Age-related Changes in the Neuronal Architecture of Caenorhabditis Elegans: A Dissertation

Anagha Khandekar
University of Massachusetts Medical School

Let us know how access to this document benefits you.
Follow this and additional works at: https://escholarship.umassmed.edu/gsbs_diss

Part of the Cellular and Molecular Physiology Commons, and the Molecular and Cellular Neuroscience Commons

Repository Citation

This material is brought to you by eScholarship@UMMS. It has been accepted for inclusion in GSBS Dissertations and Theses by an authorized administrator of eScholarship@UMMS. For more information, please contact Lisa.Palmer@umassmed.edu.
AGE-RELATED CHANGES IN THE NEURONAL ARCHITECTURE OF

CAENORHABDITIS ELEGANS

A Dissertation Presented

By

ANAGHA KHANDEKAR

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

OCTOBER 16, 2015

BIOMEDICAL SCIENCE

PROGRAM IN NEUROSCIENCE
Dedicated to my loving husband, Ashish
He Parameshwara!

Oh, Almighty god!

Ki Ghetale Wrat na He Amhi Andhatene |
Labdh - Prakash Itihas - Nisarg - Mane ||

We have undertaken this mission only after conscious thinking and thorough premeditation. While planning this path we have taken the cognizance of our glorious victories as well as painful defeats. We are determined (to fulfill our calling) but we are not temerarious. We are humble to acknowledge our limitations as a human being.

Je Diwya Dahak MhaNoni Asawayache |
BuDdhyaichi WaN Dharile Kari He Satiche ||

The path we have chosen is less-travelled, albeit, daunting and tiresome. We have vowed ourselves not to give up our mission and stand tall until our last breath!

- Veer Vinayak Damodar Savarkar

Great Indian Freedom Fighter, Leader, Poet and Writer
ACKNOWLEDGEMENTS

This journey of six years and 7,700 miles has been an incredible life experience. I remember simultaneous anxiety, fear and excitement as I stood for the first time with three huge bags in front of the house I would come to make a home in this foreign land. Everything was unfamiliar: the silence, the streets empty of people, the smooth traffic, and the culture. But though it took a while to adjust, I’ve always felt welcomed. In this amazing chapter, so many have touched my life and influenced my thoughts and perspective that it is not possible to thank all of them. But I will always remain grateful to all of those who made my stay here comfortable, pleasant, and transformative.

During my first year, after the initial rotations, I still felt that I hadn’t found a research area that truly excited me. Then I met Claire. Her enthusiasm for \textit{C. elegans} neurobiology was contagious. Her vibrant nature and her passion for her research were evident from our first meeting. Claire’s supportive yet commanding nature that propelled the lab forward always impressed me. She has been a great mentor to me, not just in my scientific research but also in my professional development. She taught me how to mentor, as I struggled in supervising and delegating tasks to different interns, high school students and technicians. She has been a patient ear to my worries, a supporting shoulder for my woes, and a comforting presence when I felt emotionally vulnerable. She always said, “I understand how difficult it is to be so far away from your loved ones”, and she
truly meant it. Claire is always open to new ideas and reasoning, but convincing her takes dedicated fact research and a thorough analysis of possible outcome. And so, meetings with Claire taught me to be a better scientist, to think with clarity and to be prepared ten steps in advance. I can never thank you enough, Claire, for your support, dedication, determination, and mentorship.

Mark Alkema has been another positive and pleasant influence in my life. One of my favorite people, his cheerful nature always brought a smile to my face. His thoughtful questions and inputs during my presentations and other discussions helped to improve and direct my research. His ukulele playing, his singing and his jolly attitude brightened many dull evenings and weekends. I always felt I could easily talk to Mark about anything, and though life did not deal me any crises, it was comforting to know I could confide in him in such times.

I have always been pleasantly surprised by David Weaver’s concern for the well-being of the students. In each and every conversation we have had, he has helped me with many questions and concerns about grad school, GSBS rules, general scientific career, etc. I feel fortunate to have had a program director like him. I am also grateful to my past and present committee members – Marc Freeman, Eric Baehrecke, Andrew Tapper, Heidi Tissenbaum, Victor Ambros and Scott Waddell - for their inputs on my research and my scientific career. I would also like to thank Dr. Mary Ellen Lane for her tremendous help as I was struggling to figure out the graduation process, thesis writing and visa procedures.
One of Claire’s many talents is to assemble a fine team of people. My lab has been a second family to me. Working in the lab has always been very enjoyable. Everyone old and new shares a collaborative spirit; sometimes even going out of one’s way to help others. Cassie, Andrea, Paola, Gillian, Devyn, Rachel, Hannah, Akusika, Marina, Akif, Mitz, Mark, Jessica and Thao, the past and present members of the lab, have all been a part of many happy moments. Thanks to the open floor plan of the LRB, I met some of the most wonderful and amazing people: Jenn, Chris, Yung-Chi, Jeremy, Amit, Raja, Navonil, Alison, Dennis, Ashleigh and Gift. Thank you all for being a part of my life. From interesting and thoughtful conversations with Mike Gorczyca, I have learned a great deal about the cultural differences, graduate school differences, and life in the U.S. Thank you Mike, for this wisdom and your help with confocal microscopy. The Neurobiology department is a unique, passionate group of people. Finally, though the list of people who have helped me in one way or the other is a long one, I would like to add that my stay in the department and the U.S. would not be the same without Gail, Tara and Colleen.

I have been extremely fortunate to receive the love of so many people in my life. My research, thesis and life in general are only possible with the infinite love and support you give me, Ashish. I can never say enough to thank you for all that you have done and you continue to do for me. As everybody always tells me – I am extremely lucky to have you! My life would also be impossible without my brain twin, my lil-big bro, my confidant Amogh! I will always thank Aai-Baba
for giving me this best gift of life. Thank you for being a part of my life for as long as I can remember and for sharing every moment of my happiness and sorrow. And thank you Aai and Baba for believing in me and for your love and support that has sustained me and carries me still.

Life is sometimes tough, but the tough times become easier with the love and support of friends. Having a shrink-like friend is the icing on the cake! Thank you Abhi for giving me a patient ear every time, for showing me the bright side when all I could see was the dark and for being an amazing person inspiring me to be a better human being. Thank you Dnyan, Vany, Madhura and Tejas for your support, love and friendship, that gave me the strength to keep working. Avery, having a friend like you in this foreign land has always meant a lot to me. Your love for me happily surprises me every time and gives me a strange confidence amidst the anxiety of being a foreigner. I have also learned a great deal from you and I feel that I have opened myself to life’s new experiences thanks to you. Your letters and cheerful laughter brighten up the dullest of my days!

I would also like to thank all the people of this country who have welcomed me and helped broaden my perspective towards people, cultures, traditions, love and happiness. I will forever be grateful to you for this experience!
ABSTRACT

Though symptoms such as loss of vision, decline in cognition and memory are evident during aging, the underlying processes that affect neuronal function during aging are not well understood. Unlike changes in other tissues and organs, age-related changes in the nervous system affect the overall physical, mental as well as social state of human beings. To start elucidating the molecular mechanisms underlying normal age-dependent brain decline, we have characterized structural neuronal changes occurring during *Caenorhabditis elegans* aging. Our analysis reveals distinct neuronal alterations that arise with age and that the types of changes and their age of onset are neuronal-type specific, highlighting the differential susceptibility of neurons to the stresses of life. We also find that these age-dependent neuronal changes are largely uncoupled from lifespan. As a first step towards understanding the neuropathological conditions manifested during senescence, we have characterized the role of the neuronal maintenance gene *sax-7/L1CAM* in normal *C. elegans* aging. Our comparison of age-related structural changes in the wild-type nervous system with that of *sax-7* mutants, indicates that loss of function of *sax-7* results in accelerated neuronal deterioration that mimics alterations occurring during normal aging. Conversely, overexpressing wild-type copies of SAX-7 delays some of the neuronal changes that accompany normal aging, indicating that SAX-7 plays a neuroprotective role. Additionally we find that
mechanical stress from body movements impacts the neuronal changes during adulthood. Taken together, our results give an entry point into the mechanisms of age-related neuroanatomical changes and neuronal protection.
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dedication</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>v</td>
</tr>
<tr>
<td>Abstract</td>
<td>ix</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>xi</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xiv</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xv</td>
</tr>
<tr>
<td>Third Party Materials</td>
<td>xviii</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xix</td>
</tr>
<tr>
<td>Chapter I: Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Theories of aging</td>
<td>4</td>
</tr>
<tr>
<td>Aging of the brain and neurodegeneration</td>
<td>11</td>
</tr>
<tr>
<td><em>C. elegans</em> as a model of aging</td>
<td>14</td>
</tr>
<tr>
<td><em>C. elegans</em> nervous system</td>
<td>16</td>
</tr>
<tr>
<td>Age-related changes in the <em>C. elegans</em> nervous system</td>
<td>18</td>
</tr>
<tr>
<td><em>C. elegans</em> models of neurodegenerative diseases</td>
<td>22</td>
</tr>
<tr>
<td>Lifespan determinant pathways and neuronal aging</td>
<td>26</td>
</tr>
<tr>
<td>Neuronal maintenance mechanisms</td>
<td>36</td>
</tr>
<tr>
<td>Mechanical stress</td>
<td>41</td>
</tr>
<tr>
<td>Chapter II: Age-related neuronal changes are uncoupled from lifespan in <em>Caenorhabditis elegans</em></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Preface to Chapter II</td>
<td>49</td>
</tr>
<tr>
<td>Chapter II</td>
<td>50</td>
</tr>
<tr>
<td>Abstract</td>
<td>51</td>
</tr>
<tr>
<td>Introduction</td>
<td>53</td>
</tr>
<tr>
<td>Experimental procedures</td>
<td>56</td>
</tr>
<tr>
<td>Results</td>
<td>66</td>
</tr>
<tr>
<td>Discussion</td>
<td>82</td>
</tr>
<tr>
<td>Figures</td>
<td>91</td>
</tr>
</tbody>
</table>

| Chapter III: SAX-7 acts as a neuroprotective molecule to counteract mechanical stress from locomotion |
|---|---|
| Preface to Chapter III | 114 |
| Chapter III | 115 |
| Abstract | 116 |
| Introduction | 117 |
| Experimental procedures | 123 |
| Results | 134 |
| Discussion | 145 |
| Figures | 154 |

| Chapter IV: Discussion and Future Directions |
|---|---|
| Discussion | 193 |
| Limitations of the work and future directions | 214 |
Bibliography
LIST OF TABLES

Chapter II

II-1 List of strains used. .................................................. 109
II-2 Sample sizes for neurons and neuronal structures examined. 111

Chapter III

III-1 List of strains used. .................................................. 187
III-2 Sample sizes for neurons and neuronal structures examined. 189

Chapter IV

I-1 Age-related neuroanatomical changes are uncoupled from lifespan. 228
LIST OF FIGURES

Chapter I
I-1 The C. elegans nervous system. 47
I-2 Schematic representation of SAX-7 protein isoforms. 48

Chapter II
II-1 Defasciculation and neurite outgrowth in wild-type animals as they age. 91
II-2 Displacement of neurons in wild-type animals as they age. 94
II-3 Longitudinal analysis of neuronal placement and behaviors in individual worms. 97
II-4 Age-associated defasciculation and neurite outgrowth in lifespan mutants. 99
II-5 Age-associated neuronal displacements in lifespan mutants. 101
II-6 The pharynx grows proportionately to the rest of the body in the wild type and lifespan mutants. 104
II-7 Neuroanatomical changes upon limiting food availability. 105
II-8 Posterior displacement of the neurons ADE in cca-1 mutants with age. 108

Chapter III
III-1 Many of the neuronal maintenance defects of sax-7 mutants are qualitatively similar to the age-related neuroanatomical changes of axons and fascicles occurring in aging wild-type animals. 154
III-2 Many of the neuronal maintenance defects of sax-7 mutants are qualitatively similar to the age-related neuroanatomical neuronal displacements occurring in aging wild-type animals. 157
III-3 Neuroanatomical changes in nerve ring and neuronal soma 160
position in young worms.

III-4 Lifespan analysis of sax-7 mutant worms. 163

III-5 SAX-7 expression in wild-type adult worms. 164

III-6 Age-related nerve ring displacement is reduced with overexpression of sax-7 in the neurons of wild-type animals. 165

III-7 Age-related displacement of ADE neurons is reduced with overexpression of sax-7 in the neurons of wild-type animals. 167

III-8 Overexpressing of sax-7 in wild-type worms does not alter locomotion or pharyngeal pumping. 169

III-9 Age-related dorsal nerve cord defasciculation is not suppressed by overexpressing sax-7 in the neurons of wild-type animals. 171

III-10 Decreased locomotion suppresses neuronal maintenance defects of sax-7 mutants. 172

III-11 Decreased locomotion suppresses some wild-type age-related neuroanatomical changes. 174

III-12 Decreased locomotion reduces some wild-type age-related neuroanatomical changes. 176

III-13 Swimming / thrashing locomotion leads to neuroanatomical changes in some neurons. 179

III-14 Neuroanatomical changes of axons and neuronal soma position when subjected to mechanical stress. 181

III-15 Neuroanatomical changes of axons and neuronal soma position at different temperatures. 183

III-16 Schematic representation of horseshoe shaped conformation of the long isoform, SAX-7L. 185

III-17 Image of the assembly of worms with agar plates atop a vortex for altered locomotion paradigm. 186
Chapter IV

IV-1 Proposed model of the role of neuronal maintenance machinery and stress in age-related neuroanatomical changes in young and old wild-type worms.
Chapter I
Fig. I-1  The *C. elegans* nervous system.
This image was taken by Claire Bénard.
LIST OF ABBREVIATIONS

C.  
Caenorhabditis genus

DiI  
lipophilic dye, (2Z)-2-[(E)-3-(3,3-dimethyl-1-octadecylindol-1-ium-2-yl)prop-2-enylidene]-3,3-dimethyl-1-octadecyldole.
CAS number 41085-99-8

DNA  
deoxyribonucleic acid

DR  
dietary restriction

E.  
Escherichia genus

GABA  
gamma-aminobutyric acid

GFP  
green fluorescent protein

L1, L2, L4  
C. elegans larval stages 1, 2, 4

N2  
C. elegans wild type strain N2 (Bristol)

Na azide  
Sodium azide

NaCl  
Sodium chloride

NGM  
nematode growth media

OP50  
Escherichia coli strain OP50

P  
Promoter

PCR  
polymerase chain reaction

RFP  
red fluorescent protein
<table>
<thead>
<tr>
<th>Abbr.</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>TBI</td>
<td>traumatic brain injury</td>
</tr>
</tbody>
</table>
Symbols and Units

C  Celsius

D  Dorsal

hr  Hour

L  Left

m  Meter

μL  Microliter

μm  micrometer, micron

μM  Micromolar

ml  Milliliter

mg  Milligram

mm  Millimeter

mM  Millimolar

min  Minutes

ns  not significant

R  right

s, sec  seconds

V  Ventral

°  Degrees
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
</tbody>
</table>
Chapter I

Introduction

"It is remarkable that after a seemingly miraculous feat of morphogenesis, a complex metazoan should be unable to perform the much simpler task of merely maintaining what is already formed."

- Evolutionary theorist George Williams [1]
Why do organisms age? Well-developed organ systems appear to function normally for certain time post-development, but start ‘wearing out’ or aging in the later part of life. Do active mechanisms exist that maintain the systems post-developmentally and during aging? Do different mechanisms exist during these phases or is mere absence of any maintenance the cause of aging? Aging is often associated with increased risk of age-related diseases in humans. Behaviors and cognition decline in older humans and there is an age-related increase in the incidence of neurodegenerative diseases. Even though neuronal death is a key feature of neurodegeneration, normal aging features subtle neuronal changes rather than death. These changes include structural changes such as age-associated increase in dendritic extent, reduction in both spine density and number of synapses as well as gene expression changes and alterations in Ca$^{2+}$ conductance of the neurons [2]. Interestingly these changes are region-specific which may affect the coordinated activation of brain regions required for cognitive functions. Age-associated gene expression changes may lead to imbalance between inhibitory and excitatory neurotransmission which could predispose individuals to cognitive decline and neurodegeneration [3]. Some neuroanatomical changes are common between normal aging and neurodegenerative disorders [4]. Therefore it becomes important to gain a broad understanding of the age-related changes in the nervous system in a non-pathological setting.
Decades of research in humans and different model organisms have aimed at understanding why we age and probably more importantly, how we age – characterizing the age-related structural and functional changes and understanding the molecular basis of these changes. The aging of the nervous system has been of particular interest.

The focus of this dissertation is to characterize the structural changes that take place in the adult nervous system with age and the impact of environmental factors and stress on the structures observed. We have also explored the role of neuronal maintenance molecules in protecting the nervous system during adulthood. Given the complexity of the mammalian brain, redundancy of genes as well as other practical disadvantages (maintenance cost, long lifespan, and time-consuming experimentation techniques), simpler model systems are ideal to study the changes in the nervous system with age, over the entire lifespan of an organism. Therefore, we have chosen to characterize the neuroanatomical changes that occur in the adult *Caenorhabditis elegans* (*C. elegans*) nervous system.
Theories of Aging

Aging can be defined as progressive deterioration of physiological function, cognition, the ability to respond to stress, loss of viability as well as increase in homeostatic imbalance and vulnerability to age-related diseases, with the ultimate consequence being death. The Gompertz-Makeham law of mortality postulates that the rate of mortality increases with age [5].

Aging has been a topic of immense interest mainly to find ways to prevent or at least delay old age. Several theories have been proposed to explain the process of aging, but due to the complexity of the process, each theory is able to explain only a part of the aging process and therefore partially overlaps with other theories. Several biological theories of aging have been proposed which argue that aging is determined by dysfunction of internal organs, cells, molecules and influence of the environment and stresses that induce progressive damage at various levels. The biological theories include Programmed aging theories, where aging is regulated in a predetermined manner by biological clocks through various stages of growth, development, maturity and old age and Damage and error theories, where aging is caused by accumulation of damage that results from environmental insults and internal errors (mitochondrial DNA damage, oxidative damage to cellular organelles, etc.). In addition to biological theories, various evolutionary theories of aging have also attempted to explain the aging process. These theories argue that a decline in the force of natural selection is
the cause of aging. A few of the biological and evolutionary theories are discussed below.

A. Biological theories of aging

1. Programmed aging theories

The Gene Regulation theory proposes that expression of genes regulating development also regulate aging. Studies in simple model organisms such as yeast, _C. elegans_ and _Drosophila melanogaster_, have identified a number of genes involved in conserved pathways of lifespan determination. Mutations in many of these genes extend lifespan, homologs of which have also been correlated with increased longevity in mammals including humans [6-8]. Interestingly, many of these genes play important roles in different biological processes of development, growth, and cell survival [9]. Studies in centenarian human populations find that siblings of centenarians have a greater chance at a long life (>85 years of age) than siblings of people dying early (at 73 years of age) [10].

The Cell Senescence theory proposes that aging of the organism is caused by aging of individual cells and organs. The research of Leonard Hayflick showed that human cells are capable of dividing only a finite number of times, called the Hayflick limit [11]. The length of the telomeres shortens with every cell division and therefore the cell division stops when the telomeres reach a critical length [12] termed as replicative senescence. However, the telomere and lifespan connection is still unresolved. Rat brain cells do not exhibit age-related
telomere shortening [13]. Additionally, telomere lengths in the human skeletal muscle remain stable between ages 23-74 years [14]. Cells could also undergo senescence in response to stresses, termed as stress-induced senescence. For instance, human melanocytes undergo senescence upon UV irradiation, that involves the activation of p53, the tumor suppressor protein [15]. Cellular senescence may play a role in preventing cancer by limiting cell proliferation.

The Neuroendocrine theory proposes that aging results from decline in neural and endocrine functions. The neuroendocrine system plays an important role in developmental processes, maintenance of tissue function and integration of biological responses to internal and external changes. The insulin/IGF-1 signaling (IIS) pathway has been shown to be important for the regulation of longevity in invertebrates and mammals [16]. In humans, defects in this pathway may lead to diseases such as Diabetes mellitus, whereas variants in one of the transcription factors (FOXO3A) of this pathway have been correlated with long lifespan [6, 8].

The Immunological theory of aging proposes that aging is caused by changes in the development and function of the immune cells. The immune system is responsible for maintaining homeostasis in the face of external threats, and is capable of reacting to a wide variety of antigens and internal threats such as cancerous cells, misfolded protein aggregates, etc. The immune system interacts with other physiological systems such as the nervous system, the endocrine system and the cardiovascular system, during activation. The immune system
function is known to decline with age [17] and there is an increase in the incidence of immunological disorders with age.

2. Damage and error theories

The Wear and Tear theory, first proposed by Dr. August Weismann in 1882, proposes that aging in humans and other animals is simply due to the ‘wearing out’ process, just like in machines, and increased use leads to quicker wearing out (certain injuries are more common in athletic people). According to this theory, the deteriorative processes such as mechanical damage, corrosion in inanimate systems or oxidative and chemical damage in biological systems, and increased friction in between different machine parts or different biological organs (for example reduction in body fluids in bone joints), result in aging. Though intuitive, there has not been enough evidence to support this theory. Interestingly, the Kornfeld group has shown that in *C. elegans*, the reproductive system undergoes age-related degeneration independent of the total progeny production and mating exposure, suggesting that this degeneration is not caused by use-dependent damage accumulation [18].

A closely related theory to the Wear and Tear theory of aging is the Somatic or Spontaneous Mutation theory. According to this theory, aging is caused by the mutations in DNA either due to errors in replication as cells divide or due to exposure to toxins or radiation. Efficient DNA damage repair systems exist in cells, but they may get overwhelmed with the accumulating damage [19].
The Rate-of-living theory, one of the first theories of aging, ascribed to Raymond Pearl (1928), rests on the idea that the lifespan of an organism is determined by its rate of energy metabolism. The theory states that every organism has a genetically determined (fixed) metabolic potential and that metabolic rate and aging are inversely proportional. Support for this theory comes from Loeb and Northdrop who found that Drosophila larval and pupal developmental rates increased with an increase in temperature and the resulting adults had shorter lifespan than at lower temperatures [20]. But the theory may not apply universally, since a detailed analysis of 1456 mammals, birds, amphibians, and reptiles for metabolic rate, and its relationship to development and longevity, revealed that basal metabolic rate does not correlate with longevity [21].

Free Radical theory of aging was developed by Denham Harman in 1956 [22]. This theory, currently one of the most popular theories, states that aging results from the accumulation of damage caused by free radicals in the cells, over time. A free radical is a molecule that has a single unpaired electron, making it highly reactive. Biologically relevant free radicals include superoxide (O$_2^-$) radicals and reactive oxygen species such as hydrogen peroxide (H$_2$O$_2$). Since mitochondria are an important source of reactive oxygen species, this theory is also referred to as the Mitochondrial Free Radical theory of aging. Additionally, mitochondria are crucial in energy metabolism that may result in reactive oxygen species production; this has linked the Free Radical theory with
the Rate-of-living theory [19]. Though multiple studies support this theory and it is clear that oxidative damage increases with age, a conclusive causal link between reactive oxygen species and aging is still missing [23].

B. **Evolutionary theories of aging**

The Mutation Accumulation theory was proposed by Peter Medawar in 1952 based on the observations that force of natural selection declines with age. Natural selection most directly favors (or works on) genes, that are beneficial early in life, through the reproductive lifespan of the organism. According to Medawar’s theory, aging is not subjected to natural selection and evolution cannot select against mutations (and/or genes) that cause detrimental effects only in old ages [24]. Genes responsible for diseases prevalent in older population, but rare in the younger (reproductively active) population, for example Huntington’s disease, Alzheimer’s disease, type-2 diabetes, rheumatoid arthritis, etc., are proposed to have escaped the natural selection and propagated.

The Antagonistic Pleiotropy theory, proposed by George Williams in 1957, proposes that some genes that are important in early life may become detrimental later [1]. This is known as the ‘pay later’ model. This theory is based on two assumptions: one, the existence of pleiotropic genes controlling more than one trait in an organism and two, these pleiotropic genes have antagonistic effects at different stages of life (required for development and detrimental in
aging). Interestingly, a mutant allele of p53, that confers enhanced resistance to spontaneous tumors, causes mice to exhibit accelerated aging phenotypes [25].

The Disposable Soma theory, proposed by Kirkwood in 1977 predicts that aging results from accumulation of damage during life that maintenance systems fail to repair [26]. The theory is based on optimal allocation of resources between different processes important for an organism’s survival: reproduction and maintenance [27]. Given finite resources, the expenditure is likely to be biased towards reproduction, as the germline must be well maintained to allow propagation to the next generation, whereas somatic maintenance will benefit only the organism in which it occurs.

Though many theories have been proposed, no single theory has been able to explain the process of aging completely. Each theory explains a part of the aging process and overlaps with other theories. It is possible that a complex process such as aging may be regulated by multiple factors such as genes, hormones and other neuroendocrine mechanisms that may lead to decline in immune function, and wear and tear of the body including oxidative damage by free radicals and reactive oxygen species.
Aging of the brain and neurodegeneration

The mammalian brain exhibits age-related decline in short term memory, spatial memory and speed of processing information [28]. Reduced spatial memory has been documented in older humans, monkeys, dogs and mice. In addition to reduced memory and processing speed, the activities of different brain regions in related tasks are also reduced in aged individuals. During aging, the most affected area to reveal altered functional activation is the prefrontal cortex. Aged adults also exhibit activation of a broader area of the prefrontal cortex, including the contralateral hemisphere [28]. Additionally, there is decline in the coordinated activation of different brain regions involved in cognitive functions [3]. This age-related decline in cognitive function may be attributed to structural changes in the aging brain since there is almost no neuronal loss in cortical regions [3, 29].

Structural changes in the aging brain appear to be region-specific. Brain regions such as frontal lobes and temporal areas have been documented to undergo reduction in volume over time but exhibit different trends of volume reduction with age [30]. For example, the cerebral cortex exhibits a linear decline in volume starting around 20 years of age, whereas hippocampal volume is almost stable till 60 years of age, but declines sharply after that [30]. Dendritic branching of pyramidal and anterior cingulate layer V neurons in rats and medial prefrontal cortex neurons in humans decreases with age, whereas increased dendritic extent in layer II of the parahippocampal gyrus and the dentate gyrus
has been observed in old humans when compared to younger adults [2]. Another age-associated region-specific alteration is the change in dendritic spine density. Significant age-related reduction in spine density is evident in the subiculum of monkeys, whereas no reduction is seen in the CA1 region of aged rats or the dentate gyrus of aged rats and aged humans [2]. Synaptic contacts also appear to be affected in the aging process. In aged rats, dentate gyrus middle and inner molecular layer exhibit significant reduction in total synapse number, which correlates with the reduction in field excitatory postsynaptic potential (EPSP) recorded in the dentate gyrus and spatial memory deficits [28]. In addition to the structural changes, gene expression changes and alterations in the biophysical properties of neurons have been observed in the aging brain. Genes involved in GABA-mediated inhibitory neurotransmission have been found to be downregulated in the prefrontal cortex of aged humans and monkeys [3]. Additionally, age-related reduction in mitochondrial function and upregulation of stress-response genes has been observed across species from C. elegans to humans [3]. Most electrophysiological properties of neurons in the hippocampus and prefrontal cortex remain stable throughout life, but aged CA1 pyramidal neurons exhibit increased calcium conductance that may lead to disruption of calcium homeostasis [2].

Even though memory loss is also a common feature of neurodegenerative diseases, the degree of impairment and the rate at which the decline occurs is strikingly different from normal aging. Additionally, volume loss in certain brain
regions is another distinguishing feature of pathological aging. Some neuronal alterations associated with neurodegeneration are different from the ones observed during normal aging, such as reduced activity in entorhinal cortex in Alzheimer's disease versus reduced activity in the subiculum and the dentate gyrus in normal aging [3]. Importantly, some changes are common between normal aging and neurodegenerative disorders, like neurofibrillary tangles found in both older non-demented and demented humans in anterior olfactory nucleus, parahippocampal gyrus and hippocampus [4]. This highlights that distinguishing features between neurodegenerative diseases and normal aging can help identify pathological conditions early in the disease process. Also identification of structural and functional changes during normal aging will allow for better treatment measures. Understanding normal aging may aid comprehension of the transition to pathological aging and thereby help diagnose and better treat the sporadic forms of neurodegenerative diseases. Sporadic cases of neurodegenerative diseases make up the majority of cases posing a significant challenge to today's medical system, as the disease progression is significant by the time of diagnosis.
**C. elegans as a model of aging**

Animal model systems have always been instrumental in studying various biological processes and the knowledge gained from these studies has helped modern medicine to find cures for many diseases. In the field of aging research, the *C. elegans* model system has provided crucial insights about different biological pathways and molecular mechanisms underlying aging, including genetic mutants that accelerate and delay aging. Major pathways of aging, to be discussed later in this chapter, have been discovered in the worm with observed conservation in many species including humans. Interestingly, features of human aging such as muscle atrophy (sarcopenia), behavioral decline, increased susceptibility to age-associated diseases and infection, are also observed in *C. elegans* aging. A multitude of features make *C. elegans* a favorite model organism for aging research: short lifespan (about three weeks at 20°C), well defined life cycle, developmental progression, completely mapped cell lineage, post-mitotic somatic cells, transparent body, genetic tractability and capability of growing isogenic populations in the lab due to its hermaphrodite body. Ability to store worm strains in frozen conditions, grow large populations on small plates, study single cells in live behaving worms due to the battery of reporters allowing fluorescent labeling of specific cells and manipulate genes using RNAi techniques, add to making *C. elegans* a practical and attractive model system for aging research studies.
A number of studies have identified the age-related changes that take place in *C. elegans*. These changes include morphological, biochemical, and behavioral changes. As worms grow old, head, pharynx, germline and body wall muscles become disorganized [31-34]. In older worms, there is an increase in DNA damage, protein carbonylation, an indicator of oxidative stress, and accumulation of ‘age pigment’ lipofuscin and yolk protein in body cavity [33-39] and reduction in metabolic and enzymatic activity as well as progeny production [37, 40-44]. Behaviors such as pharyngeal pumping, defecation, locomotion, chemotaxis, and isothermal tracking decline with age [32, 34, 40, 44-47]. Age-related decline in these physiological and behavioral parameters has been used to examine the effect of genetic mutations and treatments on lifespan.
**C. elegans nervous system**

Thanks to the extensive efforts of Sydney Brenner, John Sulston, Bob Horvitz, John White and others, the *C. elegans* nervous system has been completely mapped to comprise 302 neurons, a third of the adult hermaphrodite cells [48, 49]. The neuronal cell bodies are mainly clustered in the head or tail ganglia and the synapses are concentrated in the nerve ring, ventral and dorsal nerve cords [49] (**Fig. I-1**). The structure of *C. elegans* neurons is mostly simple; each cell body has one or two processes, though neurons with elaborately branched processes can also be seen (FLP, PVD) [49]. The axons make *en passant* synapses (side by side), with many neurons connected by gap junctions. Additionally, the entire worm connectome has been mapped and therefore all the neuronal connections, synaptic and otherwise, are known. Many neurotransmitters and their activity are conserved from worms to humans. Genes and mechanisms underlying development of neurons, synapse formation, neuronal degeneration, cell death, axonal regeneration and glial function have been identified in the worm. Many of these identified genes have homologs in vertebrates that take part in similar mechanisms as in the worms.

A battery of behaviors has been studied in the worm such as chemotaxis, thermotaxis, touch response, mating, feeding and social behavior as well as associative learning, memory and quiescence (similar to mammalian sleep). The neurons responsible for these behaviors coordinate their activity through synaptic connections using a number of neurotransmitters (acetylcholine, glutamate,
gamma-aminobutyric acid (GABA), dopamine, and serotonin), gap junctions, peptidergic signaling or a combination of these. The nervous system interacts with the external environment and executes behaviors to respond to the environmental cues – attraction towards potential food sources and aversion to potentially hazardous stimuli (toxins, predators or pathogens).

The nervous system appears to play a role in regulating the worm’s longevity. Ablation of certain gustatory and olfactory neurons as well as attenuated sensory perception increases lifespan [50]. Additionally, impairing the functionality of or ablating the interneuron AIY (downstream to the sensory neurons) negatively affects the lifespan of the worm. The nervous system also appears to play a role in regulating longevity by insulin and insulin-like signaling (IIS) and caloric restriction pathway. Loss of function mutations in IIS pathway components, daf-2 and age-1 cause lifespan extension, which can be fully rescued by expressing these proteins just in the nervous system, and a pair of chemosensory neurons ASI are required for the lifespan extension caused by caloric restriction [51]. Additionally, decreasing mitochondrial function in the nervous system by the knockdown of cytochromeC oxidase-1 subunit (CCO-1), a component of the electron transport chain (ETC), extends lifespan through the mitochondrial stress response pathway [52].
**Age-related changes in the *C. elegans* nervous system**

The nervous system is mostly laid out during embryogenesis and the birth of 80 additional neurons occurs during late first larval (L1) stage. The neurons responsible for functioning of the reproductive system undergo axonal growth and make synaptic connections during the last larval stage (L4). Thus the nervous system is completely laid out by the time the worm undergoes its last molt to become an adult. During adulthood until the worm’s death, there is no neuronal death observed in the *C. elegans* nervous system. Initially the Driscoll group reported that no structural changes were found in mechanosensory and motor neurons – neuronal cell bodies, axons, positioning and extension of neuronal processes in the older adults were similar to that found in young worms and microtubule bundles and neuronal nuclear structures were generally maintained [34]. Even electron micrograph analysis of nerves in the ventral and dorsal nerve cords and touch receptor neurons did not reveal any morphological abnormality.

