans* have two DJ-1 orthologs: djr-1.1, and djr-1.2; both encode a type of glyoxalase. In *C. elegans*, neuronal expression of DJR-1.2 in the head and ventral nerve chord neurons is elevated after exposure to acute manganese (Mn) [96] and djr-1.2 is protective against Mn-induced dopaminergic toxicity in an age-dependent manner [96]. The *C. elegans* djr-1.1, localizes to the intestine and plays a primary role in protecting animals from glyoxal-induced death [97].

Transgenic DJ-1 mouse models show loss of dopaminergic neurons in the ventral tegmental area [98] and altered DA levels in the striatum [99, 100]. Also, another transgenic DJ-1 mouse line with a C57/BL6 background demonstrates age-dependent progressive neuronal loss in the substantia nigra [101].

1.3 *C. elegans* and *M. musculus* Toxin-induced Models of Parkinson’s Disease

1.3.1 MPTP and 6-OHDA

In animal models of PD, exposure to toxins that cause an overload of reactive oxygen species (ROS) and disrupt the electron transport chain in mitochondria that leads to neuronal abnormalities and eventually cell death [102, 103]. The best studied neurodegeneration-inducing chemicals in both *C. elegans* and mice PD models are the toxins 6-hydroxy dopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [40, 48, 104-106].

MPTP was first identified as a PD-causing neurotoxin in humans in the 1980s after drug addicts in California inadvertently administered the agent present as a contaminant in synthetic heroin [107]. MPTP is lipophilic and can therefore cross the blood brain barrier. In the brain, it is
converted to 1-methyl-4-phenylpyridinium ion (MPP+) by glial monoamine oxidase B [108]. MPP+ inhibits complex I of the mitochondrial electron transport chain to induce mitochondrial dysfunction, impair autophagic degradation and ultimately cause neuronal loss [109]. In *C. elegans*, MPTP treatments selectively degenerate dopaminergic neurons [110, 111]. In mice, MPTP results in loss of dopaminergic neurons in both striatum and substantia nigra [112].

6-OHDA is a selective catecholaminergic neurotoxin; it has a chemical structure similar to DA but with the addition of a hydroxyl group that makes it toxic to dopaminergic neurons [21]. In PD research, the administration of 6-OHDA causes mitochondrial failure through inhibition of complex I of the mitochondrial electron transport chain. This leads to ATP depletion and elevated oxidative stress, which ultimately results in dopaminergic neuron damage [40, 44, 111]. In *C. elegans*, 6-OHDA administration leads to the loss of GFP-labeled dopaminergic cell bodies and processes [40, 113]. Since 6-OHDA cannot cross the blood brain barrier, mouse models require a stereotaxic injection of this toxin in the mid-brain or striatum [114] causing progressive degeneration of dopaminergic neurons in these brain regions [115].

1.3.2 Insecticides and Herbicides

Rotenone (a broad-spectrum insecticide), paraquat (an herbicide), and benomyl (fungicide) have been used to induce PD-like pathology in both *C. elegans* and mouse models of PD [2, 22, 48]. Both paraquat and rotenone trigger excessive ROS production in neurons, which leads to cellular damage [116-119]. Benomyl inhibits aldehyde dehydrogenase (ALDH), which detoxifies the DA metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL). Benomyl-treated mice accumulate DOPAL and resemble mice with knockouts of the genes encoding ALDH1A1 and 2, a mouse model of aging-related PD. Thus, there are at least two mechanisms by which pesticides
can induce PD-like symptoms.

Rotenone delivered to mice through chronic intragastric or stereotaxic administration has been used to cause slow dopaminergic neuron loss [120, 121]. Similar to rotenone, paraquat administration in mice has been used to induce Lewy body formation in DA neurons [122]. *C. elegans* strains including BY250 (dat-1p:GFP), BZ555 (dat-1p:GFP), and UA57 ([dat-1p::GFP+dat-1p::cat-2]) can be exposed to these toxins to visualize and quantify abnormalities in neuronal morphology [120, 121, 123, 124]. Thus, both animal models have advantages for studying the cellular mechanisms and pathophysiology of PD.

1.3.3 Manganese

Mn is an essential transition metal required for growth, development and cellular homeostasis [125]. It is a co-factor for multiple enzymes such as Mn superoxide dismutase, pyruvate carboxylase, arginase, and glutamine synthetase, and can substitute for magnesium (Mg) in enzymatic reactions catalyzed by kinases [126]. However, inhaling toxic levels of Mn can lead to nasal and pulmonary inflammation, renal dysfunction, and neurodegeneration [127, 128]. For example, occupational exposure through Mn mining, steel manufacturing, and welding are linked to increased risk for parkinsonian syndrome commonly known as Manganism [129]. Specifically, exposure to toxic levels of Mn can cause oxidative injury in the substantia nigra, the loss of dopaminergic neurons and motor deficits such as tremor, rigidity, and bradykinesia [130, 131]. In *C. elegans*, extracellular dopamine was found to be responsible for oxidative stress and Mn-induced dopaminergic neurodegeneration [132]. Mn neurotoxicity has also been studied in genetic DJ-1 models of *C. elegans* exposed to Mn [96].

In mice, sub-cutaneous injections of manganese chloride have resulted in behavioral deficits and metal accumulation in the basal ganglia of the brain [133, 134]. Another study reported the loss of
dopaminergic cells in the substantia nigra due to chronic manganese exposure in mice [135]. Mn exposure also triggers mitochondrial dysfunction and neurodegeneration in the nigrostriatal region and microglial activation in transgenic mice models [136, 137].

1.4 Role of Diet in Parkinson’s Disease

Environmental factors play an important role in the progression and etiology of PD [14]. Therefore, it is imperative to understand the role of diet in remission or exacerbation of the disease pathology and symptoms. Dietary fat which contributes to obesity and metabolic disorders, also plays an important role in causing neurodegenerative disorders [138, 139]. Epidemiological studies also refer to the possible link between consumption of high saturated fat and higher risk of developing PD [140, 141].

Disruption of fat metabolism can cause an obesity-like condition in a diverse range of multicellular organisms: *C. elegans* to mammals [142]. Fat metabolism in *C. elegans* includes an intricate and conserved gene network, which plays a role in food sensing and neuroendocrine signaling processes [143]. In nematodes, food sensing behavior is also controlled by a well mapped dopaminergic system, which is also a key network affected in PD [144]. Despite the lack of specific adipocytes, *C. elegans* shares common human homologues of genes that are implicated in obesity and metabolic disorders [143, 145]. In mammals, nuclear hormone receptors (NHRs) like peroxisome proliferator-activator receptor protein-alpha (PPARα) are often studied as drug target for lipid metabolism [146]. In *C. elegans*, one such receptor known as *nhr-49* promotes fatty acid desaturation and beta-oxidation leading to 'high fat' accumulation, altered mitochondrial function and shortened lifespan [142, 147]. Like mammals, mutations in *tub-1* (tubby homologue) leads to increased fat deposition and lifespan increase in *C. elegans*.
Δ9 desaturases enzymes are present in organisms from yeast to humans and are important for energy metabolism and lipid synthesis [149]. Diets high in unsaturated fatty acids decrease expression of Δ9 desaturase and high carbohydrate consumption increases its expression [149]. In C. elegans, Δ9 desaturases are encoded by genes fat-5 and fat-7. RNAi for fat-5 and fat-7 has reduced fat content in the nematodes [142]. C. elegans being a popular genetic model allows easy manipulation of these 'fat genes' using RNAi to create high and low fat content in the organisms. Moreover, fatty acid metabolism is known to be involved in stress resistance mechanisms and insulin signaling in the worm model [150]. Recently, such stress resistance mechanisms have been used to study the effect of aging and insulin signaling in C. elegans models of PD [62]. The models have been well developed to understand the symptoms of PD: α-synuclein overexpression, genetic mutations like A53T and LRRK2, and environmental toxin-induced dopaminergic neurodegeneration. Such models are also known to display phenotypic abnormalities that can be used as measure of aging, another key risk factor for PD [62]. Nematodes have emerged as a very popular in vivo model to study PD [22] and obesity [151]. However, no studies have yet investigated interactions of fat metabolism and PD-like pathology and cellular symptoms in this popular worm model.

In mice, high fat diets exacerbate the progression of PD-like symptoms by increased dopaminergic neurodegeneration in striatum and substantia nigra [152-154]. Other than worsening MPTP-induced dopaminergic loss, high fat diet also increases inflammation in mice characterized by elevated levels of cytokines and chemokines [153]. Also, genetic studies involving parkin suggest an association between fat and PD [155]. Lipid and cholesterol metabolism though highly debated may also play a key role in modifying pathogenesis of idiopathic PD [156, 157]. On the other hand, low fat diets or caloric restriction have demonstrated life span extending properties in
many organisms [158-161]. A low-calorie diet increased the stress response in neurons in \textit{in vivo} models of neurodegenerative disorders [162]. Caloric restriction is also known to enhance cognitive function by promoting synaptic plasticity and upregulating neurotrophic factors like BDNF [159, 163]. In mouse models of PD, dietary restriction improves motor function, reduces oxidative stress and enhances mitochondrial function [164]. In another study with a toxin-induced model, such restriction implicated a role of glutamate homeostasis in improving PD pathology [165]. Most of the studies investigating fat manipulations have been done using MPTP mice models; other avenues in PD pathogenesis have been sparsely explored. Also, there are epidemiological studies that refute a relationship between fat and PD [166-169]. Therefore, the complex interaction between dietary fat and PD-associated pathology and behaviors warrants further investigation.

1.5 Role of Natural Compounds in Parkinson's Disease.

Consumption of a diet containing high amounts of fruits and vegetable has been linked to lower levels of age-related neurodegenerative diseases such as PD and Alzheimer's disease [170]. Dietary supplementation with naturally available products like blueberries, spinach, grapes etc. have high levels of anti-oxidants that can combat oxidative stress [171], which is a key phenomenon that occurs in aging and age-related disorders [172]. These foods contain a specific class of compounds known as 'polyphenols' which are known to play a therapeutic role in attenuating pathology of neurological disorders [170]. Polyphenols like resveratrol have been shown to reduce the loss of dopaminergic neurons, inflammation and oxidative stress in animal models of PD [173].

In \textit{C. elegans}, several natural compounds have shown to improve symptoms of neurological disorders [174]. Extracts from the Indian plant Brahmi (\textit{Bacopa monieri}) and a
traditional Chinese herb *(Corydalis bungeana)* have exerted protection in both transgenic and toxin induced models of *C. elegans* [144, 175, 176]. Blueberry polyphenols were also found to extend lifespan in *C. elegans* [177]. Indigenous Alaskan botanicals have consistently shown to contain higher levels of polyphenolic compounds (e.g., phenolic, anthocyanin and flavonoid) than other commercially grown, temperate species [178]. Alaskan plants produce a wide variety of secondary metabolites in response to adaptation to extreme Arctic climate [178]. Interplay between these secondary metabolites of various plant species and molecular targets has been shown to have pro-health and anti-aging effects [179]. A protein target is sirtuin 1-a histone deacetylase (sirtuin 1), which can impact the progression of age-related disorders by directly influencing the transcription machinery [180]. Polyphenols from grapes and tea interact with sirtuin 1 to reduce inflammation, promote neural plasticity and reduce cell death [181, 182]. Alaskan bog blueberries have been shown to inhibit inflammation in neuronal cell culture [183] and improve age-related mechanosensory deficits in *C. elegans* [179].

Quercetin (a flavonoid polyphenol found in many fruits and vegetables, notably apples) has been shown to reduce microglial activation in a MPTP-induced cell line [184]. Polyphenols such as baicalein [185], caffeic acid [186] and epigallocatechin gallate (EGCG) [187] conferred neuroprotection in various PD-like models. Blueberries (*Vaccinium angustifolium*) in particular improve object-recognition memory loss in aging rats [172] and improve memory in humans [188]. Blueberries also have been extensively investigated for their effect on cellular signaling and neurodegeneration [189, 190].

1.6 Research Objectives

The main objective of this thesis is to explore the complex interactions of diets and
supplements in the form of Alaskan bog blueberry polyphenols on PD-like symptoms in two animal model systems: *C. elegans* and *M. musculus*, through the completion of the following aims:

1.6.1 Aim I: Study the Role of Alaskan Bog Blueberries on α-synuclein Overexpression in a Transgenic *C. elegans* Model of PD.

This study aimed to examine the effect of Alaskan bog blueberry polyphenols on protein misfolding and motility defects in the nematode model. The research also aimed to understand the underlying molecular mechanisms influenced by these blueberry polyphenols and if such mechanisms involved sirtuin 1, a key histone deacetylase needed for cell growth and survival [180].

Previous studies show that Alaskan bog blueberries ameliorate age-related deficits and improve neuronal health in both *in vivo* and *in vitro* models [179, 183]. However, no studies have been conducted to understand the role of polyphenols from these endemic berries on protein-overexpression, a key feature of neurodegenerative disorders such as PD [3]. Moreover, dietary polyphenols have been found to mitigate age-related cellular damage by influencing cell signaling pathways mediated by sirtuin 1 [191]. Based on these previous findings, I hypothesized: Alaskan bog blueberry polyphenols will reduce α-synuclein overexpression in a *C. elegans* model of PD through a sirtuin 1-mediated pathway. My experimental results will provide a better understanding of this indigenous botanical as a therapeutic option for overcoming age-related molecular stress, such as protein aggregation. They also will encourage future investigation of how phytochemicals can modulate pathways for cell survival in disorders of nervous system.
1.6.2 Aim II: Silencing Fat Metabolism Genes in Parkinson's like Models of *Caenorhabditis elegans*.

This study aimed to explore the effect of genetic knockdown of four fat metabolism genes, *nhr-49, tub-1* (high fat genes), *fat-5, and fat-7* (low fat genes) on PD-like cellular symptoms (α-synuclein overexpression wild-type and A53T mutation and toxin-induced dopaminergic neurodegeneration) in *C. elegans*.

The effect of fat metabolism on aging and related pathologies like PD is poorly understood (refer to section 3). Based on the previous interactions, I hypothesized: silencing of low fat genes will increase lifespan, decrease protein overexpression, and rescue neuronal degeneration in *C. elegans* compared to knockdown of high fat genes. This study is first of its kind to investigate the interaction of fat metabolism, aging, and PD-like symptoms in *C. elegans*. The results will also encourage future studies to characterize the fat content and further decipher downstream molecular targets in the fat metabolic pathway in the nematode PD models.

1.6.3 Aim III: Complex Interaction of Dietary Fat and Alaskan Bog Blueberry Supplementation Influences Manganese Mediated Neurotoxicity and Behavioral Impairments.

This study is aimed at understanding the complex interaction between dietary fat and supplementation on metal-induced cellular and behavioral symptoms in a mouse model.

Several studies have highlighted the stand-alone effects of diets and phytochemicals on ageing endpoints (refer to section 3 and 4). The interactions of diet, supplement, and toxic metal exposure is not well understood. Based on the previous research, I hypothesized: Mice fed a low fat diet will perform better on the behavioral and biochemical endpoints compared to those fed a high fat diet. Attenuation of manganese-induced behavioral deficits by blueberry supplements will
be more pronounced in low fat diet groups. The results from this research will strengthen the association between obesity and neurodegeneration. They will also restate the benefits of consuming a low fat diet and how health-stimulating effects of supplements like blueberries can depend on the type of diet with which they are consumed.
1. References


Trempe, J.F. and E.A. Fon, *Structure and function of Parkin, PINK1, and DJ-1, the three musketeers of neuroprotection*. Frontiers in Neurology, 2013. 4 APR.


Lu, X.H., et al., *Bacterial artificial chromosome transgenic mice expressing a truncated mutant parkin exhibit age-dependent hypokinetic motor deficits,*


Chapter 2: Sir-2.1 Mediated Attenuation of α-synuclein Expression by Alaskan Bog Blueberry Polyphenols in a Transgenic Model of *Caenorhabditis elegans*

2.1 Abstract

Misfolding and accumulation of cellular protein aggregates are pathological hallmarks of aging and neurodegeneration. One such protein is α-synuclein, which when misfolded, forms aggregates and disrupts normal cellular functions of the neurons causing Parkinson's disease. Nutritional interventions abundant in pharmacologically potent polyphenols have demonstrated a therapeutic role for combating protein aggregation associated with neurodegeneration. The current study hypothesized that Alaskan bog blueberry (*Vaccinium uliginosum*), which is high in polyphenolic content, will reduce α-synuclein expression in a model of *Caenorhabditis elegans* (*C. elegans*). We observed that blueberry extracts attenuated α-synuclein protein expression, improved healthspan in the form of motility and restored lipid content in the transgenic strain of *C. elegans* expressing human α-synuclein. We also found reduced gene expression levels of *sir-2.1* (ortholog of mammalian Sirtuin 1) in blueberry treated transgenic animals indicating that the beneficial effects of blueberries could be mediated through partial reduction of sirtuin activity. This therapeutic effect of the blueberries was attributed to its xenohormetic properties. The current results highlight the role of Alaskan blueberries in mediating inhibition of *sir-2.1* as a novel therapeutic approach to improving pathologies of protein misfolding diseases. Finally, our study warrants further investigation of the structure, and specificity of such small molecules from indigenous natural compounds and its role as sirtuin regulators.

2.2 Introduction

Neurodegeneration can lead to a multitude of pathological conditions that primarily affects the nervous system. The debilitating conditions resulting from progressive degeneration and/or death of neurons can lead to impairments in movement, memory processing, affective behaviors and emotional functioning. Several such diseases are classified as protein misfolding diseases in which certain proteins become structurally abnormal and fail to fold into their normal configuration. In the misfolded state, these proteins can become toxic and disrupt normal cellular processes. One such protein is α-synuclein whose original function is vesicular transportation in the pre-synaptic terminal of neurons. Misfolding of α-synuclein leads to its over aggregation and accumulation. This is characterized by formation of small cytoplasmic α-synuclein inclusions in the neurons known as Lewy bodies, which is one of the key pathological hallmarks of Parkinson's disease. Similar molecular pathologies are also characteristic signatures of disorders like Lewy body dementia and multiple system atrophy. Despite significant strides in unraveling some of the cellular mechanisms contributing to these disorders, treatment strategies have been elusive. Development of new therapeutics are, therefore, of utmost public health priority.

There is substantial evidence that specific dietary regimen and plant-based supplementation is closely intertwined with the aging process and related disorders including neurodegeneration. Phytochemically, fruits and vegetables are rich sources of polyphenolic compounds like anthocyanins and flavonoids. The health promoting effects of the plant-based products could be due to interactions of the active phytochemical components with key cellular machinery involving inflammation, transcription and antioxidant systems. Most of these homeostatic cellular mechanisms are disrupted in aging and neurodegeneration. At the cellular
level, sirtuins, a family of Class III NAD+ dependent histone deacetylases, are implicated in several aging, metabolic and neurodegenerative pathways. Though most of its substrates are histones, they also act as upstream regulators for transcription factors such as FOXO and P53. Dietary polyphenols such as resveratrol and catechins are known to interact with sirtuins in modulating biochemical and signaling pathways. These natural compounds have demonstrated a therapeutic role in various neurodegenerative disorders and improving age-related cognitive decline. Blueberries in particular have been shown to improve or reverse age related deficits in both animal and clinical studies. The wild Alaskan bog blueberry (Vaccinium uliginosum) is a popular subsistence species endemic to the Arctic region. These Alaskan berries contain elevated levels of polyphenolic compounds (phenolic, anthocyanin and flavonoids) compared to commercially grown and temperate counterparts. We have previously established the beneficial effects of these endemic berries in improving markers of aging and neuronal health in both in-vitro and in-vivo model systems. However, there has been no study to address the mechanistic role of berry phytochemicals in eliminating toxic misfolded proteins, a key pathological marker and endpoint for drug targets.

