RNAi STUDIES IN *CAENORHABDITIS ELEGANS* REVEAL THAT COENZYME Q PROTECTS GABA NEURONS FROM APOPTOTIC, CALCIUM-DEPENDENT DEGENERATION

By

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Dissertation
Submitted to the Faculty of the
Graduate School of Vanderbilt University
In partial fulfillment of the requirements
For the degree of

DOCTOR OF PHILOSOPHY
in
Cell and Developmental Biology

December, 2007

Nashville, Tennessee

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This thesis is dedicated to Mary-Lou Ernst-Fonberg, MD, Ph.D. for mentoring me over the past ten years. She has inspired me with her unwavering love of and excitement for science and her dedication to leading a complete and full life. She unapologetically stands out as a gifted female scientist, and has done so from a time when women were not welcome in this field. To receive advice and encouragement from such a person has made a significant positive impact in my career and my life.

I would also like to dedicate this work to my husband, JJ Westmoreland, Ph.D. for his support, frequent discussions, and for making me laugh every day.

ACKNOWLEDGEMENTS

Several people contributed significantly to the completion of this dissertation. I would like to thank my advisor, David Miller, for doing me the great service of allowing me to do research in his lab. I will always credit the challenging environment of the Miller laboratory for extensive personal and professional growth on my part. I would also like to thank my colleagues in the lab, Rebecca Fox, Stephen Von Stetina, Clay Spencer and Joseph Watson for helpful discussions, protocols, and friendship. Steve has been both friend and mentor, always open to listen to my ideas, and ready with extensive expertise and encouragement. Joseph performed the invaluable, yet extremely mundane, task of blinding all of my studies and verifying many of my results, and this work could not have taken place without his contribution. He also performed work on the coq-1 mutant described in chapter IV. I also had the pleasure of working with a very advanced, dedicated Vanderbilt undergraduate, Bittu Majmudhar. Bittu worked on the thrashing assays for the RNAi screen described in chapter III, and her focus and commitment were invaluable to completing this part of the project.

I am extremely fortunate to have had a brilliant and inspiring committee. Though Vivien Casagrande is modest about the role that she has played, I have always found her advice to be valuable and practical and her encouragement sincere and meaningful. Randy Blakely has been a mentor and role model for me throughout my graduate career. A significant portion of my development as a scientist has come from observing Randy's thought processes as he asks questions at seminars and in my committee meetings. I am

so grateful for all of his suggestions and encouragement. I should echo these sentiments for Pat Levitt and Bruce Appel. Observing them as scientists has broadened my scientific view and made me appreciate areas of neuroscience that I had previously found intimidating. As individuals, each committee member has influenced both my personal and professional life, and as a committee, they have demonstrated for me a dynamic and thoughtful ideal of scientific exchange.

The departments of Cell and Developmental Biology and Pharmacology, under the leadership of Susan Wente, Christopher Wright and Heidi Hamm, respectively have inspired and motivated me with their devotion to science, and their commitments to graduate education. I am grateful to have had so many examples to follow and so many people from both departments take an interest in my growth as a student.

I never would have made it to this point without those that gave me my first opportunities to do science. I would like to thank Drs Mary-Lou Ernst-Fonberg and Heidi Hamm for letting me do years of research in their labs, and for the encouragement that I have always received from both of them. I can be extremely cynical about my own abilities, and I therefore rely heavily on the opinions of good scientists to balance this out. In addition, having female role models, such as Lou, Vivien, Heidi, and many others, has braced me against discrimination and adversity.

I would also like to thank all of those who listened to my ideas and gave me opportunities to participate in the educational community at Vanderbilt. Chief among these are Roger Chalkley and Jim Patton, for giving me the chance to create the IGP methodology course, and Ellen Fanning and Kathie Friedman for including me in their HHMI community of scholars project. Finally, I am thankful to the Vanderbilt center for

teaching for being a pure joy to work with and for greatly advancing my teaching and professional skills. While these opportunities did not contribute directly to my research, they have been indispensable for my career as a whole, and have provided for some of the most productive and enjoyable experiences of my graduate career.

Finally, I want to thank my parents, Jeff Earls and Dianne Few, my grandparents, Joe and Edith Earls, my parents-in-law, Billy and Judy Westmoreland, and my siblings, Elijah and Heather, for seven plus years of encouragement and support. I would also like to thank my friends for their contributions, which were substantial. I could not have done this without their being there to listen, advise and cheer me on. There were times when training for races or the many potluck dinners with my friends kept me from plunging into despair from all the failed experiments. Thank you. My biggest supporter throughout this process has been my husband, JJ Westmoreland, and really there is no level of gratitude that is appropriate to his contribution to my work and life. I will just say that he has been the best friend, colleague and supporter imaginable. I could not have made it through this without him.

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Abbreviations			
3-NP	3-nitropropionic acid	GFP	green fluorescent protein
ACh	Acetyl Choline	glr-1	Glutamate receptor (AMPA-type)
ADP	Adenosine diphosphate	gpd-2	Glyceraldehyde-3-phosphate dehydrogenase
AIF	Apoptosis inducing factor	GST	glutathione-S-transferase
al	Aristaless	GTPase	Guanosine triphosphatase
alr-1	al/ARX-related	HD	Huntington's disease
AP	Adaptor protein	HSP	Heat shock protein
Apaf-1	Apoptotic protease activating factor	L1-4	Larval stage 1-4
aps-2	Adaptin small chain	MAPCel	Micro-array profiling of C. elegans cells
apt-10	Adaptin-related protein	MPTP	1-methyl-4-phenyl-1,2,3,6-terahydropyridine
ARX	Aristaless-related homeobox	myo-3	Myosin heavy chain
ATP	Adneosine triphosphate	NADH	Nicotinamide adenine dinucleotide
Bcl-2	Mutated in B-cell lymphoma	odr-4	Odorant response abnormal
BLAST	Basic local alignment and search tool	Opa-1	Mutated in optic atrophy
Ca2+	Calcium	PARL	Presenillin-associated rhomboid-like
ced-3	Cell death abnormal	PC	Purkinje cell
CEP-1	C. elegans p53-like protein	PD	Parkinson's disease
clk-1	Clock-1	pgk-1	Phosphoglycerete kinase-1
CNS	central nervous system	Pitx-2	Paired-like homeodomain-2
CoQ	Coenzyme Q	PT	Permeability transition
coq-1	CoQ synthesis enzyme 1	PTP	Permeability transition pore
crt-1	Calreticulin	RNAi	ribonucleic acid interference
DA	Dopamine	ROS	Reactive oxygen species
Dat-1	Dopamine transporter	SCA	Spinocerebellar ataxia
DD	embryonic-born D class VNC GABA neurons	sel-12	Suppressor/Enhancer of Lin-12
DIC	differential interference contrast	Shh	Sonic hedgehog
DMQ	2-polyprenyl-3-methyl-6-methoxy-1,4-benzoquinone	SOD	Superoxide dismutase
Drp-1	Dynamin-related protein	TCA	Tricarboxylic Acid
egl-36	Egglaying deficient	tph-1	Tryptophan hydroxylase
ER	Endoplasmic reticulum	ubc-20	Ubiquitin-conjuating
ERAD	ER-associated degradation	unc-25	Uncoordinated-25
ETC	Electron transport chain	UPR	Unfolded protein response
FACS	Fluorescence assisted cell sorting	vab-8	variable abnormal-8
FADH	Flavin adenine dinuclotide	VD	Larval-born D class VNC GABA neurons
Fis-1	Fission gene	vha-14	Vacuolar H ATPase
GABA	gamma amminobutyric acid	VNC	Ventral nerve cord

CHAPTER I

GABA AND NEURODEGENERATION IN HUMAN DISEASE AND MODEL ORGANISMS

Introduction

Formation of the astoundingly complex circuitry of the human brain requires neurons with different communicative properties. For example, excitatory and inhibitory neurons, arranged in different patterns, form circuits with different outputs. Neurons expressing the neurotransmitter γ-amminobutyric acid (GABA) are the major inhibitory neurons in brain circuitry. The many morphologically and electrophysiologically diverse subtypes of GABA neurons are needed to build circuits that drive the many advanced tasks of the brain, including coordinated movement and cognition. The importance of these cells is apparent in the consequences of GABA neuron dysfunction. Developmental impairment can result in psychiatric diseases as diverse as schizophrenia, epilepsy, Tourette's syndrome and autism, whereas degeneration of GABA neuron populations in the adult brain results in the symptoms of Huntington's disease and Spinocerebellar ataxia.