About a decade later though, reports from several labs confirmed that a few neurons in the aging nervous system of *C. elegans* do show age-related structural and functional decline [53-56]. The most widely studied class of neurons for the age-related structural decline is the mechanosensory neurons. There are six mechanosensory neurons in the worm: one AVM, one PVM, two ALM, and two PLM neurons. The McIntire, Kenyon and Driscoll groups found that the mechanosensory neurons exhibit age-related increase in the frequency of
aberrant protrusions from the soma (ALM), neurite outgrowths from the axons (PLM) and beading and blebbing of axonal processes (ALM and PLM) [53-55]. Electron micrograph analysis revealed that the neurite outgrowths from ALM processes were often associated with mitochondria [55]. Additionally, age-related morphological changes were also evident among the motor neurons, such as defasciculation of the cholinergic nerve fascicles and neurite outgrowth from the circumferential processes of the GABAergic motor neurons, suggesting that age-related structural changes may affect the nervous system broadly [53, 54]. It is possible that the underlying processes responsible to bring about these morphological changes may be acting in a broader sense. Do these processes affect all the neurons? There is some evidence from the worm nervous system aging studies suggesting that not all neurons exhibit morphological alterations [55], though one cannot eliminate the possibility that this may be due to the limitations in current methods of detection. It is noteworthy that not all neurons of a certain neuronal class, at a given age, exhibit these structural changes. In other words the frequency of soma projections or axonal neurite outgrowths does not reach 100% even at its maximum penetrance.

What do these structural alterations mean in terms of the function of these neurons? Is there a functional difference between neurons with structural alterations and those without? Though there is conflicting evidence arguing either way, the Kenyon group showed that mechanosensory neurons with structural aberrations correlate with impaired functional response [54] while the Driscoll
group found that structural aberrations in these neurons do not correlate with
functional impairment [55]; it is clear that *C. elegans* neurons do exhibit age-
related functional decline [56]. As evident from the electrophysiological
recordings at the neuromuscular junction, there is age-related progressive
decline in the frequency of spontaneous post-synaptic currents [PSCs], which is
a measure of frequency of spontaneous neurotransmitter release and a good
indicator of motor neuron activity [56]. This suggests that there is an age-related
progressive decline in the function of the motor nervous system. Interestingly,
this progressive decline was found to parallel the progressive decay in
locomotion as measured by locomotion speed in an automated worm tracking
system. The motor neurons signal to the muscles by releasing neurotransmitters
at the neuromuscular junction, which then contracts and relaxes in an alternate
fashion to result in the sinusoidal wave locomotion of *C. elegans*. In the worm,
structural decline in muscles, termed as sarcopenia, is evident starting at day 7
of adulthood [34]. In addition to structural deterioration, do the muscles undergo
age-related functional decline that may contribute to the decay in locomotion?
Surprisingly, this does not appear to be the case since electrophysiological
recordings from the body wall muscles of *C. elegans* did not show any decline in
the amplitude of acetylcholine (ACh) and GABA evoked muscle currents until late
in adulthood (day 11). Instead, a progressive increase in the amplitude was seen
that peaked at day 9 of adulthood. Additionally, even though there is progressive
decline in the muscle currents starting at day 11 of adulthood, the amplitude of
these muscle currents at any age did not decrease beyond that observed for day 3 suggesting that the muscles remain functional even in old worms. This further suggests that the decay observed in locomotion is largely due to the functional decline of the motor nervous system. In fact, stimulating the *C. elegans* motor nervous system to increase the release of neurotransmitters, improved the spontaneous locomotion in older adults (day 8 and day 10) [32]. Significant increase in the fraction of worms initiating movement was evident upon treating worms with Arecoline, a muscarinic agonist that can increase the release of acetylcholine from the motor neurons in the worm. It remains to be elucidated whether neuroanatomical alterations affect the function of these neurons. Are these structural alterations detrimental? Are these a sign of the neuron degenerating? Interestingly, age-related structural alterations seen in wild-type mechanosensory neurons, namely outgrowths from the soma, axons and beading of axons, are also evident in a Huntington’s disease model strain of *C. elegans* [57]. In this strain the touch-receptor neurons express the first 57 amino acids of human Huntingtin with 128 polyglutamine repeats. Additionally, the polyQ128 animals exhibit posterior touch insensitivity [57]. Thus mechanisms affected in this disease state could possibly cause the structural and functional decline in the mechanosensory neurons. It remains to be examined whether the mechanisms responsible for wild-type age-related neuroanatomical and functional alterations are the same as those underlying the morphological decline and functional impairment in polyQ128-Huntington’s disease worm model strain.
**C. elegans models of neurodegenerative diseases**

About 42% of human disease genes have been estimated to have a worm ortholog [58]. Additionally, expression of human genes in *C. elegans* has successfully replicated many biochemical, cellular and phenotypic features of the disease, establishing many worm models of human diseases. Neurodegenerative diseases are no exception. *C. elegans* disease models are being used for drug screens and testing of novel therapeutic strategies [59].

Alzheimer’s disease (AD), the most common form of dementia, is characterized by neuronal degeneration in the cerebral cortex and certain subcortical regions. Brains of people with AD contain large numbers of plaques (mainly composed of β-amyloid peptide) and neurofibrillary tangles (aggregates of hyperphosphorylated tau protein). *C. elegans* Alzheimer’s disease models express human Aβ(1-42) peptide, since the *C. elegans* β-amyloid precursor protein (APP) – related gene *apl-1*, lacks the Aβ peptide [60, 61]. These worms become paralyzed and have characteristic Aβ deposits, similar to the human condition. This model has been used in multiple studies to identify the binding partners of Aβ as well as genetic and environmental factors that influence the Aβ toxicity. These studies have found that decreased insulin/insulin growth factor 1 – like signaling and dietary restriction, can reduce the Aβ toxicity and involves heat shock proteins [59, 62]. In agreement with these findings, heat shock treatment of worms expressing human Aβ1-42 peptide causes decreased Aβ toxicity and Aβ oligomers. Additionally, maintenance of protein homeostasis and
small molecules such as tetracycline, coffee extract (but not caffeine), copper and ethanol extract of Liuwei Dihuang (Chinese medicinal formula) show protective effects against Aβ toxicity whereas posttranslational modifications namely O-GlcNAcylation may directly influence the disease progression in the C. elegans Alzheimer’s disease model [59]. These studies have opened the possibility for novel therapeutic interventions with a number of potential targets.

The second most common neurodegenerative disease, Parkinson’s disease (PD), is characterized by progressive loss of dopaminergic neurons in the substantia nigra pars compacta of the midbrain. Mutations in a number of genes including α-synuclein (SNCA), parkin (PRKN), leucine-rich repeat kinase 2 (LRRK2), PTEN-induced putative kinase 1 (PINK1), DJ-1, and ATP13A2 have been shown to cause familial PD [59]. A number of worm models have been developed either by expressing human wild-type and mutant α-synuclein in the worm, (since there is no C. elegans ortholog of α-synuclein) or by mutating worm orthologs of other PD genes such as pdr-1 (ortholog of parkin), djr-1.1, djr-1.2 (orthologs of DJ-1), pink-1 (ortholog of PINK1) and lrk-1 (ortholog of LRRK2). α-synuclein is a major component of Lewy bodies, which are a pathological hallmark of PD in humans and misfolded aggregates of α-synuclein are observed in the worm models [58]. Many RNAi screens performed on the α-synuclein transgenic worms have identified genes involved in inclusion formation, aggregation of phosphorylated α-synuclein and α-synuclein – induced
degeneration of dopaminergic neurons [63]. Additionally, worm PD models have been used to test chemical suppressors of α-synuclein toxicity [59].

Amyotrophic lateral sclerosis (ALS) is characterized by degeneration of motor neurons in the brain and spinal cord. Mutations in Cu/Zn superoxide dismutase 1 (SOD1), TAR DNA-binding protein-43 (TDP-43), fused sarcoma (FUS), ubiquilin 2 and profilin 1 have been discovered to be associated with familial cases of ALS. Many C. elegans models of ALS have been made that express the human mutant forms of these genes; the most commonly studied are transgenic worms expressing mutant forms of SOD1, TDP-43 and FUS [59]. The ALS model worms exhibit abnormalities in locomotion, degeneration of motor neurons as well as aggregation of the mutant protein [58]. Interestingly, attenuation of insulin / IGF-1 signaling by loss of daf-2, has been shown to ameliorate the motor defects and reduce the protein aggregation and toxicity [59].

Huntington’s disease (HD) is a neurodegenerative disorder caused by autosomal dominant mutation in the Huntingtin gene responsible for expansion of CAG repeats. Mutant Huntingtin has 36 or more CAG repeats (polyglutamine repeats or polyQ) causing it to misfold and aggregate, leading to neuronal degeneration [64]. C. elegans HD models express mutant human Huntingtin with transgenic worms exhibiting protein aggregates in the neurons, neurotoxicity and dysfunction. Many screens have identified modifiers of polyQ toxicity. sir-2.1 has
been shown to protect against the toxicity through the activity of *daf-16*, a transcription factor involved in the insulin-like signaling pathway [58, 59].
Lifespan determinant pathways and neuronal aging

Aging has been loosely defined as the accumulation of morphological and functional changes in different tissues over time that negatively affects the organism and increases the probability of death. The cause and effect of the loose definition in part is the challenge of clear and precise distinction between age-related changes that represent senescence. A difficulty in the process of the precise definition of aging is also the lack of characterization of a defined genetic program that would govern the age-related changes. In an attempt to ‘measure’ aging, the lifespan of an organism (usually studied as lifespan of a population) has been the most widely used assessment. Decades of research that has uncovered many genetic pathways affecting longevity, has used lifespan measurement for quantifying aging, meaning that an increase in lifespan is delayed aging and shortening of lifespan is accelerated aging.

Discovery of single gene mutations that cause lifespan extension or shortening has uncovered three major lifespan determination pathways.

Insulin-like / IGF-1 signaling (IIS) pathway

*daf-2*, the sole insulin like receptor in worms [65], was the first single gene mutant discovered, that doubled the lifespan of an organism [66]. Other downstream players in the pathway also affect lifespan [67-69]. Ligand binding activates DAF-2, which results in the activation of AGE-1, the phosphoinositide 3-kinase (PI3K) [70], which results in further activation of downstream
serine/threonine kinases (PDK-1, AKT-1, AKT-2) [71, 72] resulting in phosphorylation of DAF-16, a FOXO transcription factor, inhibiting its nuclear localization [72-74]. In *daf-2* mutant worms, in the absence of the downstream cascade events, failure to phosphorylate and thereby inactivate DAF-16 leads to nuclear translocation of DAF-16 and further transcription of target genes resulting in extended longevity. As expected, *daf-16* mutant worms are short-lived.

The insulin signaling pathway is well conserved across different species and lifespan extension effects have been documented in flies, mice and humans [75]. Flies heterozygous for *Drosophila* insulin/IGF-1 receptor, dInR, exhibit 85% lifespan extension [9]. Additionally, mutations in chico, the fly homolog of the insulin-receptor substrate, cause 48% increase in lifespan [9]. Mammalian homologs of the insulin-like growth factor-1 receptors *daf-2* and dInR modulate metabolism and growth and mutations in these genes are deleterious. Homozygous mutant mice for IGF-1 receptor (*igf1r*) are lethal but heterozygous *igf1r* mice live longer than wild-type littermates; females exhibit 33% lifespan extension and males exhibit 16% extension [76]. In dogs and humans, polymorphisms in IGF-1 or its receptor have been correlated with increased lifespan [77]. Variations in one of the mammalian homologs of DAF-16: FOXO3A have been shown to be associated with long-lived human populations [6, 8].

In *C. elegans*, age-dependent morphological alterations in touch receptor neurons and ventral nerve cord cholinergic axons are delayed in long-lived *daf-2* mutants [53-55]. Different studies in short-lived *daf-16* mutants find either
increased frequency of structural alterations or frequencies similar to the wild-type; therefore, the role of DAF-16 in wild-type age-dependent neuroanatomical alterations is still debated [53-55]. Similar to these results, the Hammarlund group found that age-dependent decline in axon regeneration upon severing GABA motor axons was suppressed in daf-2 mutants, whereas daf-16 mutants exhibited frequencies similar to wild-type [78]. Additionally, daf-2 mutants exhibit reduced rate of age-dependent motor activity decay, probably by slowing down the rate of functional decline in the motor neurons [56]. Some other behaviors that are known to decline with age in the wild-type were also better maintained in daf-2 animals such as chemotaxis, locomotion, isothermal tracking, associative learning and memory (associating cultivation temperature with food or starvation), and chemosensory learning (benzaldehyde and food association) [32, 47]. On the other hand, short-lived daf-16 mutants exhibit reduced isothermal tracking and suppress the increased isothermal tracking phenotype of daf-2 mutant worms [47]. Interestingly, long-lived daf-2 mutants show a strong defect in salt-chemotaxis learning suggesting that normal insulin signaling may be required for certain behaviors [79]. Additionally, age-dependent decline in dopamine and serotonin levels in daf-2 mutants is similar to those of wild-type [80]. This is also true for dopamine-mediated basal slowing response (BSR), where well-fed worms reduce their locomotion in the presence of bacteria, and serotonin-mediated enhanced slowing response (ESR), where food-deprived worms reduce their locomotion dramatically upon approaching the bacterial lawn.
Aged *daf-2* mutant worms fail to show BSR and ESR, similar to aged wild-type worms. These results suggest that long-lived *daf-2* mutants delay some age-related changes, but behave like wild type or worse for other age-related changes.

**Dietary Restriction Pathway**

Dietary restriction, or in simpler terms reducing the total food intake, has been shown to increase lifespan in diverse phyla from yeast to mammals [77, 81-84]. The term ‘caloric restriction’ has been used synonymously with dietary restriction, though caloric restriction involves reduction of calories without affecting the total nutritional value of the diet, often supplementing the food with extra nutrients such as vitamins and minerals to avoid malnutrition. Dietary restriction without malnutrition has been thought to slow down growth and thereby extend longevity [85]. Probably the most reproducible paradigm, dietary restriction that increases the lifespan of vertebrates is known to delay many age-associated changes that affect the physiological state. Rodents, one of the best-studied models for caloric restriction, exhibit a ‘delayed aging’ profile that includes about 300 biochemical, physiological and behavioral changes [86].

In spite of being the most unambiguous and reproducible model of lifespan extension, caloric restriction has faced the challenge of extending the treatment to human population. One of the earliest documented experimentations of caloric restriction in humans is by Luigi (Alvise) Cornaro, a 15th century
Venetian nobleman, who restricted his daily food intake starting at the age of 35 in hope to better his failing health and went onto live almost 100 years. Cynthia Kenyon is another living example of dietary restriction and has been following a low glycemic index diet that has helped her maintain an excellent blood profile with a low triglyceride level [87].

In hopes to better translate the caloric restriction research in lower vertebrates and invertebrates to humans, a 25 year long longitudinal study in non-human primates, Rhesus macaques *Macaca mulatta*, has shown that animals subjected to caloric restriction have reduced body weight, improved glucose homeostasis as well as a decrease in age-associated diseases such as diabetes, cancer and cardiovascular disease [88]. Additionally, caloric restriction also reduced age-associated brain atrophy, as measured by gray matter volume, in regions important for motor and executive functions [88]. This is similar to the attenuation of age-related cognitive decline upon caloric restriction observed in rats [89].

In the worm, the feeding-defective *eat* mutants, specifically *eat-2*, have been studied as a model of dietary restriction. About three decades ago, Klass had shown that reducing the bacterial availability causes a significant lifespan extension [37]. Study of *eat* mutants showed that the severity of eating defect, measured by the pumping rate of the worm, appears to correlate with lifespan; the slower the pumping rate, the longer the worm lives [84]. *EAT-2* encodes a ligand-gated nicotinic acetylcholine receptor subunit in the pharyngeal muscle.
that functions to regulate the rate of pharyngeal pumping [90]. About eight different dietary restriction regimes have been used in the worm that all extend lifespan, albeit to different degrees [91]. Three different regimes also appear to involve different genes to mediate longevity, although there is some overlap among these groups of genes namely the NAD-dependent deacetylase of the sir2 family, TOR signaling pathway, the Forkhead transcription factor FoxA/pha-4 and the Nrf2 transcription factor skn-1 (reviewed in [91]).

The C. elegans eat-2 mutants have significantly reduced pumping rates [92] and the worms appear pale and small [84]. Young eat-2 mutant worms exhibit increased associative thermotaxis behavior and maintain the increased consistency of isothermal tracking in old worms, suggesting a delay in age-related decline of thermotaxis learning behavior [47]. eat-2 mutants exhibit attenuation of age-dependent decline in dopamine (DA) and serotonin (5-HT) levels and the corresponding behaviors, basal slowing response (BSR: DA mediated) and enhanced slowing response (ESR: 5-HT mediated) [80]. In terms of food odorant association learning, eat-2 mutants exhibit normal learning and short-term memory similar to wild-type animals, but require longer training to achieve wild-type levels of long-term associative memory. Interestingly, eat-2 mutants are able to maintain both short-term and long-term memory with age [93]. Though eat-2 mutant worms exhibit attenuation of age-associated decline in many behaviors, they do not delay age-associated morphological changes in mechanosensory neurons [53-55].
Mitochondrial respiration pathway

Mitochondria, the power-houses of the cell, are indispensable in cellular metabolism. Though the mitochondrial genome encodes just a few genes important for mitochondrial function, a large number of genes are encoded by the genome of the cell that function in replication, transcription and maintenance of the mitochondrial genome as well as translation of mitochondrial proteins. Mitochondria are involved in a number of cellular processes such as biosynthesis of heme, lipids, and amino acids, as well as citric acid cycle, urea cycle, fatty acid oxidation and processes important for energy production.

Mitochondria are responsible for about 90% of cellular reactive oxygen species (ROS) generation, which is a consequence of oxidative phosphorylation that generates ATP. Initially, generation of reactive oxygen species was thought to be an accidental by-product, but studies have shown that reactive oxygen species may be generated to help many redox-dependent signaling processes (reviewed in [94]). According to the rate-of-living theory and the free radical theory of aging, mitochondria have been implicated in aging [95]. Mitochondrial integrity has been shown to decline with age [96], which is probably due to increased damage to the mitochondrial DNA [97]. Severe reduction in mitochondrial function appears to shorten lifespan and mice carrying mutations in the mitochondrial DNA polymerase, accumulate mutations in mitochondrial DNA and exhibit premature onset of aging-related phenotypes [98]. The nervous system appears to be particularly susceptible to defective mitochondrial function.
Many neurological and muscle related impairments are caused by deletions or mutations in mitochondrial DNA in humans and are known as mitochondrial encephalomyopathies [3].

A variety of mitochondrial mutations and their effect on lifespan has been studied and can be broadly divided into two groups: mutations that reduce lifespan probably by increasing the oxidative stress, for example gas-1 and mev-1; and mutations that increase lifespan probably by reducing mitochondrial oxidative stress, for example clk-1 and isp-1 [23]. CLK-1 encodes a hydroxylase in the electron transport chain required for ubiquinone biosynthesis [99]. clk-1 mutant worms have decreased mitochondrial function [100] and increased superoxide production potential compared to wild-type worms [101], but normal or even decreased oxidative damage levels, which is likely due to increased antioxidant defenses, since clk-1 mutants have increased mRNA levels of the superoxide dismutases, sod-2 and sod-3 [102].

*C. elegans* clk-1 mutants exhibit decreased rate of pharyngeal pumping and thrashing as well as slower defecation [103]. Interestingly, young clk-1 mutant worms exhibit increased isothermal tracking and increased learning ability upon repeated training for temperature association than wild-type worms [104]. Additionally, clk-1 mutants show thermotaxis behavior as young adults and maintain isothermal tracking consistency later in life, suggesting delay in age-related decline of thermotaxis learning behavior [47]. Age-dependent neuroanatomical alterations of mechanosensory neurons are delayed in *clk-1*
mutants [54]. Delay in age-related decline of morphological and functional changes in clk-1 mutants, suggests that learning and memory decline during aging may be influenced by mitochondrial dysfunction. Indeed, affecting mitochondrial localization and trafficking can have deleterious neurological consequences (reviewed in [105]).

In addition to these three major life span determinant pathways, the TOR signaling pathway, which has been implicated to act downstream of certain dietary restriction regimes in C. elegans, also influences lifespan. Lifespan extension by inhibition of TOR signaling is independent of the insulin/IGF-1 pathway [82]. Additionally, regulation of translation in somatic tissues has also been implicated in lifespan regulation; knockdown of ife-2, the C. elegans homolog of eukaryotic initiation factor eIF4E, a principal regulator of protein synthesis, increases lifespan [106, 107].

Interestingly, even though C. elegans lifespan appears to be regulated by different signaling pathways, these are not mutually exclusive. AMP kinase and daf-16 have been shown to be necessary for a specific dietary restriction regime induced lifespan extension [91]. Additionally eat-2; clk-1 double mutants exhibit lifespan similar to either of the single mutants, suggesting that eat-2 and clk-1 may act in the same longevity pathway. These results bring out the complexity of the aging and longevity processes highlighting the challenges faced by the
scientific community while trying to understand and ameliorate the effects of aging on the human population.
Neuronal maintenance mechanisms

Nervous systems in different organisms across species are well established by birth and the majority of neurons are born during embryogenesis. During the postnatal period, circuits develop, new neurons are added and the nervous system continues to mature. These changes continue for a long period after birth, sometimes even in adulthood and present a challenge to the maintenance of the nervous system. Additionally, the nervous system faces a number of challenges such as growth, movement, injury, aging, oxidative stress, and exposure to toxic compounds. The nerves in the arms and legs that reach up to the tip of the finger and toes need to increase their length without disrupting the existing connections as the arms and legs grow. The nervous system also experiences mechanical force from the movements of the different body parts as the organism performs voluntary functions like walking, playing, talking, etc. as well as involuntary functions such as breathing, beating of the heart, blood circulation and digestion. Maintenance of the nervous system is crucial for existence yet despite all the challenges, the nervous system structure and connectivity laid out at birth appears to be roughly maintained throughout life.

It is important that the neurons maintain their structural and functional properties as well as their position and connections with other neurons and cells. Decades of research has discovered mechanisms and factors across species involved in maintaining the cellular aspects of the neuron and integrity of its compartments – axons, dendrites and synapses that specifically affect processes
after the initial normal establishment of neurons and their features. For example, disruption of axonal transport leads to late-onset axon-degeneration in worms [108] and mice [109], fly warts mutants exhibit progressive loss of dendritic branches after initial normal dendritic tiling, mouse knockouts of the Abelson family kinases exhibit defects in dendritic arbor maintenance, and though human Down’s syndrome patients have fully formed and branched dendritic fields initially, gradual loss of dendritic branches is observed later (discussed in [110]). Additionally, some genes, whose function is important during embryogenesis, have been shown to play a role in neuronal maintenance later in life. Cyclin-dependent kinase 5 (Cdk5) has been shown to play a role in the maintenance of dendritic spines of hippocampal CA1 neurons in the adult mouse brain [111].

In *C. elegans* nervous systems a variety of studies have uncovered factors and mechanisms underlying the maintenance of neurons, axons, synapses as well as ganglia and axonal bundles. Well conserved molecules such as sax-7/L1CAM, EGL-15(5A), zig-3, zig-4, span-1, cima-1 and mec-17 as well as a worm-specific extracellular matrix protein *dig-1* [108, 110, 112-123], all contribute to nervous system maintenance. Mutations in most of the neuronal maintenance factors affect some but not other neuronal structures suggesting that neurons may have different requirements for neuronal maintenance. This specificity may underlie the susceptibility of neurons/neuronal structures to different stresses resulting in neuropathological conditions.
Neuronal maintenance factor, SAX-7

A well-studied neuronal maintenance molecule is sax-7, one of the worm homologs of human L1 cell adhesion molecule (L1CAM). SAX-7 encodes a transmembrane cell-adhesion molecule with extracellular immunoglobulin and fibronectin type III (FnIII) domains and an intracellular tail that has ankyrin, FERM domain and PDZ domain binding motifs [115, 119, 124]. Alternative splicing of sax-7 results in two isoforms solely differing in the number of immunoglobulin domains: SAX-7L (long, containing six immunoglobulin domains) and SAX-7S (short, containing four immunoglobulin domains) [119] (Fig. 1-2). sax-7 is ubiquitously expressed in the worm, as early as the two-cell stage in embryogenesis and localizes to plasma membranes of neuronal soma and processes [119].

Loss of function sax-7 mutants exhibit a variety of neuronal maintenance defects. Particularly, sax-7 mutants affect the amphid sensory neurons, many head interneurons, GABAergic, cholinergic and dopaminergic neurons and ventral nerve cord axons [115, 117-120]. sax-7 functions in the nervous system to maintain neuronal soma position and axonal positioning in the ventral nerve cord [115, 117]. Interestingly, the neuronal maintenance defects are induced by mechanical stress since suppression of the defects is seen upon genetic or pharmacologic immobilization [115, 118].

The conserved cytoplasmic tail of SAX-7 is required for the maintenance of neuronal positioning [115, 124]. The cytoplasmic tail contains three different
motifs, for FERM binding, ankyrin binding and PDZ binding respectively, that all contribute to sax-7 function in maintenance of GABA neuron position [124], but only ankyrin binding motif is required for maintaining the position of interneurons AIY and AVK [115]. Additionally, sax-7 is phosphorylated at the tyrosine residue in the ankyrin-binding motif by the worm FGF receptor and EGL-15 [119]. stn-2, a gamma-syntrophin belonging to class I PDZ proteins, interacts with sax-7 in yeast two hybrid assays and genetically in worms [124]. In addition to the nervous system, sax-7 has been shown to be required for the maintenance of other tissues such as pharynx and gonad [120, 125]. In these tissues, development occurs normally and defects become apparent post-embryogenesis or even later in life. Interestingly, the severity of maintenance phenotypes appears to vary in different mutant alleles, probably correlating with the nature of the mutation [120]. In addition to the maintenance defects, sax-7 mutants have some developmental defects. sax-7 mutants exhibit 40% embryonic lethality, probably due to defects in muscle attachment and/or morphogenesis [120], as well as defects in the development of the dendritic structure of the PVD neurons [126-128].

The mammalian homolog of sax-7, L1CAM, is a member of a subfamily (L1 subfamily) of cell surface glycoproteins that belongs to an immunoglobulin superfamily of cell adhesion molecules (CAMs). Other members of the L1 subfamily include CHL1, NrCAM and Neurofascin, Each of these proteins have
six immunoglobulin-like domains linked to five fibronectin type III (FnIII) domains extracellularly and a highly conserved intracellular tail [129]. Similar to C. elegans, mammalian L1CAM is expressed throughout the nervous system. Mutations in L1CAM have been shown to be responsible for X-linked recessive neurological disorder, CRASH: Corpus callosum hypoplasia, mental Retardation, Adducted thumbs, Spastic paraplegia and Hydrocephalus [129, 130].
**Mechanical stress**

Mechanical stress is an integral part of the organism’s life. Essential features of life such as movement of cells, rhythmic motion of organs and organ systems such as the heart, lungs and the digestive system as well as locomotion and movement of the limbs, inflict a lot of mechanical stress on the cells or tissues involved as well as structures around them. Mechanical stress plays an important role in shaping the formation of bones and joints throughout life, both prenatally as well as postnatally, and may influence the cartilage thickness in mature joints (reviewed in [131]). The importance of mechanical stress in the life of an organism is further substantiated by the fact that organisms across species have mechanosensors and mechanical stress induces downstream signaling in different cell types such as skeletal muscle and cardiac myocytes [132]. Additionally in humans, exercise has been shown to protect against age-related decline in cognition including working memory and processing speed, as well as improved cortical activation [133].

Chronic or acute increase in mechanical stress can have deleterious effects on the body, especially the nervous system. "Normal pressure hydrocephalus" is a condition in which a decrease in cerebrospinal fluid absorption causes an increased mechanical load on the brain resulting in brain damage [134]. Traumatic brain injury (TBI) is brain damage that results from external forces such as direct collision with any object or living animal, rapid acceleration and deceleration commonly occurring in a motor vehicle accident, a
penetrating object (a bullet, explosion debris) or blast waves from an explosion. TBI is major health and socioeconomic concern worldwide with incidents increasing every year. Sports athletes (football, soccer, boxing, ice-hockey etc.), motor vehicle accident victims and soldiers in military combat comprise the majority of TBI cases [135]. Moderate and severe TBI can cause primary damages such as bleeding, swelling, shearing of white matter and microhaemorrhage as well as secondary damage from inflammatory reactions, increase in calcium and free radicals and mitochondrial dysfunction [136, 137].

Mild TBI occurs in the absence of external signs of injury and comprises the majority of TBI cases. During TBI, high intracranial pressure leads to brain deformation. Rapid acceleration and deceleration of the brain that takes places during motor vehicle accidents can deform the brain, which can lead to pathological consequences to neurodegeneration [138]. TBI in early adult life has been associated with increased risk of Alzheimer’s disease [139], Parkinson’s diseases and amyotrophic lateral sclerosis (ALS) [140], as well as non-AD dementia.

It is apparent from a number of studies that active mechanisms are in place to counteract mechanical stress, since suppression of body locomotion suppresses the structural defects observed in the C. elegans neuronal maintenance mutants. This also hints at a possible role for mechanical stress in normal age-related changes.
My thesis work focuses on characterizing the anatomical changes in the *C. elegans* nervous system and the factors that may influence these alterations as age progresses. As a first step, we have characterized the neuroanatomical changes that take place in the nervous system during the lifespan of the worm. Described in chapter II, we find that *C. elegans* nervous system accumulates anatomical changes during adulthood in a number of neurons and neuronal structures. These anatomical changes include axonal bundle defasciculation, the nerve ring and head ganglia neuron displacement and neuronal acquisition of soma and axonal projections. These alterations are either absent, or present at a low frequency, in the juvenile worms. Additionally, different neurons exhibit different morphological changes. We find that the age of onset and maximum frequency of the alteration varies among the different neuronal populations analyzed suggesting underlying differential neuronal susceptibility. Most of the changes exhibit an age-related increase in their frequency. Are these changes dynamic? In other words, for instance, once displaced in the posterior direction, can the neuron displace back in the anterior direction? The McIntire group has shown that soma projections emanating from ALM neurons are transient and dynamic; the projections can retract over time [53]. We performed a longitudinal analysis of the placement of chemosensory head neurons, ASH and ASI in adult worms and found that the displacement is discontinuous. Once displaced in the posterior direction, the neuronal soma either moves further to a more posterior position, or in the anterior direction or remains in the same position over time.
Interestingly, this placement change did not affect the ASH-dependent chemoaversion behaviors, suggesting that the neuronal functionality is grossly intact.

We also address the question of whether lifespan extension or shortening intersect with the age-related neuroanatomical changes. Analysis of morphological changes in a variety of neurons in long-lived and short-lived mutants revealed that lifespan is not directly correlated with the occurrence or frequency of age-related neuroanatomical alterations. This suggests that the lifespan function of these genes can be uncoupled from their role in age-related structural changes in the nervous system.

Interestingly, we find that posterior displacement of the nerve ring and the head ganglia neurons is suppressed in feeding deficient eat-2 mutants. It is likely that reduced pharyngeal pumping or effects of dietary restriction may underlie the reduced posterior placement of head neuronal structures in eat-2 mutants. To further understand whether mechanical stress from pharyngeal pumping affects the neuronal placement in wild-type worms, we assessed the age-dependent change in neuronal placement by reducing the food availability. It is known that worms reduce their pharyngeal pumping rate in absence of food [141]. Upon subjecting wild-type worms to discontinuous feeding, we find that age-related displacement of head neuronal structures is suppressed, suggesting that mechanical stress from the movement of pharyngeal muscles may play a role in the posterior placement of the nerve ring and head neurons. On the other hand,
discontinuous feeding leads to increase in soma projections of the mechanosensory neurons ALM as well as novel soma projections in the mechanosensory neurons AVM and PVM. Since the mechanosensory neuronal cell bodies are placed posterior to the pharynx area, the soma projections may be a result of reduced food intake rather than mechanical stress from pumping.