Caenorhabditis elegans (C. elegans) is a very popular genetic model organism due to its ease of use, well defined aging properties, easy visualization and robust genetic malleability. Over the last decade, this model has also been explicitly used in studying age- and brain-related disorders, particularly protein misfolding diseases. In C. elegans, sir-2.1 (ortholog of mammalian Sirtuin 1) is a conserved regulator of aging mechanisms. We selected an α-synuclein expression/aggregation strain of C. elegans, OW13, which allows easy investigation and quantification of protein inclusions in vivo. We hypothesized that when treated with Alaskan bog blueberry extracts and polyphenolic fractions, the animals will have reduced protein
expression concomitant with improved behavioral motility and lifespan. We also hypothesized that this attenuation of protein expression is due to the interaction of polyphenols with *sir-2.1*.

2.3 Materials and Methods

2.3.1 Strains, Maintenance and Synchronization

*C. elegans* wild-type Bristol N2 and OW13 [unc54P::α-synuclein::YFP + unc-119(+)] strains used for the study were acquired from the Caenorhabditis Genetics Center (University of Minnesota). OW13 animals expresses human α-synuclein in its body wall muscle. The YFP tag is attached to the C-terminal of α-synuclein. The animals were cultured on nematode growth medium (NGM) plates using standard procedures and maintained at 22 °C. The plates were seeded with *Escherichia coli* strain OP-50-1 or HT115 as appropriate for the experiment. The synchronous population was created using the standard egg-lay method (allowing 20-30 animals to lay eggs for 4-6 hrs and then removing the adults at 22 °C) or by hypochlorite treatment (2 % sodium hypochlorite and 0.5 M NaOH). All experimental plates (except those used for fecundity assessment) contained 0.04 µg/ml Fluorodeoxyuridine (FUrD) (Sigma) for progeny control.

2.3.2 Berry Extract Preparation

Wild specimens of Alaskan bog blueberries (*Vaccinium uliginosum*) were collected from interior Alaska. Crude extracts were prepared using 80% aqueous acetone and a rotary evaporator. The specific polyphenolic fractions of anthocyanins and proanthocyanidins from the berries were extracted in the laboratory of Dr. Mary Ann Lila at Plants for Human Health Institute, North Carolina and kindly shared by Dr. Thomas Kuhn from the University of Alaska Fairbanks. All extracts were aliquoted and stored at -80 °C until further use. Instead of an extracted fraction of chlorogenic acid from blueberries, a pure form (Sigma) was used for the study to avoid
contamination with minor flavonols obtained during the fractionation process. All the specific fractions were reconstituted in deionized water with 0.1% DMSO to achieve a concentration of 400 μg/ml. A fixed volume of deionized water with 0.1% DMSO was used as control. Total phenolic content and total anthocyanin content was measured as per the methods described in (Supplemental Table 2.1).

2.3.3 Treatment Administration

To administer the Alaskan berry treatments (both crude and fractions), a fixed volume of desired concentration was prepared in deionized water (with 0.1% DMSO for fractions) and spread on top of NGM plates seeded with live OP50-1/ HT115 E. coli bacteria. The control plates were spread only with a fixed volume of deionized water (with 0.1% DMSO for fraction study). The bacteria were cultured in Miller Luria-Bertani (LB) broth containing 50 μg/ml streptomycin or carbenicillin as appropriate for the experiment. The experimental population was transferred manually with a platinum pick at late larval L4 stage allowing the animals to ingest the extract. Subsequently, the animals were transferred every other day to a fresh plate with the same constituents until the experiments were performed. OW13 animals were treated with 0, 100, 200, or 400 μg/ml of crude berry extract or 0 and 400 μg/ml of the polyphenolic fractions. A single dose of 400 μg/ml of polyphenolic fractions was selected predominantly because the dose-dependent study produced consistent attenuating effects at 400 μg/ml. Treatment started at larval stage L3 (for lipid staining) and L4 stage (for all other experiments) and continued through days 5, 7, and 12 when animals were tested.
2.3.4 Lifespan Analysis

Following egg lay, synchronous population of 22 L4 animals were transferred into two replicates of each treatment (total of 44 animals). Survival was determined daily by visual observation or gentle prodding with a platinum wire under a dissecting microscope. 'Bagged' animals and those that crawled off the plates were censored and removed from the experiment (refer to Supplemental Table 2.3). Out of 44 animals, 8-12 animals were censored from each experiment. Each experiment was repeated at least three times.

2.3.5 Motility

The motility of aging animals at time point days 5 and 12 were measured based on a class based (A-B-C) system. The animals moving in a normal sinusoidal pattern were classified as A, the ones which required gentle prodding with a platinum wire were classified as B and those which did not respond or moved just head or tail in response to touch were classified as C.

2.3.6 Fecundity

Fecundity or viable progeny count was performed by plating age-matched adults on fresh treatment plates until the end of their reproductive phase or death. The eggs laid by the adults were allowed to hatch and develop at 22 °C for 48 h. The number of progeny produced by each individual every day was manually counted under a dissecting microscope.

2.3.7 RNA Interference

RNAi plates were prepared using IPTG and 4X concentrated HT115 bacteria for each gene target (L4440, sir-2.1 and daf-16) and covered with foil to protect from light. For each replicate of the experiment, control experiments were performed to compare the knockdown of the
fluorescence between GFP treatment to empty vector (L4440) (Supplemental Fig. 2.1). All the bacterial clones were sequenced (Supplemental Table 2.2).

2.3.8 Lipid Staining

Lipid staining was performed using Nile red dye (Sigma). The stock solution was prepared by dissolving 0.5 mg of the dye in 1 ml of acetone, and then mixed with E. coli OP50-1. L3 larvae of OW13 animals were added to the treatment plates containing 0.04 μg/ml of FuDR and blueberry extracts (0 and 400 μg/ml) and the imaging was performed at day 7 to monitor the lipid content.

2.3.9 DCF-DA Assay

Reactive oxygen species (ROS) were quantified using H2DCF-DA. In a 96-well plate, 50 μl of live age-synchronous, day 7 animals in M9 buffer were added to each well. Just before loading the plate into the microplate reader, 50 μl of 100 mM 2,7-Dichlorofluorescin diacetate (DCF-DA, Sigma) in M9 was added to the wells. Basal and final fluorescence (after 1 h) was recorded at excitation of 485 nm and emission of 520 nm in a Biotek SynergyTM HT Multi-Mode Microplate Reader. Results were also normalized to total protein concentration using the BCA protein assay (Pierce Biotechnology). Each experiment was repeated three times.

2.3.10 qPCR Analysis

Total RNA was isolated from ~1000 day-7 adult animals using TRI Reagent (Life Technologies). Quantitative PCR (qPCR) experiments were performed with TaQMan gene expression analysis (Life Technologies) according to the protocol provided by the manufacturer to analyze the amount of mRNA of sir-2.1 and target genes: daf-16 and cep-1. qPCR data were analyzed as per Applied Biosystem's software protocol using cdc-42 and pmp-3 genes as an
internal reference for normalization. The primer set (Life Technologies, Grand Island, NY) used for the experiments were: sir-2.1 (Ce02459015_g1), daf-16 (Ce02422838_m1), cep-1 (Ce02410616_g1), cdc-42 (Ce02435136_g1 4) and pmp-3 (Ce02485188_m1).

2.3.11 Protein Extraction and Quantification

Day 7 animals were washed 3 times with distilled water, settled with gravity and then homogenized with a sonicator for 30-60s in 100–200 μL of a lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 0.2 mM DTT, 1% Triton X-100, v/v; 10% glycerol, v/v) with a proteinase inhibitor (1 mM PMSF). The lysate was centrifuged at 11,000× g at 4 °C for 10 min. The supernatant was collected, and protein samples were stored at -80 °C. Protein concentration was determined with the BCA assay kit (Pierce Biotechnology).

2.3.12 Western Blots

For western blots, 60 μg of total protein was mixed with 4X Laemmli loading buffer (VWR Life Sciences), heated at 100 °C for 10 min, then loaded in 12% Tris-Glycine gels (Invitrogen) consisting of a top 4% stacking gel and a bottom 12% resolving gel, and resolved by electrophoresis at 190 V in a running buffer (25 mM Tris base, 190 mM glycine, and 0.1% SDS (w/v)). Proteins were transferred to a Nitrocellulose membrane (Thermo scientific catalogue 88024) at 75 V for 3 hours and was blocked at 4 °C with 5% Bovine Serum Albumin (BSA) for 2.5 hours. The membrane was incubated in primary antibody (1:1000 dilution, rabbit anti-GFP, Invitrogen Life Technologies) overnight at 4 °C. The membrane was then washed three times with 1× Tris buffered saline with Tween 20 (TBS-T; 0.05 M Tris-HCl, 0.15 M NaCl, 0.1% (v/v) Tween 20, pH 7.5) + 1% BSA and incubated with a secondary antibody solution (1:10,000 dilution, Horse radish peroxidase conjugated goat anti-rabbit; Invitrogen) for 2.5 hours. After
three washes with 1× TBS-T, the membrane was developed with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermofisher scientific) and imaged using a ChemiDoc-It² imager at constant exposure settings. For probing with Actin as the loading control, the same membrane was incubated in a stripping buffer (0.1% SDS, w/v; 1% Tween 20, v/v; 200 mM glycine, pH 2.2) for 2 × 10 min, washed twice in PBS, then twice in 1× TBS-T + 1% BSA, prior to being blocked in 5% BSA for 2.5 hours. The membrane was treated to the primary antibody (1:1000 dilution, rabbit anti-Actin, Abcam) and the secondary antibody (1: 10,000 dilution, Horse radish peroxidase conjugated goat anti-rabbit, Invitrogen) and developed similar to the anti-GFP detection. For each treatment, protein samples were pooled from three biological replicates. The experiment was repeated twice.

2.3.13 Microscopy and Image Quantification

All images were acquired with a Zeiss LSM 510 laser scanning confocal microscope at constant magnification and exposure settings. Images were analyzed with ImageJ (National Institute of Health). Both α-synuclein and lipid content were quantified based on measurement of total fluorescence. A box of fixed height and width was drawn in the head region of the animal. Relative fluorescent intensity was determined by measuring the average integrated density after subtracting the background. Western blot lanes were imaged using ImageJ by measuring the relative band intensity and normalized to endogenous control - actin.

2.3.14 Statistical Analysis

Statistical analysis was performed in SPSS (Version 22) for motility (ordinal logistic model) and lifespan (Kaplan-Meier log rank survival) experiments. All other statistical analysis was performed in Graph Pad Prism (GraphPad Software, Inc). Treatment effect between groups were
tested using one-way or two-way analysis of variance (ANOVA) or t-test. Pairwise comparisons for significant differences between treatment groups were made using Tukey's t-tests.

2.4 Results
2.4.1 Crude extract of Alaskan bog blueberry Attenuated Expression of Human α-synuclein in C. elegans.

OW13 animals treated with different doses of crude berry extract (0, 100, 200, 400 μg/ml) were observed on day 7 of the adulthood for protein expression. Two doses of the extract (100 and 400 μg/ml) significantly reduced protein accumulation (One-way ANOVA, p<0.005). The fluorescent intensity of the protein accumulation was reduced by about 25-38 % (Fig. 2.1) for the groups administered 100 μg/ml (Tukey's post hoc t-test, p<0.01) and 400 μg/ml (Tukey's post hoc t-test, p<0.001) doses. The 200 μg/ml dose was not significantly different from the control (0 μg/ml) (One-way ANOVA, p>0.4). To further corroborate these findings, western blot analysis was conducted using antibodies probing the YFP part of the α-synuclein ::YFP chimeric protein, to measure the protein expression in whole worm extracts on the 7th day of adulthood. The protein expression was reduced by about 40% (Fig. 2.1) for the 400 μg/ml doses when compared to the control (t-test, p<0.02).

We also investigated whether these attenuating effects of blueberries were due to secondary responses in C. elegans resulting from interactions between the live food source, E. coli OP50-1, and the treatments. Transgenic C. elegans were grown on Alaskan blueberry treatment with heat-killed OP50-1 E. coli. In the presence of dead bacteria, blueberry treatment resulted in attenuated protein expression when compared to the untreated control (One-way ANOVA, p<0.02). A Tukey's post hoc t-test further indicated significant differences between control (0 μg/ml) and treatment groups (100 and 400 μg/ml) (p<0.01 and p<0.001, respectively; Supplemental Fig. 2.2).
2.4.2 Alaskan Bog Blueberry Polyphenolic Fraction Reduced Expression of Human α-synuclein in *C. elegans*.

Based on our initial findings (Fig. 2.1), we further aimed to determine which specific fraction reduced the protein expression in *C. elegans*. Chlorogenic acid (CA) had no effect on protein expression (One-way ANOVA, p>0.6) (Fig. 2.2). However, treatment with both fractions rich in proanthocyanidins (PAC) and anthocyanin (ANT) reduced α-synuclein expression significantly compared to the control (One-way ANOVA, p<0.0001). The protein accumulation was reduced by about 39% for both PAC (Tukey's post hoc t-test, p<0.002) and ANT (Tukey's post hoc t-test, p<0.0002) groups (Fig. 2.2).

2.4.3 Alaskan Bog Blueberry Polyphenols Reduced Human α-synuclein Expression in *C. elegans* through RNA Interference Mediating *sir-2.1*.

No significant effect was observed for RNAi genetic treatment and blueberry treatment (Two-way ANOVA, p>0.6 and p>0.7 respectively). However, there was a significant interaction effect between RNAi and blueberry treatment (Two-way ANOVA, p<0.0003). At day 7 of adulthood, silencing of *sir-2.1* decreased protein expression by about 30% (Tukey's post hoc t-test, p<0.001) (Fig. 2.3) compared to the empty vector, *L4440*. Also, when treated with blueberry extract (400 μg/ml), the animals fed with *L4440* demonstrated reduced protein expression by 34% (Tukey's post hoc t-test, p<0.001) (Fig. 2.3) compared to the animals treated with null control of the extract (0 μg/ml). Most strikingly, when *sir-2.1* was silenced in animals and treated with crude blueberry extract (400 μg/ml), the protein expression significantly increased by 29% compared to the control *sir-2.1* group (Tukey's post hoc t-test, p<0.01) (Fig. 2.3). There was no significance observed within *daf-16* groups (0 and 400 μg/ml) (Tukey's post hoc t-test, p>0.05) as well as between *daf-16* and other treatment groups (Tukey's post hoc t-test, p>0.05).
2.4.4 Alaskan Bog Blueberry Extract Improved Motility Mediated by sir-2.1 in C. elegans Expressing Human α-synuclein.

When treated with Alaskan blueberry crude extract (0, 100, 200 and 400 μg/ml), animals at day 5 of adulthood had normal motility compared to the controls (Fig. 2.4A). However, at day 12 of adulthood, the animals treated with 100 and 400 μg/ml of crude extract of Alaskan blueberry maintained normal motility or had less decline in motility (more Class A and B compared to immobile Class C) compared to the other groups (Ordinal logistic model, p<0.01) (Fig. 2.4B).

When animals with a silenced sir-2.1 gene were treated with crude extract of Alaskan blueberry, the α-synuclein expression significantly increased. We wanted to further investigate whether silencing sir-2.1 gene had any effect on the animals treated with the Alaskan blueberry extract (0 and 400 μg/ml). Animals in all the groups exhibited normal motility at day 5 (Fig. 2.5A). However, at day 12 of adulthood the control group treated with blueberries (L4440 + BB) had more A animals compared to the empty vector control (L4440) (ordinal logistic model, p<0.01) (Fig. 2.5B). Strikingly, at day 12 of adulthood the group with the sir-2.1 knockdown had lesser class C animals compared to empty vector, L4440. Finally, when sir-2.1 knockdown animals were treated with 400 μg/ml of crude extract of Alaskan blueberry, the motility of the animals worsened significantly compared to all other groups (Ordinal logistic model, p<0.01).

2.4.5. Alaskan Bog Blueberry Treatments did not Affect Lifespan or Total Progeny in C. elegans Expressing Human α-synuclein.

There was no significant change in mean lifespan among OW13 strain groups treated with tested doses of blueberry extract (0, 100, 200 and 400 μg/ml) (Kaplan Meir statistics, p> 0.05) (Fig. 2.6A). Interestingly, when animals were treated with genetic treatments (empty vector
control: *L4440* and *sir-2.1*) and 400 μg/ml of blueberry extract, no significant effect was found in the mean lifespan among the groups (Kaplan Meir statistics, *p* > 0.05) (Fig. 2.6B).

No significant differences were observed for the total progeny produced between groups treated with Alaskan berry extract (0, 100, 200 and 400 μg/ml) (One-Way ANOVA, *p* > 0.05) (Fig. 7A). The RNAi treatment groups (*L4440, sir-2.1*) in combination with crude extract of blueberry (0 and 400 μg/ml) also were not significantly different (Two-way ANOVA, RNAi treatment effect *p* > 0.05; blueberry treatment effect *p* > 0.05; interaction effect *p* > 0.05) (Fig. 2.7B).

2.4.6 Alaskan Bog Blueberry Restored Lipid Content in *C. elegans* Expressing Human α-synuclein.

At day 7 of adulthood, the fat levels of N2 were found to be significantly higher than the OW13 strain, which over expressed α-synuclein in its body (One-way ANOVA, *p* < 0.001) (Fig. 2.8A and 2.8B). However, treatment with blueberry extract (400 μg/ml) significantly increased total fat content compared to the untreated OW13 group (Tukey's *post hoc* t-test, *p* < 0.001) (Fig. 2.8A and 2.8B). At day 7 of adulthood, treatment with 400 μg/ml of blueberry extract, showed a trend towards elevated levels of ROS (t-test, *p* = 0.07) (Fig. 2.8C) compared to control.

2.4.7 Alaskan Bog Blueberry Treatment Reduced Gene Expression Levels of *sir-2.1* in *C. elegans* Expressing Human α-synuclein

A significant difference was observed for gene, blueberry treatment and its interaction (Two-way ANOVA, *p* < 0.03, *p* < 0.02 and *p* < 0.002, respectively). At day 7 of adulthood, the gene expression level of *sir-2.1* was significantly higher than its downstream targets: *daf-16* and *cep-1* in untreated control groups (Tukey's *post hoc* t-test, *p* < 0.01). When compared to 400 μg/ml blueberry treated group, the relative expression of *sir-2.1* was significantly decreased by almost
50% (Tukey's post hoc t-test, p<0.001) (Fig. 2.9). However, there were no significant differences (Tukey's post hoc test, p>0.5) (Fig. 2.9) between the gene expression levels of *daf-16* and *cep-1* in animals of both treatment (400 µg/ml) and control (0 µg/ml) groups.