To understand these diseases, science faces the challenge of solving the molecular and cellular components that dictate GABA neuron function starting from neuron birth and continuing throughout development and aging. Such a quest requires the integration of systems-level biology with cellular and molecular research from many laboratories,

and must involve the union of developmental biology with the molecular neuroscience of aging. Most of the progress toward this issue has been made using model systems, especially the laboratory mouse. Mice have been used to identify some of the genes involved in GABA neuron identity, migration, connectivity and survival (Anderson et al., 1997; Fode et al., 2000).

Many GABA genes are highly conserved. Therefore, invertebrate model systems, such as *Caenorhabditis elegans,* have also been useful for identifying genes that function in GABA neurons. *C. elegans* is a free-living nematode, which has been developed for genetic manipulation. These microscopic animals have a completely sequenced genome, and are amenable to genetic manipulations, including transgenic over-expression, knockout of genes and gene silencing by RNAi. The worm also has a relatively simple nervous system, which nonetheless contains a complement of many of the same neuronal sub-classes found in humans. These neurons can be visualized with GFP reporters in the intact animal throughout its life. Finally, *C. elegans* has a short life span, which makes it ideal for studies of the development and aging of neurons.

We used *C. elegans* to study GABA neurons in development and aging. The first line of research involves genomic and RNAi screening approaches to identify genes that are important for GABA neurons throughout the life cycle. The second involves the study of the Coenzyme Q metabolic gene, *coq-1*, in GABA neuron degeneration through apoptosis in aging animals. This introduction will review the literature on apoptosis in degenerative diseases, focusing on diseases with important roles for GABA neurons. Additionally, to introduce the relationship between Coenzyme Q and GABA neuron

degeneration, the current literature on Coenzyme Q in aging and disease will be reviewed.

GABA neurons from humans to worms

Diverse GABAergic neuron subtypes are present in human brain, each with distinct morphologies and synaptic connectivities. Having multiple subclasses with unique features allows for creation of elaborate circuitry (fig 1.1a) (Kubota et al., 2007). It is not surprising, therefore, that perturbations in GABA neuron development, function or survival are implicated in a variety of psychiatric diseases, including epilepsy, autism, schizophrenia and Huntington's disease.

Whereas GABA neurons act in the CNS in mammals, worm GABA neurons act peripherally to control movement, defecation, and foraging behavior in the worm. The majority of the 26 GABA neurons (73%) in *C. elegans* are the embryonically-derived DD and larvally-derived VD neurons, which reside in the ventral nerve cord. D class neurons send out single axons along the ventral cord, which branch once to send a commissure to the dorsal nerve cord of the animal. These axons synapse directly onto muscle, and the inhibitory activity of these synapses serves to prevent the simultaneous contraction of opposing muscle groups (Fig 1.1b) (White, 1986; Schuske et al., 2004). Laser ablation studies have shown that the deletion of GABA neurons has little effect on resting movement, but, when touched to elicit backward movement, these animals display a 'shrinker' phenotype, caused by the simultaneous excitation of muscles on both sides of the body (fig 1.1c) (McIntire et al., 1993b).

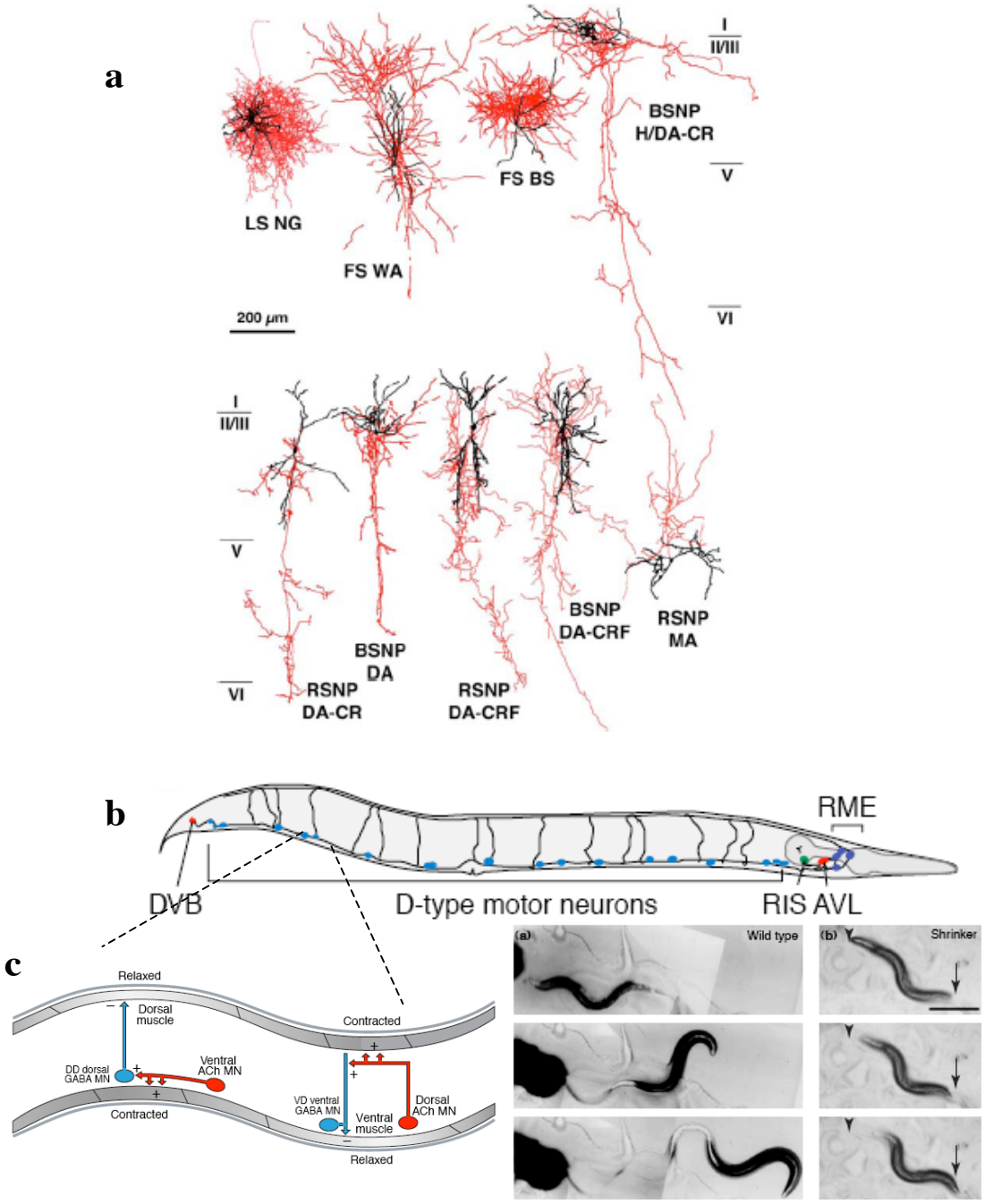


Figure 1.1 GABA Neurons from mammals to worms. (a) Cortical GABA interneuron subclasses. Reprinted from Kubota et al., 2007. (b) GABA neurons in C. elegans. (c) Function of GABA motor neurons of the ventral cord. Adapted from Schuske et al., 2004.

Many of the molecular components necessary for GABA neuron identity and function are conserved between nematodes and humans (McIntire et al., 1993a). Because of this, and because dysfunction of GABA neurons produces a readily visible phenotype, the nematode is useful for discovering genes that are important for GABA neuron function. In addition, *C. elegans* has been useful for studying neurodegeneration that results from various insults, including oxidative stress, genetic mutation and excitotoxicity. The utility of such a model for studying degeneration of GABA neurons has distinct advantages, and is ideal for addressing certain types of questions about disease. A discussion of degeneration studies in mammals and worms may bring into perspective the advantages and shortcomings of such a model.

Mammalian neurodegenerative disease with roles for GABA neurons

A common feature of neurodegenerative diseases is the occurrence of a specific age range during which symptoms of the disease appear. The factors that dictate the age of onset of disease symptoms are of interest in these diseases. Insults may build up over time, only reaching a certain threshold at the age of symptom onset. Alternatively, age-dependent changes in gene expression may create the ideal environment for pathology only at certain time points. Model systems may be useful in distinguishing such possibilities, if they recapitulate the age-dependence of the disease.