Mechanical stress has been shown to play an important role in the defects observed in neuronal maintenance mutants, since genetic and pharmacologic immobilization suppresses the neuronal maintenance defects. Chapter III explores how reduced or increased mechanical stress affects the age-related neuroanatomical alterations. Paralysis by genetic or pharmacological means suppresses the age-related placement changes in the nerve ring and head neurons in wild-type worms. On the other hand, increased mechanical stress leads to increase in soma projections of mechanosensory neuron ALM, as well as novel soma projections in AVM and PVM mechanosensory neurons, but does not affect the soma placement of head neurons.

Neuronal maintenance mechanisms have been implicated in maintenance of the neuronal structures, post-developmentally yet rather early in life. Are these mechanisms also involved in the maintenance of the nervous system later in life? In chapter III, our analysis of neuroanatomical alterations in sax-7 neuronal maintenance mutants in adulthood reveal that in most cases, the defects are similar to the age-related alterations in the wild type but the age of onset in sax-7 mutants is much earlier, suggesting that these mutants may be showing signs of
accelerated neuronal aging. Further, overexpression of SAX-7 in the wild-type nervous system, leads to suppression of age-related placement changes in nerve ring and head neurons, suggesting that SAX-7 may play a role in protecting the nervous system during aging. Occurrence of morphological alterations may be due to reduction in SAX-7 levels with age. Indeed, western blot analysis of SAX-7 reveals a reduction in the protein levels of the adhesive isoform SAX-7S and increase in disruptive isoform SAX-7L with age.

Put together, these results suggest that the *C. elegans* nervous system exhibits age-related neuroanatomical alterations in a variety of neurons. This analysis may serve as a foundation for understanding the normal changes that take place in the nervous system during aging. Current understanding of neurodegenerative diseases is limited and neuronal changes in the initial phases of the diseases are not known leading to diagnosis of the disease much later. The available treatments are not adequately effective in curing the disease or halting its progression and diagnosis in the later stage of the disease adds to the problem. Further analysis of the structural and functional changes may allow for understanding early signs of neurodegeneration, leading to better diagnosis and more effective treatment.
Figure I-1. The *C. elegans* nervous system.

An adult worm expressing a fluorescent Prgef-1::gfp reporter in most neurons; major neuronal ensembles (head and tail ganglia) and neuropils (nerve ring, ventral and dorsal nerve cord) are seen.
Figure I-2. Schematic representation of SAX-7 protein isoforms.

Schematic representation of two isoforms of transmembrane protein SAX-7 with extracellular immunoglobulin and fibronectin domains as well as intracellular binding motifs to FERM domain, Ankyrin and PDZ domain. The two isoforms, SAX-7L and SAX-7S differ in the number of extracellular immunoglobulin (Ig) domains: SAX-7L has six Ig domains, whereas SAX-7S has four Ig domains.
At the time this thesis was written, this chapter was prepared for publication. Anagha Khandekar and Claire Bénard wrote this chapter. Mike Gorczyca helped with capturing the confocal images. Strain building was a lab effort especially Andrea Thackeray, Avery Fisher, and Rachael Mazzamurro; Hannah George and Akusika Aye-Addo helped with pharynx measurements.
Chapter II

Age-related neuronal changes are uncoupled from lifespan in *Caenorhabditis elegans*
ABSTRACT

Although the age-dependency of cognitive decline is well known, the mechanisms by which brain dysfunction is triggered during aging are poorly understood. *Caenorhabditis elegans* is an excellent model to uncover molecular mechanisms underlying neuronal aging; and its nervous system development, neuronal function, and lifespan are regulated by evolutionarily conserved mechanisms. The aging nervous system of the nematode displays subtle morphological changes during normal aging, comparable to the small structural changes that characterize the aging brain in healthy humans. Age-associated neuronal changes have been recently described at the cellular level for a limited set of neuron types of the nematode. However, a broad analysis of the age-related morphological changes at the level of neuronal ensembles and for multiple neuronal types is lacking. Also, whether the response of neurons to age is modulated by lifespan regulatory pathways remains unclear. Here, we systematically characterize the structural changes occurring across the nervous system in the normally aging *C. elegans*, and test whether lifespan-determining pathways are strong determinants of neuronal aging. Our analysis reveals that, with age, neuronal ensembles become disorganized and distinct cell-type specific morphological changes arise in many neuron types. Furthermore, many age-related neuronal changes appear by early and mid-adulthood, and are uncoupled from lifespan. Our findings support the notion that, like in mammals,
the nervous system of *C. elegans* undergoes age-associated changes, suggesting that there may be conserved mechanisms underlying the responses of neurons to age. Elucidating neuronal aging mechanisms in *C. elegans* may provide insights into the molecular bases of human brain aging and age-dependent neurodegenerative diseases.
INTRODUCTION

Aging precipitates alterations in the physiology of the nervous system, including an increased incidence of age-related cognitive decline and neurodegenerative diseases, such as Alzheimer's, Parkinson's and peripheral neuropathies, among others. At the neuroanatomical level, normal brain aging is characterized by subtle changes in the morphology of specific neurons in selective brain regions [2, 28]. For instance, there is increase in dendritic branching and length in some hippocampal regions in aged individuals compared to young adults, as well as varying changes in dendritic spine and synapse number of the aging neocortex and hippocampus [142, 143], reviewed in [2]. Whereas age is known to be a strong determinant of these conditions, the etiology and molecular mechanisms leading to natural age-related neural deterioration are poorly understood.

C. elegans is a genetically tractable organism ideally suited for elucidating the mechanisms of neuronal aging. Its nervous system is simple, composed of exactly 302 identified neurons in the adult [144]. Owing to the worm's transparency and the ability to label specific neuronal types with available reporters, its nervous system is examinable in detail at any point of their lives. The worm’s life cycle is fast: it becomes an adult in 3 days, produces its progeny in the following 3 days, and reaches senescence in about two weeks. Importantly, C. elegans has been a powerful and productive model in the
identification of conserved molecular pathways that regulate lifespan, and for the unraveling of conserved mechanisms of nervous system development and function.

Approaches to study the aging nervous system in C. elegans have only recently been initiated. Earlier work had shown an absence of neuronal cell death and gross deterioration during normal aging [34]. Recently, exciting studies have examined in detail aspects of the nervous system throughout the worm's lifespan. These studies have identified subtle morphological changes in a subset of neurons (mainly mechanosensory neurons, [53-55]), synaptic deterioration (reduced synaptic density) in some neurons [55], and functional decline of motor neurons [56]. This indicates that, like in humans, the nematode's nervous system undergoes age-related neuronal alterations. However, a systematic analysis of the age-related morphological changes across the nervous system and for diverse neuronal types is lacking. Also, the relationship between organismal and neuronal aging remains vastly unexplored. Whereas body aging (e.g., reduction in body size, deterioration of muscles and the germline, slowed locomotory capacity, pharyngeal pumping, isothermal tracking, lowered stress resistance) has been investigated in long-lived mutants [32, 47, 104, 145, 146], how the nervous system is impacted by lifespan extension has started to be addressed only recently [53-55]. Three analyses report the age-related changes of mechanosensory neurons in mutants that affect lifespan [53-55]. However, the
relationship between lifespan regulation and the aging of the nervous system remains unclear.

Here we report our systematic characterization of the morphological changes occurring in multiple neuronal types and structures as C. elegans age. We find that during normal aging neuronal ensembles such as ganglia and axon bundles become disorganized and distinct morphological changes arise across diverse neuron types, including abnormal neuronal cell morphologies and excessive neurites. Thus, our results suggest that in C. elegans, like in the human nervous system, distinct neuronal classes show differential susceptibility to the stresses of life. We also find that most age-related neuronal changes already occur by early and mid-adulthood, and that the way neurons respond to age is largely uncoupled from lifespan. Our results support the notion that, like in mammals, the nervous system of C. elegans undergoes age-associated changes, suggesting that there may be conserved mechanisms underlying the responses of neurons to age. Elucidating the mechanisms underlying neuronal aging in C. elegans may provide insights into the molecular bases of neuronal decline and diseases in humans, and may help identify strategies to ameliorate the effects of nervous system aging.
EXPERIMENTAL PROCEDURES

Nematode strains and genetics

Nematode cultures were maintained at 20°C on NGM plates seeded with OP50 bacteria as described [147]. Strains were constructed using standard genetic procedures and are listed in Table II-1. The \textit{daf-16(mu86)} genotype was confirmed by PCR using primers oCB613 gacgacgacaagacaggcgg and oCB615 gtgtcgagtgaaggagccc.

Neuroanatomical analysis

\textit{Staging of animals:} All nematode strains were continuously fed for more than 3 generations at 20°C before analysis. To obtain animals of given ages, advanced fourth larval stage worms (L4) were pooled onto plates and transferred onto fresh plates daily, away from their progeny. These worms that lived for 1, 2, 5, 10 or 20 days after the L4 stage are to be 1-, 2-, 5-, 10- or 20-day old adults, respectively. Animals examined at the advanced L4 stage, just a few hours before becoming adults, are juveniles (at day 0).

\textit{Microscopy:} One to 10 worms of precise ages (0-, 1-, 2-, 5-, 10- and 20-day old adults) were mounted on 5% agarose and immobilized in 10 mM Na azide or with a nanoparticle solution of 1 µL polystyrene beads in 7.5 µL M9 buffer [148].
Worms were examined immediately after mounting under a Ziess Axioscope. z-test was used to compare the populations for statistical significance.

**Fasciculation of the ventral nerve cord.** The right fascicle of the ventral nerve cord was visualized at 1000X using the Pglr-1::gfp reporter. A worm was considered to have a defasciculated ventral nerve cord when an axon separated away from the fascicle for at least 10 µm from the right fascicle at one or multiple sites.

**Fasciculation of the dorsal nerve cord.** The dorsal nerve cord was visualized at 1000X using the Prgef-1::gfp reporter. A worm was considered to have defasciculated dorsal nerve cord when an axon separated away from the fascicle for at least 10 µm at one or multiple sites.

**Position of the nerve ring.** The nerve ring was examined at 200X using the Pmec-4::gfp or the Psra-6::gfp reporters, as well as by staining worms with Dil [149]. The position of the nerve ring in the anteroposterior axis was recorded with respect to structures of the pharynx, which do not change with age. The pharynx is composed of two bulbs, one anterior, called “anterior bulb” and one posterior called “terminal bulb”, as well as an “isthmus” that connects the two pharyngeal bulbs. The position of the nerve ring was categorized as follows: #1 anterior location, located anywhere between the posterior edge of the anterior bulb and
up to 3/4<sup>th</sup> of the isthmus in the anterior-to-posterior direction, #2 intermediate location, located anywhere from 3/4<sup>th</sup> of the isthmus in the anterior-to-posterior direction up to the anterior edge of the terminal bulb, and #3 posterior location, located adjacent to or along the length of the terminal bulb. Graphs on nerve ring position show the percentage of nerve rings in categories #2 (white bars) and #3 (grey bars). The percentage of nerve rings in category #1 is not shown on the graphs for clarity, as it can be deduced from “100% minus % in categories #2 and #3” (e.g. of the wild-type nerve rings shown in Fig. 1C, ~60% at day 0, ~12% at day 1, and 0% at days 2, 5, 10 and 20, fall in category #1).

**CAN axon:** The axon of the CAN neurons (L and R) was examined at 400X using the *Pceh-10::gfp* reporter. Each CAN neuron has two projections that extend in opposite directions from the soma along the lateral side of the animal in the anteroposterior directions. Occasionally, the CAN axon has extra neurites or branches along the main axon, which we quantified. Other anatomical alterations such as irregular soma shape and blebbing along the axon were not included in our scoring.

**Soma projections.**

**ALMs:** The two ALM neurons (L and R) were examined at 400X using the *Pmec-4::gfp* reporter. An axon that extends anteriorly from the soma of ALM, and a short posterior projection is occasionally observed in young wild-type adults.
We examined the number of projections extending from the soma (other than the main anterior axon). Projections that became split into two were counted as one projection.

PLMs: The two PLM neurons (L and R) were examined at 200X using the \(P_{mec-4::gfp}\) reporter. The PLM neuron extends an axon anteriorly, as well as a short posterior projection of unknown function. In early adults, this short posterior projection is approximately 5-10 µm in length, estimated as the equivalent of 1 to 2 cell body diameters of the neuron PLM. This posterior projection lengthens with age, and was recorded as “long” when it reached the tip of the tail, which is more than 10 times as long as in early adults.

AVM: The AVM neuron was examined at 200X using the \(P_{mec-4::gfp}\) reporter. Other than its axon, the soma does not extend any projection in wild-type adults. We examined the number of neurons with one additional projection or more in our assays. The axon was not counted as a “projection”.

PVM: The PVM neuron was examined at 200X using the \(P_{mec-4::gfp}\) reporter. Other than its axon, the soma does not have any projection in wild-type adults. We examined the number of neurons with one or more additional projections in our assays.

**Position of neuronal soma.** The position of neurons was examined at 200X. Each soma was recorded as a separate data point and data is plotted as percentage of neurons in each category at any given age. Neuronal soma position was scored
in the anteroposterior axis with respect to the terminal bulb of the pharynx and
categorized as follows.

CEPs: The four CEP neurons (D, V, L, and R) were visualized using the
Pdat-1::gfp reporter. CEPs are initially located anterior to the terminal bulb, but
with age they become displaced posteriorly. The position of the CEP soma was
categorized as follows: #1 anterior, soma located anterior to the terminal bulb, #2
intermediate, soma located on the terminal bulb and anterior to the pharyngeal
grinder, and #3 posterior, soma located posterior to the grinder. Graphs on CEP
soma position show the percentage of CEPs in categories #2 (white bars) and #3
(grey bars). The percentage of CEPs in category #1 is not shown on the graphs
for clarity, as it can be deduced from “100% minus % in categories #2 and #3”.

ADEs: The two ADE neurons (L and R) were visualized using the Pdat-
1::gfp reporter. ADEs are located just posterior to the terminal bulb, but with age
they become displaced posteriorly. We recorded their posterior displacement in
terms of units of the length of the terminal bulb in the anteroposterior direction.
The position of the ADE soma was categorized as follows: #1 anterior, soma
located on the terminal bulb, #2 intermediate, soma located just posterior of the
terminal bulb (not more posterior than half the length of a terminal bulb), and #3
posterior, soma located even more posterior. Graphs on ADE soma position
show the percentage of ADEs in categories #2 (white bars) and #3 (grey bars).
The percentage of ADEs in category #1 is not shown on the graphs for clarity, as
it can be deduced from “100% minus % in categories #2 and #3”.

60
ASEs: The two ASE neurons (L and R) were visualized using the Pceh-36::rfp reporter. ASEs are located anterior to the grinder of the terminal bulb but with age become displaced posteriorly. The position of the ASE soma was categorized as: #1 anterior, soma located anterior to the grinder, and #2 posterior, soma located posterior to the grinder. Graphs on ASE soma position show the percentage of ASEs in categories #2 only. The percentage of ASEs in category #1 is not shown on the graphs for clarity, as it can be deduced from “100% minus % in categories #2”.

AIYs: The two AIY neurons (L and R) were visualized using the Pttx-3::gfp reporter. AIYs are located close to the posterior edge of the terminal bulb, but with age they become displaced posteriorly. The position of the AIY soma was categorized as follows: #1 anterior, soma located on the terminal bulb of the pharynx, #2 intermediate, soma located just posterior of the terminal bulb (not more posterior than half the length of a terminal bulb), and #3 posterior, soma located even more posterior. Graphs on AIY soma position show the percentage of AIYs in categories #2 (white bars) and #3 (grey bars). The percentage of AIYs in category #1 is not shown on the graphs for clarity, as it can be deduced from “100% minus % in categories #2 and #3”.

ASHs and ASIs: The two ASH neurons (L and R) and the two ASI neurons (L and R) were visualized using the Psra-6::gfp reporter. These four neurons are located anterior to the terminal bulb, but with age they become displaced posteriorly. The positions of the ASH and ASI soma were categorized as follows:
#1 anterior, soma located anterior to the terminal bulb, #2 intermediate, soma located on the terminal bulb, and #3 posterior, soma located posterior to the terminal bulb. Graphs on ASH and ASI soma position show the percentage of soma in categories #2 (white bars) and #3 (grey bars). The percentage of neurons in category #1 is not shown on the graphs for clarity, as it can be deduced from “100% minus % in categories #2 and #3”.

**Shape of neuronal soma of ASH and ASI**: The two ASH neurons (L and R) and the two ASI neurons (L and R) were visualized using the *Psra-6::gfp* reporter. The somas of each of these four neurons are typically oval during larva and early adulthood [150]. With increasing age, we observed that some of these somas could become irregularly shaped. Deviation from a regular oval shape because of the appearance of protrusions from the soma was scored as abnormal.

**Statistical analysis**: Neurons and neuronal structures exhibiting structural changes are plotted on the graphs. Error bars are standard error of proportion, calculated as: \( \sqrt{\left( \frac{\text{% structural change} \times (100-\text{% structural change})}{n} \right)} \). Statistical comparisons were done using z-test and P values obtained were corrected for multiple comparisons. In case of data comparisons, where data is represented by a single category on the graph (e.g. ventral nerve cord defasciculation), or when combined data shown by the white and grey bars together was compared statistical significance is denoted by *. When data shown by white bars was
compared, the statistical significance was denoted by §, and statistical significance of comparison between data shown by grey bars was denoted by #.

**Longitudinal analysis**

Worms were picked as L4s and placed individually on NGM plates. At days 1, 3, 5, 7 and 10 of adulthood, several behaviors were assayed for each worm in the following order: pharyngeal pumping, high salt avoidance and osmotic avoidance. 1 hr later, worms were mounted one by one on 5% agarose pads in the nanoparticle solution and were immediately examined for the position of ASH and ASI soma. Worms were immediately recovered from the microscopy pads by adding an excess of M9 buffer and then transferred onto a plate.

*Pharyngeal pumping rate.* Assays were carried out at 20°C using a stereoscope at 60X magnification. Worms were scored only when they remained on the bacterial lawn for the entire duration of the assay. The time taken for the worm to carry out 40 pharyngeal grinder pulses was recorded; this measurement was carried out 4 times for each worm.

*High salt avoidance assay.* Assay plates were prepared as follows: a slight depression was made on an unseeded plate using a glass tube of 1 cm in diameter, and 16 µL of the high salt solution (1 mL of 4M NaCl freshly mixed with 20 µL of 10 mg/mL Congo Red) [151] was added to this depression to make a ring. The plate was left to stand for 5 min on the bench top for the solution to be
absorbed. A worm was then transferred to the center of the ring and was observed continuously for 5 min to determine if it crossed the ring. *osm-10* and N2 worms were used as negative and positive controls, respectively [152].

**Osmotic avoidance assay.** Assays were carried out as for the high salt avoidance assay, except that the rings were made with a high osmotic solution (1 mL of 4M fructose was freshly mixed with 20 µL of 10 mg/mL Congo Red) [151].

**Analysis of ASH and ASI soma position.** The two ASH neurons (L and R) and the two ASI neurons (L and R) were visualized using the *Psra-6::gfp* reporter. These 4 neurons are located anterior to the terminal bulb, but with increasing age they become displaced posteriorly. The positions of the ASH and ASI neurons were categorized as follows: (1) located anterior of the terminal bulb, (2) located on the terminal bulb and anterior of the grinder, (3) located on the terminal bulb and posterior to the grinder, or (4) located posterior to the terminal bulb. Each soma was given a score depending on the category it belonged to [e.g. a soma in category (1) was assigned a score of 1, a soma in category (4) was assigned score of 4]. The 4 scores for each worm corresponding to the 4 neurons were averaged for each day.

**Discontinuous feeding**
Worms were subjected to 2 cycles of feeding and non-feeding over a 48 hr period. Pools of 25 L4 worms were transferred from plates with food (OP50 bacteria) to unseeded plates, and then onto plates with food, and then again
without food. In a first group, worms were fed for 16 hr, then not fed for 8 hr, then fed again for 16 hr, and finally not fed for 8 hr. In a second group, worms were fed for 8 hr, then not fed for 16 hr, then fed again for 8 hr, and finally not fed for 16 hr. In summary, out of the 48 hr period, the first group of worms spent a total of 32 hr in the presence of food, while the second spent only 16 hr in the presence of food. Worms that crawled up the sides of the plate were discarded. At the end of the 48 hr feeding/not-feeding cycles, worms were transferred onto food for 20 minutes and mounted onto 5% agarose pads in 10 mM Na azide. Worms were examined for the position of the nerve ring and of the neurons CEP and ADE, as well as for projections extending from the soma of AVM, PVM and ALM. Each experiment was repeated 2-4 times.

**Pharynx measurements**

Day 0, 1, 2, and 5 worms were mounted on 5% agarose, with 10 mM Na azide and immediately examined at 400X under a Ziess Axioscope. DIC images were captured and analyzed using ImageJ software. The length of the pharynx (distance between the tip of the nose and the pharyngeal grinder) and its parts were measured. The body length was measured in 3 segments: (1) distance from the tip of the nose to the grinder, (2) distance from the grinder to the vulva, and (3) distance from the vulva to the anus. The total length of the pharynx was expressed as a percentage of total body length (total of the lengths of the three segments).
RESULTS

Age-related neuroanatomical changes occur across the nervous system and start manifesting early in adulthood

As a first step towards understanding the mechanisms underlying the response of the nervous system to age, we characterized the neuroanatomical changes that occur in the normally aging *C. elegans* adult hermaphrodites. We measured several neuroanatomical parameters in wild-type worms as they age, including the morphology and position of neurons and the integrity of neuronal ensembles. We examined age-synchronized populations of juveniles (just few hours before becoming adults, named “day 0”) and adults at different ages (named “day 1”, “day 2”, “day 5”, “day 10”, and “day 20” of adulthood), using transgenic reporters that allow the visualization of specific neurons. Whereas the neuroanatomy of *C. elegans* is highly stereotyped, we found that the precise position and fine branching pattern of some neurons was variable among juvenile animals (e.g. the position of the cell bodies of motor neurons along the ventral nerve cord, the branching from the axon of the HSN motor neuron) and therefore, we did not analyze these structures. We focused on neuronal structures that were invariant among juvenile worms, which included chemosensory neurons (ASH, ASI, ASE), mechanosensory neurons (ALM, AVM, PLM, PVM), dopaminergic neurons (ADE, CEP, PDE), motor neurons, and interneurons (AIY, CAN, axons in ventral nerve cord). Any deviation from the juvenile state,
including altered morphology, defasciculated axons, deformed soma, and displaced neurons was recorded. We found that the overall neuronal morphology of all examined neurons, including their cell body and neuronal processes, was well preserved into old age, even at day 20 of adulthood, an age at which the majority (~90%) of adults have already senesced and died [153]. However, all of the neuronal structures and neurons examined manifested subtle morphological change with age, often arising early in adulthood and progressively becoming more frequent as the worms age, as described below.

**Defasciculation and displacement of major axon fascicles with age**

We found that the major axon fascicles of the worm’s nervous system preserve their overall architecture but become defasciculated with age. Using a reporter that labels interneurons (Pglr-1::gfp), we examined the right fascicle of the ventral nerve cord, which contains numerous motor neurons and interneuron processes that make *en passant* synapses [144]. Whereas this fascicle runs as a tight bundle in juvenile worms, we found that with age some axons separate from the bundle ([Fig. II-1A](#)). The frequency of defasciculation of the ventral nerve cord increases with age: half of the 1-day old adults show some degree of defasciculation, 80% of the 10-day old adults, and nearly all of the 20-day old animals ([Fig. II-1A](#)). Similarly, we found that the dorsal nerve cord, composed of axons of motor neurons and interneurons and located on the dorsal aspect of the worm [144], also becomes defasciculated in aged animals. We examined the
dorsal nerve cord using a pan-neuronal reporter (Prgef-1::gfp) and whereas it runs as a tight bundle in juvenile worms, as many as 35% of the 20-day old adult animals have a defasciculated dorsal nerve cord (Fig. II-1B). In addition, we noted that with age, both the ventral and dorsal nerve cords increasingly accumulate varicosities and blebs along the length of its constituent axons (illustrated in Fig. II-1A,B).

In addition to some axons losing their precise positioning along fascicles, the position of an entire fascicle can change with age. We examined the nerve ring, which is the major neuropil of C. elegans where 180 neuronal processes engage in numerous synaptic contacts [144], and found that it becomes posteriorly displaced during adulthood. We visualized the processes of the ALML, ALMR, and AVM neurons, whose axons run along the nerve ring, using the transgene Pmec-4::gfp. We examined the antero-posterior placement of the nerve ring and used anatomical hallmarks of the pharynx (i.e., the anterior bulb; the isthmus, which is located between the two pharyngeal bulbs; and the posterior bulb) to record the nerve ring placement. As a control, we measured the length of the pharynx, its isthmus, and bulbs, compared them to the body length at juvenile and adult ages, and found that the pharynx grows proportionately to the body as the worm ages (Fig. II-6A), rendering these hallmarks reliable for our analysis. In juvenile worms the nerve ring is placed along the isthmus of the pharynx, midway or towards the terminal bulb (Fig. II-1C), and by day 5, the
nerve ring becomes much more posteriorly located, including at the level of terminal bulb (grey bars in Fig. II-1C, see Experimental Procedures for scoring criteria). We observed a similar posterior displacement of the nerve ring when we examined other sets of axons that are part of it with another transgene (Psra-6::gfp, Fig. II-5A) or by labelling chemosensory neurons with a lipophilic dye (DiI, Fig. II-5B) [149]. We also observed that the neuronal processes composing the nerve ring become less tightly bound with age (data not shown).

**Ganglia disorganization with age**

The majority of the neurons in *C. elegans* have their somas located and organized in distinct ganglia of the head and the tail of the worm, where they hold stereotypical positions. We examined the position of several sets of head neurons, using a variety of reporters. We visualized the dopaminergic neurons CEP and ADE with transgene Pdat-1::gfp, the interneurons AIY with transgene Pttx-3::gfp, the chemosensory neurons ASE transgene Pceh-36::rfp, and ASH and ASI with transgene Psra-6::gfp. We recorded the antero-posterior position of neurons with respect to the pharynx, which does not change with age (Fig. II-6A), and found that neurons, constituent of the dorsal, lateral or ventral ganglion, each can become displaced posteriorly with age, with different frequencies and severity. For instance, the dopaminergic neurons CEP (D, V, L and R) are located anterior to the terminal bulb in juvenile worms, but become increasingly posteriorly located as worms age, such that an increasing number of animals
have their CEP neurons at the level of the terminal bulb with increasing age (white bars, Fig. II-2A), and reach positions posterior to the grinder of the pharynx by day 20 day (grey bar, Fig. II-2A, see Experimental Procedures for scoring criteria). Similarly, the dopaminergic neurons ADE (L and R), which are located just posterior to the terminal bulb in juvenile worms, become posteriorly located starting at day 1, and by day 5 and day 10, as many as 80% of the ADE neurons examined were found posterior to the pharynx (Fig. II-2A, see Experimental Procedures for scoring criteria). The chemosensory neurons ASE (L and R) are located anterior to the terminal bulb in juvenile worms and become posteriorly located at the level of the terminal bulb with age (Fig. II-2B, see Experimental Procedures for scoring criteria). Likewise, the interneurons AIY (L and R) are located in the posterior region of the terminal bulb in juvenile worms (white bars, Fig. II-2B), but become posterior to the terminal bulb with age (grey bars, Fig. II-2B, see Experimental Procedures for scoring criteria): already 11% of the AIY neurons are posterior to the terminal bulb in 1-day old adults and as many as 80% of AIY are that posterior in 5-day old adults. Finally, the chemosensory neurons ASH and ASI (L and R) are located anterior to the terminal bulb or near the anterior-most part of the terminal bulb in juvenile animals, but with age become posteriorly displaced to the level of the terminal bulb or beyond (Fig. II-2C, see Experimental Procedures for scoring criteria). We noted that the chemosensory neurons ASH and ASI displayed increasingly deformed soma shapes with age. In day 0, 1 and 2 animals, these chemosensory
neurons have a stereotypical oval shape (Fig. II-2D), and by day 5 of adulthood, approximately 5% of the neurons displayed very deformed soma that appear to have cytoplasmic extensions emanating from the main cell body (Fig. II-2D). It will be interesting to determine whether soma shape changes are a result of displacement or a separate phenomenon.

**Neurite sprouting and lengthening with age**

We examined the morphology of processes at the level of single neurons and found that the neurites of some neurons became lengthened in aged animals compared to juveniles, and in other neurons, new neurites grew out of the cell body or branched out of the axon. One example is that of the neuron CAN (L and R). Each CAN neuron lies laterally in the mid body region and extends a lateral processes in both the anterior and posterior directions along the length of the animal (Fig. II-1D). We found that new neurites branch out of the CAN axon in adults, with increased frequency as the worms age (only 10% of CAN neurons have branches in juveniles, whereas 20% of the CAN neurons display long neurites in 2-day old adults, and 65% by day 10 of adulthood) (Fig. II-1D).

Mechanosensory neurons also exhibit extra neurites with age. The soma of the mechanosensory neuron ALM (L and R) lies on the sub-dorsal aspect of the animal and has one neuronal process in the anterior direction. We found that whereas most juvenile animals do not display a posterior projection (only 12% of
the ALM neurons have a short posterior neurite in juveniles), the proportion of animals with a posterior projection extending from the ALM soma increases with age. At day 1 and day 2 of adulthood already approximately 40% of the ALM neurons have posterior projection. By day 5 and onwards, more than 70% of the ALM neurons have a prominent posterior projection, and approximately 10% display two posterior projections (Fig. II-1E).

The processes of the mechanosensory neuron PLM (L and R) also change with age. The soma of each of the PLM neurons is located in the lumbar ganglion in the tail and sends an axon anteriorly along the side of the animal, and a short posterior projection. Whereas the posterior projection of PLM is short in juvenile worms, we found that at day 10 and day 20 of adulthood, nearly half of PLM neurons display very long posterior projections that reach the tip of the tail (Fig. II-1F, see Experimental Procedures). These long projections are never seen in 2- or 5-day old adults.

*Discontinuous change in soma placement in individual worms*

To characterize the evolution of neuroanatomical change in individual worms over time, we carried out a longitudinal analysis of individual animals over a period of 10 days (on days 1, 3, 5, 7 and 10 of adulthood). For each worm, we measured their performance in behavioral assays, examined the position of the chemosensory neurons ASHL, ASHR, ASIL and ASIR, and immediately...
recovered the worm to be able to continue the analysis on future days (see Experimental Procedures). Thus, we followed the position of the chemosensory neurons in each worm and plotted the average position of the 4 neurons on the antero-posterior axis over time (Fig. II-3A). As before, we found that the soma position of these neurons tends to become posterior with age (Fig. II-3A,B); however, within a given worm, the displacement is discontinuous and varies over time (Fig. 3A). Indeed, only in worms #1 and #2 (Fig. II-3A) do the neurons get steadily more posterior. In worms #3-6, the neurons get posterior initially (between days 1 and 3), but then maintain a stable position thereafter. For worms #7-10, the position of the neurons is initially stable (between days 1 and 3), but then becomes more posterior. In worms #10-13, the neurons get displaced posteriorly but then shift anteriorly, before appearing more stable for a few days or getting displaced posteriorly again.

We asked whether the position of the chemosensory neurons would correlate with the pharyngeal pumping rate of the animals. Pharyngeal pumping is known to slow down on average in aging animals [40]. We thus wondered if this pumping variability could be a factor contributing to the changes in position of the neurons, since these neurons are located right near the pharynx and might be under variable internal pressure from the pharyngeal pumping movements. We found that the speed of pharyngeal pumping, as measured on the day of the
neuroanatomical observation, did not correlate with the changes in position of the chemosensory neurons (Fig. II-3A).

To get a first indication of the relationship of neuronal placement and function, we tested the functionality of the chemosensory neurons ASH over time. The ASH neurons are nociceptors and mediate avoidance behavior from noxious stimuli (reviewed in [150, 154]). We assayed for avoidance behavior to high salt (4M NaCl) and to high osmolarity (4M fructose) in these 13 worms. We found that all worms responded normally to the high salt and fructose concentration at all ages tested (Fig. II-3A). Thus, despite the changes in the position of the ASH neurons, they appear to remain functional enough to respond to strong stimuli at days 7 and 10 of adulthood. Neuronal activity as measured by calcium transients decreases in ASH by day 5 [155], indicating that some aspects of neuronal function might be affected even if undetected in our assays.

**Age-related neuroanatomical alterations are uncoupled from lifespan**

To test the hypothesis that the lifespan regulatory pathways are also determinants of the neuronal response to aging, we examined how mutations in major lifespan pathways affect neuronal aging. Three major conserved lifespan determination pathways are: (1) insulin signaling, represented by the very long-lived mutants *daf-2(e1370)* [66], (*daf-2* is a homolog of the insulin IGF-1 receptor [65]) and by the short-lived mutants *daf-16(mu86)* (*daf-16* is a FOXO-
transcription factor downstream of the *daf-2* insulin signaling pathway [73, 83]; (2) caloric restriction, represented by the long-lived mutants *eat-2(ad465)* [84] (*eat-2* encodes a subunit of a nicotinic acetylcholine receptor that functions in the pharynx for food pumping [90], and *eat-2* loss of function is a genetic model of dietary restriction as it causes the worms to pump slower and reduce their food intake, leading to a moderate increase in lifespan [84]); and (3) mitochondrial respiration, represented by the moderately long-lived mutants *clk-1(qm30)* [153] (*clk-1* codes for the ubiquinone biosynthesis enzyme, an ubiquinone is an electron carrier in the mitochondrial electron transport chain [99]). We built strains of each of the four lifespan mutations with neuronal reporters and quantified the neuroanatomical changes at days 0, 5, 10, and 20 of adulthood in wild-type animals and these lifespan extension mutants.