2.6 Conclusion

In summary, our results identify a *sir-2.1*-mediated mechanism through which Alaskan blueberry polyphenols reduce α-synuclein expression in *C. elegans*, while also increasing lipid content and motility. These results indicate that, Sirtuin 1 can act as a double-edge sword in cellular overload conditions due to protein aggregation and partial down regulation of Sirtuin 1 that can restore homeostatic balance. Through the current study, we have also shown how xenohormetic properties of blueberries can trigger the beneficial effect of endogenous ROS signaling molecules. Overall, this study supports the future exploration of photochemical-induced alternative pathways for cell survival in various neurodegenerative disorders.

2.7 Acknowledgements

The authors thank Dr. Mitchell Reed, Ian Herriott and Noah Khalsa for their support with the experiments and Dr. Jennifer Guerard and Kristen Gagne for the blueberry extractions. We would also like to thank Drs. Thomas Kuhn and Elena Vayndorf for their intellectual contributions.
2.5 Discussion

Studies have shown that the effect of natural 'indigenous diets' have several health benefits over modern western diets\textsuperscript{54,55}. However, a comprehensive evaluation of the role of photochemical components in influencing cellular mechanisms is sparsely explored in relationship to aging and neurodegeneration. The current study resulted in several novel findings. We report for the first time that crude extract of Alaskan bog blueberry attenuated human α-synuclein aggregation and improved overall motility as expressed in a transgenic aged C. elegans model that expressed α-synuclein (OW13). Interestingly, we did not find a dose-dependent effect. Instead, we observed a bimodal or biphasic response in both aggregate reduction and motility enhancement. This pattern of response has also been reported earlier with other phytochemicals in both \textit{in-vivo} and \textit{in-vitro} models\textsuperscript{42,56,57}. The current results are of translational significance since α-synuclein aggregation is a typical pathological feature of protein misfolding diseases such as Parkinson's disease\textsuperscript{4}. A study in a yeast model suggests the therapeutic role of polyphenols like flavonoids in reducing α-synuclein toxicity\textsuperscript{58}. Other studies have also revealed the role of flavanoid sub-classes like anthocyanin and proanthocyanidin in conferring protection against oxidative stress \textit{in-vitro} and extending lifespan in \textit{C. elegans}\textsuperscript{38,59,60}. In this current study, anthocyanin and proanthocyanidin fraction of Alaskan blueberry reduced protein over expression in a transgenic strain of \textit{C. elegans}. Our results are possibly due to Alaskan blueberries growing under more extreme environmental conditions, such as extreme Arctic temperatures and 24-hours sunlight exposure (UV exposure) during summer months, and thereby having different anthocyanin content\textsuperscript{61}. In addition, doses of Alaskan blueberry extract did not influence lifespan and total fecundity in the nematode strain. Studies have shown that improved lifespan can be uncoupled from increased healthspan\textsuperscript{62}. In our study, blueberry bimodally improved one measure of healthspan (motility) with no overall effect.
on the other (fecundity). Importantly, blueberry treatment did not exhibit a uniformly toxic effect because it did not reduce lifespan and did not decrease fertility in this transgenic model of *C. elegans*.

In the past decade, sirtuins have received extensive scrutiny in relation to various age-related molecular mechanisms and have been studied across various model systems. Particularly, mammalian Sirtuin 1, due to its high abundance in the brain, has drawn much interest in the field of neuroscience including synaptic plasticity and cognition. At the same time, research that elucidates the role of natural polyphenols in activating Sirtuin 1 and affecting pathologies of protein misfolding diseases has increased. Results from these studies indicate that triggering Sirtuin 1 enhances its deacetylase activity further inducing different pro-survival pathways. While the beneficial effect of Sirtuin 1 activation has been comprehensively demonstrated, there are also studies that illuminate the role of Sirtuin 1 inhibition in neuroprotection and improving cellular functions in diseased conditions. Genetic and pharmacological studies have found that reduced Sirtuin 1 activity can be beneficial in improving neurodegenerative pathologies in *Drosophila melanogaster*, mammalian cells and mouse models of Huntington's and Alzheimer's disease. In alignment with these studies, we are reporting for the first time the protective effects of Alaskan blueberry through reduction of *sir-2.1* gene expression in a model of *C. elegans* displaying Parkinson-like molecular pathology. We found that genetically silencing (not deleting) *sir-2.1* greatly reduced the α-synuclein expression in OW13. This is further corroborated by the gene expression results suggesting higher gene expression levels of *sir-2.1* in the control group compared to the blueberry treated group and other downstream targets in OW13. Higher activity of Sirtuin 1 in metabolically compromised conditions has been linked to higher energy expenditure, which may potentially diminish the effect of the downstream
substrates \textsuperscript{76,77,83}. In our experimental model of protein overload, lower gene expression levels of downstream substrates such as \textit{daf-16} and \textit{cep-1} compared to \textit{sir-2.1} in untreated controls probably could be justified due to the same mechanism. Strikingly, the basal level of \textit{sir-2.1} was also elevated in OW13 when compared to healthy wild-type N2 (Supplemental Fig. 3). The profitable role of Sirtuin 1 activation can depend on physiological state of cells and actually can be detrimental when cells are in stress\textsuperscript{76,77}. In our experiments, we observed elevated gene expression levels of \textit{sir-2.1} in a model subjected to proteotoxic stress in the form of α-synuclein aggregation.

Research showed how studying alterations in downstream sirtuin substrates, like FOXO, P53 and PGC-1α, can be used as a treatment strategy in protein aggregating diseases \textsuperscript{27}. Based on this, we investigated the effects of blueberry extract on two molecular targets of \textit{sir-2.1}: \textit{daf-16} (\textit{C. elegans} ortholog for mammalian FOXO) and \textit{cep-1} (\textit{C. elegans} ortholog for mammalian p53). Expression of \textit{sir-2.1} gene in blueberry-treated animals showed an increase in α-synuclein expression. Results also demonstrated very low mRNA levels of \textit{sir-2.1} in blueberry-treated group compared to controls. However, we also observed that genetic silencing of \textit{daf-16} in both control and treatment groups had no effect on the protein expression. Overall, this may indicate that reduced gene expression of \textit{sir-2.1} (not complete deletion) could be a potential mechanism through which blueberry exerts its beneficial effect in reducing α-synuclein expression and such pathways may not involve changes in downstream protein targets. Reduction in Sirtuin 1 gene expression through genetic or pharmacological manipulation has been shown to improve cellular symptoms and disease-specific phenotypes in models expressing mutant huntingtin protein \textsuperscript{82}. Smith et al. (2014) showed that reducing levels of Sirtuin 1 can result in increased activity of other histone-acetylating enzymes, which can ameliorate the pathology\textsuperscript{82}. On the other hand, increased expression of Sirtuin 1 may deacetylate and repress functions of the CREB-binding protein (CBP)\textsuperscript{84} further reducing
cell activity. Impaired CREB signaling and reduced transcription also have been reported in cells that overexpress α-synuclein. However, no direct correlations have been drawn on how reduced activity of Sirtuin 1 using pharmacological interventions can possibly improve pathology of synucleinopathies through modulating transcription factors such as CREB. Though we do not speculate on the exact molecular interaction or cross talks, this study strongly advocates for future investigations of such novel mechanisms.

Protein misfolding diseases like Parkinson's are often associated with alterations in total lipid content. Increased lipid content has been used as a surrogate measure of improved cellular pathology in studies evaluating natural compounds as potential therapeutics to treat Parkinson's disease. However, these studies have not highlighted the exact mechanism behind the effect. We found higher fat levels in the wild-type N2 positive control compared to OW13 strain. The lower fat content in OW13 strain was possibly due to the metabolic burden of protein aggregation. The lipid levels were restored in OW13 animals treated with 400 μg/ml of blueberry extract. This pattern of results is consistent with those shown by others. However, much to our surprise the fat content and ROS measures did not correlate with each other as anticipated. We found that the blueberry extracts marginally increased the ROS levels, a phenomenon previously reported with Alaskan bog blueberries. Xenohormesis is a phenomenon that explains how environmentally stressed plants produce bioactive compounds that confer stress resistance and survival benefits to animals that consume them. Plants, such as blueberries, when subjected to harsh environmental stressors produce secondary metabolites such as polyphenols. Consumption of these plant metabolites by animals can activate stress response pathways other than regular antioxidant effects, thereby improving overall aging and health. Additionally, Alaskan blueberries have been shown to inhibit inflammation in neuronal cell cultures through
differential biological mechanisms other than their popular image of antioxidant ROS scavengers.

Several studies demonstrate that low levels of ROS can be beneficial in activating signaling pathways to initiate biological processes. In *C. elegans*, ROS in low concentrations can act as a secondary messenger molecule leading to fat accumulation. This increase in fat content has been found to have contrasting effects on longevity possibly due to differences in structure of the fat, its localization, or the time point of its accumulation. At day 7 of adulthood, we observed that lipid content was restored in parallel with reduction in α-synuclein expression. Important questions arise from this: What is the mechanistic approach resulting in such 'beneficial' increase in lipid levels, and; are such pathways evolutionarily conserved? Future studies aiming at further characterization of the increased fat content could be useful in answering such questions. Finally, inhibition of Sirtuin 1 has also been found to increase production of ROS due to its positive regulation of antioxidant enzymes. In the current context of investigation, we believe a marginal increase of ROS due to *sir-2.1* inhibition by blueberries might activate crucial cell survival pathways overcoming the protein overload. However, such complex interactions between proteostasis, sirtuins, ROS and lipid content should be subjected to further investigation in a variety of cellular and animal models.
2.8 Figures and Tables

Figure 2.1 Crude extract of Alaskan Blueberry Reduced Expression of α-synuclein in the OW13 Strain of C. elegans

(A) Graphical representation of fluorescence intensity of the OW13 day 7 animals fed on different concentrations of Alaskan blueberry crude extract (0, 100, 200 and 400 µg/ml) from Larval L4 stage. The data represent the mean ± SEM (n = 20-25 animals per group) with significant differences between the control and extract treatments (0 and 100 µg/ml, 0 and 400 µg/ml) *p<0.01, *** p<0.001. Dose 200 µg/ml was not significantly different from control (p>0.4). Each experiment was repeated three times. (B) Representative confocal images of the α-synuclein/YFP expression in the head region of day 7 OW13 animals, magnification 40X and scale bar 50 µm. (C) Original western blot images of α-synuclein protein of the OW13 day 7 animals fed on
concentrations of Alaskan blueberry crude extract (0 and 400 μg/ml). Blots of actin were used as a protein loading control. (D) Graphical representation of quantification of α-synuclein protein bands from Western blots. The intensity of α-synuclein protein was normalized to actin and presented as a percentage. Each experiment was repeated twice. For each treatment of each experiment, protein samples were pooled from three biological replicates. The data represent the mean ± SEM with significant differences between control and extract treatments (0 and 400 μg/ml, *p < 0.02).
Figure 2.2 Alaskan Blueberry Polyphenols Reduced Expression of α-synuclein in the OW13 Strain of *C. elegans*

(A) Graphical representation of fluorescence intensity of the OW13 day 7 animals fed on different polyphenols found in Alaskan blueberry: CA=Chlorogenic acid, PAC=Proanthocyanidins, ANT=Anthocyanins from Larval L4 stage. A concentration of 400 μg/ml was used for all extracts and 0.1% DMSO in deionized water was used as a control. The data represent the mean ± SEM (n = 20-25 animals per group) with significant differences between the control and treatments, **p<0.001, ***p<0.0001. Each experiment was repeated three times. (B) Representative confocal images of the α-synuclein/YFP expression in the head region of day 7 OW13 animals, magnification 40X and scale bar 50 μm.
Figure 2.3 Alaskan Blueberry Polyphenols Reduced Expression of α-synuclein in the OW13 strain of *C. elegans* Through a Sirtuin Mediated Pathway.

(A) Graphical representation of fluorescence intensity of the day 7 OW13 animals fed on different genetic RNA interference treatments (*L4440, sir-2.1 and daf-16*) and Alaskan blueberry (0 and 400 μg/ml) from larval L4 stage. Empty vector L4440 was considered as a control. The data represent the mean ± SEM (n = 20-25 animals per group) with significant differences between the control and treatments at **p<0.001 and ***p<0.0001. Each experiment was repeated three times. (B) Representative confocal images of the α-synuclein/YFP expression in the head region of day 7 OW13 animals, magnification 40X and scale bar 50 μm.
Figure 2.4 Alaskan Bog Blueberry Polyphenols Improved Motility in Aged (Day 12) OW13 Strain of *C. elegans*.

Percent per motility class of middle age (day 5 adults; A) and old age (day 12 adults; B) animals treated with different concentrations of Alaskan bog blueberry extract (0, 100, 200 and 400 μg/ml) from larval L4 stage. Class A animals (dark gray) moved normally and spontaneously, class B animals (light gray) moved abnormally and may have required prodding, and class C animals (black) were unable to move or moved just head or tail in response to touch (n = 20 animals per group). Each experiment was repeated at least three times.
Figure 2.5 Reduction of *sir-2.1* Expression Reverses the Motility Improving Effects of Alaskan Bog Blueberry Polyphenols in Aged (Day 12) OW13 Strain of *C. elegans*.

Percent per motility class of middle age (day 5 adults; A) and old age (day 12 adults; B) animals treated with different genetic RNA interference treatments (*L4440, sir-2.1 and daf-16*) and Alaskan bog blueberry extract (0 and 400 μg/ml) from larval L4 stage. Empty vector *L4440* was used as control. Class A animals (dark gray) moved normally and spontaneously, class B animals (light gray) moved abnormally and may have required prodding, and class C animals (black) were unable to move or moved just head or tail in response to touch (n = 20 animals per group). Each experiment was repeated at least three times.
Figure 2.6 Alaskan Bog Blueberry did not Alter Lifespan in OW13 Strain of *C. elegans*.

(A) Representative survival curves for animals treated with crude extract of blueberries (0, 100, 200 and 400 μg/ml) from larval L4 stage. There was no significant difference in lifespan extension (p > 0.05; Kaplan–Meier log-rank test) among the groups. Each experiment was repeated at least three times (n = 44 animals per treatment). (B) Representative survival curves for animals treated with RNA interference treatments (*L4440 and sir-2.1*) and crude extract of blueberries/BB (0 and 400 μg/ml) from larval L4 stage. There was no significant difference in lifespan extension (p > 0.05; Kaplan–Meier log-rank test) among the groups. Each experiment was repeated at least three times (n = 44 animals per treatment).
Figure 2.7 Alaskan Bog Blueberry and/or RNA Interference Treatments did not Influence Total Fecundity in OW13 Strain of *C. elegans*.

(A) Graphical representation of total fecundity of the OW13 animals fed on different concentrations of Alaskan bog blueberry crude extract (0, 100, 200 and 400 μg/ml) from larval L4 stage. The data represent the mean ± SEM (n = 10 animals per group) with no significant differences among the control and treatments, p>0.05. Each experiment was repeated three times.

(B) Graphical representation of total fecundity of the OW13 animals fed on different genetic RNA interference treatments (*L4440* and *sir-2.1*) and concentrations of Alaskan blueberry crude extract (0 and 400 μg/ml). The data represent the mean ± SEM (n = 10 animals per group) with no significant differences between the control and treatments, p>0.05. Each experiment was repeated three times.
Figure 2.8 Alaskan Bog Blueberry Increased Lipid Content by a Marginal Increase in Reactive Oxygen Species in OW13 Strain of *C. elegans*.

(A) Graphical representation for fluorescence intensity for Nile red staining of day 7 wild-type N2 (control) and OW13 animals fed Alaskan bog blueberry extract (0 and 400 μg/ml) from larval L3 stage. The data represent the mean ± SEM (n = 20-25 animals per group) with significant differences between the control and treatments at ***p<0.001. Each experiment was repeated at least three times. (B) Representative confocal images of the intestine of day 7 wild-type N2 (control) and OW13 animals fed Alaskan bog blueberry extract (0 and 400 μg/ml), magnification 20X and scale bar 50 μm. (C) The change in endogenous ROS was measured by DCF-DA assay
at day 7 of adulthood after treatment with crude extract of blueberries (0 and 400 μg/ml). Bars represent mean ± SEM of each replicate, with three technical replicates for the DCF-DA assay. 

*p= 0.07.
Figure 2.9 Alaskan Bog Blueberry Extract Reduced the Gene Expression of \textit{sir-2.1} in OW13 Strain of \textit{C. elegans}.

qPCR analysis of mRNA levels of \textit{sir-2.1, daf-16} and \textit{cep-1} (400 μg/ml of crude blueberry or BB extract) in day 7 OW13 animals. qPCR reactions were run in triplicates for each gene. Each experiment was repeated at least three times. The data represent the mean ± SEM at **\textit{p}<0.01 and ***\textit{p}<0.001.
Supplemental Figure 2.1 Graphical Representation of Fluorescence Intensity of the OW13 Animals Fed on Different RNA Interference Treatments: L4440 and GFP. The data represent the mean ± SEM (n = 15-20 animals per group) with a significant difference between the control and GFP treatment, p<0.01. This was used as a validation plate control for RNA interference experiments.
Supplemental Figure 2.2 Graphical Representation of Fluorescence Intensity of the OW13 animals Fed on Different Concentrations of Alaskan Bog Blueberry Crude Extract (0, 100, 200 and 400 μg/ml) and heat-killed OP-50-1 as a food source. The data represent the mean ± SEM (n = 15-16 animals per group) with significant differences between the control and treatments at *p<0.01 and **p<0.001. Each experiment was repeated two times.
Supplemental Figure 2.3 qPCR Analysis of mRNA Levels of *sir-2.1* Between Wild-type N2 and α-synuclein Overexpressing OW13 strain. qPCR reactions were run in triplicates for each strain. Each experiment was repeated at least two times. The data represent the mean ± SEM; *p<0.05.
Supplemental Table 2.1 Total Phenolic Content (Quantified by Folin–Ciocalteu assay with Gallic Acid Equivalent), and Anthocyanin Content (Quantified by pH-differential with Cyanidin-3-glucoside) of Alaskan Bog Blueberry.

<table>
<thead>
<tr>
<th></th>
<th>Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Phenolic content (mg GAE g(^{-1}) FW)</td>
<td>198.2±1.4</td>
</tr>
<tr>
<td>Total anthocyanin (mg C3G L(^{-1}) FW)</td>
<td>285.7±8.2</td>
</tr>
</tbody>
</table>

*Data is represented as mean ± SEM for each replicate. Each assay was run in triplicate. The assays were performed following the methods of Scerbak et al., 2016. The values were used for the biochemical quantification of the berry extracts to compare the content reported in Scerbak et al. (2016). The values reported are similar to that reported in previous study.
Supplemental Table 2.2 Sequencing Results of the RNA Interference Clones.