The cerebellum and basal ganglia are brain regions important for the control and coordination of complex movements and posture in humans (Kandel and Schwartz, 2000). The cerebellum governs balance, posture, motor learning and coordination. To do this, it integrates signals of intent and memory from the cerebral cortex, adjusts

movements accordingly, and communicates its modifications through efferent tracts to the motor cortex and brain stem. Similarly, the basal ganglia processes information received from the cortex, through communication between its interconnected nuclei (the striatum, globus pallidus, substantia nigra, and subthalamic nucleus), and returns output to the brainstem or the cortex for further modulation.

The importance of these two structures is illustrated by the effects of neurodegenerative diseases that result in lesions in these areas. Parkinson's Disease (PD) and Huntington's disease (HD) are degenerative disorders of the basal ganglia, which result in movement disturbances such as bradykinesia (slow movement), resting tremor, and rigidity in the case of PD (Parkinson, 2002), and uncontrolled choreoform movements in HD (Huntington, 2003). Spinocerebellar ataxias (SCAs) are a group of cerebellar neurodegenerative diseases, characterized by a gradual loss of balance and motor coordination (Duenas et al., 2006).

The symptoms of these diseases can be explained by their different pathologies. In each disease, specific brain areas and cell types within those areas are selectively vulnerable to degeneration. HD and the SCAs are both characterized by the primary degeneration of GABAergic projection neurons, which are the sole output of the affected brain region. In the case of HD, the medium spiny projection neurons of the striatum are the most vulnerable (Huntington's_Disease_Collaborative_Research_Group, 1993). Spinocerebellar ataxias are characterized by somewhat heterogeneous pathology, the best-studied being SCA type 1. The GABAergic Purkinje cells (PCs) that project from the cerebellum are the primary and most severely affected population of neurons in SCA1 (Zoghbi and Orr, 1995), and likely in many other forms of cerebellar ataxia.

The hereditary Spinocerebellar ataxias can be caused by dysfunction of a number of genes with different roles, including ion channels, protein kinase C, protein phosphatase 2 and the iron-binding protein frataxin (Duenas et al., 2006; Fogel and Perlman, 2007). In addition to the identified genetic causes, there are several metabolic, environmental, and idiopathic origins of ataxia, illustrating that many paths can lead to degeneration of cerebellar Purkinje cells. Several naturally occurring mutations in mice have also been identified, which lead to cerebellar disease. These include mouse lines such as the *purkinje cell degeneration*, *lurcher*, *weaver*, and *staggerer* mice (Grusser-Cornehls and Baurle, 2001). These mutants further emphasize an evolutionarily conserved sensitivity of these neurons to degeneration.

Degeneration: From endoplasmic reticulum to mitochondria by way of calcium

Molecular studies in the above diseases and models have provided much information about the susceptibility of GABA neurons to disease. Unifying many of these diseases are defects in the relationship between the endoplasmic reticulum and mitochondrial metabolism (Demaurex and Distelhorst, 2003; Schapira, 2006). Neurons are excitable, dynamic cells that inter-communicate to execute functions such as movement, cognition and emotion. To perform these functions, they must possess specific morphological, chemical and electrical properties, and be capable of adapting these properties in response to stimuli. Such demands result in high metabolic needs, which are met by mitochondria. Healthy mitochondria respond to electrical or morphological changes in neurons by increasing production of ATP, the energy currency of the cell. However, compromising mitochondrial function can lead to cell death (Chan,

2006). The high metabolic need of neurons may make them particularly susceptible to death as insults to our metabolic machinery accumulate during aging. This is the basis for many theories of aging and degenerative disease.

Protein folding in the endoplasmic reticulum

The relationship between neuronal metabolic need and mitochondrial energy output is mediated in part by ER calcium (Berridge, 2002; Demaurex and Distelhorst, 2003). However, the various functions of the ER are inter-connected, complicating the ER-mitochondria relationship. The endoplasmic reticulum serves three main purposes in cells. First, the cytosolic face of the ER membrane is the major site of lipid synthesis. Second, the ER is where many of the proteins made in the cell are folded and where some post-translational modifications take place. Finally, the ER is a major storage reservoir for calcium. Much research has focused on the link between ER protein folding and calcium signaling.

Eukaryotic cells have developed a highly complex series of signal transduction pathways, which monitor the status of protein folding, and adjust ER conditions accordingly. An excess of unfolded proteins is linked to the cell death pathway, such that death precedes the secretion or membrane expression of misfolded proteins. Nascent proteins are transported into the ER, where they interact with chaperones, which foster disulfide bond formation and protein folding. Several ER transmembrane proteins sense protein mysfolding with their luminal domains, and respond through their cytosolic domains by activating the unfolded protein response (UPR) (Ron and Walter, 2007). The UPR includes up-regulation of chaperones to assist in protein folding, decreasing

translation and translocation of ER proteins to reduce workload, and an increase in ERAD (ER-associated protein degradation), which exports unfolded proteins from the ER for proteasomal degradation. Failure in any one of these pathways can lead to accumulation of misfolded proteins, which can lead to cell death (figure 1.2).

ER calcium homeostasis

The exact pathways that lead from protein misfolding to degeneration are poorly understood. However, some clarity is provided by understanding another major ER function, that of calcium homeostasis. Calcium is perhaps one of the most diverse signaling molecules in the cell. However, as free calcium precipitates phosphates, the principal energy source of the cell, it is necessary to contain it for use only in distinct microdomains (Clapham, 1995). The ER performs this service for the cell, collecting Ca^{2+} from the cytosol through SERCA (Sarcoplasmic and Endoplasmic Reticular Ca) pumps, and effecting $InsP_3$ and ryanodine receptor-mediated release. Inside the lumen of the ER, the majority of Ca^{2+} is not free, but is buffered by Ca^{2+}-binding proteins. Among these are the important chaperones calreticulin and calnexin. These proteins sense protein misfolding and bind proteins accordingly to help them fold. They also act as high capacity Ca^{2+}-binding proteins, and thereby control luminal Ca^{2+} capacity (Ostwald and MacLennan, 1974; Bastianutto et al., 1995; Mery et al., 1996; Corbett et al., 1999; Mesaeli et al., 1999; Michalak et al., 1999). Knockout of calreticulin therefore decreases $[Ca^{2+}]_{ER}$, whereas its over-expression increases $[Ca^{2+}]$. The Ca^{2+} concentration in the ER lumen affects the chaperone function of these proteins and vice-versa (figure 1.2).

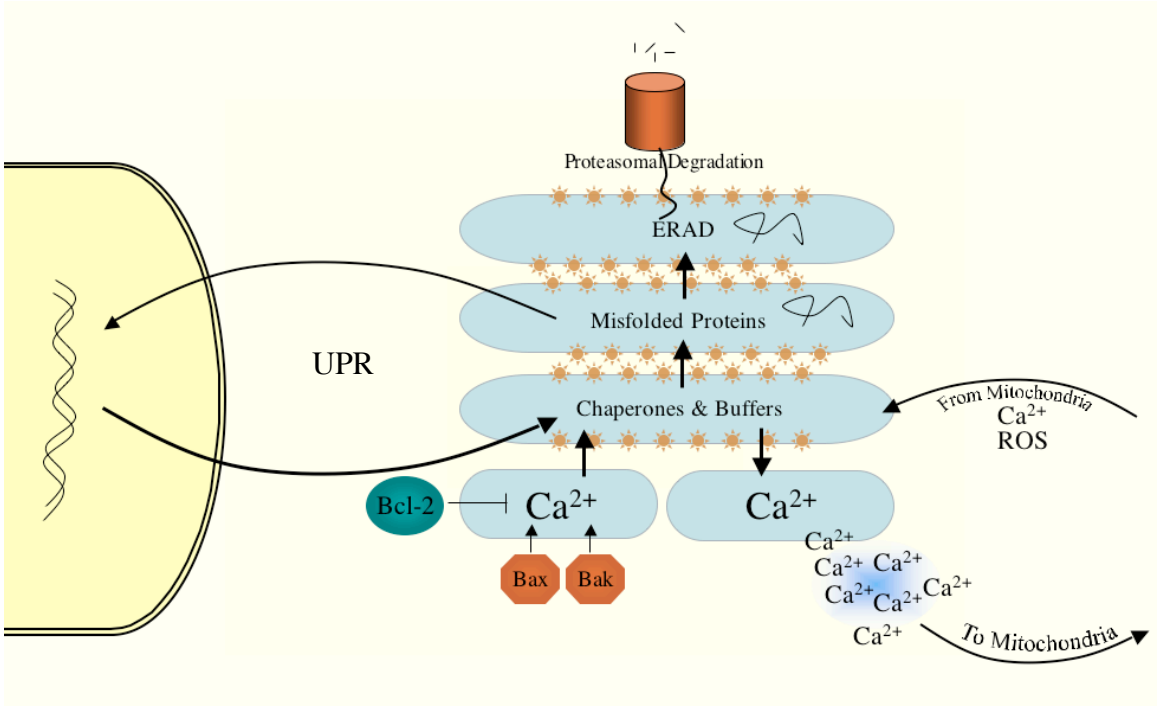


Figure 1.2 ER homeostasis pathways. Protein folding is monitored by chaperones in the ER that control the Unfolded Protein Response (UPR). This includes 1) increasing transcription of chaperones and 2) increasing the ER-Associated degradation pathway (ERAD) to remove and degrade unfolded proteins. The chaperone burden is reflected in the Ca^{2+} levels in the ER and vice-versa. Ca^{2+} levels are also controlled by anti-apoptotic Bcl-2, whereas pro-apoptotic Bax and Bak increase Ca^{2+} stores. Ca^{2+} released from the ER acts on mitochondria, which both shuttle Ca^{2+} back, and send retrograde signals such as ROS, which reflect the mitochondrial workload and further signal the need for protein production to the ER.