*Nerve cord defasciculation is uncoupled from lifespan*

If the responses of neurons to age were under the control of lifespan regulatory pathways, then one could expect that the appearance of age-related neuroanatomical changes might be delayed in long-lived mutants, and manifest earlier in short-lived mutants. However, we found this not to be the case as both long-lived and short-lived mutants exhibited most of the age-related morphological changes at similar ages and frequencies as in wild-type animals. For instance, the ventral and dorsal nerve cords became defasciculated in the long-lived mutants *eat-2*, *clk-1*, and *daf-2* at a similar age and in a comparable
manner as in wild-type worms. If anything, the defasciculation of the ventral nerve cords was accelerated in *clk-1* and *daf-2* mutants as the proportion of affected animals was higher than in the wild type at a young age (day 0, **Fig. II-4A**). Likewise, at day 20, the dorsal nerve cords of *clk-1* and *daf-2* animals were more defasciculated than wild-type animals (**Fig. II-4B**). Our results indicate that lengthening lifespan does not delay or reduce age-related nerve cord defasciculation.

A comparable situation was found with the short-lived *daf-16* mutants, for which the ventral nerve cord remained better fasciculated than in age-matched wild-type adults (day 10, **Fig. II-4A**). The dorsal nerve cord of *daf-16* mutants had the same frequency of defasciculation as in age-matched wild-type animals (day 0 and day 10, **Fig. II-4B**). This suggests that a mutation that shortens lifespan does not accelerate the appearance of age-related nerve cord defasciculation.

*Neurite sprouting and lengthening is uncoupled from lifespan*

Likewise, we observed that the age-related increase in the number of neurites projecting from the mechanosensory neurons ALM in the long-lived mutants *eat-2*, *clk-1*, and *daf-2*, and the short-lived mutant *daf-16*, was very similar to that of wild-type animals of the same age (**Fig. II-4C**). Again, far from delaying or reducing this age-related neurite increase, the life extending mutation *daf-2* led to more extra neurites than in age-matched wild-type adults (at day 10,
daf-2 mutants grow neurites more frequently than wild type, Fig. II-4C). The ALM neurites of daf-16 mutants also grew at similar frequencies than in wild-type animals (Fig. II-4C).

The age-related lengthening of the posterior projection of the mechanosensory neuron PLM (Fig. II-1F) displayed different behavior among the lifespan mutants. Like in age-matched wild-type animals, the PLM posterior projection lengthens in the long-lived mutants clk-1 and daf-2 (day 10, Fig. II-4D), and actually lengthens even more frequently in clk-1 mutants at age day 20 compared to wild-type animals. However, the PLM projection fails to lengthen altogether in both the long-lived eat-2 and short-lived daf-16 mutant adults (Fig. II-4D). Thus, with the exception of the PLM projection lengthening being suppressed in eat-2 mutants, we again found that increased lifespan does not simply delay the age-related neurite changes, and that shortened lifespan does not accelerate the age-related neurite changes.

Posterior displacement of the nerve ring and head neurons is uncoupled from lifespan, and is suppressed in eat-2 mutants

We examined the position of the nerve ring and neurons in the lifespan mutants eat-2, clk-1, daf-2 and daf-16. In general, we found that long-lived and short-lived mutant animals all exhibit age-related posterior displacement of the nerve ring, the dopaminergic neurons CEP and ADE, and the chemosensory
neurons ASH and ASI (Fig. II-5). This posterior displacement occurs with the same frequency and degree as in wild-type animals in the daf-2 and daf-16 mutants. In clk-1 mutants, the degree of displacement was either similar or more pronounced than in the wild type. Notably, the nerve ring and the soma of the ADE, ASH and ASI neurons in clk-1 mutants were more posterior than in age-matched wild-type animals. On the other hand, we found that, while the long-lived mutants eat-2 do display age-related posterior displacement of the nerve ring and head neurons, the degree and frequency of this displacement is largely suppressed. Indeed, by visualizing the nerve ring with a fluorescence reporter (Psra-6::gfp, Fig. II-5A) or by staining amphid chemosensory neurons with a lipophilic dye (Dil staining, Fig. II-5B) at days 5, 10 or 20, we found the nerve ring in eat-2 mutants retains an anterior position compared to age-matched wild-type animals. We made a similar observation with the position of the neurons CEP, ADE, ASH or ASI in the eat-2 mutants, which we found to be only mildly displaced posteriorly with age (Fig. II-5C-E). Together, our results show that extending lifespan per se does not delay the posterior displacement of the nerve ring and head neurons, quite the opposite, and that shortening lifespan does not trigger the earlier appearance of age-related neuroanatomical changes.

It is interesting that the long-lived mutants eat-2, show a different progression than other lifespan mutants and the wild-type, in that several of the age-related neuroanatomical changes are delayed and even suppressed. eat-2
mutants are somewhat smaller than wild-type animals, as a consequence of their being calorically restricted [156]. We measured the length of the pharynx with respect to the whole body in eat-2, as well as in the other lifespan mutants, compared to wild-type animals. We found that the pharynx size is proportionately similar to the body across the genotypes in juvenile and adults (Fig. II-6B), suggesting that the distinct progression of age-related neuroanatomical changes in eat-2 mutants is not due to pharynx size differences.

**Reduced pharyngeal pumping by limiting food availability suppresses some of the age-related neuronal changes**

A striking characteristic of eat-2 mutants is that the rate of pumping of their pharynx is severely reduced compared to the wild type [157] and to other lifespan extension mutants that also reduce the rate of pharyngeal pumping, namely clk-1 mutants [100, 103]. To explore the possibility that pharyngeal pumping may contribute to the structural changes observed in the nervous system during adulthood, such as the posterior displacement of neuronal structures that are located near the pharynx, we reduced the pharyngeal pumping in wild-type animals. For this, we took advantage of the fact that the pharyngeal pumping of worms is suppressed in absence of food [141]. Thus, we limited the availability of food for wild-type animals and asked whether some of the age-related neuroanatomical changes would be delayed, similar to what occurs in eat-2 mutants.
We limited the availability of food by transferring worms from culture plates with food to plates devoid of food at different time points over a 48 hr-period, starting at the juvenile stage (day 0) and ending at day 2 of adulthood (Fig. II-7A). In a first group, worms were allowed to feed normally on plates with food for 16 hr, transferred to plates without food for 8 hr, transferred back onto plates with food for 16 hr, and finally deprived of food again for 8 hr (totaling two 8 hr periods when food was absent). In a second group, worms were allowed to feed normally on plates with food for 8 hr, transferred to plates without food for 16 hr, transferred back onto plates with food for 8 hr, and finally deprived of food again for 16 hr (totaling two 16 hr periods when food was absent). In the control group, worms were continuously in the presence of food and pumping. We examined the position of the nerve ring and of the head neurons CEP and ADE in these three groups of worms and found that the posterior displacement of the nerve ring and the head neurons that normally takes place between day 0 and day 2 was partially suppressed in animals that had limited food availability and thus periods of reduced pharyngeal pumping (Fig. II-7B-D). Furthermore, the degree of suppression was stronger in the group that experienced more time without food to pump. This result suggests that reduced pharyngeal pumping correlates with delayed age-related displacement of neuroanatomical structures located near the pharynx. Interestingly, subtle reduction in pharyngeal pumping does not have any effect on the age-related displacement of head neurons, since cca-1
mutants do not suppress age-related displacement of dopaminergic head neurons ADE (Fig. II-8). cca-1 encodes a calcium channel alpha subunit and mutations in cca-1 lead to 25% reduction in pharyngeal pumping, whereas pharyngeal pumping rates are reduced by 75% in eat-2 mutants [158].

We also analyzed the effect of limited food availability on the morphology of neurons located away from the pharynx. We examined the mechanosensory neurons ALM, AVM, PVM and PLM. We found that, under conditions of limited food, the neurons AVM and PVM manifested novel morphological changes that are never seen under standard conditions as they grew new neurites (Fig. II-7F,G), which are normally absent throughout lifespan. On the other hand, the neuron ALM, which usually does acquire extra neurites during aging, displayed a subtle increase in the number of neurites stemming from its soma with limited food availability (Fig. II-7E). The length of the neuron PLM remained unchanged (data not shown) under limited food availability. Thus, limiting food availability suppresses the normal age-related posterior displacement of the nerve ring and head neurons, but had different effects on neurons located away from the head region, some of which responded by developing more neurites. These observations highlight the neuron-specific responses to different stressors of life, and indicate that the effects of eat-2 on neuroanatomy are possibly due to a combination of both mechanical stress and nutritional state of the animals.
DISCUSSION

Distinct structural changes occur across the nervous system throughout adulthood

Our analysis of normally aging C. elegans reveals distinct structural changes that arise across the nervous system with age. Age-related neuronal changes that we observed include disorganization of ganglia, defasciculation of axonal tracts, altered morphology of soma, neurite elongation, and growth of new neurites from soma and axons. Our results are in agreement with recent studies focused on mechanosensory neurons, which exhibit soma shape changes, ectopic neurites from the soma and processes, and GFP beading and blebbing along the neuronal processes [53-55, 159]. We extend the analysis of the aging nervous system to multiple neuron types (chemosensory neurons, mechanosensory neurons, interneurons, dopaminergic neurons) and to neuronal ensembles (nerve cord, neuropil and head ganglia), and find that the vast majority of neurons and structures display morphological changes over time.

Another conclusion from our study is that the age-related neuroanatomical changes are remarkably selective: subsets of neurons exhibit a specific set of structural alterations that are highly reproducible among worms. On a related note, it is interesting that while, the age-related neuroanatomical changes are
highly neuron-specific, their age of onset and frequency vary even among isogenic clonal populations of worms grown contemporarily on the same culture plate. This indicates that in addition to strong neuron-specific determinants (cell-intrinsic and -extrinsic) leading to characteristic morphological changes, stochastic factors likely influence the probability of neurons to manifest a morphological change. Stochastic differences have also been described for worms’ locomotion capacity and onset of sarcopenia [34]. The basis of selective neuronal susceptibility to certain disorders is largely unknown [160], and studying the molecular mechanisms underlying neuron-specific responses to age in *C. elegans* may help unravel principles of differential susceptibility of neurons in healthy aging and disease conditions.

Consistent with previous findings, we find that the integrity of neurons, both their somas and processes, stay intact during aging [34, 53-55]. We extend this observation to very old animals (20-day old), an age when over 90% of wild-type worm population has died under the standard growth conditions [66, 161], where we find the general organization of the nervous system architecture and the integrity of individual neurons to persist. Even when a neuronal soma is severely displaced (e.g. ADE neuron), the overall morphology of the neuron is maintained and the neuronal process remains whole, indicating that the process must extend along its length. The absence of neuronal death and the occurrence of subtle neuron-type specific morphological changes with age in *C. elegans*
parallel the neuronal changes occurring in the aging human brain [2, 3, 28],
suggesting shared mechanisms underlying age-related neuronal decline.

A common theme emerging from our analysis is that while age-associated
neuronal changes accumulate progressively and become more common among
worms of advanced age, many changes start taking place early in adulthood. For
instance, we observed defasciculation of the ventral nerve cord and displacement
of the soma of neurons (dopaminergic, chemosensory and interneurons) as early
as in days 1 and 2 of adulthood, when worms are at their most vigorous and
healthy phase of adulthood and at the peak of their reproductive period. We also
documented de novo neurite outgrowth out of the mechanosensory neuron ALM
(the second posterior neurite from the ALM soma) and branching out of the axon
of the CAN neuron as early as day 5 of adulthood, when the worms are still in
their self-fertilizing reproductive period and no age-related death occurs yet in the
population (on average, worms start dying at day 9 or 10). Our results confirm and
extend the observation that outgrowth from neuronal somas and branching from
axons can occur in early adulthood (at day 4 for ALM neurites from soma, [55]; at
day 5 and 9 for branches from neurons and axons, [53-55]). Notably, motor
neurons show functional decline with decreased synaptic transmission as early as
day 5 of adulthood [56]. Thus, changes in the nervous system start occurring early
in adulthood (with different age of onset for distinct neurons and parameters),
accumulate over time and consequently become more frequent with age.
The extension of neurites (e.g. lengthened posterior projection of PLM) and outgrowth of extra neurites (e.g. ALM, CAN) reveal a capacity of neurons to grow during adulthood, early in some neuron types or later in others. Whether these extra or lengthened neurites become functional is unknown, and the age-associated neurites that we observed extend mostly into regions devoid of possible synaptic contacts. Perhaps the propensity of neurons to sprout neurites results from altered levels of neuronal activity or of adhesiveness to their cellular environment, among other factors. Extra neurite outgrowth has been observed in the context of altered neuronal activity [162] and defective nerve attachment during development, in mutants of the extracellular matrix, can result in extra neurite sprouting later in adulthood [53, 159]. One could speculate that mechanisms that function to keep polarized growth in check during embryonic and larval development might become increasingly deregulated with age. It will be interesting to determine whether the age-related structural changes are the result of deregulated growth, an adaptive response to changes in adjacent tissues or in adhesiveness, or deterioration, all of which could be related.

It is unclear how these age-related neuroanatomical changes may impact neuronal function. At least in the context of behavioral assays in the lush laboratory conditions, the subtle morphological alterations observed do not lead to the incapacity of the neural circuits to perform in response to strong stimuli.
Indeed, we found that the ASH chemosensory neurons, while displaced from their juvenile position, are able to reliably mediate avoidance response to strong noxious stimuli. Similarly, mechanosensation is not impaired by the morphological changes of mechanosensory neurons [55]. Examination of the electrophysiological properties of neurons, however, reveals age-related functional decline of motor neurons [56, 163], and the Ca2+ transients in the chemosensory neuron ASH, decrease by day 5 [155]. Importantly, synaptic deterioration has been documented in the ventral nerve cord and the ventral ganglion of old worms (day 15), and correlates with decreased locomotory capacity [55]. It will be key to determine how specific morphological alterations occurring in the aging nervous system influence neuronal activity, synaptic transmission and function of neural circuits, and whether these are a cause or a consequence of functional decline.

**Lifespan is not a strong determinant of neuronal aging**

Our analysis of a number of neuroanatomical features using lifespan mutants indicates that the pathways regulating lifespan do not regulate the state of the aging nervous system in a simple pattern. One could have predicted that the age-related changes in the nervous system may be delayed upon lifespan extension, and that they may occur earlier in shorter-lived worms. However, we found that lifespan extension in *daf-2*, *clk-1* or *eat-2* mutants does not postpone age-related neuroanatomical changes, and that lifespan shortening in *daf-16*
mutants does not lead to early occurrence of age-related neuroanatomical changes. Complex relationships between lifespan modulation and the age of onset of specific neuronal changes emerges from previous studies as well [53-55]. For instance, age-related changes in the mechanosensory neurons that are delayed in long-lived daf-2 and clk-1 mutants are not delayed in long-lived eat-2 mutants [53, 54]. Notably, we found that loss of eat-2 function potently delays the age-related changes in another mechanosensory neuron (PLM). Our results reinforce the notion that specific longevity pathways have different effects on the aging neuronal morphology and have neuron-specific impact. The lifespan regulatory roles of daf-2 and daf-16 have also been shown to be separable from their function in axon regeneration [78]. Moreover, the aging state of the body also bears complex interactions with the lifespan pathways and extended lifespan does not correlate with extended healthspan [145].

It appears that, maximum lifespan is a poor predictor of how different tissues are aging. In these lifespan pathway mutants, change in the lifespan is just one aspect, whereas there are many other changes such as gene expression changes that may affect multiple tissues in a complex manner to bring about the structural and behavioral changes. I believe that the molecular identity of the mutants and the downstream changes that take place due to downregulation of the genes ,daf-2, clk-1 or daf-16, or systemic changes due to food deprivation (eat-2), may underlie the complex patterns of structural and behavioral changes, rather than the lifespan extension or shortening.
Mechanical stress might contribute to age-related neuroanatomical changes

A number of neuronal maintenance mutants have been identified whose neuronal architecture becomes disorganized over time (e.g. sax-7, dig-1, zig-3, zig-4; reviewed in [110]. When locomotion in these mutants is inhibited by paralysis, the neuronal maintenance defects that would normally accumulate are strongly suppressed, indicating that the mechanical stress from body movements may contribute to disorganization of the nervous system in these mutants [112, 114-118]. Axonal degeneration in mec-17 mutants, which are defective in microtubule stability, can also be suppressed by paralysis [108]. It is possible that the mechanical stress exerted on the nervous system by body movements is a contributing factor for the manifestation of age-related structural changes in wild-type animals as well. In agreement with this idea, we find that the frequency of age-related morphological changes of a number of neuronal structures is strongly reduced in eat-2 mutants. eat-2 mutants exhibit drastically reduced pharyngeal pumping rate than wild-type animals [157]. We found that all the neurons located near the pharynx retain youthful morphologies and organization even in old worms. This finding suggests that mechanical stress generated by normal pharyngeal pumping may contribute to shifting neurons to more posterior positions and lead to ganglion disorganization.
To further test the idea that mechanical stress may contribute to age-related neuroanatomical changes, we limited pharyngeal pumping by controlling the food available to the worms, and found that neuronal displacements in the head region were reduced. However, mutants that have mildly reduced rates of pharyngeal pumping such as \textit{clk-1} (Fig. II-5, [103]) and \textit{cca-1} (Fig. II-8, [158]) have wild-type rates of head neuronal displacements with age, indicating that only a severe reduction in the pharyngeal pumping can have an impact on maintaining neuronal organization. Alternatively, the strong suppression of neuronal displacement observed in \textit{eat-2} might be due to a combined action of reduced mechanical stress associated with pharyngeal pumping and the physiological effects of caloric restriction. Caloric restriction is known to decrease and delay the incidence of age-associated disease states across species [77, 81-84].

**Commonalities between \textit{C. elegans} and human neuronal aging**

The findings described above provide evidence that, like in mammals, the \textit{C. elegans} nervous system undergoes age-dependent morphological alterations. Neuronal loss is not a characteristic of the human brain aging in healthy adults, or of the normally aging nematode. Rather, nervous system aging is accompanied by subtle neuron-specific morphological alterations (e.g. increased dendrite length and reduced dendritic spines) in selective regions of the human brain [2, 3, 28], and by small neuron-type specific changes (increased length of
neuronal processes, sprouting, defasciculation and ganglia disorganization) in the worm. The *C. elegans* nervous system is much simpler and shorter lived, but these parallels between the age-related neuronal changes in nematodes and humans are intriguing. Given the evolutionary conservation of the molecular machineries regulating nervous system development and function, and more, elucidating the mechanisms underlying the neuronal responses to aging in *C. elegans* may help uncover general principles of neuronal aging and have implications in the development of strategies to ameliorate the consequences of the brain aging in humans.
Figure II-1. Defasciculation and neurite outgrowth in wild-type animals as they age.

Schematics depict the structures examined; red rectangles indicate the area in the picture.

A. Defasciculation of the ventral nerve cord with age. The right fascicle of ventral nerve cord, visualized with reporter \textit{Pglr-1::gfp}, is a tight bundle in juvenile worms.
(day 0) and it becomes defasciculated (arrows) as the worms age. Ventral nerve cord defasciculation start occurring in early adulthood and becomes more frequent with age.

B. Defasciculation of the dorsal nerve cord with age. The dorsal nerve cord, visualized with reporter Prgef-1::gfp, is a tight bundle in juvenile worms (day 0) and becomes defasciculated (arrows) with age.

C. Change in the position of the nerve ring with age. The nerve ring was visualized using the reporter Pmec-4::gfp. The nerve ring (arrows) is located anterior to the terminal bulb (dotted circle) of the pharynx in juvenile worms (day 0), and becomes posteriorly located as the worms age, to frequently reach the level of the terminal bulb. Such posterior placement of the nerve ring becomes increasingly frequent with age.

D. Branching of the axon of the neuron CAN with age. Each CAN soma has two projections that run laterally, one in the anterior direction and the other in the posterior direction. In older worms, CAN axons exhibit new branches (arrow). These axonal alterations become increasingly frequent with age.

E. Ectopic neurites on the neuron ALM with age. Each ALM neuron has one neuronal process oriented anteriorly, and in some of the juvenile worms (day 0), a short projection extends posteriorly from the soma. With age, the frequency at which the ALM soma exhibits one or two additional posterior projections (arrows) increases.
**F.** Neurite lengthening on the neuron PLM with age. The PLM soma extends one main neuronal process anteriorly and a short posterior neurite or projection in juvenile worms (day 0). In older worms, the posterior projection of PLM becomes very long (arrows).

Scale bar, 5 μm. Error bars are standard error of the proportion. Asterisks denote a significant difference between data when the graph shows only one category or when data combined from the categories shown by the white and grey bars is compared. When the data was classified into two categories, a significant difference between white bars is indicated by the ‘§’ symbol and a significant difference between grey bars is indicated by the ‘#’ symbol. ***, ###, §§§ for $P \leq 0.001$, ** for $P \leq 0.01$ and * for $P \leq 0.05$ (z-tests, P values are corrected for multiple comparisons). ns, not significant.
Figure II-2. Displacement of neurons in wild-type animals as they age.
Schematics depict the structures examined; red rectangles indicate the area in the picture.

**A.** Posterior displacement of the neurons CEP and ADE with age. In juvenile worms (day 0), the cell bodies of the 4 CEP neurons (L, R, D and V; white arrows) are located anterior to the terminal bulb (dotted circle) of the pharynx and those of the 2 ADE neurons (L and R, yellow arrows) are located just posterior to the terminal bulb (dotted circle). In older worms, CEP and ADE neurons become posteriorly located.

**B.** Posterior displacement of the neurons ASE and AIY with age. In juvenile worms (day 0), the ASE neurons (L and R, white arrows) are located anterior to the terminal bulb of the pharynx (dotted circle) and the AIY neurons (L and R, yellow arrows) are located near the terminal bulb (dotted circle). In older worms, the ASE and AIY neurons become posteriorly located.

**C.** Posterior displacement of the neurons ASH and ASI with age. In juvenile worms (day 0), ASH and ASI neurons (arrows) are located anterior to the terminal bulb of the pharynx (dotted circle). In older worms, the neurons ASH and ASI become posteriorly located, at the level of the terminal bulb of the pharynx (white bars) or even posterior to the grinder of the pharynx (gray bars).

**D.** Neuronal soma shape changes with age. The soma of the ASH and ASI neurons is oval in juveniles and most adults, but becomes misshapen with age.

Scale bar, 5 μm. Error bars are standard error of the proportion. When data is categorized into a single category, significant difference is indicated by *. When
the data was classified into two categories, a significant difference between white bars is indicated by the ‘§’ symbol and a significant difference between grey bars is indicated by the ‘#’ symbol. When data from the categories shown by the white and grey bars together is compared, significant difference is indicated by *. **, ###, §§§ for $P \leq 0.001$, ** for $P \leq 0.01$ and * for $P \leq 0.05$ (z-tests, P values are corrected for multiple comparisons). ns, not significant.
Figure II-3. Longitudinal analysis of neuronal placement and behaviors in individual worms.
A. Analysis of neuronal position and behaviors for 13 individual worms followed over several days. Each one of the 13 lines in the graph corresponds to an individual worm. The position of the cell bodies of the neurons ASHL, ASHR, ASIL and ASIR was recorded and their averaged position along the anteroposterior axis of the pharynx (drawn along the Y axis) is represented by a black dot. Overall, the neurons become more posteriorly located with age in each worm, but the displacements occur at different ages and in a discontinuous manner. Several behaviors were measured for each the 13 worms. The rate of pharyngeal pumping on a given day (indicated by the area of the square) tends to slow down with age. Worms successfully avoid the sensory stimuli of high salt (4M NaCl) and high osmolarity (4M fructose) even as they aged (indicated by “√”). Old worms that stopped moving, pumping food and defecating are indicated by “§”. The ages at which posterior displacements of the neurons occur did not correlate with the rate of pharyngeal pumping on those days.

B. Data of (A) plotted as a population (group of 13 worms, n = 52, 52, 51, 40, and 24 neurons on days 1, 3, 5, 7, and 10, respectively). In older worms, the neurons ASH and ASI become posteriorly displaced.
Figure II-4. Age-associated defasciculation and neurite outgrowth in lifespan mutants.

**A.** Defasciculation of the ventral nerve cord with age. Like in wild-type animals, the ventral nerve cord of the long-lived mutants *eat-2*, *clk-1* and *daf-2* becomes defasciculated with age.

**B.** Defasciculation of the dorsal nerve cord with age. The dorsal nerve cord of the long-lived mutants *eat-2*, *clk-1* and *daf-2* and the short-lived mutant *daf-16* becomes defasciculated with age. Rather than postponing age-associated phenotypes, the long-lived mutants *clk-1* and *daf-2* have even more
defasciculated dorsal nerve cord compared to age-matched wild-type animals (day 20).

C. Ectopic neurites on the neuron ALM with age. Similar to wild type, the soma of ALM frequently exhibits additional projections with age in the long-lived mutants *eat-2, clk-1* and *daf-2*, and the short-lived mutant *daf-16*. Rather than postponing age-associated phenotypes, the frequency of ALM soma projections is higher in *daf-2* mutants than age-matched wild-type animals (day 10).

D. Neurite lengthening on the neuron PLM with age. Similar to wild type, in the long-lived mutants *clk-1* and *daf-2*, the posterior projection of PLM becomes long in the older adults. Adults of the long-lived mutant *eat-2* or the short-lived mutant *daf-16* do not have long posterior projection of PLM.

Error bars are standard error of the proportion. When data is categorized into a single category or when data from the categories shown by the white and grey bars together is compared, significant difference is indicated by *. **P ≤ 0.01**, ***P ≤ 0.001*** (z-tests, P values are corrected for multiple comparisons). ns, not significant.
Figure II-5. Age-associated neuronal displacements in lifespan mutants.

A

Psra-6::gfp

WT eat-2 clk-1 daf-2 daf-16

% Nerve Ring in different Posterior Positions

Days of adulthood

B

Dil staining

WT eat-2 clk-1 daf-2 daf-16

% Nerve ring in different Posterior Positions

Days of adulthood

C

WT eat-2 clk-1 daf-2 daf-16

% CEP Soma in different Positions

Days of adulthood

D

WT eat-2 clk-1 daf-2 daf-16

% ADE Soma in different Positions

Days of adulthood

E

WT eat-2 clk-1 daf-2 daf-16

% ADE/AGL Soma in different Positions

Days of adulthood
A. Change in the position of the nerve ring with age (nerve ring visualized using the reporter P$sra-6::gfp$). Similar to the wild type, in the long-lived mutants $clk-1$ and $daf-2$ and in the short-lived mutant $daf-16$, the nerve ring becomes posteriorly located with age. Compared to wild type, the nerve ring is located less posteriorly in the long-lived mutant $eat-2$, and more posteriorly in the long-lived mutants $clk-1$ and $daf-2$ and short-lived mutant $daf-16$.

B. Change in the position of the nerve ring with age (nerve ring visualized by Dil staining of amphid neurons). Like in the wild type, the nerve ring location becomes more posterior with age in lifespan mutants, but to different degrees. Compared to the wild type, the posterior displacement of the nerve ring is less frequent in the long-lived mutant $eat-2$ and more frequent in the long-lived mutant $clk-1$ and short-lived mutant $daf-16$.

C. Posterior displacement of the neurons CEP with age. Like in the wild type, in the long-lived mutants $clk-1$ and $daf-2$ and in the short-lived mutant $daf-16$, the neurons CEP become located posteriorly with age, but not in the long-lived mutant $eat-2$.

D. Posterior displacement of the neurons ADE with age. Similar to the wild type, the neurons ADE become posteriorly located in the long-lived mutants $clk-1$ and $daf-2$ (albeit more severely) and in the short-lived mutant $daf-16$, but much less in the long-lived mutant $eat-2$.

E. Posterior displacement of the neurons ASH and ASI with age. As observed in the wild type, in the long-lived mutants $eat-2$, $clk-1$ and $daf-2$ and in the short-
lived mutant daf-16, the neurons ASH and ASI become posteriorly located with age. The posterior location of the neurons ASH and ASI is more pronounced in clk-1 mutants and less pronounced in eat-2 mutants, compared to the wild type. Error bars are standard error of the proportion. When the data was classified into two categories, a significant difference between white bars is indicated by the ‘§’ symbol and a significant difference between grey bars is indicated by the ‘#’ symbol. ***, ###, §§§ for $P \leq 0.001$; ** for $P \leq 0.01$; and * for $P \leq 0.05$ (z-tests, P values are corrected for multiple comparisons). ns, not significant.
Figure II-6. The pharynx grows proportionately to the rest of the body in the wild type and lifespan mutants.

A. Measurement of the pharynx at days 0, 1, 2, and 5 in the wild type. The pharynx grows proportionately to the rest of the body in the wild type.

B. Measurement of the pharynx of lifespan mutants in juvenile worms (day 0) and 5-day old adults. As observed in the wild type, the pharynx of the lifespan mutants eat-2, clk-1, daf-2 and daf-16 grows proportionate to the rest of the body.

Error bars are standard error of the proportion.
Figure II-7. Neuroanatomical changes upon limiting food availability.
A. Experimental design for discontinuous feeding. Juvenile worms (day 0) were first put on plates seeded with food (bacteria), then transferred onto plates without food, then back onto plates with food, and so on, at regular intervals. Neuroanatomical aspects of these worms were examined 48 hr later, when the animals were 2-day old adults.

B. Change in the position of the nerve ring upon discontinuous feeding. The nerve ring was visualized using the reporter *Pmec-4::gfp*. While the nerve ring becomes posteriorly located in 2-day old animals that are continuously fed, this posterior localization is suppressed under discontinuous feeding conditions.

C. Changes in the position of the neurons CEP upon discontinuous feeding. While the neurons CEP become located posteriorly in 2-day old adults that are continuously fed, this posterior localization is suppressed under discontinuous feeding conditions.

D. Changes in the position of the neurons ADE upon discontinuous feeding. While the neurons ADE become posteriorly located in 2-day old animals that are continuously fed, this posterior localization is suppressed under discontinuous feeding conditions.

E. Alterations of the ALM neurons under discontinuous feeding conditions. While the ALM soma acquires projections in 2-day old adults that are continuously fed, the frequency of these projections increases under some discontinuous feeding conditions.
F. Alterations of the AVM neurons under discontinuous feeding conditions. The AVM soma acquires projections in animals that are discontinuously fed.

G. Alterations of the PVM neuron under discontinuous feeding conditions. The PVM soma acquires projections in animals that are discontinuously fed.

Error bars are standard error of the proportion. When data is categorized into a single category, significant difference is indicated by *. When the data was classified into two categories, a significant difference between white bars is indicated by the ‘§’ symbol and a significant difference between grey bars is indicated by the ‘#’ symbol. When data from the categories shown by the white and grey bars together is compared, significant difference is indicated by *. ***, ###, §§§ for $P \leq 0.001$, ** for $P \leq 0.01$ and * for $P \leq 0.05$ (z-tests, P values are corrected for multiple comparisons). ns, not significant.
Figure II-8. Posterior displacement of the neurons ADE in *cca-1* mutants with age.

Similar to the wild type, the neurons ADE become posteriorly located in *cca-1* mutants at day 5 of adulthood.