<table>
<thead>
<tr>
<th>Name of Clones</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L4440</em></td>
<td>GGGGNCCANNNNCNATACGGGNCGANTGGNTG</td>
</tr>
<tr>
<td></td>
<td>ACCGGNCCCCCCCCNAAGG</td>
</tr>
<tr>
<td></td>
<td>CNNGGGNTNANTAACNTTGATATCCCCCTTTA</td>
</tr>
<tr>
<td></td>
<td>AAAAGAATGAGCAGNNTTT</td>
</tr>
<tr>
<td></td>
<td>TGATGCCAAAAGCTCGATANATCCCCGGGAAT</td>
</tr>
<tr>
<td></td>
<td>AACGCTTCTTACATCGAT</td>
</tr>
<tr>
<td></td>
<td>GAACGAAATCNNNNACCAAAAATTTCANNCT</td>
</tr>
<tr>
<td></td>
<td>CCAAGCGAAGCGCTCAA</td>
</tr>
<tr>
<td></td>
<td>CACAACCTCANAACATCTAAAGCGGAATTNNN</td>
</tr>
<tr>
<td></td>
<td>TTATNANNAACATTCNTC</td>
</tr>
<tr>
<td></td>
<td>ATAACCTTTGCAATGTCTCACCNNNAANCAT</td>
</tr>
<tr>
<td></td>
<td>CNCACACNTAGTTCCAAA</td>
</tr>
<tr>
<td></td>
<td>CTTNNCCATCNNATCANCATTCCATTCCACAAA</td>
</tr>
<tr>
<td></td>
<td>NCAGACTTTCGANGACCA</td>
</tr>
<tr>
<td></td>
<td>CAAATCCGNNNNAAAANNNTTACGTGGANN</td>
</tr>
<tr>
<td></td>
<td>GCTGGNCGANNNGGNNNN</td>
</tr>
<tr>
<td></td>
<td>ANNNNGNATATTCTANNGNNTATATTCCNNN</td>
</tr>
<tr>
<td>---</td>
<td>----------------------------------</td>
</tr>
<tr>
<td></td>
<td>NTTNCAACAATAACNNACT</td>
</tr>
<tr>
<td></td>
<td>NNNAGATNNTTNATTNCCNGAGCTCNNNC</td>
</tr>
<tr>
<td></td>
<td>ANNNNGNGANNAACNATC</td>
</tr>
<tr>
<td></td>
<td>CANNNTTCATAAAA</td>
</tr>
<tr>
<td></td>
<td><strong>sir-2.1</strong></td>
</tr>
<tr>
<td></td>
<td>NNNNNNNNNNNNNNNNGGCNNNNNNNNNNNG</td>
</tr>
<tr>
<td></td>
<td>GNNNNNNNNNNNNNGNNNN</td>
</tr>
<tr>
<td></td>
<td>NNGNNNNNANNNGCNNNNNNGNNCNGCANC</td>
</tr>
<tr>
<td></td>
<td>NNNNNNNNNNNNNANNNNNNN</td>
</tr>
<tr>
<td></td>
<td>NNNNNNNNNNNNNNANAGNANNNNANGNN</td>
</tr>
<tr>
<td></td>
<td>NNNNNNNNNAANNNNNNNNA</td>
</tr>
<tr>
<td></td>
<td>ANNNNTCNGNNNNNNACAAAGTCANNTAGCG</td>
</tr>
<tr>
<td></td>
<td>NNNNNNNNNTNNNNNNNNNN</td>
</tr>
<tr>
<td></td>
<td>NNNNGNNNNNGNNNGGCNNNNNGGNNNGNNNA</td>
</tr>
<tr>
<td></td>
<td>TANNNNNNACACNAGCNNN</td>
</tr>
<tr>
<td></td>
<td>CNCANNNNNNNCTCGTGATGAANNNNNCGAGCT</td>
</tr>
<tr>
<td></td>
<td>ANTNNCNACGTTTCTAGA</td>
</tr>
<tr>
<td></td>
<td>NNNAATGAGCANNCCNNTCCAGGAANGNNN</td>
</tr>
<tr>
<td></td>
<td>TTTGANGANNNNNNNCTG</td>
</tr>
<tr>
<td>AACAAAAATNATTANTTTGTAAGGCNGNNNG</td>
<td></td>
</tr>
<tr>
<td>-------------------------------------</td>
<td></td>
</tr>
<tr>
<td>TAGGTTGTTTAGAAATAT</td>
<td></td>
</tr>
<tr>
<td>TTICTAACCTTTTGACCTTTTTAATTTCAAAAACTA</td>
<td></td>
</tr>
<tr>
<td>CTCACCGCAACACAAGT</td>
<td></td>
</tr>
<tr>
<td>TATTTGCATGGAGCATCTCATGTAATGCTCAGCA</td>
<td></td>
</tr>
<tr>
<td>GCCTTGGATGCTTTATT</td>
<td></td>
</tr>
<tr>
<td>TTCTTTTAGGATAGCGTCCGAATTCCGTGCATCA</td>
<td></td>
</tr>
<tr>
<td>TCGTCATCTTCTGACTT</td>
<td></td>
</tr>
<tr>
<td>ATGCTTTTGTATTTCTGGAAATCATTTCTTCTC</td>
<td></td>
</tr>
<tr>
<td>GGAATCCATCAAATCGT</td>
<td></td>
</tr>
<tr>
<td>CAACGACCGACATTCTCGGTTTCTTCAATGTTG</td>
<td></td>
</tr>
<tr>
<td>GCTCATCCTCGAATCA</td>
<td></td>
</tr>
<tr>
<td>TCATTACTCTTCTTTCTTACATATAATTCAAA</td>
<td></td>
</tr>
<tr>
<td>AAGTTTTTCTGTGGAAT</td>
<td></td>
</tr>
<tr>
<td>AAGCTGTCTGTTTTGTTTGTAAGGCTTTTGTGA</td>
<td></td>
</tr>
<tr>
<td>TTTGATTTTCTTTGTT</td>
<td></td>
</tr>
<tr>
<td>GTTCCATAATTGAATCATAGATGTAATCAGTT</td>
<td></td>
</tr>
<tr>
<td>CAGTGAAGGAGCCTCCN</td>
<td></td>
</tr>
</tbody>
</table>

75
| AGACTGAAACAGATATCTCTGATGGTGCCATC  |
| AGACTGAAACAGATATCTCTGATGGTGCCATC  |
| ACAATTTCGAGCAATTTC                |
| ACAATTTCGAGCAATTTC                |
| NATATCCGCATTATNATGTTGGAGGGANTCTCT |
| NATATCCGCATTATNATGTTGGAGGGANTCTCT |
| GTTGANTAGAATTCGTG                 |
| GTTGANTAGAATTCGTG                 |
| GACCATTCTTTATCNACACNATGAGGAATTNAT |
| GACCATTCTTTATCNACACNATGAGGAATTNAT |
| GCNACTGGTGGAACCTCT                |
| GCNACTGGTGGAACCTCT                |
| ANAGAANATCCNATCACTACNTAANATCTACT  |
| ANAGAANATCCNATCACTACNTAANATCTACT  |
| TNNTGCTTNNCTTCTGTAT               |
| TNNTGCTTNNCTTCTGTAT               |
| NCATGNTGATGAANTNCCNTCNANANCNNNC   |
| NCATGNTGATGAANTNCCNTCNANANCNNNC   |
| NAAGATANANATTNNNNTT               |
| NAAGATANANATTNNNNTT               |
| GATGACNNNNNNANNNNTTNCATGNNANNCN   |
| GATGACNNNNNNANNNNTTNCATGNNANNCN   |
| NNTCANACTNNNCNCONNN               |
| NNTCANACTNNNCNCONNN               |
| NNNNTCNNNANTNNNATNTNNCCNNNNCN     |
| NNNNTCNNNANTNNNATNTNNCCNNNNCN     |
| NNNGNNNCNNNTNNNTNNA               |
| NNNGNNNCNNNTNNNTNNA               |
| NANNNGNNNTNCCCNNNNNNNNNNNNNNN    |
| NANNNGNNNTNCCCNNNNNNNNNNNNNNN    |
| NNNNNNNNNNNNNNNNNNNNNNNNNNN      |
| NNNNNNNNNNNNNNNNNNNNNNNNNNN      |
| NNNNNNNNNNNNNNNNNNNNNNNNNNN      |
| NNNNNNNNNNNNNNNNNNNNNNNNNNN      |
| NNNNNNNNNNNNNNNNNNNNNNNNNNN      |
| daf-16 | NNNNNNNNNNNNNNNGNNCNNNNNNNNNNNNNNGGNCCNNCCTCGAGGTCG  

ACGGTATCGAATAAGCTTGATATCACAAGTTTGT  
ACAAAAAAGCAGGCTTG  
ATGGAGATGTGGTAGAGTACAGGAACTGATGC  
ATCGTCATCCGCCTCCAC  
GTCCACCTCATCTGTTCGAGATTCGGAGCGGA  
CACGTTATGAATACAC  
CGGATGATGTGTGATGATGAATGATGATATGGAA  
CCGATTCCTCGTGATCGG  
TGCAATACGTGGCCAATGCGTAGGCCGCAACT  
CGAACCACCACTCAAACCT  
GAGTCCCATATTCATGAACAAATTCCTGAAG  
AAGATGCTGACCTATACG  
GGAGCAATGAGCAATGTGGACAGCTCGCGGA  
GCATCTTCAAACGGGTCG  
ACAGCAATGCTTCTATACGATGGAAGCAA  
TTCTCATCAGACATCGTT |
<table>
<thead>
<tr>
<th>CCTTCACTCGACACTTAATGGAAATTGATTTCGATG</th>
<th>CGGATCGATTCAAACGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTTTCACGATTTTGTATGATGATGATTCAATGC</td>
<td>AAGGAGCATTTGATAAC</td>
</tr>
<tr>
<td>GTTCCATCATCTTTCCGTCCCCGAACCTCAATCG</td>
<td>AACCTCTCGATTCCCTG</td>
</tr>
<tr>
<td>ATCGTCGTCTCGTGTTTCTCCAGCTATTGGGAAG</td>
<td>TGATATCTATGATGATC</td>
</tr>
<tr>
<td>TAGAATTCCCATCATGGGGTTGGNCGAATCCGG</td>
<td>TTCCAGCAATTTCGAGT</td>
</tr>
<tr>
<td>GANNTTGGTTGATAAAGAACTTGGATCAAATGNN</td>
<td>NNNCNGATGNANCTACNC</td>
</tr>
<tr>
<td>NNTNTTGGTGGNNNTNNAGATTANNCCGGNAG</td>
<td>TCCAANGCCCNNTTAANA</td>
</tr>
<tr>
<td>ACNNACCNNANTTGNNNNNNNNNNNNNNNN</td>
<td>NNCCNNNNNGNTTAAAAAN</td>
</tr>
<tr>
<td>NNNNCANNGGNNANCAGGGGGGGNTTCCNNANNC</td>
<td>NNNTNNNNNNCTNNNNTNCC</td>
</tr>
</tbody>
</table>
This was performed to ensure the validity of the dsRNA and the sequences were verified using BLAST nucleotide search tool.
Supplemental Table 2.3 Total Number of Animals Used for Lifespan Experiments.

<table>
<thead>
<tr>
<th>Lifespan (Crude extract)</th>
<th>Total number of animals at the beginning of the experiment</th>
<th>Total Censored</th>
<th>Actual number of animals used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate 1</td>
<td>176</td>
<td>41</td>
<td>135</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>176</td>
<td>37</td>
<td>139</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>176</td>
<td>42</td>
<td>134</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lifespan (RNAi)</th>
<th>Total number of animals at the beginning of the experiment</th>
<th>Total Censored</th>
<th>Actual number of animals used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate 1</td>
<td>176</td>
<td>44</td>
<td>132</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>176</td>
<td>39</td>
<td>137</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>176</td>
<td>41</td>
<td>135</td>
</tr>
</tbody>
</table>
2.9 References


Chapter 3: Silencing Fat Metabolism Genes in Parkinson's like Models of *Caenorhabditis elegans*

3.1 Abstract

Fat metabolism plays an important role in modulating key molecular pathways, such as insulin signaling associated with aging. Aging is considered a risk factor for developing Parkinson’s disease (PD). Previous studies in the nematode model of *Caenorhabditis elegans* (*C. elegans*) show that delaying aging can rescue some of the deleterious PD-like pathologies. However, the role of fat metabolism in the pathogenesis of PD is not clear. Using RNA interference, we studied the effects of silencing of ‘fat genes’ on PD pathology in models of *C. elegans*. We used four candidate genes: *nhr-49 and tub-1* (silencing of either one leads to ‘high fat’ content), and *fat-5 and fat-7* (silencing of either one leads to ‘low fat’ content) to manipulate fat levels in the nematode. The silencing of the *fat-5 and fat-7* genes rescued both degeneration of dopamine neurons and deficits in dopamine-dependent behaviors, including basal slowing and ethanol avoidance in worm models of PD. Similarly, silencing of these genes also decreased the formation of protein aggregates in a nematode model of PD expressing α-synuclein in the body wall muscles and rescued deficits in resistance to heat and osmotic stress. However, the silencing of all four fat genes did not change the total lifespan of any of the models. Overall, our research suggests that low fat content can be beneficial in ameliorating PD-like pathology and improving functionality, even though it did not extend lifespan.

Authorship for this chapter is as follows: Malabika Maulik, Ajiel Mae Basmayor, Brianna Lu, Swarup Mitra, Barbara E Taylor and Abel Bult-Ito. Submitted to Human Molecular Genetics.
3.2 Introduction

Parkinson’s disease (PD) is one of the most prevalent age-related neurodegenerative diseases causing motor impairments and cognitive dysfunction\(^1\). PD usually results in degeneration of dopaminergic neurons within the substantia nigra (SN) region of the brain. Such degeneration may happen due to the formation of protein aggregates known as Lewy bodies\(^2\). Although there are treatments available to reduce individual symptoms such as motor deficits, there are currently no preventive therapies that can target and lessen PD progression.

Both genetic and environmental factors have been implicated in the pathogenesis of PD\(^3,4\). The α-synuclein (α-syn) gene SNCA was the first to be associated with PD\(^5\). Both mutations in the SNCA gene and increased copy number result in PD pathology, which is characterized by formation of protein aggregates\(^6\). The A53T mutation, substitution of threonine for alanine at position 53, is linked with the autosomal dominant form of PD\(^5\). Exposure to environmental toxins such as rotenone, a broad-spectrum insecticide, impairs mitochondrial function and causes oxidative stress, causes the death of dopaminergic neurons in the SN and subsequent development PD pathophysiology\(^7\). Aging is also considered a major risk factor as the number of alterations that take place during normal aging have been implicated in the progression of PD\(^8\).

*Caenorhabditis elegans* (*C. elegans*) offers several advantages for investigating both aging and neurodegenerative disease\(^9\). These nematode models have been used to characterize protein aggregation and dopaminergic neuron degeneration, key molecular hallmarks of PD\(^8\). In *C. elegans*, fat metabolism involves a complex and conserved gene network, which regulates food sensing and neuroendocrine signaling processes\(^10\). Food sensing behavior in worms is also controlled by the dopaminergic system, which is also the crucial neural network affected in PD\(^11\). Despite lack of specific adipocytes, *C. elegans* shares common human homologues of genes that
are implicated in obesity and metabolic disorders\textsuperscript{10}. In mammals, nuclear hormone receptors (NHRs), such as peroxisome proliferator-activator receptor protein-alpha (PPAR\textalpha{}), are often studied as drug targets for lipid metabolism\textsuperscript{12}. One such receptor known as \textit{nhr-49} promotes fatty acid desaturation and beta-oxidation leading to 'high fat' accumulation on gene silencing, altered mitochondrial function and shortened lifespan in nematodes\textsuperscript{13,14}. Like in mammals, mutations in \textit{tub-1} (tubby homologue) leads to increased fat deposition and increased lifespan in \textit{C. elegans}\textsuperscript{15}.

\(\Delta 9\) desaturase enzymes are present in organisms from yeast to humans and are important for energy metabolism and lipid synthesis\textsuperscript{16}. Diets high in unsaturated fatty acids decrease expression of \(\Delta 9\) desaturase and high carbohydrate consumption increases its expression\textsuperscript{17}. In \textit{C. elegans}, \(\Delta 9\) desaturases are encoded by the genes: \textit{fat-5} and \textit{fat-7}. RNA interference (RNAi) studies with \textit{fat-5} and \textit{fat-7} have shown that silencing of these genes reduces fat levels in the nematodes\textsuperscript{13}. \textit{C. elegans} is a popular genetic model that allows for easy manipulation of these fat metabolism genes using RNAi to create high and low fat content in these organisms. Moreover, fatty acid metabolism is known to be involved in stress resistance mechanisms and insulin signaling in the worm model\textsuperscript{18}. Such stress resistance mechanisms have been used as a parameter to study the effect of aging and insulin signaling in PD models of \textit{C. elegans}\textsuperscript{8}. However, the direct relationship between fat metabolism and PD pathology in nematodes have been sparsely explored.

In this study, we chose to examine the effects of silencing of fat metabolism genes in different worm models of PD. Silencing these candidate genes manipulated the level of fats in the nematodes. Using this genetic approach, we examined the effects of high and low fat content on \(\alpha\)-synuclein protein aggregation (wild-type and A53T mutation) and dopaminergic neuron degeneration in \textit{C. elegans}. In addition, we also studied the overall effects on lifespan, health span
and stress mechanisms. We hypothesized that that silencing of fat-5 and fat-7 gene will improve the pathophysiology and rescue several deficits present in worm models of PD.

3.3 Materials and Methods

3.3.1 C. elegans Strains and Maintenance

Strains used for the study were OW13 (Punc54: α-syn) and TG2435 (pDAT::GFP), which were acquired from the Caenorhabditis Genetics Center (University of Minnesota). The other strains for the study, i.e., JVR203 (pDAT::mut A53T- α-syn) and JVR208 (pDAT:: WT- α-syn), were kindly donated by Drs. Shohei Mitani and Jeremy Van Raamsdonk. The OW13 strain expresses human wild-type α-synuclein in its body wall muscle. The JVR203 and JVR208 strains express human wild-type and mutant A53T α-synuclein, respectively, in the dopaminergic neurons.

The animals were cultured on nematode growth medium (NGM) plates using standard procedures and maintained at 20 °C. The plates were seeded with Escherichia coli strains OP-50-1 or HT115 depending on the experiments. Synchronous populations were created using the standard egg lay method (allowing 20-30 animals to lay eggs for 4-6 h and then removing the adults) or by hypochlorite treatment (2% sodium hypochlorite in 0.5 M NaOH). All experimental plates (except for fecundity studies) contained 0.04 μg/ml fluorodeoxyuridine (FUdR) for progeny control.

3.3.2 RNA Interference

RNAi plates were prepared using isopropyl β-D-thiogalactoside (IPTG) and 4X concentrated HT115 bacteria for each gene target (L4440, fat-5, fat-7, nhr-49 and tub-1) and covered with foil to protect from light. For each replicate of the experiment, control
experiments were performed to compare the mechanosensory touch function between mec-7 silenced to empty vector (L4440)\textsuperscript{22} groups, to ensure that the RNAi procedure worked properly (Supplemental Fig. 1). Nile red staining was done to measure the fat content of the worms \textsuperscript{21}.

3.3.3 Rotenone Administration

Rotenone (Sigma; 4 μM) in DMSO was added to bacteria-seeded NGM plates\textsuperscript{23}. Larval L4 worms were then loaded onto the rotenone-coated plates until further experimentation. An equal volume of DMSO bacteria-seeded NGM plates were used as vehicle controls.

3.3.4 Lifespan Analysis

Following egg lay, synchronous populations of 22 L4 animals were transferred into two replicates of each treatment. The survival was determined daily by visual observation under dissecting microscope or by gentle prodding with a platinum wire. 'Bagged' animals and animals crawling off the plates were censored\textsuperscript{24}. Each experiment was repeated at least 3 times.

3.3.5 Body Bends

Body bends were measured in Day 3 adult worms by counting forward and complete sigmoidal movement of the nematodes for 1 min\textsuperscript{8}. Spontaneous reversals were excluded.