Ca^{2+} levels in the ER lumen are important not only for cell signaling, but also dictate the apoptotic decision. High $[Ca^{2+}]_{ER}$ is known to sensitize cells to apoptosis, whereas low $[Ca^{2+}]$ is protective. Therefore, over-expression of calreticulin sensitizes cells to apoptosis (Arnaudeau et al., 2002), whereas calreticulin knockout is protective (Nakamura et al., 2000). The link between protein folding and $[Ca^{2+}]$ is proposed to explain the degenerative effects of many mutations that affect protein folding and degradation.

Mitochondria as energy producers of the cell

Ca^{2+} exerts its apoptotic effects though mitochondria (Mattson et al., 2000). Mitochondria are the energy factories of cells, and excitable cells such as neurons have more mitochondria than other cells. Mitochondria are composed of an outer membrane and an inner membrane, which is extensively folded into structures called cristae. The inner mitochondrial membrane is relatively impermeable, whereas the outer membrane is highly permeable and renders the intermembrane space practically continuous with the cytosol. The cristae of the inner membrane allow ample surface area in which to embed the components of the electron transport chain (ETC). These include complexes I-V, cytochrome c and coenzyme Q. NADH and $FADH_2$ are the ETC's electron donors and are produced by metabolic reactions like those of the TCA cycle. Electrons are passed along the ETC and ultimately are added to molecular oxygen to make water. The movement of electrons through the assembly line allows protons to be pumped across the inner membrane, establishing a proton gradient between the enclosed matrix and intermembrane space. This gradient (denoted $\Delta\Psi m$) provides potential energy to drive

protons back into the mitochondrial matrix, which drives the conversion of ADP to ATP by complex V/ATP synthase (Alberts, 2002).

Mitochondrial Ca^{2+} in apoptosis

Cell signaling and membrane activity can induce Ca^{2+} release from the ER. Normally, ER-released Ca^{2+} is taken up by nearby mitochondria, where it translates the activity of the cell into a need for increased energy production. Ca^{2+}, either through direct binding or indirect means, activates several rate-limiting metabolic enzymes and components of the ETC to boost ATP production (McCormack et al., 1990; Rizzuto et al., 1993; Hajnoczky et al., 1995; Rizzuto et al., 1998; Jouaville et al., 1999). Mitochondria recycle Ca^{2+} back to the ER, thus contributing to Ca^{2+} buffering. Mitochondria also communicate the increased burden on their resources through retrograde signals to the ER. Some of these may include the reactive oxygen species (ROS/free radicals) that are produced as by-products of the ETC. As electrons are passed through the complexes of the ETC, it is estimated that as much as 2% of them are passed in a side-reaction to oxygen, producing partially reduced free oxygen radicals (Adam-Vizi and Chinopoulos, 2006). These are highly reactive molecules capable of damaging the macromolecules that make up the cell. High ROS levels are dangerous to cells, but small increases in ROS may signal the ER to up-regulate stress response genes and increase protein folding and lipid synthesis. In this way, small increases in a cellular activity prepare the ER for additional stress and are actually cyto-protective (Kaufman, 1999).

Release of high levels of Ca^{2+} from the ER, or Ca^{2+} signals to an already stressed mitochondrion, however, act as pro-apoptotic signals (Szalai et al., 1999; Pacher and Hajnoczky, 2001; Hajnoczky et al., 2002; Rapizzi et al., 2002). This occurs through an increase in mitochondrial permeability, a process referred to as the permeability transition (PT). Scientists have long hypothesized that the formation of a pore complex (permeability transition pore, PTP) is responsible for the transition. However, the molecular components of this pore have been elusive, and permeability transition has been shown to persist in knockout mice for candidate pore-forming proteins, such as VDAC and ANT (Kokoszka et al., 2004; Krauskopf et al., 2006). Whatever the mechanism, PT causes a disruption of inner membrane cristae structure, dissipation of $\Delta\Psi m$ and release of cytochrome c, one of the electron carriers in the ETC, into the cytoplasm (Norenberg and Rao, 2007). Cytochrome c binds to a protein called apoptotic protease activating factor-1 (Apaf-1), forming the 'apoptosome.' This complex then starts a cascade of activation of caspase enzymes that execute the cell death program. This is the basic pathway of apoptosis, but the complete story is more complex. In reality, mitochondria integrate information from several sources before making the decision to execute the cell. Our knowledge of factors that influence the apoptotic decision is ever growing. A few that are relevant to this work are described below and in figure 1.3.

Apoptosis-regulating pathways

Among regulators of apoptosis, Bcl-2 and the Bcl family of proteins are among the best studied. Bcl-2 was originally discovered as an oncogene mutated in B cell