Error bars are standard error of the proportion. ns, not significant (z-tests).
Table II-1. List of strains used.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Transgene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK4005</td>
<td>zdIs4 IV</td>
<td>Pmec-4::gfp; lin-15(+)</td>
<td>Gift from Scott Clark [164]</td>
</tr>
<tr>
<td>BY200</td>
<td>vtIs1 V</td>
<td>Pdat-1::gfp; rol-6</td>
<td>[165]</td>
</tr>
<tr>
<td>VQ43</td>
<td>oyls14 V; 6X outcross of PY1058</td>
<td>Psra-6::gfp; lin-15(+)</td>
<td>This study, [166]</td>
</tr>
<tr>
<td>VH15</td>
<td>rhls4 III</td>
<td>Pglr-1::gfp; dpy-20(+)</td>
<td>[167]</td>
</tr>
<tr>
<td>NW1229</td>
<td>evls111</td>
<td>Prgef-1::gfp; dpy-20(+)</td>
<td>[168]</td>
</tr>
<tr>
<td>LE311</td>
<td>lqls4</td>
<td>Pceh-10::gfp; lin-15(+)</td>
<td>[169-171]</td>
</tr>
<tr>
<td>VQ448</td>
<td>oyls264 III; mglsls18 IV</td>
<td>Pceh-36::rfp; Ptx-3::gfp</td>
<td>This study</td>
</tr>
<tr>
<td>VQ140</td>
<td>eat-2(ad465) II; rhls4 III</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ155</td>
<td>eat-2(ad465) II; evls111</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ86</td>
<td>eat-2(ad465) II; zdls4 IV</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ152</td>
<td>eat-2(ad465) II; vtIs1 V</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ120</td>
<td>eat-2(ad465) II; oyls14 V</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ141</td>
<td>clk-1(qm30) III rhls4 III</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ70</td>
<td>clk-1(qm30) III; evls111</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ83</td>
<td>clk-1(qm30) III; zdls4 IV</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ136</td>
<td>clk-1(qm30) III; vtIs1 V</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ112</td>
<td>clk-1(qm30) III; oyls14 V</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ127</td>
<td>daf-2(e1370) III rhls4 III</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ78</td>
<td>daf-2(e1370) III; evls111</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ72</td>
<td>daf-2(e1370) III; zdls4 IV</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ50</td>
<td>daf-2(e1370) III; vtIs1 V</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>OH7247</td>
<td>daf-2(e1370) III; oyls14 V</td>
<td></td>
<td>Claire Bénard</td>
</tr>
<tr>
<td>VQ210</td>
<td>daf-16(mu86) I; rhls4 III</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ185</td>
<td>daf-16(mu86) I; evls111</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ186</td>
<td>daf-16(mu86) I; zdls4 IV</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ232</td>
<td>daf-16(mu86) I; vtIs1 V</td>
<td></td>
<td>This study</td>
</tr>
</tbody>
</table>
VQ174  

daf-16(mu86) I; oyls14 V

This study
Table II-2. Sample sizes for neurons and neuronal structures examined

<table>
<thead>
<tr>
<th>Age</th>
<th>Neuronal structure examined</th>
<th>VNC</th>
<th>DNC</th>
<th>Nerve Ring</th>
<th>CAN</th>
<th>ALM</th>
<th>PLM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. II-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td></td>
<td>33</td>
<td>36</td>
<td>53</td>
<td>69</td>
<td>90</td>
<td>92</td>
</tr>
<tr>
<td>1-day old adult</td>
<td></td>
<td>36</td>
<td>55</td>
<td>75</td>
<td>54</td>
<td>87</td>
<td>44</td>
</tr>
<tr>
<td>2-day old adult</td>
<td></td>
<td>93</td>
<td>75</td>
<td>54</td>
<td>61</td>
<td>87</td>
<td>44</td>
</tr>
<tr>
<td>5-day old adult</td>
<td></td>
<td>103</td>
<td>54</td>
<td>65</td>
<td>61</td>
<td>130</td>
<td>128</td>
</tr>
<tr>
<td>10-day old adult</td>
<td></td>
<td>46</td>
<td>53</td>
<td>50</td>
<td>83</td>
<td>94</td>
<td>102</td>
</tr>
<tr>
<td>20-day old adult</td>
<td></td>
<td>27</td>
<td>46</td>
<td>29</td>
<td>48</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Fig. II-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td></td>
<td>696</td>
<td>348</td>
<td>140</td>
<td>138</td>
<td>147</td>
<td>703</td>
</tr>
<tr>
<td>1-day old adult</td>
<td></td>
<td>428</td>
<td>100</td>
<td>110</td>
<td>110</td>
<td>147</td>
<td>283</td>
</tr>
<tr>
<td>2-day old adult</td>
<td></td>
<td>560</td>
<td>278</td>
<td>130</td>
<td>130</td>
<td>147</td>
<td>198</td>
</tr>
<tr>
<td>5-day old adult</td>
<td></td>
<td>412</td>
<td>155</td>
<td>88</td>
<td>88</td>
<td>524</td>
<td>1795</td>
</tr>
<tr>
<td>10-day old adult</td>
<td></td>
<td>391</td>
<td>189</td>
<td>112</td>
<td>112</td>
<td>538</td>
<td>174</td>
</tr>
<tr>
<td>20-day old adult</td>
<td></td>
<td>116</td>
<td>56</td>
<td>56</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fig. II-4A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td></td>
<td>33</td>
<td>27</td>
<td>36</td>
<td>28</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>10-day old adult</td>
<td></td>
<td>46</td>
<td>46</td>
<td>39</td>
<td>31</td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td>20-day old adult</td>
<td></td>
<td>27</td>
<td>26</td>
<td>37</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fig. II-4B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td></td>
<td>36</td>
<td>28</td>
<td>24</td>
<td>27</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>10-day old adult</td>
<td></td>
<td>53</td>
<td>38</td>
<td>41</td>
<td>48</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>20-day old adult</td>
<td></td>
<td>46</td>
<td>21</td>
<td>30</td>
<td>42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fig. II-4C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td></td>
<td>90</td>
<td>54</td>
<td>59</td>
<td>84</td>
<td>61</td>
<td>61</td>
</tr>
<tr>
<td>10-day old adult</td>
<td></td>
<td>94</td>
<td>53</td>
<td>87</td>
<td>57</td>
<td>66</td>
<td>66</td>
</tr>
<tr>
<td>20-day old adult</td>
<td></td>
<td>48</td>
<td>68</td>
<td>66</td>
<td>84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fig. II-4D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td></td>
<td>92</td>
<td>58</td>
<td>60</td>
<td>58</td>
<td>55</td>
<td>55</td>
</tr>
<tr>
<td>10-day old adult</td>
<td></td>
<td>102</td>
<td>58</td>
<td>86</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>20-day old adult</td>
<td></td>
<td>56</td>
<td>68</td>
<td>74</td>
<td>94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fig. II-5A</td>
<td>Nerve ring – Psra-6::gfp</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>--------------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>eat-2</td>
<td>clk-1</td>
<td>daf-2</td>
<td>daf-16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td>43</td>
<td>29</td>
<td>49</td>
<td>96</td>
<td>45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-day old adult</td>
<td>136</td>
<td>68</td>
<td>54</td>
<td>120</td>
<td>41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-day old adult</td>
<td>19</td>
<td>29</td>
<td>34</td>
<td>102</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fig. II-5B</th>
<th>Nerve ring - Dil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td>59</td>
</tr>
<tr>
<td>5-day old adult</td>
<td>151</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fig. II-5C</th>
<th>CEP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td>696</td>
</tr>
<tr>
<td>10-day old adult</td>
<td>391</td>
</tr>
<tr>
<td>20-day old adult</td>
<td>116</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fig. II-5D</th>
<th>ADE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td>348</td>
</tr>
<tr>
<td>10-day old adult</td>
<td>189</td>
</tr>
<tr>
<td>20-day old adult</td>
<td>56</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fig. II-5E</th>
<th>ASH/ASI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td>147</td>
</tr>
<tr>
<td>10-day old adult</td>
<td>538</td>
</tr>
<tr>
<td>20-day old adult</td>
<td>154</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fig. II-6</th>
<th>Pharynx</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td>14</td>
</tr>
<tr>
<td>1-day old adult</td>
<td>12</td>
</tr>
<tr>
<td>2-day old adult</td>
<td>12</td>
</tr>
<tr>
<td>5-day old adult</td>
<td>15</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fig. II-7</th>
<th>Nerve ring</th>
<th>CEP</th>
<th>ADE</th>
<th>ALM</th>
<th>AVM</th>
<th>PVM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td>60</td>
<td>264</td>
<td>132</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-day old adult</td>
<td>48</td>
<td>360</td>
<td>180</td>
<td>91</td>
<td>46</td>
<td>43</td>
</tr>
<tr>
<td>Discontinued feeding 8 hr</td>
<td>78</td>
<td>452</td>
<td>226</td>
<td>74</td>
<td>63</td>
<td>61</td>
</tr>
<tr>
<td>Discontinued feeding 16 hr</td>
<td>66</td>
<td>352</td>
<td>176</td>
<td>76</td>
<td>74</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>ADE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>cca-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td>348</td>
<td>110</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-day old adult</td>
<td>155</td>
<td>86</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
At the time this thesis was written, this chapter was prepared for publication. Anagha Khandekar and Claire Bénard wrote this chapter. Mike Gorczyca helped with capturing the confocal images. Avery Fisher and Hannah George helped with building strains, Andrea Thackeray and Lauren Goodale helped with transgenics, Jessica Acosta and Thao Tran helped with behavioural experiments (pumping and thrashing).
Chapter III

SAX-7 acts as a neuroprotective molecule to counteract mechanical stress from locomotion
ABSTRACT

Brain decline and the high incidence of neuropathological conditions such as Alzheimer’s and Parkinson’s disease are known to be associated with old age and injury from accidents and high impact sports. The mechanisms by which aging and mechanical stress trigger these conditions are largely unknown. The nervous system of *C. elegans* is an ideal model to study the impact of age and of locomotive wear and tear on neuronal structure and function. SAX-7 is a key molecule required to maintain the precise positioning of neurons and axons in *C. elegans* during maturation and early adulthood. It is homologous to the L1CAM family of cell adhesion molecules in mammals where, in addition to its developmental roles, it is required in the brain of adults to preserve cognitive abilities. Here, we analyze the functional requirements of *sax-7* in maintaining the organization of the neuronal ensembles in adults across the nervous system, and find that loss of function of *sax-7* results in accelerated neuronal deterioration that is qualitatively comparable to that in normally aging adults. We show that SAX-7 protein is present at high levels during adulthood, and that increasing its levels in otherwise wild-type animals confers protection of the neuronal organization. Moreover, we provide evidence that locomotive movements are a trigger of age-dependent nervous system disorganization. Elucidating the mechanisms underlying the response of neurons to age and mechanical stress will have implications to ameliorate functional decline in age- and injury-associated neurodegenerative conditions.
INTRODUCTION

Aging leads to cognitive and neurological decline as well as an increased incidence of neurodegenerative diseases. Traumatic brain injury in contact sport athletes, motor-vehicle accident victims and soldiers in military combat, alters brain function and increases the risk of developing neurodegenerative diseases such as Alzheimer’s, Parkinson’s, neuropathies, as well as dementia [137], conditions usually associated with advanced age in healthy individuals. In humans, activities that subject the brain to mechanical stresses (e.g., boxing and football) dramatically increase susceptibility to age-related degenerative diseases, such as Alzheimer’s disease [139]. In children, the shaken baby syndrome leads to a number of abnormalities of speech, vision, behavior and cognition [172]. Whereas many neuronal alterations associated with neurodegeneration and normal aging are different, normal aging and neurodegenerative disorders share a number of characteristics, such as the neurofibrillary tangles that occur in older non-demented and demented humans in anterior olfactory nucleus, para-hippocampal gyrus and hippocampus [4]. Cellular and molecular responses of neurons to aging and mechanical stress are being actively investigated in a number of systems with the hope of understanding why age and brain injury act as risk factors and to help develop strategies to ameliorate human life with these conditions.

C. elegans is an excellent system to identify molecular mechanisms underlying neuronal responses to stresses associated with aging and mechanical
stress. The hermaphrodite nervous system has been completely mapped, including its 302 neurons connected by 7000 synapses and 600 gap junctions [144]. Its neuroanatomy can be examined in detail, even in live worms, owing to the transparent body of the worm and the numerous available fluorescent reporters that allow visualization of neurons at single cell resolution. The worm is genetically tractable, which combined with advanced techniques for activating or repressing neuronal activity, provides great opportunities to interrogate the processes underlying neuronal morphology and function. The nervous system of *C. elegans* undergoes age-dependent decline and the neuronal aging consequences at the level of neuronal morphology are comparable to the structural changes that take place in the human brain. Indeed, in healthy human adults, while no neuronal loss is observed, aging brain exhibits altered neuronal morphology including lengthened dendrites, change in dendritic spine and synaptic density [2, 3, 28]. Similarly normally aging nematodes do not exhibit neurodegeneration, but rather display outgrowth and extension of neurites, reduced synaptic density and function and disorganization of neuronal ensembles ([53-56], Chapter II).

Several studies in the worm have elucidated the role of the nervous system in lifespan determination [50, 51, 173]. When mitochondrial function is decreased in the entire nervous system, lifespan is extended, via unknown mechanisms that trigger mitochondrial stress response in non-neuronal tissues [52]. Additionally, lowering insulin signaling in all neurons can extend lifespan
Ablation of amphid sensory neurons can increase lifespan, suggesting that the interaction of the nervous system with the environment plays an important role in the worm’s life [50]. The nervous system also interacts with lifespan determination pathways in lifespan extension; two sensory neurons, the ASIs, are required for lifespan extension in response to caloric restriction [51]. These findings suggest that the nervous system regulates lifespan, and that specific subset of neurons perform this function. However, age-related changes in the nervous system are not simply coupled to lifespan (Chapter II, [53-55]), and the effect of lifespan determination pathways on age-related neuronal features may vary for different neurons. Prior studies have suggested that extending lifespan delays the age-related structural and functional changes of neurons, though mutation in the caloric restriction pathway that extend lifespan, failed to delay such neuronal changes [53]. Interestingly, lifespan extension mediated by the insulin-signaling pathway has been shown to be separable from its function in age-related structural changes in mechanosensory neurons as well as in axon regeneration [54, 78]. This suggests that mechanisms other than lifespan determination may underlie age-related neuronal changes.

Mechanisms that maintain the nervous system after initial development of neuronal architecture have been identified in *C. elegans*. The nervous system faces a number of challenges after the initial establishment, such as maturation of circuits, addition of new neurons, growth of the body, and mechanical stress from body movements throughout life, and active maintenance is required to
preserve the neuronal organization (reviewed in [110]). A number of genes have been implicated in neuronal maintenance and mutations in these molecules result in loss of the precise positioning of neurons and axons post development. Defects are specific to neurons, as other tissues (skin, muscles, intestine, and pharynx) remain normal, as determined by using tissue reporters and electron microscopy [112, 114]. In addition, rather than exhibiting general disorganization of the neuronal structures, the defects in these mutants are specific to particular neurons. For instance, (1) the axon of PVQ becomes displaced with age, while other axons of the ventral nerve cord remain normal [114-116]; (2) certain cell bodies of a ganglion get displaced (PVQ in the tail ganglion) while the other soma in the same ganglion (PHA, PHB) remain unaffected (C. Bénard, unpub, [175]). Molecules implicated in the process of neuronal maintenance, include members of the immunoglobulin super family (SAX-7, DIG-1, ZIG-3, ZIG-4, EGL-15) as well as CIMA-1, a homolog of SLC17 family of transporters [112-117, 119-122]. Many of these molecules are conserved in humans, providing a proof of principle for the use of the worm as a model system to understand the molecular mechanisms underlying neuronal aging.

One of the neuronal maintenance factors in the worm is the homolog of the mammalian L1 family of cell adhesion molecules, SAX-7. L1CAM is a transmembrane cell-adhesion molecule, and in humans, congenital disorders in the L1CAM gene lead to severe neurological disorders, including mental retardation (CRASH syndrome) [176, 177]. Moreover, depletion of L1CAM
specifically in the brains of adult mice renders them unable to learn \[177\], indicating that the function of L1CAM is crucial in the adult mammalian brain to maintain normal neuronal function. In worms, sax-7 is required for proper neuronal development and functions in neurons to maintain neuronal organization during larval and adult life [115, 118, 120]. SAX-7 is composed of an extracellular region containing six immunoglobulin domains and five fibronectin III domains, a transmembrane domain, and an intracellular tail containing ankyrin, FERM domain and PDZ domain binding motifs. sax-7 is ubiquitously expressed and required for maintaining the position of many head ganglia neurons including ASH, ASI, AFD, AWB, AWC, RIA and AIY, the ventral nerve cord axons such as PVP, PVQ and RMEV and commissures of the motor neurons, as well as, for maintaining the direct cell contact between AIY and AVK neuronal soma, but is dispensable for the maintenance of some neuronal structures such as position of AVK axons in the ventral nerve cord [115, 117-120]. Additionally sax-7 is required in the neurons for neuronal maintenance [115].

Previous findings suggest that the mechanical stress associated with body movements is a major trigger of neuronal disorganization in the neuronal maintenance mutants, and support the notion that the neuronal maintenance genes function to counteract the effects of such mechanical stress. The defects in these mutants manifest only if the animals are locomoting normally, as paralysis suppresses the defects [112, 114-118]. For instance, displacement of chemosensory neurons ASH and ASI as well as the axon flip over defect of
ventral nerve cord axons observed in sax-7 mutants can be suppressed by paralysis [115, 117]. Whether, mechanical stress from locomotion may also contribute to the age-related neuroanatomical changes in the wild-type remains to be investigated.

Here we have examined neuronal maintenance defects of sax-7 mutants across the nervous system compared to normally aging wild-type animals. We confirm and extend the findings of neuronal maintenance defects of sax-7 mutants and find remarkable parallels between defects of sax-7 mutants and age-related changes of normal adults. Since loss of function of sax-7 results in accelerated neuronal disorganization that mimics alterations occurring during normal aging, it is possible that dysregulated function of sax-7 may contribute to the changes observed in aging wildtype worms. We find that providing increased levels of sax-7 in neurons helps the wild-type nervous system preserve neuronal architecture. Moreover, we find that age-related neuroanatomical alterations seen in wild-type animals can be reduced by decreasing the animal's movements. Taken together, our results give an entry point into the mechanisms of life-long neuronal protection from aging and mechanical stress. Elucidating the genetic programs that protect the nervous system from wear and tear may lead to strategies to help prevent and treat neurodegenerative conditions.
EXPERIMENTAL PROCEDURES

Nematode strains and genetics

Nematode cultures were maintained at 20°C on NGM plates seeded with OP50 bacteria as described [147]. Strains were constructed using standard genetic procedures and are listed in Table III-1. The sax-7(nj48) genotype was confirmed by PCR using primers oCB1022 tggtgtagcgatgtgtag and oCB208 gagttatggtatgttagcg.

Neuroanatomical analysis

Staging of animals: All nematode strains were fed for at least 3 generations at 20°C before analysis. To obtain adult animals of given ages, advanced fourth larval stage worms (L4) were pooled onto plates and transferred onto fresh plates daily, away from their progeny. These worms that lived for 2, 5 or 10 days after the L4 stage were considered to be 2-, 5- or 10-day old adults, respectively. Animals examined as L4 were considered to be juveniles (at day 0). To obtain first larval stage worms, eggs were smeared on a plate and larvae were picked after 1-2 hr. To obtain second larval stage worms, freshly hatched larvae (< 30 min post hatch) were pooled and grown at 20°C for 18 hr.

Microscopy: 5-10 worms of precise adult ages (0-, 2-, 5- and 10-day old adults) or larval stages were mounted on 5% agarose and immobilized in 10 mM Na
Fasciculation of the ventral nerve cord. The right fascicle of the ventral nerve cord was visualized at 1000X using the Pglr-1::gfp reporter. A worm was considered to have a defasciculated ventral nerve cord when an axon separated away from the fascicle for at least 10 µm from the right fascicle at one or multiple sites.

Fasciculation of the dorsal nerve cord. The dorsal nerve cord was visualized at 1000X using the Prgef-1::gfp reporter. A worm was considered to have defasciculated dorsal nerve cord when an axon separated away from the fascicle for at least 10 µm at one or multiple sites.

Position of the nerve ring. The nerve ring was examined at 200X using the Pmec-4::gfp or the Psra-6::gfp reporters. The position of the nerve ring in the anteroposterior axis was recorded with respect to parts of the pharynx. The pharynx is composed of two bulbs, one anterior, called “anterior bulb” and one posterior called “terminal bulb”, as well as an “isthmus” that connects the two pharyngeal bulbs. The position of the nerve ring was categorized as follows: #1 anterior location, located anywhere between the posterior edge of the anterior
bulb and up to 3/4\(^{th}\) of the isthmus in the anterior-to-posterior direction, #2 intermediate location, located anywhere from 3/4\(^{th}\) of the isthmus in the anterior-to-posterior direction up to the anterior edge of the terminal bulb, and #3 posterior location, located adjacent to or along the length of the terminal bulb. Graphs on nerve ring position show the percentage of nerve rings in categories #2 (white bars) and #3 (grey bars). The percentage of nerve rings in category #1 is not shown on the graphs for clarity, as it can be deduced from “100% minus % in categories #2 and #3” (e.g. of the wild-type nerve rings shown in Fig. III-1C, ~80% at day 0, and 0% at days 2, 5, and 10, fall in category #1).

**Soma projections.**

ALMs: The two ALM neurons (L and R) were examined at 400X using the \textit{Pmec-4::gfp} reporter. In addition to the axon that extends anteriorly from the soma of ALM, a short posterior projection is occasionally observed in early wild-type adults. We examined the number of projections extending from the soma (other than the anterior axon). Projections that became split into two were counted as only one projection.

AVM: The AVM neuron was examined at 200X using the \textit{Pmec-4::gfp} reporter. Other than its axon, the soma does not extend any projection in early wild-type adults. We examined the number of neurons with one projection or more in our assays.
PVM: The PVM neuron was examined at 200X using the P*me*c-4::gf*p reporter. Other than its axon, the soma does not have any projection in early wild-type adults. We examined the number of neurons with one projection or more in our assays.

*Position of neuronal soma.* The position of neurons was examined at 200X. Each soma was recorded as a separate data point and data is plotted as percentage of neurons in each category at any given age. Neuronal soma position was scored in the anteroposterior axis with respect to the terminal bulb of the pharynx and categorized as follows.

CEPs: The four CEP neurons (D, V, L, and R) were visualized using the P*da*t-1::gf*p reporter. CEPs are initially located anterior to the terminal bulb, but with age become displaced posteriorly. The position of the CEP soma was categorized as follows: #1 *anterior*, soma located anterior to the terminal bulb, #2 *intermediate*, soma located on the terminal bulb and anterior to the pharyngeal grinder, and #3 *posterior*, soma located posterior to the grinder. Graphs on CEP soma position show the percentage of CEPs in categories #2 (white bars) and #3 (grey bars). The percentage of CEPs in category #1 is not shown on the graphs for clarity, as it can be deduced from “100% minus % in categories #2 and #3”.

ADEs: The two ADE neurons (L and R) were visualized using the P*da*t-1::gf*p reporter. ADEs are located just posterior to the terminal bulb, but with age become displaced posteriorly. We recorded their posterior displacement in terms
of units of the length of the terminal bulb in the anteroposterior direction. The position of the ADE soma was categorized as follows: #1 anterior, soma located on the terminal bulb, #2 intermediate, soma located just posterior of the terminal bulb (not more posterior than half the length of a terminal bulb), #3 posterior, soma located posterior of the terminal bulb, as far posterior as half to two terminal bulb lengths, and #4 very posterior, soma located posterior of the terminal bulb for more than two terminal bulb lengths. Graphs on ADE soma position show the percentage of ADEs in categories #2 (white bars), #3 (grey bars) and #4 (black bars). The percentage of ADEs in category #1 is not shown on the graphs for clarity, as it can be deduced from “100% minus % in categories #2, #3 and #4”.

ASEs: The two ASE (L and R) neurons were visualized using the Pceh-36::rfp reporter. ASEs are located anterior to the grinder of the terminal bulb but with age become displaced posteriorly. The position of the ASE soma was categorized as: #1 anterior, soma located anterior to the grinder, and #2 posterior, soma located posterior to the grinder. Graphs on ASE soma position show the percentage of ASEs in categories #2 only. The percentage of ASEs in category #1 is not shown on the graphs for clarity, as it can be deduced from “100% minus % in categories #1”.

AIYs: The two AIY neurons (L and R) were visualized using the Pttx-3::gfp reporter. AIYs are located close to the posterior edge of the terminal bulb, but with age become displaced posteriorly. The position of the AIY soma was
categorized as follows: #1 anterior, soma located on the terminal bulb of the pharynx, #2 intermediate, soma located just posterior of the terminal bulb (not more posterior than half the length of a terminal bulb), and #3 posterior, soma located even more posterior. Graphs on AIY soma position show the percentage of AIYs in categories #2 (white bars) and #3 (grey bars). The percentage of AIYs in category #1 is not shown on the graphs for clarity, as it can be deduced from “100% minus % in categories #2 and #3”.

ASHs and ASIs: The two ASH neurons (L and R) and the two ASI neurons (L and R) were visualized using the Psra-6::gfp reporter. These four neurons are located anterior to the terminal bulb, but with age become displaced posteriorly. The positions of the ASH and ASI soma were categorized as follows: #1 anterior, soma located anterior to the terminal bulb, #2 intermediate, soma located on the terminal bulb, and #3 posterior, soma located posterior to the terminal bulb. Graphs on ASH and ASI soma position show the percentage of soma in categories #2 (white bars) and #3 (grey bars). The percentage of neurons in category #1 is not shown on the graphs for clarity, as it can be deduced from “100% minus % in categories #2 and #3”.

Statistical analysis: Neurons and neuronal structures exhibiting structural changes are plotted on the graphs. Error bars are standard error of proportion, calculated as: sqrt((% structural change*(100-% structural change))/n). Statistical comparisons were done using z-test and P values obtained were corrected for
multiple comparisons. In case of data comparisons, where data is represented by a single category on the graph (e.g. ventral nerve cord defasciculation), or when combined data shown by the white and grey bars together was compared statistical significance is denoted by *. the statistical significance of data shown by white bars was denoted by §, and statistical significance of comparison between data shown by grey bars was denoted by #.

**Levamisole treatment**

Levamisole was added to unseeded plates (final concentration was 50 µM) and allowed to dry overnight. These plates were then seeded with *E.coli* (OP50) bacteria and allowed to dry overnight. Pools of 25 L4 worms were transferred onto these levamisole plates for 48 hr at 20°C. Worms were then mounted onto 5% agarose pads in 10 mM Na azide and examined for the position of the nerve ring and the neurons CEP, ADE, ASE and AIY. Each experiment was repeated 2-3 times. Data pooled together from all the experiments was plotted.

**Growth in liquid medium**

25 mL of *E.coli* (OP50) culture grown overnight was centrifuged 5 min at 3000 rpm and the bacterial pellet was resuspended in 5 mL of M9 buffer (M9-OP50 medium). Pools of 25 L4 worms were transferred into 2 mL microcentrifuge tubes containing 1 mL of the M9-OP50 medium and were grown on a nutator for 48 hr at 20°C. Worms were then mounted onto 5% agarose pads in 10 mM Na azide
and examined for the position of the ADE neurons as well as for projections extending from the soma of neurons ALM, AVM and PVM. Each experiment was repeated 2-3 times.

**Growth with continuous agitation**

Pools of 25 L4 worms were transferred onto regular seeded plates. These plates were taped onto a plastic rack that was taped onto the vortex (to avoid heating of the plates) (Fig. III-17) and worms were allowed to grow for 48 hr at 20°C with the vortex set at maximum speed. Worms were then mounted onto 5% agarose pads in 10 mM Na azide and examined for the position of the ADE neurons as well as for projections extending from the soma of ALM. Each experiment was repeated 2-3 times.

**Growth at different temperatures**

Worms were grown for at least 3 generations at 20°C. 25 L4 worms were pooled onto plates and grown at 15°C, 20°C or 25°C for 2 or 5 days. Worms were transferred onto fresh plates daily, away from their progeny. Animals examined as L4 were considered to be juveniles (at day 0). At the end of 2 or 5 days, worms were mounted onto 5% agarose pads in 10 mM Na azide and examined for the position of the ADE neurons. Each experiment was repeated 2-3 times.

**Overexpression of sax-7 in wild-type worms**
A construct encoding the short isoform of *sax-7* was expressed under a pan-neuronal *unc-14* promoter [115] and transgenic animals were generated by standard microinjection techniques [178]. pRP100 (*Punc-14::sax-7S*) was injected at 15 ng/μL with *Punc-122::rfp* (100 ng/μL) and *Pttx-3::mCherry* (85 ng/μL) as coinjection markers into BY200. Transgenic lines were established in BY200 background (VQ498, VQ499, VQ500) and then crossed into SK4005 to transfer the extrachromosomal array (VQ634, VQ635, VQ636).

**Locomotion analysis of *sax-7* overexpressing lines**

Transgenic worms from lines VQ498, VQ499 and VQ500 were assayed for locomotion by the thrashing assay [179]. Briefly, 10 juvenile worms (day 0) were transferred onto fresh plates and were assayed for locomotion 24 hr later. Adult worms were individually placed in a well of a 24-well plate containing 1 mL of M9 buffer and assayed using a stereoscope at 45X magnification. Measurements were carried out by counting the numbers of thrashes occurring in 30 sec. Measurements were repeated four times for each worm. One thrash was counted as a single C-shape movement of the worm.

**Pharyngeal pumping rate measured of transgenic worms overexpressing *sax-7***

Transgenic worms from lines VQ498, VQ499 and VQ500 were assayed for pharyngeal pumping. Assays were carried out at 20°C using a stereoscope at
45X magnification. Worms were scored only when they remained on the bacterial lawn for the entire duration of the assay. The pharyngeal pumping rate was measured as the number of pharyngeal grinder pulses occurring during 15 sec, and was measured four times for each worm.

**Western blot analysis of SAX-7 in worms**

0-, 2-, 5- and 10-day old wild-type (N2) worms were treated with serotonin to get rid of the embryos. 25 worms of a given age were put on a plate with a small food spot in the center and 50 µL of 30 mg/mL serotonin in M9 buffer was added on top of the worms. The plates were allowed to stand for 1 hr on the bench. 100 worms were collected in 50 µL M9 buffer and 50 µL 2x Laemmli sample buffer (Bio-Rad). Worms were then flash frozen in liquid nitrogen and stored at -80°C. Samples were prepared by boiling at 95°C for 15 minutes, centrifugation at 13000 rpm for 10 minutes. Samples were separated by SDS-PAGE on a 10% Mini-Protean TGX gel (Bio-Rad), and transferred to PVDF membrane. Membranes were incubated with rabbit anti-SAX-7 primary antibody (1:12000) (6991 antibody, gift from Dr. Chen) and goat anti-rabbit HRP secondary antibody (1:10000) (Bio-Rad #166-2408EDU). For the loading control, membranes were incubated with rabbit anti-HSP90 antibody (1:5000) (CST #4874) and goat anti-rabbit HRP secondary antibody (1:10000) (Bio-Rad #166-2408EDU). Signals were revealed using Clarity Western ECL Substrate (Bio-Rad), and imaged using film (Lab Scientific). ImageJ software was used to quantify the bands.
**Lifespan analysis**

Wild-type and sax-7 mutant L4 worms (day 0) were pooled and grown on regular NGM plates with *E.coli* OP50 at 20°C (10 worms per plate). Worms were transferred daily away from their progeny onto fresh plates, for as long as progeny was produced. 50 worms were assayed for lifespan and another 50 were used as spare to substitute for worms that died due to bagging or crawling on the side of the plate. All assay and spare worms were treated identically. Worms that died a natural death were counted everyday till the death of the last ‘assay’ worm. Worms that did not move were tested by lightly tapping the agar next to the nose of the worm and twitching of the nose was observed. Lifespan assay was performed twice for each genotype.
RESULTS

Loss of sax-7 leads to neuronal maintenance defects that are largely similar, yet occur earlier than the morphological changes occurring in wild-type aging nematodes

Previous findings from the lab and others [115, 117-121] revealed that the gene sax-7 is required for maintaining the neuronal architecture of animals. sax-7 mutants display cell-specific defects, such as the loss of the precise positioning of axons along the ventral nerve cord and the displacement of some neurons in the head ganglia during larval stages and early adulthood. During our previous work (Chapter II), we noticed that some of the age-related morphological changes that occur in the neurons of wild-type aging nematodes are reminiscent of defects displayed by sax-7 mutant animals. Therefore, we tested the hypothesis that mechanisms required for maintaining the nervous system architecture during early life may also protect the nervous system during aging. Loss of sax-7 function may (1) accelerate the deterioration that takes place during normal aging, or alternatively (2) lead to defects akin to neurodegenerative conditions, leading to age-dependent insults that are more severe or different than the age-related changes in the wild type.

To start addressing this question, we examined and compared the neuroanatomical changes occurring across the nervous system in both wild-type and sax-7 mutant worms during adulthood. The allele we use, nji48, is a strong
loss of function, a deletion that eliminates the fifth exon of the long isoform (SAX-7L) and the first exon of the short isoform (SAX-7S). Age-synchronized populations of larvae, juveniles (day 0 adults) and adult worms aged 2-, 5- and 10-day were examined for neuronal alterations in the wild type and sax-7 mutants using transgenic reporters (Table III-1) that label specific neurons and neuronal structures.

We found that some sax-7 mutant defects are similar to wild-type age-related neuronal changes. For instance, the frequency of age-related ventral nerve cord defasciculation is similar in sax-7 mutants and age-matched wild-type (Fig. III-1A) animals. The right fascicle of the ventral nerve cord contains numerous motor neuron and interneuron processes that make en passant synapses [49], and runs as a tight bundle in juvenile worms, but defasciculates with age (Fig. II-1A). The frequency of defasciculation of the ventral nerve cord increases with age, in both the wild-type and sax-7 mutants (Fig. III-1A), suggesting that for the ventral nerve cord, loss of sax-7 function does not lead to more severe consequences than what occurs in normal aging.