3.3.6 Fecundity

Fecundity or viable progeny count was performed by plating Day 2 adults (peak fecundity) on fresh treatment plates\textsuperscript{25}. The eggs laid by the adults were allowed to hatch and develop at 20 °C for 48 h. The number of progenies produced was manually counted under a dissecting microscope.
3.3.7 Pharyngeal Pump Rate

The pharyngeal pump rate was assessed by manual counting in pharyngeal compressions for 30 s in Day 3 adult worms by placing them on a seeded NGM assay plates.25

3.3.8 Stress Resistance Assays: Heat Stress and Osmotic Stress

Sensitivity to heat stress was determined by assessing survival of young adult worms incubated at 35 °C.26 Survival was assessed after four hours. Sensitivity to osmotic stress was determined by transferring young adult worms to NGM plates containing 500mM concentration of NaCl. The survival of worms was determined after 24 h. Both stress assays were conducted on Day 3 of adulthood. The survival was determined by visual observation of motility under dissecting microscope or by gentle prodding with a platinum wire. The worms who failed to move on prodding were counted as dead.

3.3.9 Dopamine Dependent Behaviors

3.3.9.1 Basal Response to Food

Day 3 worms were washed in M9 buffer and transferred to either unseeded NGM plates or NGM plates seeded with OP50-1 bacteria over the entire plate. After 3 min of acclimatization, the rate of movement was assessed by counting body bends for 30 s. Basal slowing was calculated as the (rate of movement off food – rate of movement on food) / rate of movement off food.

3.3.9.2 Ethanol Avoidance Assay

Day 3 adult worms were transferred to the center of NGM plates divided into four quadrants: two quadrants seeded with 40 μl ethanol and two others contained no ethanol. After 30 min, the worms were scored for their presence in each quadrant. Ethanol avoidance was
assessed as (number of worms in control quadrants – number of worms in ethanol seeded quadrants)/ total number of worms\(^8\).

3.3.11 Statistical Analysis

The Kaplan-Meier log rank analysis for lifespan survival experiments was performed with SPSS (Version 22, IBM, Inc.). All other statistical analyses were performed with Graph Pad Prism 7 (GraphPad Software, Inc). Treatment effects among groups were assessed using one-way or two-way analysis of variance (ANOVA). Pairwise comparisons to identify significant differences between treatment groups were tested using Tukey's post hoc tests.

3.4 Results

3.4.1 Silencing of fat-5 and fat-7 Genes Ameliorated α-synuclein Overexpression and Improved Dopamine-related Behaviors in Transgenic Models of C. elegans

At day 7 of adulthood, silencing of fat-5 and fat-7 genes significantly reduced human wild-type α-synuclein expression in the OW13 model (Figs. 3.1A and 3.1B) by about 30\% (F\(_{4,145} = 15.89, p<0.0001\)) when compared to the L4440 control (p<0.0001 and p<0.0001, respectively). There were no significant differences among control, nhr-49 and tub-1 groups (p>0.05).

At day 3 of adulthood, dopaminergic functions were tested in another transgenic model (Figs. 3.1C and 3.1D), JVR208, in which human wild-type α-synuclein was expressed specifically in dopaminergic neurons. Basal slowing was increased significantly (F\(_{4,70} = 16.5, p<0.0001\)) in both fat-5 and fat-7 treated animals when compared to the L4440 control group (p<0.0008 and p<0.007, respectively). Similarly, the ethanol avoidance index was significantly increased (F\(_{4,10} = 6.884, p<0.001\)) in animals in the fat-5 and fat-7 groups compared to the empty
vector control (p<0.008 and p<0.03, respectively). There were no significant differences among control, nhr-49 and tub-1 groups (p>0.05) for either of the behaviors.

3.4.2 Genetic Silencing of fat-5 and fat-7 Genes Improved Resistance to Heat in a Human Wild-type α-synuclein Overexpression Model of C. elegans

Following RNAi treatment with the candidate fat metabolism genes in a human wild-type α-synuclein overexpression model of C. elegans, no significant effects (p>0.05) were observed on the overall lifespan (Fig 3.2A), the number of body bends per second (Fig. 3.2D), and osmotic tolerance (Fig. 3.2E). Silencing the nhr-49 and tub-1 genes significantly reduced peak fecundity (F (4,45) = 5.777, p<0.001) compared to the L4440 control (Fig 3.2B; p<0.003 and p<0.0009, respectively). The pharyngeal pump rate was significantly increased (F4,45 = 3.124, p<0.001) in the fat-7 group compared to the L4440 control group (Fig. 3.2C; p<0.04). The fat-5 group showed significantly increased (F4,10 = 8.4, p<0.001) heat tolerance compared to the L4440 group (Fig. 3.2F; p<0.002), while the fat-7 group showed a strong trend in improving heat tolerance (Fig. 3.2F; p<0.06).

3.3.10 Microscopy and Image Quantification

All images of dopaminergic neuron degeneration were acquired with an Axiovert FX100 fluorescent microscope and all images of protein overexpression were acquired with a Zeiss LSM 510 laser scanning confocal microscope. Magnification and exposure settings were kept constant for each microscope. Images were analyzed with ImageJ (National Institutes of Health). The α-synuclein levels were quantified based on measurement of total fluorescence. Dopaminergic neuron degeneration was quantified on the basis of scoring animals for presence
of normal and abnormal neurons. Any neuron with missing soma or dendrite, or with any morphological alterations like outgrowths or blebs were considered abnormal\textsuperscript{27,28}.

3.4.3 Genetic Silencing of \textit{fat-5} and \textit{fat-7} Genes Improved Dopaminergic Neuron Degeneration and Related Behaviors in a Model of \textit{C. elegans} Expressing Human Mutated $\alpha$-synuclein (A53T Mutation)

Genetic silencing of \textit{fat-5} and \textit{fat-7} genes in a model of \textit{C. elegans} expressing human mutated $\alpha$-synuclein (A53T mutation) significantly increased the percentage of normal dopaminergic neuron morphology in \textit{C. elegans} ($F_{4,10} = 11.27$, $p<0.001$) expressing human mutated $\alpha$-synuclein in their dopaminergic neurons at Day 7 of adulthood (Fig. 3.3A and 3.3B) compared to the \textit{L4440} control group ($p<0.02$ and $p<0.02$, respectively). When compared to the \textit{L4440} control group, both \textit{fat-5} and \textit{fat-7} groups showed significantly increased basal slowing behavior ($F_{4,70} = 6.7$, $p<0.001$) (Fig. 3.3C; $p<0.003$ and $p<0.0007$, respectively) and ethanol avoidance index ($F_{4,10} = 15.33$, $p<0.01$) (Fig. 3.3D, $p<0.0007$ and $p<0.002$, respectively). Silencing of the \textit{nhr-49} and \textit{tub-1} genes (Fig. 3.3) had no significant effects on either dopaminergic neuron health ($p>0.05$) or its associated functional behaviors ($p>0.05$).

3.4.4 Defects in Stress Resistance was Improved by Silencing of \textit{fat-5} and \textit{fat-7} Genes in \textit{C. elegans} Expressing Human Mutated $\alpha$-synuclein (A53T Mutation)

Silencing of the four fat genes in a model of \textit{C. elegans} expressing human mutated $\alpha$-synuclein (A53T mutation) had no significant impact ($p>0.05$) on the overall lifespan, (Fig. 3.4A), the pharyngeal pump rate (Fig. 3.4C) and the number of body bends per second (Fig. 3.4D). Silencing the \textit{nhr-49} and \textit{tub-1} genes significantly reduced peak fecundity compared to the \textit{L4440} control group ($F_{4,45} = 21.71$, $p<0.001$) (Fig 3.4B; $p<0.0002$ and $p<0.0002$, respectively). The heat
tolerance was significantly increased in the fat-5 and fat-7 groups compared to the L4440 control group (F_{4,10} = 8.78, p<0.002) (Fig 3.4E; p<0.002 and p<0.005, respectively). Silencing of the fat-5 and fat-7 genes also significantly increased osmotic tolerance compared to the L4440 control group (F_{4,10} = 35.65, p<0.001) (Fig 3.4F; p<0.0002 and p<0.0002, respectively).

3.4.5 Dopaminergic Degeneration and Related Behaviors were Rescued by Silencing fat-5 and fat-7 Genes in a Rotenone-induced Model of C. elegans

There was significant rotenone treatment (F_{1,20} = 231.2, p<0.0003), RNAi treatment (F_{4,20} = 9.925, p<0.0001) and interaction (F_{4,20} = 14.07, p<0.0001) effects observed for dopaminergic degeneration at day 7 of adulthood (Fig. 3.5A and 3.5B). The L4440 (p<0.001), nhr-49 (p<0.02) and tub-1 (p<0.05) groups had significant neuronal degeneration after rotenone treatment compared to their vehicle control groups. However, there were no significant differences between rotenone and control groups for fat-5 (p>0.5) and fat-7 (p>0.5) genetic treatments, which explains the significant interaction effect.

Significant rotenone treatment (F_{1,20} = 29.64, p<0.0003), RNAi treatment (F_{4,20} = 6.064, p<0.001) and interaction (F_{4,20} = 3.295, p<0.04) effects were found for basal slowing behavior (Fig. 3.5C). Rotenone treatment significantly reduced basal slowing in all groups except in the fat-5 (p>0.5) and fat-7 (p>0.5) groups. Rotenone treatment did not have any negative effect on nhr-49 (p>0.5) and tub-1 (p>0.5) groups compared to their vehicle control groups,

For the ethanol avoidance index, significant rotenone (F_{1,20} = 8.736, p<0.0001), RNAi treatment (F_{4,20} = 5.509, p<0.008) and interaction (F_{4,20} = 20.08, p<0.004) effects were observed (Fig. 3.5D). Animals in the L4440 (p<0.0003), nhr-49 (p<0.02) and tub-1 (p<0.002) groups showed significant reductions in the ethanol avoidance index compared to their vehicle control groups. However, there were no significant differences between rotenone and vehicle control
groups for fat-5 (p > 0.5) and fat-7 (p > 0.5) genetic treatments, which explains the significant interaction effect. Even though the rotenone dosage did not have any negative effect on nhr-49 (p > 0.5) and tub-1 (p > 0.5) groups compared to their vehicle control groups, the total scores for both these groups were significantly lower than the rotenone treated fat-5 (p < 0.01) and fat-7 (p < 0.01) groups.

3.4.6 Lifespan or Healthspan Measures were Unaltered by Silencing of Fat genes in the Rotenone-induced Model of C. elegans

Significant rotenone treatment effects were found for the overall lifespan (Fig. 3.6A; F_{1,20} = 59.65, p < 0.0001) and fecundity (Fig. 3.6C; F_{1,40} = 144.8, p < 0.004), while not significant for the pharyngeal pump rate (Fig. 3.6B; p > 0.1) and number of body bends per s (Fig. 3.6D; p > 0.1). The RNAi treatment, and interaction between rotenone treatment and RNAi treatment effects were not significant for lifespan (p > 0.1), pharyngeal pump rate (p > 0.1), peak fecundity (p > 0.1), and number of body bends per second (p > 0.1). After rotenone treatment, the lifespan of all the RNAi groups were significantly reduced compared to the untreated controls (p < 0.05), without significant differences among the RNAi groups (p > 0.05). Similarly, rotenone administration reduced the total progeny produced for all the RNAi groups compared to the vehicle control groups (p < 0.0001), without significant differences among the RNAi groups (p > 0.05).

3.5 Discussion

Age being the greatest risk factor for neurodegenerative disorders like PD leads to pathological changes in brains such as loss of neurons, oxidative stress, and reduced protein degradation to name a few. Fat metabolism plays an important role in cell physiology and molecular signaling. PD is often characterized by dysfunction of cell homeostasis and energy
metabolism. Recent research also shows the role of insulin in neuronal growth and survival and how insulin signaling can impact PD. Though there has been extensive research on the molecular mechanisms leading to cell death in PD pathology, the role of fat metabolism in influencing such molecular hallmarks is not clear. In *C. elegans*, aging is represented by healthspan measures which resembles humans. This includes reduced pharyngeal pump rate, decline in motility and fertility and changes in neuromuscular junction. Moreover, environmental stress responses, such as heat shock and osmotic stress, have been used in nematodes to unravel genetic machineries of many diseases. The transgenic PD models of *C. elegans* have shown to have deficits in the form of reduced lifespan, healthspan and increased sensitivity to environmental stressors. These parameters were also used as parameters to characterize aging in our study. Through genetic manipulation, we for the first time demonstrate how silencing of *fat-5* and *fat-7* in worms can improve key features of PD: protein aggregation, neuronal degeneration and dopamine-dependent behaviors.

Like others, our experiments showed how expression of a toxic protein (α-synuclein) or using a neurotoxin (rotenone) to induce dopaminergic degeneration in *C. elegans* can modify dopaminergic functions or healthspan and make them stress sensitive. We also demonstrated that silencing of *fat-5* and *fat-7* genes resulted in lower fat content (Supplemental Fig. 3.2). Most strikingly, our study highlighted that such genetic manipulation of *fat-5* and *fat-7* genes was beneficial for the PD worm strains (wild-type or mutated α-synuclein or toxin-induced model). Silencing these genes reduced α-synuclein overexpression and protected against neuronal damage. In addition, it also improved dopamine-dependent behaviors, including basal slowing and ethanol avoidance in the nematodes. Previous studies have recognized multiple genes and pathways that are able to suppress aggregate formation, protect from dopamine-mediated toxicity and regulate
cell death in DA neurons\textsuperscript{28,38}. Interestingly, the association between fat metabolism and these degenerative molecular pathologies in worm models has not been previously explored. This exploration of a connection between genes that regulate fat metabolism and physiological assessments of PD symptoms in established \textit{C. elegans} models has yielded interesting results, and our studies now add fat content and specific fat genes to these protective mechanisms.

Research shows that stress-resistance mechanisms are controlled by fatty acid metabolism\textsuperscript{18}. Moreover, PD models can have different sensitivity towards different stressors\textsuperscript{8}. Our research showed that RNAi of \textit{fat-5} and \textit{fat-7} increased heat tolerance in protein overexpression (both wild-type and A53T \textit{α}-synuclein) models of \textit{C. elegans} probably due to reduced unsaturated fats which are known to impact thermotolerance\textsuperscript{18}. This was also shown to improve osmotic tolerance in A53T \textit{α}-synuclein model but not in the wild-type \textit{α}-synuclein strain. These differential effects might be attributed to varying changes in the mitochondrial proteome and function due to different protein expression (wild-type vs mutated)\textsuperscript{39}. Such altered mitochondrial function is often considered as a key factor for osmotic stress adaptation\textsuperscript{40}.

Silencing of the \textit{nhr-49} and \textit{tub-1} genes significantly reduced ethanol avoidance behavior only in the wild-type strain, TG2435. In mammals, PPAR\textit{α}, a \textit{nhr-49} homologue, is known to negatively modulate dopamine cell activity\textsuperscript{41}. Moreover, ethanol avoidance in worms is regulated by both dopamine and serotonin and such an environmental response is co-dependent on olfactory neurons\textsuperscript{42}. Our results also show that only silencing \textit{tub-1} (and not \textit{nhr-49}) increases fat levels in the adult worms. A previous study indicates that fat content due to silencing of \textit{nhr-49} may depend on the age of worms\textsuperscript{13}. Genetic manipulation of both these genes led to reduced fertility in protein overexpression worm models\textsuperscript{13}. However, silencing of either of these genes had no effect on \textit{α}-synuclein protein overexpression strains (both wild-type and A53T). One of the limitations of our
study is that we did not create mutants by deleting these candidate genes. We rather chose to genetically silence the genes and reduce gene expression levels rather than completely deactivating them. Therefore, for follow up studies it would be interesting to assess whether complete deletion of these fat metabolism genes have the same effects on the tested end points used in the study.

Finally, results from our research reveal that that silencing of fat-5 and fat-7 may influence neuronal health or protein expression greatly, while the effects on lifespan and general health can be marginal to none. Cooper et al. have primarily used lifespan as the key parameter for measuring anti-aging interventions\(^8\). However, there are studies who have clearly shown that lifespan and healthspan can be considered as different events in organisms and increased lifespan often is accompanied by frail health\(^43\). This could explain our differences in results where none of the fat genes had any impact on longevity of the PD models. Hence, based on our results we may conclude that modulating lifespan should not be the sole focus for anti-aging treatments, especially for therapeutic options of age-related disorders such as PD.

3.6 Conclusion

Overall, our results show that silencing of fat-5 and fat-7 genes, which results in reduced fat content, can improve a diseased pathology by reducing toxic protein expression and rescuing neuronal loss in the nematode models. However, it also poses further questions on the intricate interplay between fat metabolism pathways and neurodegenerative disorders.

3.7 Acknowledgements

The authors would like to thank Dr. Shohei Mitani and Dr. Jeremy Van Raamsdonk for the C. elegans strains.
3.8 Figures

Figure 3.1 Silencing of fat-5 and fat-7 Genes Reduced Human Wild-type α-synuclein Overexpression and Dopamine-related Behaviors in Models of C. elegans.

Graphical representation of (A) fluorescence intensity of the OW13 (n=20-30 animals per group), (C) basal slowing and (D) ethanol avoidance of JVR208 animals (n=10-15 animals per group) fed on different genetic RNAi treatments (L4440, fat-5, fat-7, nhr-49 and tub-1). The empty vector L4440 was considered the control. (B) Representative confocal images of the α-synuclein/YFP expression in the head region of day 7 OW13 adults. Magnification is 40X and scale bar is 50 μm. The data represent the mean ± SEM with significant differences between the control and treatments at *p<0.05, **p<0.001 and ***p<0.0001. Each experiment was repeated three times.
Figure 3.2 Genetic Silencing of *fat-5* and *fat-7* Genes Conferred Heat Resistance in a Human Wild-type α-synuclein Overexpression Model of *C. elegans*.

Graphical representation of (A) mean lifespan (n=44 animals per group), (B) peak fecundity, (C) pharyngeal pump rate, (D) body bends, (E) osmotic stress and (F) heat tolerance of JVR208 animals (n=10-15 animals per group) fed on different genetic RNAi treatments (*L4440, fat-5, fat-7, nhr-49* and *tub-1*). Empty vector L4440 was the control treatment. The data represent the mean ± SEM with significant differences between the control and treatments at \*p<0.05 and \**p<0.01. Each experiment was repeated three times.
Figure 3.3 Silencing of *fat-5* and *fat-7* Genes Rescued Dopaminergic Degeneration and Related Behaviors in a Model of *C. elegans* Expressing Human Mutated α-synuclein (A53T Mutation).

Graphical representation of (A) percentage of normal dopaminergic neurons (n=25-30 animals per group), (C) basal slowing and (D) ethanol avoidance behaviors (n=10-15 animals) in JVR203 animals fed on different genetic RNAi treatments (*L4440, fat-5, fat-7, nhr-49 and tub-1*). Empty vector L4440 was the control treatment. The data represent the mean ± SEM with significant differences between the control and treatments at *p<0.05, **p<0.01 and *** p<0.001. Each experiment was repeated three times. (B) Representative images of dopaminergic neurons with
outgrowths and blebs. Any neurons having either of these morphological features were considered as not normal. Magnification is 40X and scale bar is 100 μm.
Figure 3.4 Defects in Stress Resistance was Ameliorated by Silencing of \textit{fat-5} and \textit{fat-7} Genes in \textit{C. elegans} Expressing Human Mutated $\alpha$-synuclein (A53T Mutation).