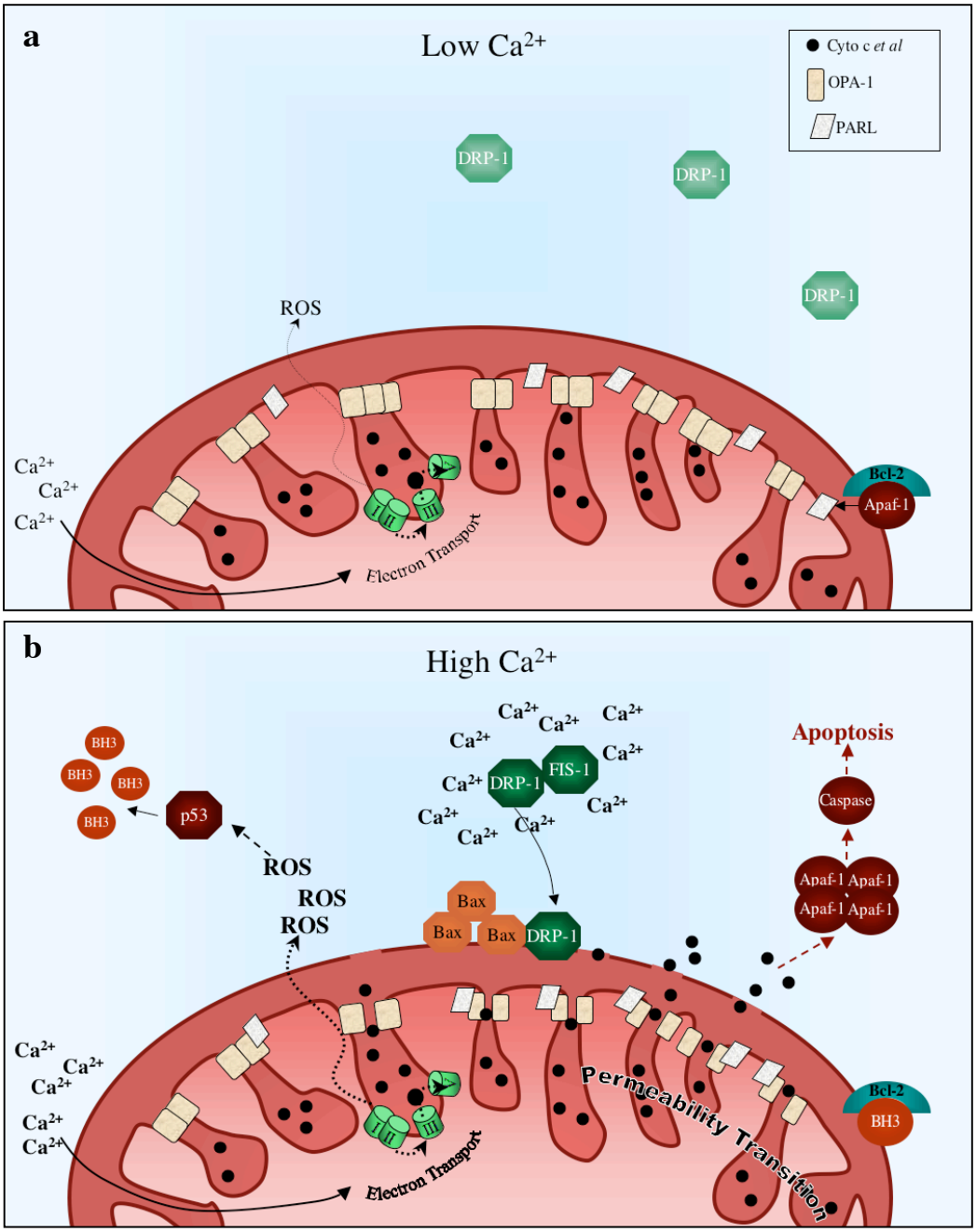


Figure 2.3 Mitochondrial response to Ca^{2+}. **(a)** Normally, when the ER releases Ca^{2+}, this acts as a signal to increase energy production. A low level of ROS is released, which acts as a retrograde signal to the ER. Bcl-2 proteins maintain cristae structure, through modulation of fusion proteins, such as OPA-1 and PARL. **(b)** When Ca^{2+} release is very high, ROS levels increase, which can damage the cell, activating damage pathways, such as the p53 pathway, which up-regulates BH3-only proteins. Also, high Ca^{2+} results in DRP-1 translocation to the mitochondria, and activation of outer membrane fission. BH3-only proteins antagonize Bcl-2, causing activation of PARL and OPA-1, and cristae remodeling to release pro-apoptotic proteins, such as cytochrome c.

lymphomas (Bcl) (Bakhshi et al., 1985; Cleary and Sklar, 1985; Tsujimoto et al., 1985). Cancer promotion by Bcl-2 was subsequently linked to its anti-apoptotic properties (Vaux et al., 1988; McDonnell et al., 1989). Bcl-2 and other anti-apoptotic members of its family contain 4 domains (BH1-4), which create a hydrophobic pocket. Pro-apoptotic, 'multidomain' members of this family, like Bax and Bak, are sequestered by the binding of their BH3 domain in this pocket (Muchmore et al., 1996; Sattler et al., 1997). 'BH3-only' members, like Bid, Bad, and Bim, contain only the BH3 domain, and can bind competitively in this pocket, releasing the Bax/Bak members of the family, and are thus pro-apoptotic as well. In addition to Bcl-2 binding, the BH3-only proteins are controlled by numerous mechanisms, including through transcriptional control and phosphorylation. For example, the increased ROS released by stressed or compromised mitochondria damages macromolecules in the cell, including DNA. DNA damage can activate the oncogene p53, which can promote transcription of BH3-only proteins, leading to apoptosis. The involvement of Bax and Bak in apoptosis is apparently complex. They are known to act either in mitochondria to promote permeability transition (Shimizu et al., 1999) or in the ER to increase releasable Ca^{2+} (Pinton et al., 2000; Scorrano et al., 2003) or both. The pathways leading to activation of any given arm of the apoptotic machinery appears to depend on the original apoptotic stimuli.

An additional pathway linking Ca^{2+} to apoptosis through the mitochondrial fission and fusion machinery is currently being elucidated. Mitochondria are often depicted as solitary organelles within the cell. However, they are actually dynamic structures, dividing by a process called fission and are often connected to one another by fusion into a tubular network of organelles, allowing for the intercommunication between

mitochondria in different regions of the cell (Nakada et al., 2001; Ono et al., 2001). The proteins involved in fission and fusion have not all been elucidated, but some of the major players have been discovered. One of the key players in fission is dynamin-related protein 1 (*drp-1*). Dynamin is a GTPase, which acts during vesicle endocytosis to help pinch off vesicles budding from the membrane. Drp-1 may act in a similar manner to effect fission of the outer mitochondrial membrane. Drp-1 is normally found in the cytoplasm. In response to Ca^{2+} release from the ER (Hinshaw, 2000; Breckenridge et al., 2003), another fission protein, Fis-1, escorts Drp-1 to selected sites on the mitochondrial outer membrane (Mozdy et al., 2000; Yoon et al., 2003). Another dynamin-related GTPase, OPA-1 is involved in fusion of mitochondria and may be activated by the fusion protein Presenilin-associated rhomboid-like protease (PARL) (McQuibban et al., 2003). These fusion proteins are thought to reside at cristae junctions, holding the junction closed to inhibit the release of apoptotic factors, such as cytochrome c (Wong et al., 2000; Olichon et al., 2002).

The links between the fission/fusion machinery and apoptosis are numerous. Taken together, there seems to be a balance between fission and fusion, such that an increase in fission proteins is pro-apoptotic, whereas an increase in fusion proteins is protective against apoptosis (Frank et al., 2001; Scorrano et al., 2002; Olichon et al., 2003; Lee et al., 2004; Sugioka et al., 2004; Cipolat et al., 2006; Frezza et al., 2006). ER-to-Mitochondrial Ca^{2+} has recently been shown to stimulate mitochondrial fission-related apoptosis (Breckenridge et al., 2003). Furthermore, Bcl-2 family members, such as Bax, have been associated with this process, indicating yet another means by which these

proteins can carry out apoptosis and an additional link between Ca^{2+} and fission/fusion-related apoptosis (Karbowski et al., 2002; Youle and Karbowski, 2005).

The changes in membrane permeability and cristae structure that are governed by fission/fusion proteins may finally explain the permeability transition phenomenon, and replace or complement the idea of the transition pore as the true mechanism of mitochondrial release of apoptogenic factors. This is a novel viewpoint, and the involvement of the fission/fusion machinery in apoptosis is still a young and controversial field. One model even suggests that fission of the tubular mitochondrial network *protects* cells from some pro-apoptotic insults by preventing the efficient conductance of Ca^{2+} waves throughout all mitochondria in the cell (Szabadkai et al., 2004). However, it is clear that the fusion/fission pathways, or some of the components thereof can be party to the mitochondria's decision to either undergo apoptosis or maintain survival.

Developmental and pathological apoptosis in *C. elegans*

The mammalian apoptotic pathways described above are summarized in figures 1.2 and 1.3. The degree to which these pathways are conserved throughout evolution is debated, but it is becoming increasingly clear that many of these pathways, or similar ones, are in fact shared between mammals and nematodes. The original programmed cell death pathway elucidated in the nematode is employed during developmental patterning. In this model, *C. elegans* CED-4/Apaf-1 is sequestered at the mitochondrial outer membrane by the Bcl-2 ortholog, CED-9. In cells destined to die, EGL-1, a BH3-only protein, is up-regulated and binds to CED-9/Bcl-2, thereby freeing CED-4/Apaf-1 to

activate CED-3, the only known caspase in *C. elegans*. This well-established developmental apoptosis model does not involve Ca^{2+} or cytochrome c release. In fact, cytochrome c is hypothesized to be irrelevant to the worm apoptotic pathway, as CED-4/Apaf-1 lacks the canonical cyto c binding site and has been shown to form the apoptosome *in vitro* (Rolland and Conradt, 2006).

Since the discovery of developmental apoptosis, several examples of pathological apoptosis have been identified in *C. elegans*. Pathological apoptosis, which occurs in response to disruption of certain genes or environmental insults, can be distinguished from developmental apoptosis. While this form of cell death uses the basic machinery described above, it also shares many of the known mammalian modulating pathways. For example, DNA damage can activate the worm ortholog of p53, CEP-1, which activates transcription of BH3-only proteins EGL-1 and CED-13, resulting in apoptosis (Schumacher et al., 2005). Furthermore, DRP-1 in *C. elegans* has been shown to be necessary for apoptosis in response to certain insults (Jagasia et al., 2005). In addition, worm CED-9 (Bcl) has been shown to antagonize fission-related death in mammalian cells, whereas EGL-1 (BH3-only) promotes it, indicating a highly conserved role for these proteins in apoptotic fission (Delivani et al., 2006). While cytochrome c may not be downstream of fission-related mitochondrial changes (although it has never been tested), it is possible that some other factors are released, which mediate this form of apoptosis in the worm, such as the apoptosis inducing factor (AIF) orthologs F20D6.11 and WAH-1. An alternative possibility is that the structural changes in the mitochondrial membrane themselves are sufficient to disrupt CED-4 (Apaf-1) binding to CED-9 (Bcl).