Most of the sax-7 defects, however, are qualitatively similar to the age-related neuroanatomical changes occurring in the wild type, but occur earlier. Many abnormalities of neuronal architecture arise in young and middle-age sax-7 mutant adults and resemble those found in older wild-type animals. For instance, we found that in 2-day old adults, sax-7 mutants but not wild-type, exhibit an...
increased frequency of dorsal nerve cord defasciculation (Fig. III-1B). The degree of defasciculation of 2-day old sax-7 adults is similar to that observed in 10-day old wild-type adults (Fig. III-1B). In addition, we observe defects in age-related displacement of a number of head neurons and the nerve ring (Fig. III-1C, III-2). In the wild type, the nerve ring and the head neurons become placed posteriorly with age (Chapter II). In sax-7 mutants, the nerve ring and several neurons located in head ganglia (e.g., CEP, AIY, ASH and ASI) become progressively displaced starting at the juvenile stage (Fig. III-1C, III-2). In the wild type, the displacement is observed later by day 2 or day 5 of adulthood (Fig. III-1C, III-2). We find that day 2 sax-7 mutant adults exhibit higher frequency of this posterior displacement than age-matched wild type for the head neuronal structures (Fig. III-1C, III-2). Thus, sax-7 mutants exhibit neuronal alterations similar to those exhibited by older wild-type animals, suggesting that sax-7 is “protective” for the structures of the nervous system during adulthood. sax-7 mutants may exhibit signs of early neuronal aging.

Some of the neuronal structures, which we examined in sax-7 mutants, exhibit defects already in juveniles. To determine when these defects appear in sax-7 mutants, we analyzed the neuroanatomy in earlier larval stages of sax-7 mutants. We find that the position of the nerve ring and dopaminergic neurons CEP is wild type at the first larval stage, and that by the second larval stage it becomes defective in sax-7 mutants (Fig. III-3A, B). This is not the case for interneuron AIY, where the displacement defect is present even in the first stage
sax-7 mutant larvae (Fig. III-3D), suggesting that either sax-7 may be involved in the maintenance of the placement of interneurons AIY earlier, probably right after its establishment during embryogenesis, or sax-7 mutants may be defective in the development of AIY neurons. Indeed, sax-7 has been shown to be involved in the development of other neurons [126-128].

In addition to some defects of sax-7 mutants occurring earlier than in the wild type, we found that other defects are more severe than in the normally aging nematodes. We found that the dopaminergic head neurons ADE in sax-7 mutants appear much more displaced that the normal age-related displacement of these neurons in the wild type. In 2-day old adults, 15% of sax-7 mutant ADE neuronal soma are displaced very posteriorly (see Experimental Procedures for details), whereas no wild-type ADE neurons are displaced this far even in old age (Fig. III-2A, Chapter II, Fig II-2A). Since, the displacement defect is visible in juveniles, we analyzed larval stages of sax-7 mutants for the placement of ADE neurons. We find that the placement of ADE neurons is defective in sax-7 mutants as early as two hours post hatch in first larval stage animals (Fig. III-3C), suggesting that sax-7 may be involved in the maintenance of ADE neuronal position earlier during embryogenesis or that sax-7 mutants may be defective in ADE neuronal development.

Together, our results suggest that, the function of sax-7 is required across the nervous system and with great cell-specificity for the maintenance of the
neuronal architecture during larvae and adults, in such a way that many of the
defects accumulated by wild-type aging animals are observed in sax-7 mutants
at an earlier age.

**Loss of neuronal maintenance in sax-7 mutants does not shorten lifespan**

The nervous system plays a role in lifespan determination [50, 51, 173]. We examined whether the early neuronal disorganization displayed in sax-7 mutants would correlate with shortened lifespan. For this, we performed standard lifespan assays, to determine the survival curves of sax-7 mutants in comparison to wild type. We find that the lifespan of sax-7 mutants is similar to that of wild type (Fig. III-4), suggesting that neuronal disorganization does not affect the lifespan of the worm. This result, together with our findings of Chapter II, suggests that neuronal aging may be uncoupled from organismal aging and that loss of sax-7’s function does not affect the function of the nervous system in organismal lifespan.

**SAX-7 is expressed during adulthood**

sax-7 is expressed ubiquitously, throughout embryogenesis, larval
development and adults [119]. Yet, how sax-7 expression levels change with age
was unknown. We analyzed the levels of the protein SAX-7 in 0, 2, 5 and 10-day
old wild-type adult worms by Western blot analysis. We found that the protein
SAX-7 is expressed during adulthood, at least until the worms are 10 days old. A
number of SAX-7 products can be detected by Western blot analysis of worm lysates using the 6991 antibody [119], of which the highest molecular weight band (about 200 kD) is thought to be the long isoform of SAX-7, SAX-7L and the lower molecular weight band (about 185 kD) is the short isoform of SAX-7, SAX-7S [119]. We find that SAX-7L and SAX-7S are expressed in juvenile and adult worms of all ages examined. Additionally, we find that the cleavage product of SAX-7 can be detected throughout adulthood, at least till day 10 of adulthood (Fig. III-5). There is a conserved cleavage site in the third fibronectin domain of SAX-7 and the product generated from the cleavage (about 65 kD) can be detected in worm lysates as well as in cell culture [115, 119]. Our results suggest that the expression level of the two isoforms of SAX-7 may be regulated during adulthood.

**Overexpression of SAX-7 in wild-type adults helps preserve neuronal architecture**

SAX-7 is expressed during adulthood, and the expression of SAX-7L and SAX-7S changes in 2-day old adults (Fig. III-5), that coincides with the appearance of age-related neuronal changes in the wild type. To test whether the decline in SAX-7S levels may underlie the wild-type age-related neuroanatomical alterations, we provided excess SAX-7S in wild-type animals, and tested whether this could protect the nervous system architecture during aging. Pan-neuronal expression of SAX-7S rescues the neuronal maintenance defects of sax-7
mutants [115, 117] and promotes adhesion in cellular assays [118], suggesting that SAX-7S is the isoform that functions to maintain neuronal architecture. SAX-7L actually promotes neuronal disorganization, as overexpression of SAX-7L leads to maintenance defects [117]. We therefore overexpressed sax-7S in all neurons using a pan-neuronal unc-14 promoter [115]. We find that the age-related displacement of both the nerve ring and dopaminergic neurons ADE are suppressed in 2-, 5- and 10-day old transgenic adults that overexpress SAX-7S (Fig. III-6, III-7). As a control, we measured the locomotion and pharyngeal pumping rates of the transgenic worms overexpressing sax-7 and found that they were normal (Fig. III-8). Notably, overexpression of sax-7S did not delay all age-related changes, namely the defasciculation of the dorsal nerve cord in aging wild type (Fig. II-1B) was not suppressed in animals overexpressing wild-type copies of sax-7 (Fig. III-9). Our findings suggest that the protective effect of sax-7S overexpression is specific to certain neuronal structures. Our results suggest that overexpression of SAX-7S is protective during aging.

**Reduced locomotory movements decrease the incidence of age-related neuroanatomical changes.**

Mechanical stress from body movements is a trigger of neuronal disorganization in neuronal maintenance mutants (e.g. sax-7). Indeed, genetic or pharmacological immobilization of sax-7 mutants strongly suppresses their neuronal displacement defects [115, 117, 118]. This is also true for other
neuronal maintenance factors [112, 114, 116], suggesting that mechanical stress may affect nervous system maintenance broadly. Additionally, my results in Chapter II suggest that reduction in pharyngeal pumping correlates with suppression of the age-related placement change in head neuronal structures (Fig. II-5, II-7). We have extended the observation that sax-7 neuronal maintenance defects are suppressed by immobilization and found that the displacement of the nerve ring and dopaminergic neurons ADE in sax-7 mutants can be suppressed by unc mutations and by paralyzing the animals with levamisole (Fig. III-10). To test whether mechanical stress also plays a role in the neuroanatomical changes during normal aging, we examined the effect of immobilization on the age-related neuroanatomical changes.

We paralyzed wild-type worms by genetic or pharmacological means. Genetic paralysis was carried out by building strains of several unc mutations with reporters and examining their neuroanatomy. We used unc-2, unc-13 and unc-75 mutants. unc-2 encodes an α₁ subunit of the calcium channel in C. elegans, homologous to mammalian N/P/Q (Ca_2) voltage gated calcium channel subunit and controls locomotion [180]. unc-13 encodes a protein important for synaptic vesicle priming and regulates neurotransmitter release [181-183]. unc-75 encodes a neuronal RNA-binding protein [184] that regulates the relative abundance of unc-64/Syntaxin isoforms and controls cholinergic synaptic transmission [185]. We analyzed age-related displacement of the nerve ring and chemosensory neurons ASH and ASI, as well as dorsal nerve cord fasciculation.
and soma projections of mechanosensory neurons ALM (Fig. III-11). We found
that whereas in juveniles, the placement of the nerve ring and head neurons ASH
and ASI is similar in the wild type and the unc mutants, 10-day old unc adults are
strongly suppressed for the age-related displacement of these structures
compared to age-matched wild-type adults (Fig. III-11A,B). Paralysis did not
suppress the age-related dorsal nerve cord defasciculation and the soma
projections of mechanosensory neurons ALM (Fig. III-11C,D). Overall, our
results suggest that mechanical stress from body movements plays a role in
many of the age-related neuroanatomical changes.

One caveat to the use of unc mutants is that the worms are paralyzed
from birth and we cannot exclude any effect that this may have on the age-
related changes during adulthood. To analyze the effect of an acute paralysis
during adulthood only, we paralyzed the worms using levamisole from day 0 to
day 2 of adulthood (Fig. III-12A). Levamisole is an acetylcholine agonist that
causes hypercontraction and therefore paralysis of wild-type C. elegans [186].
We find that paralyzing animals for their first 48 hr of adulthood suppresses the
displacement of the nerve ring and the head neurons ADE, ASE and AIY (Fig. III-
12B-E). As observed earlier for unc mutants, paralysis by levamisole does not
suppress age-related increase in the frequency of soma projections of
mechanosensory neurons ALM (Fig. III-12F), suggesting that mechanical stress
from body movements may impact specific age-related neuroanatomical
alterations.
Altered locomotion leads to neuroanatomical changes

To further explore the effect of mechanical stress from body movements on morphological changes in the adult nervous system, we analyzed the neuroanatomy in worms subjected to altered locomotion during adulthood. We used two different experimental paradigms: (1) growth in liquid, causing the worms to swim and (2) constant agitation by growth on agar plates atop a vortex.

In the first experimental paradigm, adults were grown in liquid for 48 hours (Fig. III-13A). We found that the worms grown in liquid exhibited increased soma projections from their mechanosensory neurons. We observed more neurite sprouting on the mechanosensory neuron ALM (Fig. III-13B), as well as de novo soma projections of mechanosensory neurons AVM and PVM (Fig. III-13C,D), compared to the control worms that were grown on agar plates. Liquid growth did not affect the soma placement of the dopaminergic neurons ADE (Fig. III-13E), suggesting specificity of the effect of liquid growth on aspects of adult neuroanatomy.

In the second experimental paradigm, adults were grown on agar plates atop a vortex running at the highest speed setting for 48 hours (Fig. III-14A). We found that growth under constant agitation led to increased frequency of soma projections of mechanosensory neurons ALM (Fig. III-14B). No projections for the mechanosensory neurons AVM and PVM were observed under these conditions. Additionally, worms grown in constant agitation do not exhibit any
placement change for dopaminergic neurons ADE (Fig. III-14C), similar to the observation in the liquid growth paradigm.

Together these results suggest that altered locomotion leads to structural changes in the mechanosensory neurons. It is interesting to note that dopaminergic neurons were not affected by these growth conditions, highlighting the specificity of the effect.

**Locomotory speed may influence age-related neuronal changes**

Temperature regulation is known to affect a variety of physiological processes in all animals. We examined the effect of change in growth temperature on age-related neuroanatomical changes. Worms were grown at the standard temperature of 20˚C and were shifted to lower or higher temperatures (15˚C and 25˚C) for 2 or 5 days. They were then analyzed for the placement of dopaminergic neurons ADE as day 2 or day 5 old adults. We find that at day 2 of adulthood, the age-related displacement of ADE neurons is suppressed in worms grown at 15˚C and enhanced in worms grown at 25˚C compared to worms grown continuously at 20˚C (Fig. III-15). In contrast, at day 5 of adulthood, there is no difference in worms grown at different temperatures (Fig. III-15), suggesting that temperature may only mildly affect the age-related neuroanatomical changes.
DISCUSSION

In this study we have carried out a comparative analysis of the neuroanatomy of wild-type and sax-7 mutant adult worms and revealed that many of the structural changes arising with age often occur earlier in sax-7 mutants and are largely qualitatively similar in both genotypes. We also find that the SAX-7 protein is expressed during adulthood, at least until animals are 10-day old, an age by which wild-type animals have already started to senesce and die [66, 161]. Providing extra copies of sax-7S(+) to the neurons of wild-type animals prolongs the youthful appearance of several aspects of neuronal architecture, suggesting that sax-7S can function in adults to counteract the effects of aging and protect the nervous system. Reducing locomotion in otherwise wild-type animals suppresses the occurrence of age-related neuroanatomical changes, whereas different locomotory paradigms lead to increased changes in the wild-type neuronal architecture. Together, our findings suggest that sax-7 not only plays a role in maintaining neuronal architecture during larval stages and early adulthood, but also during mid- and advanced adulthood. Our results lend support to the notion that sax-7 protects the nervous system from the damage of wear and tear that accumulates with age.

Our analysis reveals that sax-7 mutants exhibit age-related neuroanatomical changes in a wide variety of neuronal structures. The alterations we observed in sax-7 mutants can be categorized into three groups:
(a) First, changes similar to those seen in wild-type aging, such as the defasciculation of the ventral nerve cord with age, which occurs at a similar frequency in both wild type and sax-7 mutants. That sax-7 mutants age similar to wild-type animals with respect to the fasciculation of the ventral nerve cord, suggests that, sax-7 does not play a role in the maintenance of the ventral nerve cord fasciculation.

(b) Second, alterations similar to those seen in wild type but that occur earlier in sax-7 mutants. This category includes most of the neuronal structures examined: dorsal nerve cord, nerve ring, head ganglia neurons (except ADE neurons) and mechanosensory neurons ALM. I found that for these neurons, the neuroanatomical changes seen in sax-7 mutants were similar to the ones observed in wild type, but the onset of these changes was significantly earlier in sax-7 mutants than wild type. This suggests that sax-7 may be involved in maintaining the architecture of these neuronal structures.

(c) Third, neuroanatomical changes that were more severe than what we observed in the same neurons in wild type; dopaminergic head neurons ADE become more posteriorly displaced in sax-7 mutants than in wild type. These results suggest that sax-7 plays a role in the age-related morphological changes.

Age-related ventral nerve cord defasciculation observed in sax-7 mutants is similar to wild type, suggesting the specificity of neuronal maintenance mechanisms including sax-7, in age-related neuroanatomical alterations. Additionally, the penetrance of age-related neuroanatomical changes that I
observed in sax-7 mutants was partial. This suggests that additional factors may be involved in the maintenance process, and that the different players may act redundantly. It is also possible that small amounts of wild type sax-7 product is still being made in these mutants, since sax-7 (nj48), the allele used in this study is not a null allele.

For the majority of the neuronal structures that I studied, juvenile sax-7 mutant worms did not show any defects, and appeared similar to juvenile wild type, but for some neuronal structures, namely nerve ring, head interneurons AIY and dopaminergic head neurons CEP and ADE, I observed defects in juvenile (day 0 adults) sax-7 mutant worms. All these structures are established during embryogenesis. To understand whether these defects are developmental, I analyzed first and second stage larvae. I found that for all these structures, displacement defects could be seen even in second stage sax-7 mutant larvae. When analyzed in first stage larvae, I found that placement of the nerve ring and the CEP neurons is similar in sax-7 mutant and wild type worms, but in the case of ADE and AIY neurons even first larval stage sax-7 mutant worms exhibit displacement defects. These results suggest that sax-7 is required for the maintenance of the nerve ring and the CEP neurons rather early in life. In the case of ADE and AIY neurons, it is possible that sax-7 may be required for maintenance even earlier, possibly, right after the neurons are established, or it is possible that sax-7 may be involved in the development of these structures. This is not surprising given that sax-7 is expressed during embryogenesis and
the expression can be detected as early as the 2-cell stage [119]. Recently it has been shown that *sax-7* is required for the development of the dendritic arbors of mechanosensory and thermosensory PVD neurons [126-128].

**Differential susceptibility of neurons to the effect of age**

The nervous system of *C. elegans* manifests morphological changes with age that are remarkably neuron-specific (Chapter II and this chapter). This cell-type specificity may be due to a combination of cell-intrinsic and extrinsic factors that may influence how distinct neuron types respond to age. Similarly, the defects of *sax-7* mutants are highly neuron-specific: for instance, some but not all axons of the ventral nerve cord defasciculate and ectopic neurites sprout out of the mechanosensory neuron ALM, but not from other neurons examined. This neuron-type specificity is also characteristic of the larval neuronal maintenance defects of *sax-7* mutants, where only particular axons of the ventral nerve cord flip over to the opposite fascicle [115]. The specificity of the defects arising from the loss of function of *sax-7* in the mutants or in aging wild-type animals may be due to context-dependent interactions of SAX-7 with extracellular and membrane bound molecular partners. Vertebrate homologues of SAX-7 have been shown to interact not only homophilically, but also heterophilically with multiple molecules, including other cell adhesion molecules, the fibroblast growth factor receptor, among others (reviewed in [187]).
SAX-7S may play a protective role in the adult nervous system

We show that the SAX-7 protein is expressed during young-, mid- and advanced adulthood (i.e., 10-day old adults). The two isoforms of SAX-7, SAX-7S and SAX-7L are both expressed in juvenile and adult wild-type worms, at least till day 10 of adulthood. We hypothesize that, the relative abundance of the two isoforms may underlie some of the age-related neuroanatomical changes exhibited by wild-type aging adults. Consistent with this notion, we found that artificially elevating the levels of SAX-7S(+) in the neurons leads to preserving neuronal architecture, indicating a neuroprotective role of sax-7 during adulthood. Further temporal analysis of the requirements of SAX-7 during adulthood may help in understanding the specific role of sax-7 in normal neuronal aging.

Earlier studies have shown that the two isoforms have opposite effects on neuronal maintenance. Pan-neuronal expression of sax-7L does not rescue the maintenance defects of loss-of-function sax-7 mutants whereas the defects are efficiently rescued by expressing sax-7S pan-neuronally [115]. Additionally, wild-type worms over-expressing pan-neuronal sax-7L display soma and axon displacement defects that are similar to the defects seen in loss-of-function sax-7 mutants, but pan-neuronal expression of sax-7S in the wild-type background does not cause any defects [117]. This suggests that SAX-7L may have disruptive activity that may be suppressed in larval and juvenile worms, but loss of this suppression during adulthood may result in neuroanatomical alterations. We observe that SAX-7L is expressed throughout adulthood till day 10, in whole
worm lysates. It will be interesting to see if there are nervous system specific changes in the expression levels of the disruptive isoform SAX-7L, that may be correlated to the anatomical changes in the nervous system. Additionally, an age-related decrease in the expression levels of SAX-7S may also underlie the occurrence of structural changes in the nervous system. SAX-7S has been proposed to be the adhesive isoform and shown to efficiently rescue the positional defects in sax-7 mutants [115, 118, 119].

Changes in the expression of SAX-7S and SAX-7L may result from progressive deregulation of sax-7 gene expression, changes in translation or protein stability. Analysis of transcript levels of SAX-7S and SAX-7L during adulthood may provide additional clues, though in addition to changes in transcript levels, a combined effect of changes in translation, transcript stability as well as protein stability of the two isoforms may lead to differential expression levels of SAX-7S and SAX-7L. Interestingly, difference in the protein structure of the two isoforms has been proposed to underlie the differential ability of the two isoforms to rescue the sax-7 mutant neuronal maintenance defects. SAX-7L has six immunoglobulin (Ig) domains and five fibronectin type III domains extracellularly, a transmembrane domain and an intracellular tail (Fig. I-2). The region between the Ig domains Ig2 and Ig3 is called the hinge region, and SAX-7L has been proposed to form a horse-shoe model, where Ig1 and Ig2, fold back onto Ig3 and Ig4, [115] (Fig. III-16). Inhibiting this possible folding by shortening the hinge region, improves the ability of SAX-7L to rescue the sax-7 mutant.
defects [115]. SAX-7S, the adhesive isoform, has four Ig domains (Ig3 to Ig6), and it is possible that, the protein remains functionally active due to absence of inhibition by folding of Ig1 and Ig2. Additionally, SAX-7L could bind SAX-7S and reduce the effective functional pool of SAX-7S.

The third fibronectin III-type domain of SAX-7 has a conserved, predicted cleavage site for Furin and PC-type proprotein proteases and a cleaved C-terminal fragment can be detected by Western blot analysis in worms and in cell culture ([115, 119], Fig. III-5). It is possible that this cleavage reaction may play a role in the stability of the SAX-7L and SAX-7S proteins. Identification of proteases that would cleave these isoforms and age-related changes in the expression of these proteases may be able to reveal some information about the relative abundance of the two isoforms, in older adults. The Bulow group has reported that kpc-1, a C. elegans homolog of Furin genetically acts in the same pathway as sax-7 for dendritic arborization of PVD mechanosensory neurons [127]. Analysis of kpc-1 and other members of the kex-2/ subtilisin-like proprotein convertase family for changes in their expression levels during adulthood, interactions with sax-7, and effects on age-related neuroanatomical changes may help in understanding the role of SAX-7 in maintenance of the adult nervous system. The mammalian homolog L1CAM is cleaved by a number of proteases and the resulting fragment has been shown to be important for many developmental functions of L1CAM such as neural cell adhesion, cell migration and neurite outgrowth (reviewed in [188]). Additionally, cleavage of L1CAM has
been shown to promote structural and functional recovery post injury to the femoral nerve and spinal cord in mice [188]. These results suggest that the proteolytic cleavage of sax-7 may be important for its function, may be another conserved feature of the L1CAM family proteins.

**Mechanical stress may be a trigger for the disorganization neuronal architecture**

We found that locomotory activity correlates with the appearance of age-related neuronal alterations. Paralyzing animals by genetic means (unc mutants) suppresses the nervous system changes that would otherwise be displayed with age. Reducing the locomotion even for two days in early adulthood is also able to suppress neuroanatomical changes. This indicates that the mechanical stress from body movements contributes to neuroanatomical changes. Consistent with this notion, swimming leads to increased neuronal changes and even new defects that are not seen under normal laboratory conditions.

From the first larval stage, nematodes are constantly locomoting in their environment, and yet, neuroanatomical changes such as defasciculation of nerve cords, disorganization of ganglia, and ectopic neurite outgrowth occur only later in life. This suggests, that young animals can cope with the stresses of locomotion through neuronal maintenance mechanisms, but that the protective systems become overwhelmed with age and as a result, the nervous system progressively accumulates damage over time. The human brain is also subjected
to numerous mechanical stresses during post-natal life and life-long activities (sports, shocks), and therefore our findings may provide information about mechanisms operating to protect human neurons as well.

Like in mammals, the nervous system of *C. elegans* undergoes age-associated changes, suggesting that there may be conserved mechanisms underlying the responses of neurons to age. Genetic analysis in *C. elegans* has shown that a conserved cell adhesion molecule, which is a part of the neuronal maintenance machinery, may play a role in protecting the organization of the nervous system during aging. Further studies using this tractable model will help elucidate principles underlying neuronal maintenance and identify the processes that contribute to or protect from neural disorders during maturation and aging.
Figure III-1. Many of the neuronal maintenance defects of sax-7 mutants are qualitatively similar to the age-related neuroanatomical changes of axons and fascicles occurring in aging wild-type animals.
Schematics depict the structures examined; red dotted rectangles indicate the area in the picture.

A. Defasciculation of the ventral nerve cord with age. The ventral nerve cord is a tight bundle in juvenile worms (day 0) and it becomes defasciculated (arrows) with age. Ventral nerve cord defasciculation is more frequent in older wild-type and sax-7 mutant worms. The frequency of ventral nerve cord defasciculation is similar in wild-type and sax-7 mutant worms.

B. Defasciculation of the dorsal nerve cord with age. The dorsal nerve cord is a tight bundle in juvenile worms (day 0) and it becomes defasciculated (arrows) with age. Dorsal nerve cord defasciculation is more frequent in older wild-type and sax-7 mutant worms. 2-day old sax-7 mutant worms exhibit increased frequency of dorsal nerve cord defasciculation than age-matched wild-type worms.

C. Change in the position of the nerve ring with age. The nerve ring was visualized using the reporter Pmec-4::gfp. The nerve ring (arrows) is located anterior to the terminal bulb of the pharynx (dotted circle) in juvenile worms (day 0) and becomes posteriorly located in older adults, including being at the level of the terminal bulb. Such posterior location of the nerve ring becomes increasingly frequent with age in wild-type and sax-7 mutant worms. 2-day old sax-7 mutant worms exhibit increased frequency of more posterior location of the nerve ring than age-matched wild-type worms.
D. Changes in the neurons ALM with age. Each ALM neuron has an axon oriented anteriorly, and in some of the juvenile worms (day 0) a short projection extends from the soma. The frequency, at which the soma of ALM exhibits one or two additional projections (arrows), increases with age in wild-type and sax-7 mutant worms. 2-day old sax-7 mutant worms exhibit slightly increased frequency of soma projections than age-matched wild-type worms.

Scale bar, 5 μm. Error bars are standard error of the proportion. Asterisks denote a significant difference. When the data is classified into two categories, a significant difference between white bars is indicated by the “§” symbol and a significant difference between grey bars is indicated by the “#” symbol. ***, ###, §§§ for \( P \leq 0.001 \), **, ##, §§ for \( P \leq 0.01 \) and *, #, § for \( P \leq 0.05 \) (z-tests, \( P \) values are corrected for multiple comparisons). ns, not significant.
Figure III-2. Many of the neuronal maintenance defects of sax-7 mutants are qualitatively similar to the age-related neuroanatomical neuronal displacements occurring in aging wild-type animals.
Schematics depict the structures examined; red dotted rectangles indicate the area in the picture.

A. Changes in the position of the neurons CEP and ADE with age. In juvenile worms (day 0), the cell bodies of the four CEP neurons (white arrows) are located anterior to the terminal bulb of the pharynx (dotted circle) and those of the two ADE neurons (yellow arrows) are located just posterior to the terminal bulb (dotted circle). In older wild-type and sax-7 mutant worms, the neurons CEP become posterior, including to the level of the terminal bulb, and the neurons ADE become more posterior. In older worms, the frequency of posterior location for soma of CEP and ADE increases; sax-7 mutant worms exhibit increased frequency of posterior location for the soma of CEP and ADE than age-matched wild-type worms.

B. Changes in the position of the neurons ASE and AIY with age. In juvenile worms (day 0), the neurons ASE (white arrows) are located anterior to the terminal bulb of the pharynx (dotted circle) and the neurons AIY (yellow arrows) are located near the terminal bulb (dotted circle). In older wild-type and sax-7 mutant worms, the neurons ASE and AIY are located in more posterior locations. sax-7 mutant worms exhibit increased frequency of posterior location for the soma of ASE and AIY than age-matched wild-type worms.

C. Changes in the position of the neurons ASH and ASI with age. In juvenile worms (day 0), the neurons ASH and ASI (arrows) are located anterior to the terminal bulb of the pharynx (dotted circle). In older wild-type and sax-7 mutant
worms, the neurons ASH and ASI become posteriorly located, at the level of the terminal bulb of the pharynx (white bars) or even posterior to the grinder of the pharynx (gray bars). sax-7 mutant worms exhibit increased frequency of posterior location for the soma of ASH and ASI than age-matched wild-type worms.

Scale bar, 5 μm. Error bars are standard error of the proportion. Asterisks denote a significant difference. When the data is classified into two categories, a significant difference between white bars is indicated by the “§” symbol and a significant difference between grey bars is indicated by the “#” symbol. ***, ###, §§§ for \( P \leq 0.001 \), **, ##, §§ for \( P \leq 0.01 \) and *, #, § for \( P \leq 0.05 \) (z-tests, \( P \) values are corrected for multiple comparisons). ns, not significant.
Figure III-3. Neuroanatomical changes in nerve ring and neuronal soma position in young worms.
A. Changes in the position of the nerve ring in young worms. In worms as young as L2, the nerve ring is more posteriorly located in sax-7 mutants compared to wild type. Data for 0-, 2-, 5- and 10-day wild-type and sax-7 mutant worms is same as in Fig. III-1C.

B. Changes in the position of the neurons AIY in young worms. In worms as young as L1, the neurons AIY are more posteriorly located in sax-7 mutants compared to wild type. Data for 0-, 2-, 5- and 10-day wild-type and sax-7 mutant worms is same as in Fig. III-2B.

C. Changes in the position of the neurons CEP in young worms. In worms as young as L2, the neurons CEP are more posteriorly located in sax-7 mutants compared to wild type. Data for 0-, 2-, 5- and 10-day wild-type and sax-7 mutant worms is same as in Fig. III-2A.

D. Changes in the position of the neurons ADE in young worms. In worms as young as L1, the neurons ADE are more posteriorly located in sax-7 mutants compared to wild type. Data for 0-, 2-, 5- and 10-day wild-type and sax-7 mutant worms is same as in Fig. III-2A.

Error bars are standard error of the proportion. Asterisks denote a significant difference. When the data was classified into two categories, a significant difference between white bars is indicated by the “§” symbol and a significant difference between grey bars is indicated by the “#” symbol. ***, ###, §§§ for $P \leq 0.001$, **, ##, §§ for $P \leq 0.01$ and *, #, § for $P \leq 0.05$ (z-tests, P values are
corrected for multiple comparisons). ns, not significant. L1, first larval stage; L2, second larval stage.
Wild-type and sax-7 mutant juvenile worms (day 0) were pooled and lifespan of the worms was assessed by counting the number of worms alive everyday till all the worms died. Lifespan of sax-7 mutants (20.49 ± 5.1 days) was similar to wild type (20.5 ± 4.5 days). (n=100 total worms for each genotype, 50 for each experiment)
Protein extracts of 100 0-, 2-, 5-, and 10-day old worms were probed against polyclonal 6991 SAX-7 antibody by western blot analysis. We observed three different bands on the gel that were absent in the protein extracts from sax-7 mutant worms. The top two bands are probably the 2 isoforms of SAX-7 – SAX-7 long and SAX-7 short, that run between 150kD and 250kD protein standards. The third band is suspected to be the cleavage product of SAX-7 protein that runs between 50kD and 75kD protein standards. HSP-90 is the loading control. The bands were quantified using ImageJ software and the normalized to the loading control and expressed as fold change with respect to the bands observed for day 2.
Figure III-6. Age-related nerve ring displacement is reduced with overexpression of sax-7 in the neurons of wild-type animals.
A. Change in the position of the nerve ring upon overexpression of sax-7. The cDNA of the short isoform of sax-7 was expressed extrachromosomally under the pan-neuronal unc-14 promoter and unc-54 3'UTR. The nerve ring was visualized using the reporter Pmec-4::gfp. Schematic depicts the structures examined; red rectangle indicates the area in the picture. The nerve ring (arrows) is located anterior to the terminal bulb of the pharynx (dotted circle) in wild-type juvenile worms (day 0) and becomes posteriorly located in 2-day old wild-type worms, including being at the level of the terminal bulb. Such posterior localization of the nerve ring is not observed in 2-day and 5-day old wild-type worms overexpressing sax-7.

B-D. Change in the position of the nerve ring upon overexpression of sax-7. The nerve ring becomes located in posterior positions as wild-type worms age; such posterior localization is suppressed in wild-type worms overexpressing sax-7. This suppression is observed in 2-day (B), 5-day (C) and 10-day (D) old worms. Scale bar, 5 μm. Error bars are standard error of the proportion. Asterisks denote a significant difference. When the data was classified into two categories, a significant difference between white bars is indicated by the “§” symbol and a significant difference between grey bars is indicated by the “#” symbol. ***, ###, §§§ for $P \leq 0.001$, **, ##, §§ for $P \leq 0.01$ and *, #, § for $P \leq 0.05$ (z-tests, $P$ values are corrected for multiple comparisons). ns, not significant.
Figure III-7. Age-related displacement of ADE neurons is reduced with overexpression of sax-7 in the neurons of wild-type animals.
A. Changes in the position of the neurons ADE upon overexpression of sax-7. sax-7 was expressed extrachromosomally under the pan-neuronal unc-14 promoter. The neurons ADE were visualized using the reporter Pdat-1::gfp. Schematic depicts the structures examined; red rectangle indicates the area in the picture. The cell bodies of the two ADE neurons (arrows) are located just posterior to the terminal bulb (dotted circle) in wild-type juvenile worms (day 0) and become posteriorly located in 2-day wild-type worms. Such posterior localization of the neurons ADE is not observed in 2-day and 5-day old wild-type worms overexpressing sax-7.