Graphical representation of (A) mean lifespan (n=44 animals per group), (B) peak fecundity, (C) pharyngeal pump rate, (D) body bends, (E) heat stress and (F) osmotic stress of JVR208 animals (n=10-15 animals per group) fed on different genetic RNA interference treatments (\textit{L4440}, \textit{fat-5}, \textit{fat-7}, \textit{nhr-49} and \textit{tub-1}). Empty vector L4440 was the control treatment. The data represent the mean ± SEM with significant differences between the control and treatments at $^* p<0.05$, $^{**} p<0.01$ and $^{***} p<0.001$. Each experiment was repeated three times.
Figure 3.5 Silencing of \textit{fat-5} and \textit{fat-7} Genes Rescued Dopaminergic Degeneration and Associated Behaviors in a Rotenone Induced Model of \textit{C. elegans}.

Graphical representation of (A) percentage of normal dopaminergic neurons (n=25-30 animals per group), (C) basal slowing and (D) ethanol avoidance behaviors (n=10-15 animals) in TG2435 animals fed on different genetic RNA interference treatments (\textit{L4440}, \textit{fat-5}, \textit{fat-7}, \textit{nhr-49} and \textit{tub-1}) and rotenone (0 and 4\textmu M). Empty vector L4440 was the control treatment. The data represent the mean ± SEM with significant differences between the rotenone treated and untreated groups at *p<0.05, **p<0.01 and *** p<0.001. Each experiment was repeated three times. (B) Representative images of dopaminergic neurons with rounding neurons or axons with blebs or
missing dendrites. Any neurons having either of these morphological features were considered as not normal. Magnification is 40X and scale bar is 50 μm.
Figure 3.6 Lifespan or Healthspan Measures were Unaltered by Silencing of fat-5 and fat-7 genes in Rotenone-induced Model of *C. elegans*.

Graphical representation of (A) mean lifespan (n=44 animals per group), (B) pharyngeal pump rate, (C) peak fecundity and (D) body bends of TG2435 animals (n=8-10 animals per group) fed on different genetic RNA interference treatments (*L4440, fat-5, fat-7, nhr-49* and *tub-1*) and rotenone (0 and 4□M). Empty vector L4440 was the control treatment. The data represent the mean ± SEM with significant differences between the control and rotenone treatments at #p<0.05, **p<0.01 and ***p<0.001. Each experiment was repeated three times.
Supplemental Figure 3.1 Graphical Representation of the Touch Response as a Measure of Batch Control for the RNAi Plates.

The worms (n=10 per group) were grown on two different RNAi treatments: *L4440* and *mec-7*. Treatment with *mec-7* showed significantly diminished mechanosensory touch response compared to the L4440 control (**p<0.001).
Supplemental Figure 3.2 Lipid Staining to Validate the Effect of Silencing of Fat Metabolism Genes

(A) Confocal images and (B) graphical representation of the Nile red staining as a measure of total fat content in TG2435 Day 3 worms after gene silencing of fat metabolism genes. The worms (n=12-13 per group) were grown on different RNAi treatments: *L4440*, *fat-5*, *fat-7*, *nhr-49* and *tub-1* genes. Treatment with *fat-5* and *fat-7* genes showed significantly lower fat content compared to the *L4440* control (**p<0.01 and * p<0.05 respectively). Treatment with *tub-1* shows
significantly higher fat content than the *L*4440 control (**p<0.01). The experiment was repeated twice. Scale bar is 200 μm.
3.9 References

1 Beitz, J. M. Parkinson's disease: a review. *Front Biosci (Schol Ed)* 6, 65-74 (2014).


Chapter 4: Complex Interaction of Dietary Fat and Alaskan Bog Blueberry Supplementation Influences Manganese Mediated Neurotoxicity and Behavioral Impairments.

4.1 Abstract

Dietary fat is known to modulate neuronal health that contributes to age related nervous system disorders. However, the complex interaction between dietary fat and supplementation and its consequences on neurotoxic pathophysiology has been sparsely explored. The indigenous Alaskan bog blueberry (BB), *Vaccinum uliginosum*, is known to have anti-inflammatory properties, mostly attributed to its rich polyphenolic content. Here, we evaluate the interplay between dietary fat and BB supplementation in a sub-chronic manganese (Mn) exposure that inflicts neurotoxicity and behavioral impairments. When supplemented with BB, both low-fat and normal-fat diet ameliorated the behavioral and the molecular hallmarks of Mn-induced neurotoxicity. On the contrary, high-fat diet was found to exacerbate these Mn-induced pathological features. Furthermore, BB supplementation failed to recover the aggravation catalyzed by high fat diet in Mn-treated mice. Overall, our results demonstrate the importance of including dietary regimen comprising polyphenolic rich supplements with low-fat content in combating age related neurodegenerative disorders.

Authorship for this chapter is as follows: Malabika Maulik, Swarup Mitra, Mckenzie Sweeney, Brianna Lu, Barbara E Taylor, and Abel Bult-Ito. Submitted to Journal of Functional Foods.
4.2 Introduction

Manganese (Mn) is an essential trace metal which governs physiological processes that support healthy growth, development and brain function (1). Particularly, Mn is essential for maintaining redox status, energy balance and metabolism of key biomolecules, including proteins, lipids and carbohydrates (1). Additionally, Mn functions as a cofactor for crucial enzymes such as pyruvate carboxylase, arginase and Mn-superoxide dismutase, which are required for synthesis of neurotransmitters as well as functioning of neuronal and glial cells in the brain (2). However, excessive and chronic exposure to Mn results in pathological alterations in the brain, particularly in the striatal pallidum and substantia nigra (SN) accompanied by loss of dopaminergic neurons (3). Mn induced toxicity and degeneration includes motor dysfunction similar to those observed in Parkinson’s disease (PD) (4). The symptoms include gait imbalance, rigidity, tremors, dystonia, and bradykinesia (5). Studies have elucidated the role of neuroinflammatory factors, such as, nitric oxide synthase 2 (iNOS), that aggravate neuronal injury due to Mn exposure via microglial activation (6). Additionally, in vitro studies have demonstrated that such Mn-induced inflammatory microglial activation can result from and interaction with signaling pathways involving nuclear factor kappa B (NF-κB) (6).

Since environmental factors play a critical role in age-related neurodegenerative disorders, it is imperative to examine the concerted action diet and supplementation play in the disease etiology and progression. Previous research indicates that long term consumption of high fat in the diet has been linked to increased inflammation and oxidative stress in the brain (7). A high fat diet (HFD) leads to increased microglial activation (8), which in turn can promote pro-inflammatory responses in the brain resulting in impaired neurogenesis and cognitive function, similar to what is seen in aging (9). Studies also associate a HFD and reduced neurogenesis because of decreased
levels of brain-derived neurotrophic factors (BDNF) (7). A low fat diet (LFD) on the contrary is known to be protective against cognitive decline in older subjects (10) and other age-related chronic diseases (11). LFDs reduce inflammatory microglial activation in the ageing mouse brain (12), upregulate BDNF and enhance neurogenesis (13).

Blueberries (BB) are rich in antioxidant/anti-inflammatory polyphenolic compounds like anthocyanins and proanthocyanidins (14). Diets supplemented with such phytochemicals, impact the aging brain (15), modulate stress signaling pathways and reduce microglial activation, a key marker of inflammatory response (7). Moreover, diets rich in such natural compounds can attenuate age-related behavioral deficits (16) and enhance neurogenesis by elevating neurotrophic growth factors (17). Indigenous Alaskan botanicals like bog blueberry (BB), Vaccinium uliginosum, contain higher amounts of polyphenolic compounds relative to Vaccinium sp. grown in temperate regions (18, 20). These endemic species of berries primarily have been beneficial in combating age-related cellular symptoms in C. elegans and in in vitro models (19, 20).

The current study aimed at determining and comparing the behavioral effects and underlying molecular mechanisms modulated by consumption of different diets in the presence/absence of BB supplementation and when introduced to a neurodegenerative toxic insult. We investigated the hypothesis that a LFD in the presence of BB will be most neuroprotective among all the diet groups in improving behavioral and molecular markers in this metal-induced neurotoxic mouse model. Further, HFD will cause decline in behavioral measures and result in increased neurotoxic responses in the animals compared to the ones fed with a LFD and a NFD. Based on a previous study (7), it was also postulated that BB supplementation will alleviate the deficits induced by a HFD in this metal-induced neurotoxic mouse model.
4.3 Materials and Methods

This project was conducted as per the University of Alaska Fairbanks Institutional Animal Care and Use Committee approved animal care and experimental procedures (IACUC assurance number 1024315).

4.3.1 Experimental Animals

Three month old C57BL6/J male mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and were housed in groups of 4 in a 12:12 L:D cycle. Mice were given two weeks to acclimatize before introducing them to the experimental regimen.

4.3.2 Experimental Design

Following the two week acclimatization period, mice were placed on one of the six diets and fed ad libitum for a period of four months (n=24-26 animals per diet). The diets comprised of NFD, LFD, HFD, NFD + 5% BB, LFD + 5% BB and HFD + 5% BB. Alaskan bog blueberries were collected from interior Alaska and freeze dried. Freeze dried berries were crushed into a powder and shipped to LabDiet (St Louis, MO) where they were added to food pellets at 5% of the total pellet weight. The diet compositions are included in Table 4.2.

After four months on the diet, the mice were subjected to vehicle and subcutaneous manganese injections (n=12-13 animals per diet and injection group) on every other day for a total of 3 injections. We used a sub chronic dosage of manganese chloride (MnCl₂) that is known to inflict a neurotoxic state that often precedes neurodegeneration (6, 21, 22). Twenty-four hours after the last injection, mice were subjected to a series of behavioral tests comprised of the open field test on day 1 to measure anxiety-like behavior and locomotor activity, the novel object recognition test on day 3 to measure short term memory, and the cylinder test on day 5 and parallel rod floor
test on day 7 to measure locomotor agility (Fig 4.1). All data were collected by experimenters blinded to the study design and hypothesized outcomes.

4.3.3 Drugs
MnCl₂ was purchased from Sigma-Aldrich (Catalogue no. 203734) and dissolved in 0.9% sterile physiological saline (pH=7.4). Sterile saline was used as vehicle for control groups. Animals in the manganese treatment group received subcutaneous injections of 50 mg/kg MnCl₂ for three days (day 1, day 3 and day 5). The vehicle group received saline injections on the same days. Injection volume of 0.3 mL per 40 g mouse was adjusted proportionally according to the body weight of the animals.

4.3.4 Behavioral Tests
4.3.4.1 Open Field

Animals were assessed for anxiety-like behavior and locomotor activity in the open field test (23). Animals undergoing testing were transported in home cages and were housed outside the testing room prior to testing. The open field apparatus consisted of an arena (40 cm x 40 cm x 30 cm) with opaque plexiglass walls. Testing was conducted for a 3-min duration (24-26). Animals were individually placed in the center of the field and allowed to explore the arena. The total number of central entries into the central zone was evaluated as an anxiety-like measure (24). Total distance traveled was used to assess locomotor activity (27). All experimental parameters were recorded by the ANYMaze video tracking system (Stoelting Co., Wood Dale, IL, USA). The apparatus was cleaned with a dilute chlorhexidine solution and dried before each test.
4.3.4.2 Novel Object Recognition

The novel object recognition test was performed to evaluate object recognition memory (28). Mice were trained in the open field arena (40 × 40 × 30 cm). The mice were given an opportunity to explore the open field arena without objects present for 3 min. Twenty-four hours later, the training comprised of allowing the mice to explore two identical objects (plastic toys) within a 5 cm distance for 3 min with the ANYMaze video tracking program (Stoelting Co.). Mice were then removed from the apparatus and returned to their home cages for 4 hours. After four hours, one of the objects was replaced with a novel object of different shape and size. In the testing phase mice were reintroduced into the apparatus for 3 min to investigate the familiar and the novel object. Time spent exploring the familiar and novel objects were recorded. The preference of one object over another was assessed through the recognition index (RI), which is determined by the time spent on the novel object relative to the time spent on both novel and familiar objects: [RI = TN/(TN + TF)] where TN is time spent on the novel object and TF is time spent on the familiar object (29). Objects and exploration arena were cleaned with a dilute chlorhexidine solution after each animal.

4.3.5.3 Cylinder Test

Forelimb locomotor asymmetry was measured with the cylinder test. Mice were placed in a glass cylinder (8 cm in diameter and 12 cm in height) for 3 minute duration. The number of wall contacts with both the forelimbs together was counted and a lower number indicated a larger level of forelimb locomotor asymmetry (30).
4.3.6.4 Parallel Rod Floor

The parallel rod floor apparatus was used to measure motor coordination in the mice (31). Mice were placed in a 20 x 20 cm acrylic box with a floor made of steel rods spaced 8mm apart from each other. The floor of the box was raised at a height of 1 cm above a base steel plate. The total testing time was for 15 min during which motor coordination deficits were assessed by the total number of paw slip errors normalized to the distance travelled. The parameters were calculated through the AnyMaze behavioral acquisition software. The apparatus was cleaned with 70% ethanol after each animal.

4.3.5 Brain Tissue Collection and Molecular Analysis

4.3.5.1 Perfusion and Brain Sectioning

Twenty-four hours after the last behavioral assessment mice (n=4-5 from each of 12-13 per treatment groups) were subjected to whole body transcardial perfusion with 4% paraformaldehyde in phosphate buffered saline (PBS), following a rinse with PBS to remove the blood (32, 33). Brains collected after perfusion were fixed overnight in 4% paraformaldehyde. Fixed brains were then transferred to 30% sucrose solution in PBS and stored at 4 °C until they submerged completely. Brains were then placed in Tissue-Tek OCT compound in beaker cups (VWR International) and frozen in chilled hexane. Coronal sections of 20 μm between -4.84 Bregma and -5.02 Bregma were obtained from the frozen brains on a freezing microtome (Leica CM1900) as per the mouse brain atlas. Brain slices on gelatin coated slides (VWR Cat. no. 48311-703) were then stored at -20 °C until further use.
4.3.5.2 Nissl Staining

Slides containing brain sections were kept on a slide drier for 20 minutes. The slides were dipped in 100% alcohol (twice for 5 min) followed by 70% alcohol (twice for 5 min). Samples were stained with a 0.1% warm Cresyl Violet solution (37 °C) for 3-10 mins. The slides were washed in distilled water and subjected to dehydration in 95% and 100% alcohol (twice for 5 min each). Finally, the slides were cleared with xylene and mounted with vectamount. The slides were visualized and the total number of stained cells in the substantia nigra pars compacta were counted using the ImageJ software (NIH) cell counter.

4.3.5.3 Immunohistochemistry

Slides containing brain sections were first washed in PBS (five 5-min washes). The slides were then incubated in 30% hydrogen peroxide in PBS for 30 min at room temperature followed by PBS washes (six 5-min washes). Blocking buffer (PBS containing 5% normal goat serum, 2% BSA, and 0.4% Triton X-100) was then added to the slides for 1 h. After 1 h, slides were washed in PBS (three 5-min washes) and incubated with Tyrosine Hydroxylase (TH) primary antibody (Millipore Catalogue AB152; 1:500 in PBS and 0.4% Triton X-100) overnight for 18 hours. On day two, slides were washed in PBS (four 5-min washes) and were incubated with secondary biotinylated goat anti-rabbit antibody (1:600, Vector laboratories Cat. no. BA1000; in PBS and 0.04% Triton X-100) for 1 h. Sections were finally processed using the Vectastain Elite ABC immunoperoxidase system (Vector Laboratories) as per the manufacturer instructions and visualized with Ni²⁺-DAB enzyme substrate. For analysis of TH reactive cells, ImageJ software (NIH) was used. All brain sections were analyzed by measuring the optical density of the neurons positively for TH in the substantia nigra pars compacta by subtracting the background staining.
4.3.5.4 Brain Tissue Harvesting

After 24 hours of the last behavioral test, mice from each treatment group were sacrificed through cervical dislocation and brains were extracted and snap frozen in liquid nitrogen at 80 °C until further processing. Hippocampal and substantia nigra regions were obtained using a micropuncher and homogenized in chilled RIPA buffer (with added Proteinase/Phosphatase inhibitors cocktail, Roche). Homogenates were then centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was collected in fresh Eppendorf® tubes and stored at -80 °C until further analysis. Total protein was measured by Pierce BCA kit.

4.3.5.5 ELISA

Total BDNF protein was measured in hippocampal homogenates using a commercially available ELISA kit following the manufacturer's protocol (BDNF Emax ImmunoAssay System, Promega Southampton, UK, Cat No. G7611), NOS2 (iNOS) and NF-κB levels were measured in the homogenates from substantia nigra region using kits from Cloud-Clone Corporation (Catalog No. ABIN415396 and ABIN425131, respectively). Finally, Iba-1 levels were measured in the substantia nigra region using a kit from Lifespan BioSciences Inc. (Catalog No. LS-F16598). All samples were assayed in duplicate (n= 5 per group). Data collection was performed at 450 nm using a Biotek EL808 spectrophotometric plate reader.

4.3.6 Statistical Analysis

All statistical analyses were performed in the Statistical Analysis Software (SAS version 9.4, Cary, NC). Behavioral assessments were tested in a general linear model (GLM) analysis of variance (ANOVA) for the main effects of diet, BB, Mn treatment and interaction effects (diet x
BB, diet x Mn treatment, BB x Mn treatment and diet x blueberry x Mn treatment). Wherever significance was found appropriate post hoc pair-wise comparisons were conducted using the studentized range test.

4.4 Results

4.4.1 Alaskan BB Improved OF Locomotor Activity in Mn Treated Animals when Supplemented with LFD

There were significant main effects of diet, BB and Mn treatment on the total distance travelled by the mice in the open field (Table 4.1 and Fig. 4.2A). The interaction effect between diet and BB on the assessed behavior was also significant (Table 4.1 and Fig. 4.2A). In the vehicle groups, mice consuming the HFD travelled shorter distances compared to ones in the NFD ($t_{23}=3.174$, $p<0.03$) and the LFD ($t_{23}=5.664$, $p<0.0001$) groups. In the vehicle groups, animals consuming the LFD with BB travelled greater distances compared to ones fed the NFD with BB ($t_{24}=6.756$, $p<0.0001$) and the HFD with BB ($t_{22}=2.194$, $p<0.0002$). All animals in on a NFD ($t_{23}=3.88$, $p<0.002$), LFD ($t_{23}=3.542$, $p<0.004$) and LFD with BB ($t_{23}=3.432$, $p<0.005$) groups displayed reduced locomotor activity after administration of Mn injections when compared to the vehicle groups. Mice in the NFD with BB group ($t_{23}=0.2482$, $p>0.1$), the HFD with ($t_{23}=1.468$, $p>0.1$) and without BB ($t_{22}=1.387$, $p>0.1$) groups when exposed to Mn were not significantly different from the vehicle control groups and showed comparatively reduced locomotor deficits. Also, when exposed to Mn, the LFD group without BB was significantly different from the HFD group without BB ($t_{22}=3.468$, $p<0.02$). In presence of BB, animals treated with Mn in the LFD group travelled greater distances compared to the NFD ($t_{22}=3.369$, $p<0.02$) and HFD ($t_{22}=3.651$, $p<0.006$) groups.
The significant diet and BB interaction effect was due to the mice on the LFD and HFD, both in the vehicle and MN treatment groups, tending to travel longer distances with BB treatment compared to without BB, while no BB effect was observed in the NFD groups (Fig. 4.2A).