Interestingly, while the role of calcium has been extensively studied in pathological apoptosis in mammals, few studies of apoptosis exist in the worm literature in which a role for Ca^{2+} has been probed. One notable example is the *sel-12 (ar131)* mutant. SEL-12 is the *C. elegans* homolog of human Presenilin-1, an ER protease that has been implicated in Alzheimer's disease. The *ar131* allele of *sel-12* causes ectopic apoptosis in embryogenesis, which is blocked by the inhibitor of ER Ca^{2+} release, dantrolene (Kitagawa et al., 2003). This provides an example of Ca^{2+}-dependent pathological apoptosis in the worm.

Apoptosis in neurodegenerative disease

Apoptotic cell death is associated with at least some of the degeneration that occurs in these diseases. For example, mutant ataxin-3 and ataxin-7 cause up-regulation of Bax and down-regulation of Bcl-2-like Bcl-XL, leading to caspase activation and apoptotic death of cerebellar neurons (Chou et al., 2006; Wang et al., 2006). Use of chemical caspase inhibitors or expression of dominant negative caspases delays symptom onset in mouse models of HD (Ona et al., 1999; Chen et al., 2000). As outlined above, nuclear (p53), cytosolic (proteasome), ER (UPR and ERAD), and mitochondrial (PTP/fusion/fission) components all contribute to apoptosis. Mutations or toxins that target elements of ER-Mitochondria-Ca cycle can cause pathological apoptosis, and are associated with neurodegenerative disease. Examples of the disruption of these pathways are summarized Table 1.1.

One major point of disruption in degenerative diseases is thought to be the area of protein folding and degradation (Aigelsreiter et al., 2007). For example, in the case of

Table 1.1 Degenerative diseases affecting the ER-mitochondrial calcium cycle		
Disease	**gene**	**Mechanism**
Heritable Parkinsonism	*parkin*	*parkin* is an E3 Ubiquitin ligase-deletion may lead to accumulation of unfolded proteins
	PINK1	Kinase activity of PINK1 effects mitochondrial membrane structure and cytochrome c release
Alzheimer's Disease	presenilin	mutant presenilin-1 affects amyloid peptide levels and ER calcium release
Friedreich's ataxia	frataxin	frataxin mutation affects Iron homeostatsis in mitochondria, which is devastating to mitochondrial function
SCA3	ataxin-3	ataxin-3 is a ubiquitin protease associated with the unfolded protein response
SCA6	CACNA1A	CACNA1A encodes a calcium channel; it's mutation is thought to perturb calcium homeostasis
Coenzyme Q deficiency	coq-1 and coq-2	mutation in these genes leads to a reduction in CoQ and reduced electron transport
Huntington's disease	*huntingtin/htt*	The function of htt is unknown, but calcium, protein folding and mitochondrial function are all compromised in this disease
ALS	Superoxide Dismutase	SOD mutation decreases cellular defenses against ROS

HD and some SCAs, expansions of polyglutamine tracts within the coding region of the disease-related genes are associated with the formation of multi-protein aggregates. This aggregation is currently thought to be protective in many such diseases, as expansion of polyglutamines may confer a toxic gain-of function, and aggregation may sequester these dysfunctioning proteins. Consistent with this idea, aggregates form more readily in surviving neurons, and less so in degenerating populations in SCA1 (Klement et al., 1998), SCA7 (Bowman et al., 2005) and HD (Saudou et al., 1998). Differences in either protein folding or proteasomal degradation in sensitive cell populations may be the cause of selective aggregate formation. In keeping with this idea, the over-expression of chaperones has been shown to reduce SCA1-associated toxicity in a number of models (Cummings et al., 1998; Cummings et al., 2001; Bonini, 2002). Furthermore, proteasomal degradation and ubiquitination are directly affected in SCA3, as Ataxin 3 is a proteasomal cysteine protease involved in de-ubiquitination (Chai et al., 2004; Nicastro et al., 2005).

The ER has been implicated in degenerative disease not only at the protein folding level, but also at the level of Ca^{2+} storage and release. Ca^{2+} homeostasis has been shown to be disrupted in several forms of SCA (Duenas et al., 2006) as well as in HD (Bezprozvanny and Hayden, 2004). For example, several Ca^{2+} homeostasis genes have been shown to be down-regulated in Purkinje cells in both a mouse model of Spinocerebellar ataxia type1, and in human patients post-mortem (Lin et al., 2000). In addition, the polyglutamine expansion in SCA6 occurs in a voltage-dependent Ca^{2+} channel (Zhuchenko et al., 1997), and may alter channel function to affect cellular Ca^{2+} levels (Matsuyama et al., 1999; Restituito et al., 2000; Toru et al., 2000). GABAergic

PCs are highly sensitive to degeneration in SCA types 1 and 6, perhaps indicating an importance for Ca^{2+} in GABA neuron sensitivity. Furthermore, mitochondria isolated from lymphoblasts of Huntington's disease (HD) patients and brains from a mouse model show heightened sensitivity to Ca^{2+}, as manifested by an increased tendency to undergo permeability transition (Panov et al., 2002).

It should be noted that Ca^{2+} does not only signal apoptotic cell death. Excessive release of Ca^{2+} from the ER can also result in necrosis, through activation of cytosolic Ca^{2+}-dependent enzymes, such as calpain proteases and phospholipases. This process has been extensively studied in mammals and in *C. elegans*, particularly in the paradigm of glutamate excitotoxicity (Choi, 1992; Coyle and Puttfarcken, 1993; Driscoll and Gerstbrein, 2003). Apoptosis may be less damaging to the cell than necrosis, which ends in cell rupture, and the release of its toxic contents into the surrounding tissue. In contrast, apoptotic cells activate both autophagic and phagocytic engulfment pathways, which contain their toxic contents. The choice of cell death mode depends on the intensity of the insult (Bonfoco et al., 1995). Contribution of both necrotic and apoptotic cell death is likely in the neurodegenerative diseases discussed above (Beal, 1994; Serra et al., 2004).

Finally, sensitivity to degeneration can also be conferred at the level of the mitochondria. As the mediators of ROS release, aging and apoptosis, mitochondrial dysfunction is prominent in most neurodegenerative diseases. This may be because, regardless of the molecular insult that triggers disease, death pathways converge on the mitochondria. For example, much evidence links mitochondrial dysfunction with Huntington's disease. First, the neurotoxin 3-nitropropionic acid (3-NP) targets complex

II of the ETC, and poisoning with 3-NP recapitulates many of the symptoms of HD (Beal et al., 1993; Wullner et al., 1994; Brouillet et al., 1995). Ca^{2+}-induced mitochondrial permeability transition has been shown to be a consequence of *huntingtin* mutation in mouse models of HD (Choo et al., 2004). PGC-1α, a master regulator of mitochondrial biogenesis is down-regulated in HD patients and animal models, and PGC-1α over-expression in cultured cells or in HD mouse models protects neurons from 3-NP toxicity or mutant *huntingtin* protein (Cui et al., 2006; St-Pierre et al., 2006; Weydt et al., 2006).

Coenzyme Q and its role in mitochondrial function and disease

Coenzyme Q in human disease

One notable example of mitochondria-related neurodegeneration is the form of cerebellar ataxia, which results from deficiency of Coenzyme Q (CoQ). Human CoQ_{10} deficiency is a rare genetic disease that is caused by mutation in one of the synthetic enzymes in the CoQ pathway. The most common outcome of this deficiency is cerebellar ataxia (Quinzii et al., 2007), but symptoms can also be the result of myopathy (Ogasahara et al., 1989; Lalani et al., 2005; Horvath et al., 2006). Thus far, only mutations in the *coq-2* gene have been positively identified as causes of CoQ deficiency, although *coq-1* dysfunction is suspected to underlie some cases (Rotig et al., 2000). CoQ deficiency can also participate in the pathology of diseases, in which it is not the primary feature. For example, patients carrying mutations of the apraxin gene (APTX) display secondary CoQ_{10} deficiency and cerebellar ataxia, which improves with CoQ_{10} supplementation (Quinzii et al., 2005). These human genetic disorders provide powerful evidence that

CoQ_{10}, though important for respiration in all cells, seems to be most vital for the survival of neurons or muscle, which have high metabolic needs. Furthermore, the symptoms of the disorders associated with loss of CoQ reveal that some regions of the brain are more sensitive than others to metabolic compromise. The common loss of function of the cerebellum, the output of which is exclusively GABAergic, may indicate selective sensitivity of the efferent Purkinje neurons from this region, similar to that observed in SCA.