B-D. Changes in the position of the neurons ADE upon overexpression of sax-7. The neurons ADE become located in posterior positions as wild-type worms age; such posterior localization is suppressed in wild-type worms overexpressing sax-7. This suppression is observed in 2-day (B), 5-day (C) and 10-day (D) old worms.

Scale bar, 5 μm. Error bars are standard error of the proportion. Asterisks denote a significant difference. When the data was classified into two categories, a significant difference between white bars is indicated by the “§” symbol and a significant difference between grey bars is indicated by the “#” symbol. ***, ###, §§§ for \( P \leq 0.001 \), **, ##, §§ for \( P \leq 0.01 \) and *, #, § for \( P \leq 0.05 \) (z-tests, P values are corrected for multiple comparisons). ns, not significant.
Figure III-8. Overexpressing of sax-7 in wild-type worms has little effect on locomotion or pharyngeal pumping.

A. Locomotion analysis of wild-type worms overexpressing sax-7. Wild-type juvenile worms overexpressing sax-7 (day 0) were pooled and 24 hr later, these worms were assayed for locomotion using the thrashing in liquid assay (10 worms per line assayed). The wild-type worms overexpressing sax-7 performed similar to wild-type worms.

B. Pumping analysis of wild-type worms overexpressing sax-7. Wild-type juvenile worms overexpressing sax-7 (day 0) were pooled and 24 hr later, these worms were assayed for pumping on food (10 worms per line assayed). The wild-type worms overexpressing sax-7 showed a slight reduction in pumping rate in two lines, when compared with wild-type worms.
Error bars are standard error of the proportion. Asterisks denote a significant difference. *** for $P \leq 0.001$, and * for $P \leq 0.05$ (Student’s t-test, P values are corrected for multiple comparisons). ns, not significant.
Figure III-9. Age-related dorsal nerve cord defasciculation is not suppressed by overexpressing sax-7 in the neurons of wild-type animals.

sax-7 was expressed extrachromosomally under the pan-neuronal unc-14 promoter. The dorsal nerve cord was visualized using the reporter Prgef-1::gfp. The dorsal nerve cord is a tight bundle in juvenile worms and becomes defasciculated with age. The frequency of dorsal nerve cord defasciculation is similar in 10-day old wild-type and sax-7 overexpressing wild-type worms. Error bars are standard error of the proportion. ns, not significant. (z-tests)
Figure III-10. Decreased locomotion suppresses neuronal maintenance defects of sax-7 mutants.

A. Experimental design for levamisole treatment. Juvenile sax-7 mutant worms (day 0) were put on plates with levamisole for 48 hr. Neuroanatomical characteristics of these worms were examined at the end of the incubation, when the animals were 2-day old adults.
B. Change in the position of the nerve ring in paralyzed sax-7 mutants. The nerve ring was visualized using the reporter $P_{mec-4::gfp}$. sax-7 mutant worms were paralyzed throughout their life by creating double mutants with Unc mutants $unc-2$ or $unc-13$, or just in adulthood by levamisole treatment. The nerve ring becomes located in more posterior positions in 2-day old sax-7 mutant worms. However, this posterior localization is suppressed by paralysis.

C. Changes in the position of the neurons ADE in paralyzed sax-7 mutants. sax-7 mutant worms were paralyzed throughout their life by creating double mutants with Unc mutants $unc-2$ or $unc-13$, or just in adulthood by levamisole treatment. The neurons ADE become located in more posterior positions in 2-day old sax-7 mutant worms. However, this posterior localization is suppressed by paralysis.

Error bars are standard error of the proportion. Asterisks denote a significant difference. When the data was classified into two categories, a significant difference between white bars is indicated by the “§” symbol and a significant difference between grey bars is indicated by the “#” symbol. ***, ###, §§§ for $P \leq 0.001$, **, ##, §§ for $P \leq 0.01$ and *, #, § for $P \leq 0.05$ (z-tests, P values are corrected for multiple comparisons). ns, not significant.
Figure III-11. Decreased locomotion suppresses some wild-type age-related neuroanatomical changes.

A. Change in the position of the nerve ring in paralyzed worms. The nerve ring was visualized using the reporter Psra-6::gfp. Worms were paralyzed from birth
using Unc mutations. While the nerve ring becomes more posteriorly located in 10-day old wild-type worms, this posterior localization is suppressed by the unc-2, unc-13 and unc-75 mutations.

B. Changes in the position of the neurons ASH and ASI in paralyzed worms. Worms were paralyzed from birth using Unc mutations. While the neurons ASH and ASI become more posteriorly located in 10-day old wild-type worms, this posterior localization is suppressed by the unc-2, unc-13 and unc-75 mutations.

C. Dorsal nerve cord defasciculation in paralyzed wild-type worms. Worms were paralyzed from birth using Unc mutations. The dorsal nerve cord becomes defasciculated in 10-day old wild-type worms, and this defasciculation is not suppressed by the unc-2 mutations.

D. Soma projections of mechanosensory neuron ALM in paralyzed wild-type worms. Worms were paralyzed from birth using Unc mutations. The frequency, at which the soma of ALM exhibits one or two projections, is similar in 10-day old wild-type and unc-2 mutant worms.

Error bars are standard error of the proportion. Asterisks denote a significant difference. When the data was classified into two categories, a significant difference between white bars is indicated by the “§” symbol and a significant difference between grey bars is indicated by the “#” symbol. ***, ###, §§§ for $P \leq 0.001$, **, ##, §§ for $P \leq 0.01$ and *, #, § for $P \leq 0.05$ (z-tests, P values are corrected for multiple comparisons). ns, not significant.
Figure III-12. Decreased locomotion reduces some wild-type age-related neuroanatomical changes.
A. Experimental design for levamisole treatment. Juvenile worms (day 0) were put on plates with levamisole for 48 hr. Neuroanatomical characteristics of these worms were examined at the end of the incubation, when the animals were 2-day old adults.

B. Change in the position of the nerve ring in paralyzed wild-type worms. The nerve ring was visualized using the reporter Pmec-4::gfp. Wild-type worms were paralyzed by levamisole treatment for 48 hr in adulthood (See Fig. III-12A for experimental design). While the nerve ring becomes more posteriorly located in 2-day old wild-type worms, this posterior localization is suppressed by paralysis.

C. Changes in the position of the neurons ADE in paralyzed wild-type worms. Wild-type worms were paralyzed by levamisole treatment for 48 hr in adulthood (See Fig. III-12A for experimental design). While the neurons ADE become more posteriorly located in 2-day old wild-type worms, this posterior localization is suppressed by paralysis.

D. Changes in the position of the neurons ASE in paralyzed wild-type worms. Wild-type worms were paralyzed by levamisole treatment for 48 hr in adulthood (See Fig. III-12A for experimental design). While the neurons ASE become more posteriorly located in 2-day old wild-type worms, this posterior localization is suppressed by paralysis.

E. Changes in the position of the neurons AIY in paralyzed wild-type worms. Wild-type worms were paralyzed by levamisole treatment for 48 hr in adulthood (See Fig. III-12A for experimental design). While the neurons AIY become more
posteriorly located in 2-day old wild-type worms, this posterior localization is suppressed by paralysis.

E. Soma projections of mechanosensory neuron ALM in paralyzed wild-type worms. Wild-type worms were paralyzed by levamisole treatment for 48 hr in adulthood (See Fig. III-12A for experimental design). While the frequency, at which the soma of ALM exhibits one or two projections, increases in 2-day old wild-type worms, this increase is not suppressed by paralysis. Error bars are standard error of the proportion. Asterisks denote a significant difference. When the data was classified into two categories, a significant difference between white bars is indicated by the “§” symbol and a significant difference between grey bars is indicated by the “#” symbol. ***, ###, §§§ for $P \leq 0.001$, **, ##, §§ for $P \leq 0.01$ and *, #, § for $P \leq 0.05$ (z-tests, $P$ values are corrected for multiple comparisons). ns, not significant.
Figure III-13. Swimming / thrashing locomotion leads to neuroanatomical changes in some neurons.

A. Experimental design. For growth in liquid medium, juvenile worms (day 0) were incubated in M9 containing *E. coli* (OP50) for 48 hr (day 0 to day 2 of adulthood) on a nutator at 20°C. Neuroanatomical characteristics of these worms were examined at the end of incubation, when the animals were 2-day old adults.

B. Changes of the neurons ALM when worms are grown in liquid medium. While the ALM soma acquires projections in 2-day old adults that are grown on solid
medium, the frequency of these projections increases when grown in liquid medium.

C. Changes of the neuron AVM when worms are grown in liquid medium. The AVM soma acquires projections in animals that are grown in liquid medium.

D. Changes of the neuron PVM when worms are grown in liquid medium. The PVM soma acquires projections in animals that are grown in liquid medium.

E. Changes in the position of the neurons ADE when worms are grown in liquid medium. The neurons ADE become located in more posterior positions in 2-day old animals in a similar trend when the worms are grown in liquid medium or regular plates.

Error bars are standard error of the proportion. Asterisks denote a significant difference. When the data was classified into two categories, a significant difference between white bars is indicated by the “§” symbol and a significant difference between grey bars is indicated by the “#” symbol. ***, ###, §§§ for $P \leq 0.001$, **, ##, §§ for $P \leq 0.01$ and *, #, § for $P \leq 0.05$ (z-tests, P values are corrected for multiple comparisons). ns, not significant.
**Figure III-14.** Neuroanatomical changes of axons and neuronal soma position when subjected to mechanical stress.

**A.** Experimental design for mechanical stimulation. For growth on vortex, juvenile worms (day 0) were incubated in regular NGM plates, taped on top of a vortex set at maximum speed for 48 hr (day 0 to day 2 of adulthood) at 20°C. For control, juvenile worms (day 0) were grown in regular NGM plates for 48 hr (day 0 to day 2 of adulthood) at 20°C. Neuroanatomical characteristics of these worms were examined at the end of the incubation, when the animals were 2-day old adults.
**B.** Changes of the neuron ALM when worms are grown on plates taped to the vortex. The ALM soma acquires projections in 2-day old adults that are grown under control conditions. The frequency of these projections increases when the worms are grown under the mechanical stimulation of the vortex.

**C.** Changes in the position of the neurons ADE when worms are grown on plates taped to the vortex. The neurons ADE become posteriorly located in 2-day old adults in a similar trend when the worms are grown under control conditions or under the mechanical stimulation of the vortex.

Error bars are standard error of the proportion. Asterisks denote a significant difference. When the data was classified into two categories, a significant difference between white bars is indicated by the “§” symbol and a significant difference between grey bars is indicated by the “#” symbol. ***, ###, §§§ for \( P \leq 0.001 \), **, ##, §§ for \( P \leq 0.01 \) and *, #, § for \( P \leq 0.05 \) (z-tests, P values are corrected for multiple comparisons). ns, not significant.
Changes in the position of the neurons ADE upon variation of the growth temperature. Worms were grown at 20°C from birth until the juvenile stage (L4). Juvenile worms (day 0) were transferred to regular plates and grown at 15°C, 20°C or 25°C for 2 or 5 days. These 2-, 5-day adults were analyzed for ADE soma position. In 2-day adult animals, while the neurons ADE become posteriorly located in animals grown at 20°C, this posterior localization is less pronounced in animals grown at 15°C and more pronounced in animals grown at 25°C. The posterior localization of the neurons ADE was similar in 5-day adult animals grown at 15°C, 20°C or 25°C.
Error bars are standard error of the proportion. Asterisks denote a significant difference. When the data was classified into two categories, a significant difference between white bars is indicated by the “§” symbol and a significant difference between grey bars is indicated by the “#” symbol. ***, ###, §§§ for \( P \leq 0.001 \), **, ##, §§ for \( P \leq 0.01 \) and *, #, § for \( P \leq 0.05 \) (z-tests, \( P \) values are corrected for multiple comparisons). ns, not significant.
Figure III-16. Schematic representation of horseshoe shaped conformation of the long isoform, SAX-7L.

Schematic representation of horseshoe shaped conformation of the long isoform of SAX-7, SAX-7L, speculated to be formed by folding of the first two immunoglobulin (Ig) domains, Ig1 and Ig2 onto Ig3 and Ig4.
Figure III-17. Image of the assembly of worms with agar plates atop a vortex for altered locomotion paradigm.
Table III-1. List of strains used.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Transgene / Extrachromosomal array</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK4005</td>
<td>zdIs4 IV</td>
<td>Pmec-4::gfp; lin-15(+)</td>
<td>Gift from Scott Clark [164]</td>
</tr>
<tr>
<td>BY200</td>
<td>vtIs1 V</td>
<td>Pdat-1::gfp; rol-6</td>
<td>[165]</td>
</tr>
<tr>
<td>VQ43</td>
<td>oyls14 V</td>
<td>Psra-6::gfp; lin-15(+)</td>
<td>This study, [166]</td>
</tr>
<tr>
<td>VH15</td>
<td>rhl4 III</td>
<td>Pgir-1::gfp; dpy-20(+)</td>
<td>[167]</td>
</tr>
<tr>
<td>NW1229</td>
<td>evls111</td>
<td>Prgef-1::gfp; dpy-20(+)</td>
<td>[168]</td>
</tr>
<tr>
<td>VQ448</td>
<td>otls264 III; mgl18 IV</td>
<td>Pceh-36::rfp; Ptx-3::gfp</td>
<td>This study</td>
</tr>
<tr>
<td>VQ292</td>
<td>sax-7(nj48) IV; rhl4 III</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ295</td>
<td>sax-7(nj48) IV; evls111</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ294</td>
<td>sax-7(nj48) zdIs4 IV</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ293</td>
<td>sax-7(nj48) IV; vtls1 V</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>OH7984</td>
<td>sax-7(nj48) IV; oyls14 V</td>
<td></td>
<td>[115]</td>
</tr>
<tr>
<td>VQ643</td>
<td>sax-7(nj48) mgl18 IV; otls264 III</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ226</td>
<td>unc-2 (e55) X; oyls14 V</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>OH8808</td>
<td>unc-13 (e1091) I; oyls14 V</td>
<td></td>
<td>[114]</td>
</tr>
<tr>
<td>VQ228</td>
<td>unc-75 (e950) I; oyls14 V</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ229</td>
<td>unc-97 (su110) X; oyls14 V</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ585</td>
<td>unc-2(e55)X; sax-7(nj48) zdIs4 IV</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ642</td>
<td>unc-13(e1091)I; sax-7(nj48) zdIs4 IV</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ641</td>
<td>sax-7(nj48) IV; unc-2(e55)X; vtls1 V</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ630</td>
<td>unc-13(e1091)I; sax-7(nj48) IV; vtls1 V</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VQ500</td>
<td>vtls1 V; qvEx96</td>
<td>Punc-14::sax-7S; Punc-122::rfp</td>
<td>This study</td>
</tr>
<tr>
<td>VQ498</td>
<td>vtls1 V; qvEx97</td>
<td>Punc-14::sax-7S; Punc-122::rfp</td>
<td>This study</td>
</tr>
<tr>
<td>VQ499</td>
<td>vtls1 V; qvEx98</td>
<td>Punc-14::sax-7S; Punc-122::rfp</td>
<td>This study</td>
</tr>
<tr>
<td>VQ634</td>
<td>zdIs4 IV; qvEx96</td>
<td>Punc-14::sax-7S;</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td>---------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>VQ635</td>
<td>zdis4 IV; qvEx97</td>
<td>Punc-14::sax-7S; Punc-122::rfp</td>
<td>This study</td>
</tr>
<tr>
<td>VQ636</td>
<td>zdis4 IV; qvEx98</td>
<td>Punc-14::sax-7S; Punc-122::rfp</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table III-2. Sample sizes for neurons and neuronal structures examined

<table>
<thead>
<tr>
<th>Age</th>
<th>Neuronal structure examined</th>
<th>WT</th>
<th>sax-7</th>
<th>WT</th>
<th>sax-7</th>
<th>WT</th>
<th>sax-7</th>
<th>WT</th>
<th>sax-7</th>
<th>WT</th>
<th>sax-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. III-1</td>
<td>VNC</td>
<td>73</td>
<td>33</td>
<td>40</td>
<td>50</td>
<td>60</td>
<td>32</td>
<td>115</td>
<td>63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td></td>
<td>39</td>
<td>31</td>
<td>46</td>
<td>49</td>
<td>61</td>
<td>31</td>
<td>108</td>
<td>58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-day old adult</td>
<td></td>
<td>42</td>
<td>27</td>
<td>41</td>
<td>70</td>
<td>72</td>
<td>36</td>
<td>143</td>
<td>69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-day old adult</td>
<td></td>
<td>55</td>
<td>73</td>
<td>37</td>
<td>69</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-day old adult</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fig. III-2A-B</td>
<td>CEP</td>
<td>263</td>
<td>277</td>
<td>132</td>
<td>52</td>
<td>114</td>
<td>110</td>
<td>114</td>
<td>110</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td></td>
<td>220</td>
<td>169</td>
<td>110</td>
<td>88</td>
<td>132</td>
<td>110</td>
<td>132</td>
<td>110</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-day old adult</td>
<td></td>
<td>248</td>
<td>224</td>
<td>124</td>
<td>109</td>
<td>143</td>
<td>100</td>
<td>142</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-day old adult</td>
<td></td>
<td>245</td>
<td>124</td>
<td>152</td>
<td>152</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-day old adult</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fig. III-2C</td>
<td>ASH/ASI</td>
<td>252</td>
<td>151</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td></td>
<td>199</td>
<td>166</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-day old adult</td>
<td></td>
<td>396</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-day old adult</td>
<td></td>
<td>174</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-day old adult</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fig. III-3</td>
<td>Nerve ring</td>
<td>28</td>
<td>36</td>
<td>112</td>
<td>108</td>
<td>78</td>
<td>139</td>
<td>166</td>
<td>154</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larval stage 1 (L1)</td>
<td></td>
<td>47</td>
<td>48</td>
<td>244</td>
<td>256</td>
<td>122</td>
<td>128</td>
<td>58</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larval stage 2 (L1)</td>
<td></td>
<td>60</td>
<td>32</td>
<td>263</td>
<td>277</td>
<td>132</td>
<td>52</td>
<td>114</td>
<td>110</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td></td>
<td>61</td>
<td>31</td>
<td>220</td>
<td>169</td>
<td>110</td>
<td>88</td>
<td>132</td>
<td>110</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-day old adult</td>
<td></td>
<td>72</td>
<td>36</td>
<td>248</td>
<td>224</td>
<td>124</td>
<td>109</td>
<td>142</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-day old adult</td>
<td></td>
<td>37</td>
<td>245</td>
<td>124</td>
<td>152</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-day old adult</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fig. III-6B-D</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Punc-14::sax-7(+</td>
<td>line1 line2 line3</td>
<td>60</td>
<td>61</td>
<td>57</td>
<td>56</td>
<td>55</td>
<td>54</td>
<td>56</td>
<td>55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-transgenic control line2</td>
<td>line1 line2 line3</td>
<td>132</td>
<td>123</td>
<td>122</td>
<td>122</td>
<td>116</td>
<td>116</td>
<td>116</td>
<td>116</td>
<td></td>
<td></td>
</tr>
<tr>
<td>line3</td>
<td>line1 line2 line3</td>
<td>124</td>
<td>123</td>
<td>122</td>
<td>122</td>
<td>116</td>
<td>116</td>
<td>116</td>
<td>116</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-day old adult</td>
<td></td>
<td>124</td>
<td>132</td>
<td>84</td>
<td>122</td>
<td>124</td>
<td>90</td>
<td>124</td>
<td>124</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fig. III-9</td>
<td>DNC</td>
<td>\textit{Punc-14::sax-7(+)}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>-----</td>
<td>---------------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>line1</td>
<td>line2</td>
<td>line3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-day old adult</td>
<td>53</td>
<td>31</td>
<td>42</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fig. III-10B</th>
<th>Nerve ring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (sax-7)</td>
<td>sax-7 on levamisole</td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td>32</td>
</tr>
<tr>
<td>2-day old adult</td>
<td>56</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fig. III-10C</th>
<th>ADE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (sax-7)</td>
<td>sax-7 on levamisole</td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td>52</td>
</tr>
<tr>
<td>2-day old adult</td>
<td>88</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fig. III-11A</th>
<th>Nerve ring</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>unc-2</td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td>43</td>
</tr>
<tr>
<td>10-day old adult</td>
<td>136</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fig. III-11B</th>
<th>ASH/ASI</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>unc-2</td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td>252</td>
</tr>
<tr>
<td>10-day old adult</td>
<td>174</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fig. III-11C-D</th>
<th>DNC</th>
<th>ALM</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>unc-2</td>
<td>WT</td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td>40</td>
<td>22</td>
</tr>
<tr>
<td>10-day old adult</td>
<td>73</td>
<td>44</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fig. III-12B-D</th>
<th>Nerve ring</th>
<th>ADE</th>
<th>ASE</th>
<th>AIIY</th>
<th>ALM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control: Juvenile (day 0)</td>
<td>60</td>
<td>132</td>
<td>114</td>
<td>114</td>
<td>90</td>
</tr>
<tr>
<td>Control: 2-day old adult</td>
<td>61</td>
<td>110</td>
<td>132</td>
<td>132</td>
<td>129</td>
</tr>
<tr>
<td>Levamisole d0-d2</td>
<td>43</td>
<td>108</td>
<td>116</td>
<td>116</td>
<td>83</td>
</tr>
<tr>
<td>Fig. III-13B-E</td>
<td>ALM</td>
<td>AVM</td>
<td>PVM</td>
<td>ADE</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td></td>
</tr>
<tr>
<td>Control:</td>
<td>79</td>
<td>40</td>
<td>40</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>2-day old adult</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth in liquid</td>
<td>129</td>
<td>48</td>
<td>49</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>2-day old adult</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fig. III-14B-C</th>
<th>ALM</th>
<th>ADE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control:</td>
<td>79</td>
<td>100</td>
</tr>
<tr>
<td>2-day old adult</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth on vortex</td>
<td>113</td>
<td>92</td>
</tr>
<tr>
<td>2-day old adult</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fig. III-15</th>
<th>ADE</th>
</tr>
</thead>
<tbody>
<tr>
<td>20°C</td>
<td>25°C</td>
</tr>
<tr>
<td>Juvenile (day 0)</td>
<td>132</td>
</tr>
<tr>
<td>2-day old adult</td>
<td>106</td>
</tr>
<tr>
<td>5-day old adult</td>
<td>97</td>
</tr>
<tr>
<td>15°C</td>
<td>110</td>
</tr>
<tr>
<td>25°C</td>
<td>90</td>
</tr>
</tbody>
</table>
Chapter IV

Discussion and Future Directions
Discussion

*C. elegans* has been a favorite model system for aging studies. Age-dependent decline of the whole body, at both the anatomical and behavioral levels, has been well studied in the worm and conserved lifespan determination pathways have been shown to be involved in these age-related changes [31-47, 189]. Additionally, several neurodegenerative diseases have been modeled in *C. elegans*, such as Alzheimer’s disease, Parkinson’s disease, Amyotrophic Lateral Sclerosis (ALS), tauopathies, muscle dystrophies, etc. [58]. Though such C. *elegans* models of neurodegeneration have been investigated extensively, how the normal nervous system is affected by aging in the wild-type worm has only been recently studied.

Aging of the mammalian brain includes morphological changes such as subtle region-specific changes in dendritic branching and spine density, changes in the Ca$^{2+}$ conductance, synapse number, gene expression, as well as behavioral and cognitive decline (e.g. memory loss) [2, 3, 28]. Comparable changes have been observed in *C. elegans*. In both mammals and *C. elegans*, significant neuronal death is not a characteristic of normal aging and neuronal loss does not play a role in age-related cognitive decline in humans ([2, 34, 53-55], our results).

The age-related structural changes observed in aging mechanosensory neurons are similar to those observed in the Huntington's disease model strain of
C. elegans [57] suggesting that these age-related anatomical changes may be the first steps in the disease progression. Therefore a broad analysis of different age-related neuronal changes in the wild type becomes important. Insights into normal as well as neurodegenerative changes in the C. elegans nervous system, may allow us to differentiate normal aging from pathological aging as well as draw parallels between them to begin understanding the underlying age-related causes of neurodegeneration.

As a first step towards understanding the age-related normal neuronal changes, my thesis research explored the morphological changes that take place in the adult C. elegans nervous system with age and factors that may influence the structural alterations. My results show that the C. elegans nervous system exhibits age-related neuroanatomical changes in a wide variety of neurons and neuronal structures. These morphological changes occur in early adulthood and many reach a maximum penetrance by day 5 or day 10 of adulthood. My results suggest an underlying neuronal specificity as the age of onset and type of morphological change appears to be specific to a given neuron or neuronal structure. Additionally, I find that these age-related structural changes in the nervous system are not affected by organismal aging in a simple linear fashion. Although the frequency of these structural changes in the adult nervous system increases with age, extending or shortening lifespan does not simply correlate with delaying or accelerating neuroanatomical changes. Exploring factors that may influence normal age-related neuronal changes, I found that the cell
adhesion molecule, SAX-7, required for maintenance of the nervous system architecture post-establishment, also plays a role in the occurrence of age-related neuroanatomical changes. It is known that the mechanical stress from body movements plays a role in the occurrence of maintenance defects in sax-7 mutants. Since my results suggest that SAX-7 plays a role in age-related neuroanatomical changes and given the involvement of mechanical stress in sax-7 mutant defects, we hypothesized that mechanical stress from body movements may play a role in age-related morphological changes. Indeed, I found that altered body movements affect the neuroanatomical changes in the adult C. elegans nervous system. We speculate that during the aging process, a functional reduction of mechanisms in place to maintain the neuronal architecture contributes to the emergence of age-related neuroanatomical changes, as some of these changes can be suppressed by overexpression of SAX-7. This could suggest that a decline in SAX-7 activity during adulthood (I found that the protein levels are stable during adulthood) may play a role in the changes we have documented in the adult nervous system, and point to an area in need of future investigation.

**Heterogeneity of age-related changes in the nervous system**

The C. elegans nervous system is a unique and complex structure that comes into contact with a variety of tissues as it spans the entire length of an animal. Whether a specific neuron or neuronal structure develops age-related
morphological changes may be influenced by neighboring tissues, the extracellular environment, as well as intrinsic cellular physiology. These variables may make some neurons more susceptible than others, to the stresses the nervous system faces throughout a lifetime. I find that a given neuronal structure generally exhibits only one type of change. For instance, although dopaminergic neurons CEP and ADE exhibit age-related changes in their positioning, I did not observe any age-associated ectopic soma or axon projections. These examples highlight that within the aging nervous system, neurons within different contexts exhibit specific differences in regards to age-related neuroanatomical changes. Although C. elegans lab strains are isogenic, within a population there is heterogeneity in spontaneous locomotion and response to prodding of same-age worms, as well as, in age of onset and rate of age-related decline in locomotory behavior [34]. Similar to the heterogeneity in mobility, I observe individual differences in the positioning of chemosensory neurons ASH and ASI. Susceptibility of a neuron to age-related neuroanatomical changes appears to be more complex than just the identity of the neuron. For each specific neuron examined within a population of isogenic worms, I observed that a fraction of neurons did not exhibit age-related neuroanatomical changes. I also find that these age-related changes to the nervous system occur at specific frequencies in different neuronal classes.
Age-related neuroanatomical alterations: Neuronal plasticity or deterioration?

We and others have shown that the *C. elegans* nervous system exhibits different structural changes with age [53-55] that start to appear in early adulthood and reach peak penetrance by mid-adulthood. This peak often occurs before the worms start dying [161]. What do these structural changes mean in terms of function? Do these structural changes affect the functionality of the neurons? To examine whether age-related change in soma position of ASH and ASI neurons correlated with functional loss, I assessed the worm’s chemoaversion response to high salt concentration (4M NaCl) and high osmolarity (4M fructose). All the worms avoided the compounds at all ages, even despite the soma position change. Thus, these behavioral assays revealed no major functional abnormalities associated with age-related soma position changes and suggest that the neuroanatomical changes may correlate with subtle changes in the functionality of the neurons. Indeed, calcium imaging of ASH neurons shows that calcium transients are detected in ASH neurons in response to glycerol or electric current until day 5 of adulthood [155, 190]. Additionally, there is conflicting evidence in the field about correlation between age-related functional and structural changes in mechanosensory neurons. The Kenyon group found that the inability of worms to respond normally in a gentle touch assay correlated with increased neurite branching of their mechanosensory neurons [54], whereas the Driscoll group found no correlation between defective
touch response and structural changes in the mechanosensory neurons [55]. One possibility is that these morphological changes could be a part of normal growth and development of the neuronal structure and its maintenance throughout life with no effect on the functional aspects of the neuron. [53] A second possibility is that these changes could be adaptive, either in response to a changing environment or compensating for a decline in the neuron’s functional capacity. Neurons in older worms may send out extra branches to be able to take in more signals, possibly to compensate for the loss in sensitivity. Neurite outgrowths from the mechanosensory neuron ALM contain acetylated microtubules [53]. Acetylation of microtubules is considered to be stabilizing the microtubules and local stabilization of microtubules can induce axon formation [191]. Additionally, kinesin 1, which is involved in transporting tubulin oligomers to the tip of the growing axon, binds to acetylated microtubules (reviewed in [192]). These results suggest that the neurites emanating from the mechanosensory ALM neurons may be a sign of adaptive changes in the neuron. Even with these structural changes, the overall neuronal architecture remains almost intact; I did not observe breaking of axons despite the displacement of neuronal cell bodies suggesting that active mechanisms may exist that allow extension of the axon structure to accommodate for the displacement. Mitochondria and cytoskeletal machinery may play a role in the maintenance of axonal structure as well as age-related structural changes such as projections from the neuronal soma and axons [53, 55]. Stable microtubule structure has
been shown to be important for maintenance of the axonal structure during adulthood [108]. Destabilization of microtubule organization leads to adult-onset axonal degeneration possibly through mitochondrial mislocalization [108]. Additionally, mitochondria have been found associated at the branch sites of novel outgrowths in mechanosensory neurons of old worms [55]. A third possibility is that these changes could be degenerative and may be early signs of deterioration. Examination of mechanosensory neurons in C. elegans model of Huntington’s disease has revealed structural changes in these neurons that are similar to those seen for mechanosensory neurons in the aged wild-type worms [57].

Does the nervous system undergo any age-related functional decline? Behaviors such as pumping, locomotion, chemotaxis, and thermotaxis have been shown to undergo age-related decline [32, 34, 40, 44-47]. The pharynx and the body wall muscles also exhibit age-related decline, which had been speculated to underlie the age-related behavioral decline [32, 34]. However, recent studies suggest that age-related decline in the neuronal function may underlie the behavioral decline. Electrophysiological recordings at the neuromuscular junction show that motor neuron activity declines with age, starting as early as day 5 of adulthood, and parallels the progressive decay in locomotion, whereas the body wall muscles remain functional even in old worms [56].

Age-related morphological alterations, such as soma position changes and outgrowths from the soma and axon have not been correlated with a
complete loss of neuronal function. It remains to be explored, whether the structural changes cause partial loss of function or subtle changes in the other aspects of the neuron.

Age-related subtle functional changes have been observed in ASH neurons. Glycerol-evoked calcium transients of ASH neurons exhibit a subtle but significant decline in day 5 adults compared to day 1 adults [155]. Age-related changes in the intrinsic and extrinsic factors may influence structural changes as well as subtle changes in the functional capacity of the neuron. It is possible that these functional changes may contribute to the neuron’s susceptibility to age-related intrinsic and extrinsic changes, and may thereby influence further age-related functional decline.

Organismal lifespan and age-related neuroanatomical changes are uncoupled

As *C. elegans* ages different tissues exhibit age-related changes. Work in a number of labs has identified mutants that extend as well as shorten the lifespan of *C. elegans*. Long-lived mutants have been shown to delay age-dependent structural and functional changes such as sarcopenia [34] and aging of the pharynx [31, 33]. Based on these results, one might expect long-lived mutants to delay the onset of age-related changes in the nervous system, and conversely that short-lived mutants may exhibit these changes earlier. Instead, we find that for a number of neurons, the appearance of age-related
neuroanatomical changes is not delayed in long-lived mutants or accelerated in short-lived mutants and that the correlation is anything but linear. My analysis of long-lived \textit{daf-2} (insulin/IGF-1 signaling pathway), \textit{clk-1} (mitochondrial respiration pathway) and \textit{eat-2} (dietary restriction pathway) mutants and short-lived \textit{daf-16} (insulin/IGF-1 signaling pathway) mutants reveals that head ganglia neurons, axonal bundles and single neurons exhibit age-related neuroanatomical alterations in these mutants. For a number of neuronal structures, long-lived \textit{daf-2} and \textit{clk-1} as well as short-lived \textit{daf-16} mutants exhibit age-related neuronal changes similar to wild type. Long-lived \textit{eat-2} mutants exhibit ventral and dorsal nerve cord defasciculation frequencies similar to wild type but a significant reduction in the frequency of age-related placement change of nerve ring and head neurons. My findings support the idea that change in lifespan is one of the many altered aspects in these mutants, which is separable from their effect on age-related structural and functional changes in the nervous system. Mutants that extend or shorten lifespan affect other aspects of cellular biology and may therefore influence these age-related neuroanatomical changes in unanticipated ways.