4.4.2 Alaskan BB with LFD Reduced Anxiogenic OF Behavior Inflicted by Mn Insult.

Significant main effects of the diet, BB and Mn treatment on the anxiety-like OF behavior, i.e., number of central entries, were found (Table 4.1 and Fig. 4.2B). The interaction effects between diet and Mn and diet and BB on the number of central entries were also significant (Table 4.1 and Fig. 4.2B). HFD animals were most anxiogenic in comparison to the NFD vehicle group ($t_{23} = 6.342, p<0.0001$). Vehicle animals consuming BB with a NFD ($t_{24} = 3.286, p < 0.02$) and a LFD ($t_{24} = 4.168, p < 0.0009$) were less anxiogenic compared to their controls without BB. Mice fed with a NFD ($t_{23} = 6.15, p<0.0001$), a NFD with BB ($t_{23} = 6.646, p<0.0001$) and a LFD ($t_{23} = 3.046, p<0.02$) scored lower numbers of central entries and hence were more anxious-like after administration of Mn injections when compared to their vehicle groups. Animals on a LFD with BB ($t_{23} = 0.2482, p > 0.1$) when exposed to Mn were not significantly different from the control group and displayed similar anxiolytic behaviors as the latter. Moreover, in the presence of Mn mice on a LFD with BB performed better than the mice on a LFD without BB ($t_{22} = 4.756, p<0.0001$). The mice on a HFD with or without BB had the lowest number of central entries compared to the mice on the NFD or LFD. In addition, Mn did not affect this anxiety-like behavior any further.

The significant diet and Mn interaction effect was due to the mice on the NFD with and without BB and on the LFD significantly decreasing the number of central entries after Mn treatment, while the mice on the LFD with BB and on the HFD with and without BB did not show a Mn effect. The significant diet and BB interaction effect was due to the mice on the NFD and LFD
with BB performing better in the OF than the mice on the NFD and LFD without BB, while BB treatment had no effect on the mice on a HFD (Fig. 4.2B).

4.4.3 LFD in Combination with Alaskan BB Improved Novel Object Recognition Memory.

Significant main effects of the diet, BB and Mn on the recognition index in OR behavior was found (Table 4.1 and Fig. 4.2C). There was also a significant interaction effect between BB and Mn treatment (Table 4.1 and Fig. 4.2C) on the memory function test. HFD mice had the lowest recognition index in comparison with NFD ($t_{23}= 3.971, p<0.002$) and LFD ($t_{23}= 3.5, p<0.001$) groups. Animals consuming BB with a NFD ($t_{24} = 4.272, p<0.0006$) and a LFD ($t_{24} = 3.427, p < 0.02$) showed enhanced memory compared to their controls without BB. Mice on a NFD ($t_{23}=3.244, p<0.009$), a NFD with BB ($t_{23}=7.146, p<0.0001$) and a LFD ($t_{23}=4.208, p<0.0004$) had a significantly lower recognition index and had cognitive impairment after administration of Mn injections when compared to their vehicle groups. Mice on a LFD with BB ($t_{23} = 1.061, p>0.1$) when exposed to Mn were not significant different and displayed similar recognition index as the vehicle control mice. Also, when exposed to Mn, animals on the LFD with BB group overcame the memory deficits and performed better than both mice on a NFD with BB ($t_{22}=5.36, p<0.0001$) and a LFD with BB ($t_{22}=6.451, p<0.0001$).

The significant BB and Mn interaction effect was due to the mice on the LFD with BB and the HFD with and without BB showing no significant effect of Mn treatment, while the mice on the NFD with and without BB and on the NFD significantly decreasing the recognition index after Mn treatment compared to their vehicle controls (Fig. 4.2C).
4.4.4 Alaskan BB Ameliorated Mn-induced Sensory Motor Deficits in the Cylinder Test

Significant main effects of the diet, BB and Mn treatment on the sensory motor function in the cylinder test, i.e., number of rears, were observed (Table 4.1 and Fig. 4.3A). A significant BB and Mn interaction effect was also found (Table 4.1 and Fig. 4.3A) for the number of rears. Mice on a HFD had a significant higher sensory motor deficit score, i.e., ataxia index, compared to mice on a NFD ($t_{23} = 4.088$, $p<0.002$), with the mice on a LFD having an intermediate rearing score. Mn induced sensory defects in mice on a NFD ($t_{23} = 6.468$, $p <0.0001$), a LFD ($t_{23} = 4.834$, $p <0.0001$) and a HFD ($t_{22} = 3.77$, $p <0.001$) compared to their vehicle controls. These Mn-induced deficits were rescued by BB supplementation for mice on a NFD ($t_{22}= 4.219$, $p<0.0008$) and a LFD ($t_{22}= 3.95$, $p<0.002$) compared to the mice not receiving BB. The Mn-induced deficit of mice on a HFD was also ameliorated by BB supplementation as the sensory defects of mice on a HFD with BB were not significantly different between the Mn and vehicle groups ($t_{22}= 2.355$, $p>0.1$).

The significant BB and Mn interaction effect was due to Mn treatment significantly reducing the number of rears of the mice on the NFD, LFD, and HFD without BB, while Mn had no effect on these mice on the diets with BB (Fig. 4.3A).

4.4.5 Alaskan BB Mitigated Mn-induced Motor Co-ordination Defects in the Parallel Rod Floor Test.

Significant main effects of the diet, BB and Mn treatment on the motor co-ordination function, i.e., the ataxia index, in the parallel rod floor test (Table 4.1 and Fig. 4.3B). The interaction effects between the diet and Mn treatment and between BB and Mn treatment (Table 4.1 and Fig. 4.3B) for the ataxia index were also significant. Overall, vehicle treated mice consuming a HFD scored higher ataxia index scores compared to mice on a NFD ($t_{23}=3.184$, $p<0.004$) and on a LFD ($t_{23}=1.384$, $p<0.04$). After administration of Mn, all three diets groups: NFD ($t_{23}=4.417$, $p<0.005$),
LFD (t_{23}=3.528, p<0.004) and HFD (t_{23}=4.991, p<0.0001) showed impaired motor co-ordination with higher ataxia index scores compared to their vehicle treated groups. BB improved motor coordination because no significant differences were found between vehicle and Mn treated mice for all the BB consuming diet groups (NFD: t_{23}= 0.8201, p>0.1; LFD: t_{23}= 0.5526, p>0.1, HFD t_{23}= 0.5526, p>0.1). Consumption of BB ameliorated motor dysfunction in the Mn-treated NFD groups (t_{22}= 3.737, p<0.005). Similarly, mice on a LFD with BB displayed improved motor functions in both vehicle (t_{24}=3.073, p<0.04) and Mn (t_{22}=6.94, p<0.0001) treated groups compared to mice on a LFD.

The significant Diet and Mn interaction effect was due to the mice on the NFD and LFD with BB significantly reducing their ataxia index after Mn treatment compared to their diet without BB groups after Mn treatment, while the mice on the HFD with BB after Mn treatment did not significantly differ from their HFD without BB group after Mn treatment (Fig. 4.3B). The significant BB and Mn interaction effect was due to all the diet groups without BB significantly increasing their ataxia index scores after Mn treatment compared to their vehicle groups, while all the diet groups with BB after Mn treatment were not significantly different from their vehicle groups (Fig. 3B).

4.4.6 A LFD Diet in Presence of Alaskan BB Elevated BDNF Levels in Mn Exposed Animals

Significant main diet, BB and Mn treatment effects were found for the total BDNF levels in the hippocampal region (Table 4.1 and Fig. 4.4A). No significant interaction effects were found. Mice on a NFD with BB had elevated hippocampal BDNF levels compared to mice on a NFD without BB (t_{8}= 3.314, p<0.03) and a similar trend was observed in the LFD vehicle groups. Mn administration reduced the BDNF levels in the LFD without BB group (t_{8}= 3.36, p<0.001) and showed a similar trend in the NFD without BB group (t_{8}= 2.447, p<0.1). There was significant
difference between control and Mn treated NFD with BB group (t₈ = 4.319, p<0.0006) group. However, after Mn treatment the LFD group with BB showed significantly higher BDNF levels than the LFD group without BB (t₈ = 4.18, p<0.002). There were no significant differences between control and Mn treated LFD with BB groups (t₈ = 1.066, p>0.1). Animals on a HFD had very low levels of BDNF compared to mice on a NFD (t₈ = 1.81, p<0.008) and a LFD (t₈ = 5.542, p<0.0001). But there was no significant difference observed between either BB consuming HFD groups (t₈ = 0.035, p>0.5) or between Mn treated HFD without BB and Mn treated HFD with BB group (t₈ = 2.569, p>0.1).

4.4.7 Attenuation of Inflammatory Markers by Alaskan BB in Mn Treated Animals

4.4.7.1 Iba1

Significant main BB and Mn treatment effects were found for the total iba1 levels in the SN region (Table 4.1 and Figure 4.4B). There was also an interaction effect between BB and diet and between BB and Mn treatment on this inflammatory marker (Table 4.1 and Fig. 4.4B). BB decreased the iba1 levels in mice on a HFD (t₈ = 4.561, p<0.0006) only in the vehicle groups. After Mn treatment, there was a significant increase in iba1 levels for mice fed NFD (t₈ = 3.624, p<0.005), LFD (t₈ = 2.814, p<0.05) and HFD with BB (t₈ = 4.357, p<0.0005). The level of iba1 in the NFD with BB group (t₈ = 3.755, p<0.008) after Mn treatment was significantly lower compared to the NFD without BB group. A similar trend was shown in mice on a LFD with BB (t₈ = 2.959, p<0.08) after Mn treatment compared to the non BB treated LFD group.

4.4.7.2 NOS2/iNOS

A significant main BB effect was found for the total NOS2/iNOS levels in the substantia nigra region (Table 4.1 and Fig. 4.4C). No significant interaction effects were found. After Mn
treatment, mice on a NFD \((t_8 = 3.681, p<0.009)\) and LFD \((t_8 = 4.106, p<0.003)\) with BB had lower levels of NOS2/iNOS levels compared to their respect diet group without BB. Mn administration significant decreased NOS2/iNOS levels in the mice on a LFD with BB compared to the LFD with BB vehicle group \((t_8 = 2.948, p<0.03)\).

4.4.7.3 NF-κB

Significant main BB and Mn treatment effects were found for the total NF-κB levels in the SN region (Table 4.1 and Fig. 4.4D). No significant interaction effects were found. The level of NF-κB in the SN region of mice on a HFD without BB was significantly higher compared to the mice on the HFD with BB \((t_8 = 3.613, p<0.02)\) only for the vehicle group. In the NFD and LFD vehicle groups, BB did not change NF-κB levels compared to their respective without BB groups. Mn treatment tended to increase NF-κB levels in the NFD and HFD without BB, while these differences were significant for the other comparisons compared to their respective vehicle controls; Mn treatment significantly increased NF-κB levels in mice on a NFD with BB \((t_8 = 3.104, p<0.02)\), LFD without BB \((t_8 = 2.793, p<0.05)\), and HFD with BB \((t_8 = 3.416, p<0.008)\), while it decreased NF-κB levels in mice on a LFD with BB \((t_8 = 2.956, p<0.008)\). BB supplementation significantly reduced NF-κB levels in mice on a LFD compared to the LFD without BB group after Mn treatment \((t_8 = 3.844, p<0.006)\).

4.4.8 A HFD Resulted in Neuronal Loss in the Substantia Nigra

4.4.8.1 Nissl Staining

A significant main diet effect (Table 4.1 and Fig. 4.5A and 4.5C) was found on the total number of neurons estimated through Nissl staining in the substantia nigra pars compacta (SNPC) region of the mid brain. No significant interaction effects were found. The HFD vehicle group had
significantly smaller numbers of stained neurons compared to the NFD (t₈ = 3.401, p<0.03) vehicle group. After Mn administration, the HFD group showed a significant reduction in number of cells compared to the NFD (t₈ = 3.791, p<0.007) and the LFD (t₈ = 4.045, p<0.003) groups. In presence of vehicle, HFD with BB group was also significantly different from LFD with BB group (t₈ = 3.733, p<0.008).

4.4.8.2 TH Staining

A significant main diet effect (Table 4.1 and Fig. 4.5B and 4.5D) was found for the total number of dopaminergic neurons assessed through tyrosine hydroxylase staining in the substantia nigra pars compacta (SNPc) region. No significant interaction effects were found. Overall, the HFD groups tended to have smaller numbers of TH positive neurons in the SNPc compared to the NFD and LFD groups. The HFD vehicle group had a significant smaller number of TH neurons compared to NFD vehicle group (t₈ = 3.106, p<0.05). Moreover, HFD with BB vehicle group had lesser number of neurons compared to NFD vehicle groups (t₈ = 3.376, p<0.03).

4.5 Discussion

Results from our study reveals that endemic Alaskan BB consumption significantly improved anxiety-like (open field), motor coordination (parallel rod floor test), sensory (cylinder test) and cognitive (novel object recognition) behaviors for mice on a LFD and NFD diet when introduced to neurotoxic challenge with Mn. Among all the diet groups, mice fed on HFD performed the least in all behavioral tasks. These animals were more anxiogenic and had impaired memory, sensory and motor co-ordination possibly due to metabolic burden as examined by others (34, 35). Mn treatment triggered reduced locomotor, memory and motor functions in tested behaviors for all the three diet groups and this effect was most pronounced in mice fed on HFD. Interestingly, Mn
treated HFD animals that consumed BB performed better in parallel rod floor (motor coordination) and cylinder test (sensory motor) when compared to HFD animals that were not fed with BB but received Mn insult. This suggests that when combined with toxic insult HFD can dampen the beneficial effect of BB. We also observed that BB was not able to reverse the memory impairment in the HFD animals as shown by Carey and colleagues (7) who used a temperate species of BB: *Vaccinium ashei* for their experiments. As mentioned earlier, the polyphenolic profile of Alaskan BB largely varies from the non-arctic species (18). This difference in result could have resulted from using a different *Vaccinium* sp. growing under the influence of varying environmental conditions and thereby having alternative anthocyanin content.

BDNF is abundant in brain regions that modulate neuroplasticity underlying learning and memory (36). The hippocampus serves as an important hub for controlling memory acquisition, storage and retrieval (37). Prior studies have indicated that the levels of BDNF in the hippocampus directly correlate to performance in memory tasks for rodents (7, 35). There is also evidence that obesity triggered by diet can lead to memory impairments in adult rodents (38). Furthermore, polyphenols are shown to enhance cognition by regulating extracellular signal-regulated kinase pathways (39). In our study, BB improved the BDNF levels either in combination with NFD or LFD. However, this effect was dependent on the diet of the animals and the type of treatments they received (Mn or Vehicle). These results show a novel interaction where a certain diet (NFD) in combination with a supplement (BB) can be beneficial in the normal physiological state. The same supplement (BB) when combined with a different diet formulation (LFD) can improve molecular markers in a neurotoxic condition. Consumption of a HFD has been linked with reduced BDNF levels in the cortex and hippocampus of rodent brains (17, 40). Contrary to prior investigations which have revealed elevated BDNF levels in presence of BB and a HFD (7), we found increased
BDNF in the hippocampus of animals fed with BB and LFD. This is not surprising since the mechanistic action of BB is linked to the complex interactions between BB anthocyanins with molecular pathway intermediates (7). Because our study utilizes an endemic BB species with a very different anthocyanin profile that is largely influenced by environmental conditions, the current results corroborates the hypothesis of differential regulation of BDNF. Though, our experiments do not investigate any specific BDNF pathway, such complex associations between BB polyphenols, BDNF expression coupled with selection of diet type is subjected to further scrutiny.

Microglial cells are key players of neuroinflammation and their activation is often associated with aging and neuronal dysfunction (41). Such changes further cause nitrosative stress causing NOS2 production and elevated levels of the stress-sensitive protein, NF-κB (6). A HFD is notable for aggravated expression of microglia and associated factors (42). Previous research elucidates the role of blueberries in ameliorating microglial activation and levels of NF-κB in aged mice and rats (43). Studies also elaborate on the mechanistic actions of BB in attenuating microglial mediated release of nitric oxide (7, 44). Our study showed spikes in microglial and nitrosative stress markers (NOS2/iNOS and NF-κB) in the HFD group compared to other diets (7). Interestingly, our finding highlights the novel interactions of Alaskan BB with different diets in combating neurotoxicity. In the case of Mn treatment, BB in combination with NFD conferred protection by reducing only levels of NOS2/iNOS and not NF-κB. On the other hand, BB in presence of LFD was beneficial in reducing both NOS2/iNOS and NF-κB and thereby improving the neurotoxic pathology. This preventative action of BB on Mn-toxicity was not reflected in the HFD group. Expression of NOS2 in response to Mn necessitates the up regulation of NF-κB (6). Research also shows mechanisms of NO expression occurring independent of NF-κB activation.
However, it is unknown whether such independent actions occur during Mn-induced neurotoxicity.

Dysregulation of the neuronal function due to a disease pathology can result in synaptic loss, impaired signaling and progressive change of neuronal structures (46). These transitions often occur before the actual neuronal cell loss or cell death in chronic neurodegenerative diseases (46). Mn vulnerability is often linked with changes in cytoskeletal structures which cause damage to the neuronal circuits in the long axons of the dopaminergic neurons of the SN (4). The neuropathological alterations observed with such exposure also indicate that Mn toxicity is not just restricted to dopaminergic neurons in the SN, but also can occur in the globus pallidus and striatum (4, 47). Few studies with rare human post mortem tissue samples also show that modified morphology resulting from excessive environmental exposure of Mn treatment can vary from no obvious changes to massive atrophy of neurons and glial structures in brain regions (47). This could possibly explain our results which demonstrated no differences in total cell number / TH positive neurons between the manganese and vehicle treated NFD and LFD groups in the SN region. Furthermore, only HFD group showed significant loss of neurons in comparisons with the other diet groups. Studies have suggested that HFD can possibly lead to dysfunction of dopamine and opioid neurotransmitter systems and related variations in gene expression level (48). The animals for this study were euthanized for brain sample collection within a 7-10 day of the sub-chronic Mn administration. Hence, the experimental period post exposure may be enough to present remarkable modifications in behaviors, biochemical markers and possibly neurite length structure rather than complete neuronal loss. Though there has been research to characterize the dosage and neurotoxicity of Mn (49, 50), little work has been done to study the interaction of diet and environmental toxins. Further research should target investigating the neuronal structure
changes occurring due to different diets. Future investigations should focus on whether such cytoskeletal changes are limited to neurite structure or causing overall circuit level disruption. Our study can form the basis for such future exploration.

In summary, our study illustrates a complex interaction between dietary fat and BB in ameliorating metal induced neurotoxicity and concomitant behavioral correlates. The data reiterates the deleterious effects of consuming HFD bolstering the evidence that associate metabolic overload and neurotoxic pathophysiology. Importantly, it reflects that consuming HFD and or obesity can mask the positive health promoting effects of BB supplementation, showing that a change of diet and loss of weight is imperative for health and supplements alone cannot mitigate the effects of HFD and obesity.