CoQ structure and biosynthesis

CoEnzymeQ (also ubiquinone, CoQ or Q), is a ubiquitous molecule found in the membranes of all cells. CoQ is composed of a benzoquinone ring attached to an isoprenoid side chain (fig 1.5a). The hydroxy groups on the quinone ring act as the acceptors and donors of electrons, making this part of the molecule ideal for electron transport. The side chain makes CoQ highly lipophillic, and varies in length from species to species. The predominant form in humans is 10 isoprenoid units in length (denoted CoQ_{10}), whereas CoQ_9 predominates in rats and nematodes, and CoQ_8 is made preferentially by bacteria.

The 8 'COQ' enzymes responsible for synthesis of CoQ were originally discovered in yeast. Yeast mutants of these enzymes arrest due to respiratory failure in the absence of CoQ. Although each gene is important for CoQ synthesis, a direct biochemical mechanism has not been established for most of these enzymes (Tran and Clarke, 2007). For example, COQ-4 and COQ-8 have no known or predicted biochemical function. COQ-1 is a polyprenyl synthetase important for the metabolism of many different species of lipids. *coq-2* encodes a polyprenyl-transferase that joins the

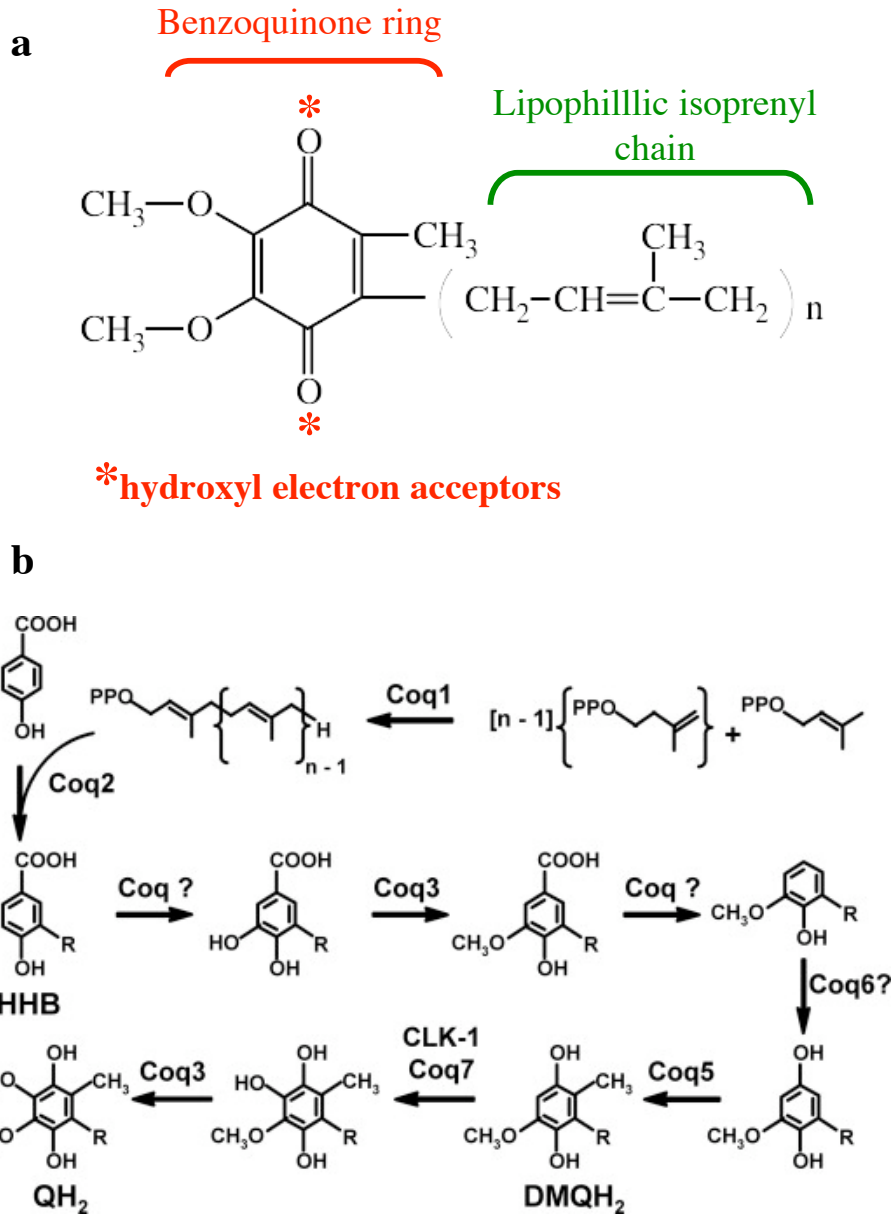


Figure 1.4 Structure and Synthesis of Coenzyme Q
a. Structure of CoQ adapted from http://home.caregroup.org
b. Synthesis of CoQ adapted from Tran and Clarke, 2007.

isoprenyl tail to the electron-transferring portion of the molecule, 4-hydroxybenzoic acid. COQ-3 is an O-methyltransferase, and transfers two methoxy groups to make the quinone ring. The current understanding of CoQ synthesis is shown in figure 1.5b.

Roles of CoQ in the cell

CoQ plays many roles in the cell, but it is perhaps best known for its role in the electron transport chain to produce energy in mitochondria. CoQ in the mitochondrial inner membrane accepts electrons from complex I and II of the electron transport chain, and transfers them to complex III. This makes CoQ essential for ATP production in the cell. Also, because complex I, and to a lesser degree complex II, may also pass electrons out of the ETC to create free radicals, CoQ must be present at appropriate levels to counteract creation of ROS.

CoQ also prevents ROS damage by acting as an antioxidant itself. CoQ is found, not only in mitochondria, but in all membranes of the cell. Thus, CoQ is strategically placed in all organelles, where it acts as an important antioxidant. CoQ has been shown to block oxidative damage of lipids, proteins, and DNA (Bentinger et al., 2007). For this reason, it has been tested as a therapeutic agent in a number of disorders in which mitochondrial dysfunction and reactive oxygen species have been implicated. For example, prolonged, high dose, CoQ_{10} treatment has been shown to protect dopaminergic neurons from MPTP toxicity in mice (Beal et al., 1998) and primates (Horvath et al., 2003) and to slow the progression of Parkinson's disease early on in humans (Shults et al., 2002). CoQ_{10} treatment has also been shown to slow disease progression in mouse

models of HD (Matthews et al., 1998; Ferrante et al., 2002) and human patients (Huntington_Study_Group, 2001).

There is some evidence to suggest that CoQ inhibits the Ca^{2+}-dependent opening of the mitochondrial permeability transition pore through a mechanism that is independent of its roles in electron transport or as an anti-oxidant (Fontaine et al., 1998; Fontaine and Bernardi, 1999; Martinucci et al., 2000; Walter et al., 2000). This finding indicates that CoQ deficiency may sensitize mitochondria to opening of the PTP and subsequent apoptosis directly.

Because CoQ has many functions, the molecular mechanisms of cell death in CoQ deficiency may be complex. The use of a simple, easily-manipulated model of CoQ deficiency could be useful in sorting out this complexity. The work in Chapter IV will describe the establishment of such a model in the nematode *C. elegans.*

Studies of CoQ in *C. elegans*

C. elegans expresses 8 genes homologous to those which have been shown to be responsible for CoQ synthesis in yeast. Only mutants of the *coq-7* homolog are viable. COQ-7 catalyzes the second-to-last step in CoQ biosynthesis, the monooxygenation of 2-polyprenyl-3-methyl-6-methoxy-1,4-benzoquinone (DMQ) to produce 2-polyprenyl-3-methyl-5-hydroxy-1,4-benzoquinone (see fig 1.5b). Deletion of this enzyme causes the build up of the DMQ precursor, which appears to have some activity of its own, as these animals do not die, but live with reduced mitochondrial metabolic rates (Kayser et al., 2004; Nakai et al., 2004). The lowered metabolic rates of *coq-7* leads to an increase in lifespan, and *coq-7* is thus also referred to as the 'Clock' gene, *clk-1* (Wong et al., 1995).

Deletion of the gene responsible for the step in synthesis after the *coq-7/clk-1* step, *coq-3*, is lethal, likely because the COQ-3 gene product is also responsible for an earlier synthesis step (Hihi et al., 2002). Likewise, mutants for other genes responsible for earlier steps in synthesis are reported to result in arrest at larval stages and death, due to massive breakdown of vital tissues, such as pharyngeal muscle (which ultimately leads to starvation) and intestine (Gavilan et al., 2005). Prior to larval arrest, some of these mutants (*coq-1* and *coq-2*) show slowed movement that progresses into paralysis. The mechanism of this paralysis may simply be the tissue atrophy seen in these animals, but has never been studied.