Recent work also suggests that lifespan regulation does not directly correlate with age-associated changes in the nervous system. Age-related changes in the mechanosensory neurons that are delayed in long-lived \textit{daf-2} mutants are not delayed in long-lived \textit{eat-2} mutants [53, 54]. Also the function of \textit{daf-2} and \textit{daf-16} in lifespan determination has been shown to be separable from
their function in age-related neurite branching of mechanosensory neurons as well as in axon regeneration [54, 78]. Additionally, age-dependent decline in 5-HT and DA levels observed in long-lived daf-2 and clk-1 mutants was observed to be similar to wild type, whereas long-lived eat-2 mutants did not exhibit this decline [80].

A number of studies in worms, flies, and rodents show that lifespan determination pathways affect age-related neuronal dysfunction and cognitive impairment in different ways [105]. Decreased insulin signaling leads to an increase in lifespan and also improved thermotaxis learning ability, but impaired salt chemotaxis learning [47, 79]. Dietary restriction (DR) has been shown to increase the lifespan in many organisms including yeast, worms, flies, and rodents [105], and DR has been shown to improve learning in aged flies [193] and also suggested to reduce age-related cognitive decline in rats [89]. In worms, DR influences long-term memory during aging but not short-term memory [93]. These examples suggest that lifespan-impacting pathways may affect aspects of the aging nervous system differently and organismal lifespan may not be an indicator of neuronal aging. A recent study suggests that lifespan may not be a good measure for aging studies [145], and instead healthspan should be considered. Healthspan does not correlate with extended lifespan and old long-lived mutants perform poorly than old wild-type worms when assayed for locomotion and resistance to heat and oxidative stress [145].
Mechanical stress might contribute to age-related neuroanatomical changes

It is known that mechanical stress from body movements contributes to the defects in maintenance mutants such as *sax-7*, *dig-1*, and *egl-15* [117] [114] [113] since suppression of locomotion by paralysis suppresses neuronal maintenance defects in these mutants. Additionally, age-related axonal degeneration in *mec-17* mutants can also be suppressed by paralysis [108]. Since mechanical stress from body movements contributes to the maintenance defects of *sax-7* mutants [115, 117, 118] and since my results suggest that *sax-7* may play a role in the age-related neuroanatomical changes in the adulthood, we hypothesized that age-related wild-type structural alterations may be affected by mechanical stress. I subjected worms to paralysis either by genetic or pharmacological means. My results in Chapter III show that paralysis by either method suppresses age-related positioning changes of nerve ring and head ganglia neurons, suggesting that mechanical stress from body movements may play a role in age-related positioning changes. Some of the paralyzed mutants we used such as *unc-2*, *unc-13* and *unc-75*, may affect pharyngeal pumping of the worms and therefore we cannot rule out that reduced pharyngeal pumping may play an important role in the suppression of these changes [92, 194]. Additionally, levamisole is known to inhibit pharyngeal pumping [195] when the worms are exposed to levamisole for up to 30 min. In our assays the worms are exposed to levamisole for 48 hours and effect of prolonged levamisole exposure
on pharyngeal pumping is not known. Therefore, it is unclear whether reduction in pharyngeal pumping plays a role in the suppression of age-related positioning changes of nerve ring and head ganglia neurons seen in worms immobilized using levamisole. Neuroanatomical analysis of structures away from the pharynx may help in differentiating between the effect of mechanical stress from pharyngeal pumping and from body locomotion. However, paralysis by genetic or pharmacological means did not have any effect on age-related soma projections of mechanosensory ALM neurons (Chapter III). These results suggest the following possibilities: mechanical stress affects age-related changes in positioning of head neuronal structures but does not affect age-related structural changes in mechanosensory neurons, or mechanical stress from body movements has a rather weak effect on age-related changes and these possibilities may not be mutually exclusive. Additionally, it is possible that the inherent nature of the structural change may affect the frequency observed; a placement change may be easier to bring about than a soma projection.

In support of the idea that mechanical stress from pharyngeal pumping may affect age-related changes in positioning of head neuronal structures, we find that the frequency of age-related morphological changes in neuronal structures around the pharynx is reduced in eat-2 mutants, which exhibit drastically reduced pharyngeal pumping than wild type [157]. However, this is a chronic reduction in pumping that eat-2 mutants experience throughout their life, and we cannot rule out any developmental effects this may have. To explore the
effect of acute reduction in pharyngeal pumping, we manipulated the availability of food to wild-type worms. *C. elegans* reduce pharyngeal pumping in absence of food [141]. In our assay of ‘discontinued feeding’, adult worms, starting day 0 of adulthood, were alternated between growth conditions with food and without food and examined as day 2 adults. I observed a significant reduction in the frequency of nerve ring and soma placement. Reduction in mechanical stress from pharyngeal pumping may underlie this observed reduction in age-related position change of neuronal structures near the pharynx. In line with this idea, we hypothesized that structures situated away from the pharynx would not exhibit any change in age-related structural changes. Surprisingly, I observed a subtle increase in soma projections of mechanosensory ALM neurons as well as novel soma projections for mechanosensory AVM and PVM neurons. This suggests that, in addition to mechanical stress from pumping, the neuroanatomical alterations may be affected by systemic effects of food deprivation. It is noteworthy, that the effect of discontinued feeding appears to cause a strong effect on age-related position change of neuronal structures located near the pharynx and a rather subtle effect on frequency of soma projections of mechanosensory neurons. This difference in the effect of discontinued feeding on the different neuronal structures is similar to that observed in the paralysis assay. These observations highlight the underlying differential neuronal susceptibility to various stresses.
The difference in the effect of reduced mechanical stress on different neuronal structures motivated us to explore the effect of altered locomotion paradigms on neuroanatomical changes. Juvenile worms (day 0 adults) were subjected to growth for 48 hours either in liquid on a nutator or on agar plates atop a vortex. In the liquid growth assay, worms were grown in M9 buffer with *E. coli* as food in microcentrifuge tubes on a nutator to allow aeration and mixing of the medium. Worms exhibit swimming / thrashing locomotion in liquid distinct from the sinusoidal wave motion / crawling on agar. In the constant agitation assay, worms were grown on NGM agar plates with *E. coli* as food, and the plates were taped on top of a test tube rack that was taped to the top of the vortex. The vortex was run at high speed for 48 hours continuously. In both these experimental paradigms, I observed an increase in soma projections of mechanosensory neurons. We hypothesize that the growth in liquid and growth under constant agitation are a form of increased mechanical stimulation that may cause constant or increased activation of the mechanosensory neurons resulting in increased soma projections. Interestingly, neither altered locomotion paradigm had any effect on the soma placement of head neurons. These results support the idea that neuronal structures respond differently to mechanical stress from different body movements highlighting the underlying differential neuronal susceptibility.
Environmental factors such as temperature may also affect the neuroanatomical changes through a wide range of intracellular and extracellular changes. I find that in early adulthood (day 2), age-related changes in the position of dopaminergic neurons ADE, are suppressed at a temperature lower than cultivation temperature, yet enhanced at a higher temperature. Interestingly, this suppression at lower temperature and enhancement at higher temperature is no longer observed in day 5 of adults. Additionally, though significant, the suppression or enhancement is rather subtle. This suggests that temperature may have weak effect on the age-related changes that may be lost later in life in the face of different age-related stressors. It is also possible that, since temperature affects a number of processes, the change in these intracellular and extracellular processes that may then affect the age-related neuronal changes may add up to a weak effect in early adulthood or no effect later. Change in temperature is a complex phenomenon and understanding its effect on morphological changes in the nervous system will require additional analysis.

**Loss of function sax-7 mutants exhibit age-related neuroanatomical changes similar to wild type albeit earlier**

Neuronal maintenance mechanisms exist to preserve the structure and function of the nervous system throughout the life of an organism. One important component of the neuronal maintenance mechanism in *C. elegans* is *sax-7*. *sax-7* is the *C. elegans* homolog of mammalian L1 cell adhesion molecule (L1CAM).
sax-7 mutants exhibit neuronal architecture maintenance defects in a number of neurons, including displacements of axons within fascicles and neuronal cell bodies within ganglia [115, 117-120]. These neuronal structures develop normally in sax-7 mutants and display these positioning defects in axons and cell bodies only after their proper establishment. Although, several classes of neurons have been examined in sax-7 mutants, a thorough neuroanatomical analysis through adulthood can provide information as to whether in a wild-type animal sax-7 plays a role in normal aging, and also whether these sax-7 defects are early signs of normal aging. My analysis reveals that sax-7 mutants exhibit age-related neuroanatomical changes in a variety of neuronal structures. The alterations I observed in sax-7 mutants were either similar to those seen in wild-type aging, such as the defasciculation of the ventral nerve cord with age; or similar to those seen in wild type but that occur earlier in sax-7 mutants, such as defasciculation of the dorsal nerve cord, displacement of nerve ring and head ganglia neurons as well as soma projections of mechanosensory neurons ALM; or more severe than what I observed in the same neurons in wild type such as displacement of dopaminergic head neurons ADE. These results suggest that, sax-7 plays a role in some age-related morphological changes suggesting the specificity of neuronal maintenance mechanisms, in age-related neuroanatomical alterations. Additionally, I found that for maintenance of some neuronal structures such as the nerve ring and dopaminergic head neurons CEP, sax-7 was required even earlier in life during larval stages since the displacement defects were
observed in second larval stage worms. This is similar to the observation for ASH and ASI neurons, where the displacement defects are seen in third larval stage worms (C. Bénard, unpub.). In case of dopaminergic head neurons ADE and interneurons AIY, sax-7 mutants exhibit displacement defects as early as two hours post hatch. Therefore, it is possible that sax-7 may be required for maintenance of these structures even earlier, possibly during embryogenesis, right after the neurons are established, or that sax-7 may be involved in the development of these structures. sax-7 is expressed starting 2-cell stage of embryogenesis [119] and is involved in the development of the dendritic arbors of mechanosensory and thermosensory PVD neurons [126-128].

Put together these results suggest that sax-7 is involved in the maintenance of neuronal structures post-establishment during larval stages and later during adulthood. This may be a conserved feature, since the mammalian homolog L1CAM function is required for learning and memory in the adult mouse brain [177]. My results also suggest that the requirements of sax-7 function vary among the different neurons and neuropils. This highlights the differences among the neuronal structures that may contribute to differential neuronal susceptibility to different stressors including aging.

**SAX-7 protein levels in the adult wild type**

In most of neuroanatomical alterations that we observed, the defects are similar to wild type but appear earlier in adulthood. Does this suggest that these
are signs of accelerated aging? Is it possible that the occurrence of age-related neuroanatomical changes is due to reduction in the expression and / or activity of the maintenance machinery with age? To explore this possibility, I analyzed the SAX-7 protein levels in wild-type adults by western blot analysis of worm lysates of age-synchronized adult populations aged day 0, 2, 5, and 10. I found that SAX-7 is expressed in wild type worms until day 10 of adulthood. Interestingly, in mammals L1CAM levels are low in the femoral nerve segments in adult mice, which have been correlated to poor recovery after injury [188]. Pan-neuronal expression of sax-7S, but not sax-7L, rescues the maintenance defects of loss-of-function sax-7 mutants [115], and wild-type worms over-expressing pan-neuronal sax-7L, but not sax-7S, display soma and axon displacement defects [117], suggesting that SAX-7S may be the functional isoform and SAX-7L may have disruptive activity. It is possible that a net reduction in the amount of functional SAX-7S together with a net increase in the amount of functional SAX-7L, may underlie the occurrence of age-related changes. Differential regulation and protein stability, as well as cleavage by Furin and PC-type proprotein proteases with differential affinities may contribute to the difference in the protein levels of the two isoforms. Additionally, possible differences in the protein structure (proposed horseshoe shaped folding of SAX-7L) may render the two isoforms differentially susceptible to protease cleavage. It may be interesting to see, whether the levels of the proteases that cleave SAX-7, are altered during aging and therefore affect protein levels of SAX-7S and SAX-7L. Additionally,
one could test, whether the proteases cleave the isoforms with different affinities. SAX-7 has been implicated to engage in homophilic interactions [118]. It is possible that SAX-7L may bind to SAX-7S and therefore, reduce the functional pool of SAX-7S in adult animals.

I hypothesize that SAX-7S protects the nervous system in young worms and deregulation at the level of transcription and/or translation, differential protein stability, protease cleavage and interaction with SAX-7L, affect SAX-7S levels in adult worms leading to neuroanatomical alterations.

**SAX-7 may protect the nervous system against age-related neuroanatomical changes**

My results suggest that there is an age-related decline in the levels of SAX-7S, which coincides with many of the age-related neuroanatomical changes we observed in wild-type aging animals. Possibly, the decline in SAX-7S in wild-type aging animals plays a role in the appearance of these age-related neuroanatomical changes. We hypothesized that providing excess of sax-7S to wild-type animals may prevent or at least delay these age-related neuronal changes (**Fig. IV-1**). Indeed, I find that pan-neuronal overexpression of SAX-7S does suppress age-related changes in the placement of the nerve ring and the positioning of head neurons up to day 10 of adulthood. This suggests that providing the nervous system with more SAX-7S protects against these age-related changes and that a decline in the levels of SAX-7S may play a role in
these age-related neuroanatomical changes. Is this a direct effect? In other words, does SAX-7S affect body locomotion or pharyngeal pumping, which leads to the suppression of age-related changes? To address this possibility, I measured the pumping rate and thrashing rate of the sax-7S overexpressing worms, and found that these worms exhibit slight reduction in the pharyngeal pumping rate and wild-type locomotion. A drastic reduction in pharyngeal pumping affects age-related neuroanatomical changes (as seen in eat-2 mutants) but a subtle reduction may not affect the age-related neuroanatomical changes (as seen in clk-1 and cca-1). The ability of increased SAX-7S expression to suppress age-related neuroanatomical changes appears to be neuron specific, since we do not observe suppression of age-related dorsal nerve cord defasciculation upon pan-neuronal overexpression of SAX-7S. The neuroprotective effect of sax-7 appears to be conserved since adult mice show enhanced recovery after spinal cord injury when the mammalian homolog L1CAM is overexpressed or activated just after the injury [188, 196-205]. It remains to be tested whether SAX-7 overexpression protects the nervous system against age-related functional and behavioral decline.

SAX-7 is expressed ubiquitously and is required in multiple tissues for the maintenance of GABAergic neuronal soma placement and commissural axon trajectories, whereas neuronal expression is sufficient to rescue the head soma placement and axon flip-over defects of sax-7 mutants, suggesting that maintenance of different neuronal structures may have specific spatial
requirements for SAX-7 function [115, 117, 120]. Additionally we find that age of onset of the neuroanatomical defects in sax-7 mutants varies for the different neuronal structures assessed, suggesting that there may be specific temporal requirements for SAX-7 function. A recent study highlighted the temporal requirement of SAX-7 in synaptic regulation by using a heat-shock inducible construct [121]. Use of this heat-shock inducible expression system with cell specific promoters may help to identify the spatial and temporal requirements of SAX-7 function in wild-type neuroanatomical changes.

The wild-type nervous system exhibits age-related changes and these changes are not regulated by length of the lifespan. Examination of sax-7 mutants and studies with SAX-7S overexpression in the wild type, suggest that the nervous system may be maintained with age, and occurrence of the structural changes may be an indication that the maintenance machinery may have become overwhelmed in the face of stresses such as mechanical stress from body movements. These results together suggest that age-related changes in the nervous system may be explained by a combination of the Damage and error theories of aging specially, the Wear and tear and the Free-radical theory. Additionally, since neuronal maintenance mechanisms protect the nervous system even after reproductive senescence, it may be possible to rule out the Antagonistic Pleiotropy theory for explaining the changes that take place in the nervous system with age.
Limitations of the work and future directions

My results suggest that a number of neuronal structures exhibit neuroanatomical changes in the adult wild-type *C. elegans* nervous system. Mechanical stress from body movements appears to be a contributing factor to these neuroanatomical changes. However, certain limitations in our experimental paradigms limit the inferences that can be drawn from the work. Additionally, I observe that temperature change has a mild effect on age-related neuroanatomical changes in early adulthood suggesting that changes in the intracellular and extracellular environments may affect the neuronal changes. Involvement of cell adhesion molecule SAX-7 in the occurrence of age-related changes further suggests that changes in the basement membrane proteins, extracellular matrix proteins as well as other adhesion molecules may shape the age-related structural changes in the nervous system. SAX-7 is also known to interact with the cytoskeletal machinery downstream via interaction with Ankyrin, PDZ domain protein gamma-syntrophin and dystrophin [124, 206]. Analysis of age-related changes in the intracellular components such as cellular organelles, cytoskeletal components, and in extracellular components such as adhesion proteins, basement membrane components as well as extracellular matrix proteins may help better understand the complex nature age-related changes that lead to age-related structural and functional changes in the nervous system. It is highly likely that changes in the intracellular and extracellular environment of
the neuron lead to structural and functional changes in the neurons that lead to further changes in the intracellular and extracellular components, causing a cascading effect, which eventually may lead to deterioration of the nervous system and other tissues.

Nervous system structural changes may correlate to subtle changes in functionality

In our longitudinal analysis, we observed that worms exhibiting age-related changes in the position of chemosensory neurons ASH and ASI were still able to avoid high salt and high osmolarity compounds. These chemoaversive cues are very strong indeed and the ability of the worms to be able to respond to these even at older ages, merely suggests that the neurons do not lose functionality completely. It may be informative to assess the functionality by analyzing the calcium transients in old neurons and attempting to correlate the structural changes with changes in calcium transients. Additionally, analysis of chemoaversion to salt concentrations much lower (250 or 500 mM NaCl) than the one used in our study (4M NaCl), may allow for finer behavioral analysis and may correlate better with the structural changes.
Effect of mechanical stress from pharyngeal pumping on age-related positional changes of head neuronal structures

My results show that age-related structural changes in the position of head neuronal structures are affected by the mechanical stress from body movements, evidenced by either paralyzing the worms or altering body locomotion. Paralysis, either by genetic or by pharmacological means, causes a reduction in pharyngeal pumping [92, 194, 195]. Therefore, it becomes difficult to separate the effect of mechanical stress due to body locomotion from that of pharyngeal pumping, on the age-related morphological alterations. *eat-2* mutants have significantly reduced pharyngeal pumping rates and I find that age-related positional changes in the nerve ring and head neurons are suppressed in these worms. Since *eat-2* mutants are calorically restricted, it is difficult to draw a clear relationship between the mechanical stress of pharyngeal pumping and age-related structural changes.

It may be possible to analyze the effect of mechanical stress from pharyngeal pumping solely by feeding *eat-2* worms an ‘easier-to-eat’ bacterium, *Comamonas*, which is known to suppress the lifespan extension of *eat-2* mutants as well as their small body size [93]. This suggests that *eat-2* mutant worms that are fed *Comamonas*, no longer experience dietary restriction [93]. Thus, this experimental paradigm helps separate the effect of pharyngeal pumping based mechanical stress from dietary restriction with respect to age-related neuronal changes. While the pumping rate of *eat-2* mutant worms that are fed
*Comamonas* has not been measured, I speculate that it is similar to that of *eat-2* mutants fed *E. coli*, since, EAT-2 is a subunit of the ligand-gated ion channel in the pharyngeal muscle and regulates the rhythmic motion of the pharyngeal pump. It is possible to calorically restrict worms only in adulthood, which is sufficient to cause lifespan extension [37]. *eat-2* mutants fed *E. coli* exhibit slower body locomotion than wild type [207]. Yet it is not known whether feeding *Comamonas* rescues this phenotype. Therefore, analysis of body locomotion of *eat-2* mutants on *Comamonas* will help to dissect the effect of mechanical stress from pharyngeal pumping versus that from body locomotion.

Feeding *eat-2* mutants *Comamonas* till adulthood and then feeding them *E. coli* to cause caloric restriction can give us a temporal handle to tease apart the possible effects of mechanical stress and dietary restriction on age-related neuronal changes.

**Nervous system is capable of accommodating subtle changes in pharyngeal pumping rate**

Age-related positional changes of the nerve ring and head neurons are suppressed in *eat-2* mutants that have reduced pharyngeal pumping. *clk-1* mutants also exhibit reduced pumping (1.5 fold reduction), though the reduction is not as drastic as seen in *eat-2* mutants (4 fold reduction) [103, 158]. Despite the reduction in pumping seen in *clk-1* mutants, the age-related neuroanatomical changes of the nerve ring and head ganglia neurons observed in *clk-1* mutants
do not resemble those observed in eat-2 mutants (Table IV-1). Additionally, mutants of cca-1, a calcium channel alpha subunit, exhibit about a 1.3 fold reduction in pharyngeal pumping [158]. In cca-1 mutants placement of the nerve ring and head neurons is similar to wild type. These results suggest that the nervous system may be flexible enough to accommodate subtle changes in pharyngeal pumping and have a certain capacity to bear mechanical stress. Therefore only a drastic reduction in pumping is able to bring out a significant change in the placement of head neurons.

**Discontinued feeding paradigm: Starvation?**

In our discontinued feeding paradigm, juvenile worms are alternated between food and no food for periods of 8 or 16 hours. I find that discontinued feeding leads to suppression of age-related positional changes in the nerve ring and head neurons as well as increased soma projections of mechanosensory neurons. Though worms suppress their pharyngeal pumping in absence of food, extended periods of food deprivation cause a modest reduction, about 1.5 fold, in pharyngeal pumping [141]. Therefore it is possible that the effects on age-related neuronal changes actually stem from starvation rather than reduction in pharyngeal pumping. Reduction in the length of food deprivation may allow us to analyze the effect of reduction in pharyngeal pumping without starving the worms. Two hours off food reduces pharyngeal pumping by 2-fold, the maximum reduction observed in the food deprivation paradigms [141].
Vortexing the worms: a model of Traumatic Brain Injury?

Traumatic brain injury (TBI), one of the major causes of death and disability in the world today, is often caused by an acute external mechanical force which may be from direct impact, rapid acceleration / deceleration, explosion or a penetrating object [136]. In addition to the primary damage caused by the physical impact, secondary brain damage due to excitotoxicity or inflammation, as well as increase in calcium and free radicals plays an important role in the deterioration that follows after the initial impact. Several models of TBI have been developed in a variety of animals including flies, mice, rats and pigs. Though a number of studies in the worm have provided useful insights into the cellular and molecular processes following neurotoxic stress, cytoskeletal disruptions and axonal injuries [208], models of TBI in worms are just beginning to be formulated. Recent study has used shock waves to simulate blast waves from explosion in worms, an increasing cause of traumatic brain injury in military combat and terrorist attacks [209]. There is increasing interest in sports related brain injuries, the majority of which are mild TBI caused by direct impact to the brain but without any external injury signs [133]. I hypothesize that constant agitation of worms atop a vortex, may serve as a model for mild TBI. I find that worms under these conditions exhibit increased soma projections of mechanosensory neurons. Further investigation of molecular and cellular changes in these worms, may provide more evidence to support this
experimental paradigm to model TBI in sports personnel such as footballers, boxers, soccer players amongst others.

**SAX-7 expression levels during adulthood**

The wild-type nervous system exhibits age-related structural changes and earlier occurrence of the neuroanatomical changes in sax-7 mutants as well as delayed occurrence of these changes in the wild type upon overexpression of SAX-7 suggests that neuronal maintenance mechanisms may play a role in the occurrence of age-related changes. An age-related downregulation of SAX-7 may underlie these changes, though we observed that SAX-7 protein was expressed in adult worms at least till day 10 of adulthood, assessed by western blot analysis of age-synchronized whole worm lysates. Since SAX-7 is ubiquitously expressed, a caveat of this assessment is that the SAX-7 expression levels detected are from the entire worm body and may not reflect the age-related expression changes that may take place in the nervous system. SAX-7 is a transmembrane protein, which can be cleaved, raising a possibility of secretion of the cleaved fragment of SAX-7 that may be functionally active. Therefore, it is possible that changes in the expression of SAX-7 in the worm and not just the nervous system may be able to influence the age-related changes in the nervous system. Nevertheless, assessment of age-related change in SAX-7 expression levels in the nervous system alone will be informative. Neuronal cell bodies can be isolated by labeling neurons with a pan-neuronal fluorescent marker and then
FACS sorting them. SAX-7 protein levels may then be analyzed by western blot analysis of the neuronal populations. It may be technically challenging to isolate neuronal structures such as the nerve cords and therefore the analysis may not reflect the true protein levels in the nervous system, since SAX-7 expression is detected on the plasma membrane of the neuronal cell bodies as well as neuronal processes. Additionally, use of protease inhibitors in the sample preparation may help avoid cleavage of SAX-7 and allow for a better estimate of the expression levels of the full-length proteins, SAX-7S and SAX-7L.

Tagging the C- and N- termini of native SAX-7S and SAX-7L proteins using CRISPR/Cas9 targeted genome editing or inserting tagged versions of these proteins in the worm’s genome using mosSCI single-copy insertion techniques, may allow for analysis of temporal and spatial changes in the expression of the two isoforms in the adult *C. elegans* nervous system.

**Temporal and spatial resolution of the protective effect of increased SAX-7S levels in delaying the age-related neuronal changes**

In our current experimental design, SAX-7S is overexpressed in all neurons throughout life, from embryo through adulthood. Therefore, it is difficult to understand the temporal and spatial requirements of SAX-7S for delaying the age-related changes. Experiments are underway, where SAX-7S is overexpressed from a small subset of neurons, the ciliated sensory neurons (*P*osm-6), and the effect of this overexpression is being tested on the age-related
change in the position of the nerve ring. With the help of a variety of cell-specific promoters, it is possible to narrow down the spatial requirement for SAX-7S in delaying the age-related changes. It has been observed that the requirement of SAX-7S in single or in multiple cells for rescuing the sax-7 mutant defects is context dependent. In sax-7 mutants, defects in AFD neuronal placement can be rescued by expressing SAX-7S only in AFD neurons [118], whereas, defects in AVK-AIY attachment require expression of SAX-7S in both the neurons for the rescue [115].

To gain insights into the temporal requirement for SAX-7S overexpression, SAX-7S can be expressed under the heat shock promoter (Phsp-16.2). These overexpression worms are grown and maintained at 15°C, to avoid expression of the promoter. The adult worms are heat shocked for a few hours, which leads to expression of the Phsp-16.2 promoter and drives SAX-7S overexpression, and the neuroanatomy may be assessed a few hours or days after the heat shock.

It would be further interesting to understand, which domains of SAX-7S are required for protecting the nervous system during aging. It is known that expression of the first three Ig domains of SAX-7L has a dominant negative effect and leads to a number of defects in locomotion, gonadal morphology as well as embryogenesis [119]. Additionally, the third and fourth Ig domains have been shown to be important for the ability of SAX-7S in rescuing the sax-7 mutant defects [115]. The role of the intracellular tail domains in the rescue of sax-7 mutant defects by SAX-7S expression is context-dependent [115, 118]. Analysis
of the effect of overexpression of various constructs deleting specific domains of SAX-7S on age-related changes, may help narrow down the domain(s) of SAX-7S functioning in protecting the nervous system during aging. This may further open possibilities for developing treatments for age-related nervous system decline in higher animals and eventually humans.

**Other neuronal maintenance factors may be involved in the occurrence of age-related neuroanatomical alterations**

I find that *sax-7* is involved in maintenance of some neuronal structures but not all, suggesting that additional neuronal maintenance factors may play a role in age-related neuroanatomical changes. *zig-3* and *zig-4* are members of the *zig* family of two-Ig domain proteins and have been shown to be important for the maintenance of ventral nerve cord axons but not for the maintenance of head neuron positioning of neurons tested [112, 116]. It would therefore be interesting to see whether overexpression of these proteins may in fact delay the age-related defasciculation of the ventral nerve cord. Another neuronal maintenance factor, EGL-15 (5A), the *C. elegans* fibroblast growth factor receptor (FGFR) isoform 5A, has been shown to affect the maintenance of a small subset of ventral nerve cord axons [113]. It will be interesting to see if the ventral nerve cord defasciculation can be suppressed by providing excess of EGL-15(5A), though overexpression of EGL-15 (5A) in epidermal cells in the wild-type has been shown to result in abnormal glial morphology and ectopic presynaptic
specialization in AIY neurons [122], suggesting that EGL-15(5A) levels may be under tight regulation. The Colon-Ramos group also identified a novel factor involved in the maintenance of presynaptic distribution of AIY neurons post-development, a SLC17 family transporter, CIMA-1 [122].

It is not known whether these different maintenance factors interact with each other [110]. Additionally, for most of these molecules, only one or a few neuronal classes have been analyzed for phenotypes of defects in the maintenance of neuronal architecture. An extensive anatomical analysis of different neurons and neuronal structures affected in these mutants may help understand the specific requirements of different neurons for the neuronal maintenance machinery. It is not known whether these molecules are required in adulthood, whether they are expressed in adulthood, and whether their expression levels change with age.

Results from my thesis work suggest that normal age-related anatomical changes affect the nervous system broadly yet with neuron-type specificity. Though these structural changes have not yet been correlated with functional changes, the nervous system has been shown to undergo age-related functional and behavioral decline [47, 56, 163]. My results also suggest that mechanical stress and environmental factors such as temperature may impact on these age-related changes. This gives us a handle to study the impact of various stressors on age-related neuronal changes. Analysis of age-related intracellular changes
together with changes in the extracellular environment of the aging neuron may
further our understanding of the mechanisms that may underlie age-related
neuronal changes.

Though age-related changes in the brain have not been shown to cause
neurodegeneration, it is evident that old age is associated with higher risk of
neurodegenerative diseases. It is possible that biological processes in the
younger organisms protect the nervous system against all the different stressors
but become less and less effective in older organisms (Fig. IV-1). I find that
mechanisms known to protect the nervous system early in life, post-
development, may also be involved in the age-related alterations. Overexpression of SAX-7S suppresses the age-related changes in the aging
wild-type nervous system. This is the first evidence of increased expression of a
gene leading to delay in age-related changes in the wild-type. This protective
function of SAX-7/ L1CAM has been shown to be important for better recovery
from spinal cord injury in mice [202], suggesting a conserved functional role.
Further analysis of neuronal maintenance mechanisms may unravel the
underlying biological processes that become affected during aging.
Figure IV-1. Proposed model of the role of neuronal maintenance machinery and stress in age-related neuroanatomical changes in young and old wild-type worms.

Model proposing that the balance between neuronal maintenance machinery and stress may lead to maintenance of the neuronal architecture in young worms, whereas the maintenance machinery may get overwhelmed with increased stress leading to age-related neuroanatomical changes in older wild-type worms.
Worms deficient in the neuronal maintenance machinery (sax-7 mutants) exhibit neuroanatomical alterations earlier in life suggestive of accelerated aging, whereas increasing expression of sax-7S, probably leading to increased activity of the maintenance machinery suppresses age-related neuroanatomical changes in older wild-type worms.
Table IV-1. Age-related neuroanatomical changes are uncoupled from lifespan.

<table>
<thead>
<tr>
<th>Neuronal structure</th>
<th>Wild type</th>
<th>eat-2</th>
<th>clk-1</th>
<th>daf-2</th>
<th>daf-16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsal nerve cord defasciculation</td>
<td>Increases with age</td>
<td>ns</td>
<td>↑</td>
<td>↑</td>
<td>ns</td>
</tr>
<tr>
<td>Ventral nerve cord defasciculation</td>
<td>Increases with age</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Nerve ring posterior displacement</td>
<td>Increases with age</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>ns</td>
</tr>
<tr>
<td>ALM posterior projections</td>
<td>Increased in older worms</td>
<td>ns</td>
<td>ns</td>
<td>↑</td>
<td>ns</td>
</tr>
<tr>
<td>PLM posterior projections</td>
<td>Increased in older worms</td>
<td>↓</td>
<td>ns</td>
<td>ns</td>
<td>↓</td>
</tr>
<tr>
<td>CEP soma posterior displacement</td>
<td>Increases with age</td>
<td>↓</td>
<td>ns</td>
<td>ns</td>
<td>↓</td>
</tr>
<tr>
<td>ADE soma posterior displacement</td>
<td>Increases with age</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>ns</td>
</tr>
<tr>
<td>ASH/ASI soma posterior displacement</td>
<td>Increases with age</td>
<td>↓</td>
<td>↑</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

Comparative analysis of wild-type and long-lived (eat-2, daf-2, clk-1) as well as short-lived (daf-16) mutants suggesting uncoupling of age-related neuroanatomical changes from lifespan. ‘Wild type’ column lists the findings in day 10 adults compared to day 0 wild-type adults. Long-lived and short-lived mutant columns list results of day 10 mutant adults compared to day 10 wild-type adults. ns, not significant; upward arrow, increase in the frequency of the alteration; downward arrow, decrease in the frequency of the alteration.