4.6 Acknowledgments

We thank the Biological Research and Diagnostics (BIRD) Facility animal quarters staff for excellent routine animal care and Timothy Howe from Alaska Stable Isotope Facility for Blueberry lyophilization. The authors would also like to thank Abigail Moran, Tandi Marth and Sequoia Tyadalma for their support with the study.
3 month old male C57BL/6 mice were fed on Normal fat diet (NFD), Low fat diet (LFD) and High fat diet (HFD) for 4 months in presence or absence of Alaskan blueberry (BB). All experimental animals were then administered single subcutaneous injections of 0 mg/kg or 50 mg/kg MnCl₂ (in saline) on 1, 3 and 5th day. Following which the animals were tested for open field (OF), object recognition (OR), cylinder test (CT) and parallel rod floor (PR) test. After the last behavioral assays, the animals were euthanized, and brain samples were collected for the biochemical experiments.
Figure 4.2 Alaskan Blueberry Supplementation Enhanced Locomotor, Anxiety and Memory Functions in Mice on a LFD

(A) Locomotor activity in open field test was measured as the total distance travelled in the open field test, (B) anxiogenic behavior was assessed as the number of central entries in the open field test and (C) memory function was measured as the recognition index in the object recognition test.

* p<0.05, ** p<0.01 and *** p<0.001 represents significant differences between vehicle and Mn treated or BB and non BB treated groups. 1, 2, 3 and 4 represents significant difference between HFD with other diet groups in vehicle condition, 6, 7 and 8 represents significant difference between HFD with other diet groups in MnCl₂ condition (where 1 = NFD, 2/6 = NFD + BB, 3/7 =
LFD and 4/8 = LFD + BB) (n=12-13 animals per group, p<0.05, Bonferroni multiple comparison test).
Figure 4.3 Alaskan Blueberry Supplementation Ameliorates Mn-induced Sensory and Motor Co-ordination Deficits in Mice Consuming NFD and LFD

(A) Sensory function was assessed by measuring 'number of rears' in the cylinder test and (B) motor co-ordination was measured in form of 'ataxia index' in parallel rod floor test. * p<0.05, ** p<0.01 and *** p<0.001 represents significant differences between vehicle and Mn treated or BB and non BB treated groups. 1, 2, 3 and 4 represents significant difference between HFD with other
diet groups in vehicle condition, 6 and 8 represents significant difference between HFD with other diet groups in MnCl₂ condition (where 1 = NFD, 2/6 = NFD + BB, 3 = LFD and 4/8 = LFD + BB) (n=12-13 animals per group, p<0.05, Bonferroni multiple comparison test).
Figure 4.4 Alaskan Blueberry Improves Hippocampal BDNF and Attenuates Levels of Inflammatory Markers in the SN Region

(A) Brain homogenates from hippocampus were measured using ELISA for BDNF levels. Brain homogenates from substantia nigra region were measured using ELISA for (B) iba1 levels (C) NOS2 levels and (D) NF-KB levels. * p<0.05, ** p<0.01 and *** p<0.001 represents significant differences between vehicle and Mn treated or BB and non BB treated groups. 1, 2, 3 and 4 represents significant difference between HFD with other diet groups in vehicle condition; 6, 7 and 8 represents significant difference between HFD with other diet groups in MnCl2 condition (where 1 = NFD, 2/6 = NFD+ BB, 3/7 = LFD and 4/8 = LFD+ BB) (n=5 animals per group, p<0.05, Bonferroni multiple comparison test).
Figure 4.5 HFD Consumption Decreases Total Cell Count and Tyrosine Hydroxylase Immunoreactive Neurons in the SN region

(A) Bright field images of Nissl staining of coronal brain sections in the SNpc region of the mid brain area. Scale bar 200μm. (B) Bright field images of DAB-TH staining of coronal brain sections in the SNpc region of the mid brain area. Scale bar 200μm. (C) Graphical representation revealed the significant effect of HFD on reduced cell counts in the SNpc region of the mid brain, 1, 3 and 4 represents significant diet differences from NFD, LFD and LFD + BB respectively (n=5, p<0.05, Bonferroni multiple comparison test). (D) Graphical representation revealed the significant effect of HFD on reduced TH immunoreactivity in the SNpc region of the mid brain. 1 represents significant differences from NFD (n=4-5, p<0.05, Bonferroni multiple comparison test).
Tables

Table 4.1 Summary of Statistical Effects of Individual Factors (Diet, BB and MnCl₂ treatment) and their Interactions on Behavioral and Molecular Measures used in the Study

<table>
<thead>
<tr>
<th>Measure</th>
<th>Diet effect</th>
<th>Blueberry effect</th>
<th>Mn treatment effect</th>
<th>Interaction effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total distance</td>
<td>F₂,₁₃⁷ = 49.86, p&lt;0.001</td>
<td>F₁,₁₃⁷ = 11.76, p&lt;0.0009</td>
<td>F₁,₁₃⁷ = 32.41, p&lt;0.0001</td>
<td>Diet and BB</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F₂,₁₃⁷ = 3.85, p&lt;0.03</td>
</tr>
<tr>
<td>Central entries</td>
<td>F₁,₁₄¹ = 90.72, p&lt;0.001</td>
<td>F₁,₁₄¹ = 36.44, p&lt;0.001</td>
<td>F₁,₁₄¹ = 55.95, p&lt;0.001</td>
<td>Diet and Mn</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F₁,₁₄¹ = 30.45, p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Diet and BB</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F₁,₁₄¹ = 6.04, p&lt;0.02</td>
</tr>
<tr>
<td>Recognition index</td>
<td>F₁,₁₄¹ = 58.35, p&lt;0.0001</td>
<td>F₁,₁₄¹ = 32.42, p&lt;0.0001</td>
<td>F₁,₁₄¹ = 30.10, p&lt;0.0001</td>
<td>BB and Mn</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F₁,₁₄¹ = 13.01, p&lt;0.0005</td>
</tr>
<tr>
<td></td>
<td>$F_{1,141} = 41.75, \ p&lt;0.0001$</td>
<td>$F_{1,141} = 6.81, \ p&lt;0.02$</td>
<td>$F_{1,141} = 66.65, \ p&lt;0.0001$</td>
<td>BB and Mn $F_{1,141} = 6.76, \ p&lt;0.02$</td>
</tr>
<tr>
<td>-----------------------</td>
<td>--------------------------------</td>
<td>--------------------------------</td>
<td>--------------------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>Ataxia</td>
<td>$F_{1,141} = 54.97, \ p&lt;0.0001$</td>
<td>$F_{1,141} = 14.98, \ p&lt;0.0003$</td>
<td>$F_{1,141} = 68.04, \ p&lt;0.0001$</td>
<td>Diet and Mn $F_{1,141} = 5.15, \ p&lt;0.03$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BB and Mn $F_{1,141} = 20.83, \ p&lt;0.0001$</td>
</tr>
<tr>
<td>BDNF</td>
<td>$F_{1,52} = 26.10, \ p&lt;0.0001$</td>
<td>$F_{1,52} = 12.70, \ p&lt;0.0009$</td>
<td>$F_{1,52} = 10.20, \ p&lt;0.003$</td>
<td>-</td>
</tr>
<tr>
<td>IBA1</td>
<td>-</td>
<td>$F_{1,48} = 6.83, \ p&lt;0.02$</td>
<td>$F_{1,48} = 11.28, \ p&lt;0.002$</td>
<td>BB and Mn $F_{1,48} = 11.21, \ p&lt;0.002$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Diet and BB $F_{1,48} = 16.26, \ p&lt;0.0001$</td>
</tr>
<tr>
<td>NOS2</td>
<td>-</td>
<td>$F_{1,52} = 10.36, \ p&lt;0.003$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$F_{1,52} = 5.35$, p&lt;0.03</td>
<td>$F_{1,52} = 5.75$, p&lt;0.03</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>----------</td>
<td>--------------------------</td>
<td>--------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>NfkB</td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Total cells</td>
<td>$F_{1,52} = 41.53$, p&lt;0.0001</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TH immunoreactivity</td>
<td>$F_{1,52} = 18.55$, p&lt;0.0001</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 4.2 Diet Composition.

<table>
<thead>
<tr>
<th></th>
<th>10 Kcal% fat diet (Low-fat diet)</th>
<th>60 Kcal% fat diet (High-fat diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>20.5</td>
<td>18.4</td>
</tr>
<tr>
<td>carbohydrate</td>
<td>69.1</td>
<td>21.3</td>
</tr>
<tr>
<td>Fat (Lard)</td>
<td>10.4</td>
<td>60.3</td>
</tr>
<tr>
<td>Kcal/g</td>
<td>3.6</td>
<td>5.1</td>
</tr>
</tbody>
</table>

The diet composition in the study has been used as per Carey et al. (2014). The Alaska blueberries were added to the pellet at 5% of the pellet weight. The pellets were prepared by PMI Nutrition International, St. Louis, MO, (http://www.labdiet.com/Products/CertifiedDiets/).
4.8 References


20. Gustafson SJ, Dunlap KL, McGill CM, Kuhn TB. A Nonpolar Blueberry Fraction Blunts NADPH Oxidase Activation in Neuronal Cells Exposed to Tumor Necrosis Factor-


Chapter 5: General Conclusions

5.1 General Overview

Neurodegenerative disorders, such as Parkinson’s disease (PD), are debilitating disorders affecting an increasing number of populations throughout the world [1]. The exact etiology is unknown and several molecular factors like oxidative stress, inflammation, mitochondrial dysfunction, α-synuclein aggregation and cell death have been reported to be associated with PD pathology [2]. Environmental influences, such as diet, play a major role in influencing the progressive pathophysiology [3]. The treatment options in PD are limited and most of the translational research often fails to reach the further stages of drug development [4, 5]. Plant-derived natural products have been tested extensively in in vitro and in vivo models for treatment of this chronic neurodegenerative disorder [6]. Nonetheless, the interplay between natural therapy and external factors, such as diet, in addressing this complex diseased condition has been rarely explored. Moreover, either the mechanistic approach for the treatment options remains understudied or the treatment itself lacks reproducibility in different physiological systems. Furthermore, absence of a single animal model to reflect complete pathogenesis and progressive nature of PD creates hindrance in determining an exact disease modifying therapy [7]. Through this thesis, I examined the effect of natural therapy, diet (specifically fat levels) and underlying molecular mechanisms of neurodegenerative pathologies in two animal models: Caenorhabditis elegans and Mus musculus.
5.2 Effect of Alaskan Bog Blueberry on α-synuclein Overexpression in a Transgenic Model of Caenorhabditis elegans.

In the experiments described in Chapter 2, we tested the effects of indigenous Alaskan bog blueberry (BB) on α-synuclein overexpression in a transgenic model of Caenorhabditis elegans (C. elegans). Previous research from our laboratories reported that endemic Alaskan botanicals, including BB, had anti-ageing and anti-inflammatory effects in in vitro and in C. elegans [8, 9]. Our study further examined the role of the BB polyphenols in the context of protein misfolding. We for the first time, reported that BB polyphenols reduced expression of the toxic protein α-synuclein and improved movement deficit in the nematode model of C. elegans [10]. It was also observed that specific anthocyanins and proanthocyanidins fractions were the key polyphenolic compounds mediating this beneficial outcome. Furthermore, our data revealed the novel molecular mechanism through which BB inhibits Sirtuin 1, a key molecular target and relives protein overexpression. The results from this research enhance the knowledge of the use of indigenous botanicals as alternative medicine for neurodegenerative disorders. As a continuation to the study, it would be interesting to characterize the chemical composition of berry extracts and identify which specific molecule(s) contribute to such neuroprotective pathways leading to future translational research.

5.3 Silencing Fat Metabolism Genes in Parkinson's like Models of Caenorhabditis elegans.

Through Chapter 3, we evaluated the role of fat metabolism on key features of PD-like pathology such as α-synuclein overexpression (both wild-type and A53T mutation) and environmental toxin-induced dopaminergic neuron degeneration in models of C. elegans. Fat metabolism in general is known to impact longevity [11, 12] and stress response pathways mediated through insulin signaling [13]. Besides, delaying aging by modulating insulin signaling
has been implicated to be profitable in PD-like models of *C. elegans* [14]. There have been no studies conducted so far to examine the relationship between fat metabolism and PD-like pathology in nematode models. Through RNA interference (rather than diet), we genetically manipulated the nematodes to create high and low fat levels in the organisms. We found that low fat content due to silencing of *fat-5* and *fat-7* genes was most beneficial in reducing the α-synuclein expression, rescuing DA neuron degeneration and improving DA-dependent behaviors. Interestingly, the high fat content due to genetic silencing of *nhr-49* and *tub-1* genes had no significant effect on any of the PD-like molecular features in the worm models. Our results also demonstrated no alteration in lifespan of the transgenic PD models on silencing of these candidate genes suggesting decoupling between longevity and general health. Overall, our data highlighted the beneficial effect of low fat diet in ameliorating PD-like symptoms in models of *C. elegans*. In the current research, we did not examine the different fat molecules and the downstream molecular targets that could be responsible for this profitable effect. These questions can be further addressed in follow up studies.

5.4 Complex Interaction of Dietary Fat and Alaskan Bog Blueberry Supplementation Influences Manganese Mediated Neurotoxicity and Behavioral Impairments.

Finally, in Chapter 4, we investigated the combined role of dietary fat (normal, low and high fat) and supplementation (in form of Alaskan BB) on the neuronal health and behavioral outcomes in a manganese-induced neurotoxic model of *Mus musculus* (mice). Previous studies have highlighted the anti-oxidant and anti-inflammatory role of BB in combating features of the ageing brain (such as memory loss, protein aggregation etc.) [15-21]. Recent research also elucidates the role of high fat diet and blueberry supplementation in improving neural plasticity in aging mice [22, 23]. Moreover, obesity is considered a risk factor for PD [3], but there have been
conflicting data reporting the same. Our study is the first to compare different diets in combination with Alaskan BB on neurotoxic end points in a metal induced mice model, which presents clinical features also seen in PD. Here, we reported that overall a low fat diet was neuroprotective and in the presence of BB it improved behavioral phenotypes (locomotion, memory and motor coordination) and molecular markers (inflammatory IBA1, NF-KB, NOS2/iNOS and neurotrophic growth factor BDNF). However, we did not find any major differences in number of dopaminergic neurons between the control and MnCl₂ treated groups. We speculate that considering the experimental time frame, MnCl₂ exposure was not enough to cause complete cell loss. Probably, the exposure to MnCl₂ induced neurotoxic conditions and led to changes in neuronal morphology and probably cytoskeletal structures as well. This was not addressed in our current research and requires future investigations. On the contrary, and supporting our hypothesis, a high fat diet compared to other treatments was found to be deleterious in inducing a pro-inflammatory response, worsening behaviors and triggering dopaminergic cell loss in the mouse model supporting our hypothesis. The results from this chapter further bolstered the association between PD and obesity and substantiated our understanding that the benefit of supplements, such as BB, cannot overcome the detrimental effects of a high fat diet. This further reiterates the importance of consuming a balanced diet with low fat content along with therapeutic interventions for ensuring treatment success in progressive disorders like PD.

5.5 Overall conclusion

Our results demonstrated for the first time the effect of culturally relevant Alaskan botanical in displaying protection against neurodegeneration in two different in vivo model systems. Importantly, the studies showed how neurodegenerative pathology can be modulated by fat content either genetically (e.g., RNAi of fat metabolism) or through dietary interventions (e.g.,
feeding different ‘fat diets’). We also uncovered novel underlying mechanistic approaches influenced by consumption of Alaskan bog blueberry. Different cellular symptoms, such as protein overexpression and neuronal morphology, and molecular markers, such as inflammation and neurotrophic growth factors, were affected by external environmental factors, i.e., diet and supplementation, or intrinsic processes, i.e., aging and neurodegenerative physiology. This supports the explanation that a reciprocity between multiple factors and genetic mechanisms is what fosters health-promoting benefits of therapeutic inventions. Lastly, use of subsistence species like Alaskan BB as potential treatment recapitulates the traditional knowledge on consuming the endemic plants and further deciphers the molecular pathways underlying aging and neurodegeneration process.
5.6 References


Appendix

July 26, 2017

To: Abel Bult-Ito, PhD
    Principal Investigator
From: University of Alaska Fairbanks IACUC
Re: [1024315-8] Role of high and low fat diet with Alaskan blueberry supplementation on behavior and neuronal health in a 6-OHDA induced Parkinson model of Mus musculus

The IACUC reviewed and approved the Amendment/Modification referenced above by Designated Member Review.

Received: July 25, 2017
Approval Date: July 26, 2017
Initial Approval Date: February 21, 2017
Expiration Date: February 21, 2018

This action is included on the August 10, 2017 IACUC Agenda.

**PI responsibilities:**

- Acquire and maintain all necessary permits and permissions prior to beginning work on this protocol. Failure to obtain or maintain valid permits is considered a violation of an IACUC protocol and could result in revocation of IACUC approval.
- Ensure the protocol is up-to-date and submit modifications to the IACUC when necessary (see form 006 "Significant changes requiring IACUC review" in the IRBNet Forms and Templates)
- Inform research personnel that only activities described in the approved IACUC protocol can be performed. Ensure personnel have been appropriately trained to perform their duties.
- Be aware of status of other packages in IRBNet; this approval only applies to this package and the documents it contains; it does not imply approval for other revisions or renewals you may have submitted to the IACUC previously.
- Ensure animal research personnel are aware of the reporting procedures on the following page.
HOW DO I REPORT CONCERNS ABOUT ANIMALS IN A UAF RESEARCH FACILITY?

- All "live" animal concerns related to care and use should be reported to the IACUC
- Email: uaf-iacuc@alaska.edu  Phone: 474-7800
- Report form: www.uaf.edu/iacuc/report-concerns/
- IACUC Committee Members: www.uaf.edu/iacuc/iacuc-info/
- Additional information: www.uaf.edu/ori/responsible-conduct/research-misconduct/ and www.uaf.edu/ori/responsible-conduct/conflict-of-interest/

WHAT SHOULD I DO IF AN ACCIDENT OR INCIDENT OCCURS IN AN UAF ANIMAL FACILITY?

- For all immediate human emergencies call 911 or UAF Dispatch at 474-7721 for less immediate emergencies.
- If you have suffered an animal bite or other injury, complete an "Accident/Incident Investigation form" (personal injury) form available at www.uaf.edu/safety/incidentreport-2012.pdf.
- If an accident such as a chemical spill occurs, contact the Environmental Health, Safety, and Risk Management (EHS&RM) Supervisor at 474-5617 or the Hazmat Coordinator at 474-7889.

WHO DO I CONTACT IF I FIND A DEAD, INJURED, OR DISTRESSED ANIMAL IN A UAF RESEARCH FACILITY?

- During regular business hours, immediately contact facility staff and/or Veterinary Services Staff at 474-7020.
- After hours or on weekends, immediately contact facility staff and/or Veterinary Services Staff using the contact numbers posted on the "Emergency Contact Information" in the facility or call UAF Dispatch at 474-7721.
- Contact the IACUC at 474-7800 or uaf-iacuc@alaska.edu if an "Emergency Contact Information" sign is NOT posted in the facility.
- Contact the IACUC if you are not satisfied with the response from Vet Services.

HOW DO I REPORT ANY CONCERNS REGARDING WORK HAZARDS OR ANY GENERAL UNSAFE CONDITIONS?

- Complete an "Unsafe Condition Reporting Program" form, available at the EHS&RM website: www.uaf.edu/safety/unsafe-condition/

WHERE CAN I OBTAIN GENERAL OCCUPATIONAL SAFETY INFORMATION?

- www.uaf.edu/iacuc/occupational-health/