RNAi knockdown of the coq genes has been achieved in *C. elegans*. Whereas knockdown animals show a drastic (though not complete) reduction on CoQ concentrations, they do not die or show defects in pharyngeal pumping or development, but are, on the contrary, long-lived (Asencio et al., 2003). This again emphasizes the fact that, although ablation of key genes for mitochondrial function is lethal, a reduction in metabolism positively affects lifespan. However, a long life is not necessarily a quality life. The organism may pay for these metabolic reductions by losing tissues that are dependent on high metabolic rates to function and survive, namely neurons and muscles. Both the aforementioned human diseases and the result of CoQ knockdown in worms support this idea.

In the process of genomic and RNAi screening approaches to better understand GABA neuron genes in development and aging (described in chapters II and III), we discovered that knockdown of the *coq-1* gene, resulted in the progressive degeneration of GABA neurons in *C. elegans*. We have developed this discovery to establish a useful

model for CoQ deficiency in humans. Chapter IV will describe this research. Chapter V will discuss the implications of this work and possible directions for future research.

CHAPTER II

MICROARRAY STUDIES OF GABA NEURONS AND THE ARISTALESS TRANSCRIPTION FACTOR IN *C. ELEGANS*

Introduction

The majority of GABA neurons in the human brain are local circuit interneurons. In the developing brain, GABA expressing cells migrate tangentially from their birthplace in the medial germinal eminence to the intermediate and subventricular zones of the neocortex. From here, they migrate radially into the layers of the cortex (Wonders and Anderson, 2006). Although it is unclear at what point or by what mechanism, these neurons become further fated to be specific subclasses of GABAergic neurons. These vary greatly in both morphology and synaptic features (Fig 1.1a). Once situated in the cortex, these neurons begin morphologic and synaptic maturation, an extensive process that isn't completed until late adolescence in mice and primates (Morales et al., 2002; Chattopadhyaya et al., 2004; Jiao et al., 2006). GABA released from GABA neurons early in development acts on other neuronal populations affecting proliferation, migration and differentiation throughout the brain (Ben-Ari, 2002). Therefore, disruption of GABA neuron development has widespread consequences (Di Cristo, 2007).

Aristaless-related homeobox domain (ARX) is a paired-like homeodomain transcription factor that has been shown to both repress and activate gene transcription throughout development (Schneitz et al., 1993; Seufert et al., 2005; McKenzie et al.,

2007). Several lines of evidence have linked ARX to GABA neurons. First, ARX expression has been localized at the transcript and protein levels to areas rich in GABA neuron progenitor proliferation and migration throughout development in mice (Miura et al., 1997; Bienvenu et al., 2002; Colombo et al., 2004; Poirier et al., 2004). Second, co-staining against ARX and GABA neuron markers has shown the presence of ARX in ~70% of GABA-containing cells *in situ* (Colombo et al., 2004), and in ~90% of primary cultured GABA neurons (Poirier et al., 2004). Third, numerous human genetic studies have linked mutations in ARX to a range of neural and reproductive deficits, including mental retardation, epilepsy, autism, and abnormal genitalia development (fig2.3g) (Bienvenu et al., 2002; Scheffer et al., 2002; Stromme et al., 2002a; Stromme et al., 2002b; Uyanik et al., 2003; Hartmann et al., 2004; Stepp et al., 2005; Wohlrab et al., 2005; Spinosa et al., 2006; Chaste et al., 2007). Finally, the creation of a knock-out mouse has provided more detailed cellular information as to what occurs when ARX is lost. Arx-mutant males display decreased migration of GABA neurons both from the MGE to the intermediate zone of the neocortex and from the intermediate and subventricular zones to the cortical plate, resulting in small brain size (fig 2.3e-f,b). These mice also have smaller testes and hypoplasia of the seminal vesicles (fig 2.3c-d), reminiscent of the reproductive defects seen in some human patients with lesions in the ARX gene (Kitamura et al., 2002). Taken together, these data suggest that the ARX transcription factor plays an important role in the development of the mammalian GABAergic system.

Though *C. elegans* GABA neurons are simplified in comparison to mammalian GABA neurons, the nematode has the advantage over the mouse that is is highly

genetically tractable, and has a short life cycle. Some of the key genes needed for GABA neuron identity have been discovered in the worm. *unc-30* (Pitx-2), for example, encodes a homeodomain transcription factor that regulates the transcription of GABA-specific genes (McIntire et al., 1993a). *unc-25* encodes the glutamic acid decarboxylase enzyme necessary for GABA synthesis (Jin et al., 1999). The vesicular transporter for GABA, *unc-47*, and GABA receptors (eg *unc-49*) have also been characterized in the worm (McIntire et al., 1997; Bamber et al., 1999).

A previous postdoctoral fellow in the lab, Dr. Susan Barlow, developed a list of genes that are enriched in GABA neurons through genomic profiling. Importantly, she found a high degree of conservation in the genomic profile of GABA neurons. As shown in fig 2.1i, close to 50% of genes in *C. elegans* show conservation with human genes (conservation being determined by a human BLAST hit with an E-value of e^{-10} or lower). Whereas previously published panneural (Von Stetina et al., 2007a) and DA cholinergic neuron (Fox et al., 2005)-specific datasets are also around 50% conserved, the GABAergic dataset showed 68% conservation. This distinguishes *C. elegans* as a good model system for studying GABA neurons. Consequently, I have used bioinformatics to mine this dataset for interesting trends and hypothesis development (see below).

A previously-described *C. elegans* homolog of ARX, termed *alr-1*, for ARX/al-related-1, is expressed in 24 of the 26 GABAergic neurons of the worm. Three mutants of this gene have been created; a point mutation in the splice site for the first exon, *alr-1(oy56)*; a deletion mutation in which part of the homeodomain is deleted, *alr-1(ok545);* and a complex rearrangement which abolishes *alr-1* gene function, *alr-1(oy42)*. These mutant lines have chemosensory defects, and their larvally-derived VD GABAergic

neurons are partially mis-fated, expressing a marker normally specific to embryonic-born DD GABA neurons (Melkman and Sengupta, 2005). The Barlow micro-array profile verified *alr-1* enrichment in GABA neurons. Dr. Barlow also discovered that *alr-1* mutations cause morphological defects in the D-class GABA motor neurons (fig2.3h). This is a significant finding, as it suggests a conservation of the role for *alr-1* in GABA neuron development.

The search for aristaless target genes is essential for understanding the cellular events that lead to the devastating pathogenesis of aristaless-associated syndromes in humans. *C. elegans* may be an ideal organism in which to conduct this search, due to the high conservation of its GABAergic system, the apparently related role of the aristaless ortholog, and the ease with which GFP-labeled GABA neurons can be observed *in vivo* and isolated for micro-array profiling. By comparing expression profiles of cells that express mutant *alr-1* with those in which the *alr-1* gene is wildtype, we can identify transcripts that are up- or down-regulated in the *alr-1* mutant. To this end, I attempted a similar micro-array strategy to determine *alr-1* transcriptional targets. I also performed additional phenotypic characterization of *alr-1* mutant worms. Along with the aforementioned bioinformatic studies, these experiments are described below.

Materials and Methods

Strains: Nematode strains were maintained at 20-25°C using standard culture methods (Brenner, 1974). The wild type strain was N2. Strains included *unc-25*::GFP (*juIs76)*, *C04G2.1*::GFP(*NC916*), and *alr-1* alleles *oy56(PY3019), ok545(RB762)*, and *oy42(PY1598).*

MAPCel profiling of GABA neurons: Cell culture, sorting, RNA isolation and micro-array experiments were performed as previously described, with slight modifications (Christensen et al., 2002; Fox et al., 2005). Briefly, adult worms were bleached to release eggs. Chitinase was used to dissolve egg shells, and cells were dissociated and plated in L-15-10 media and incubated overnight at 25°C. Cells were washed off plates with M9 buffer, filtered to remove debris and clumps, and then sorted by FACS to an 80-90% GFP-positive population of cells. RNA was isolated from sorted cells using the Absolutely microRNA isolation kit® (Stratagene), and amplified by the pico-low kit, a proprietary technology under development by NuGen, prior to hybridization on a *C. elegans* affimetrix gene array.

Data Analysis: Robust Multi-Array Analysis (RMA) was used to normalize hybridization intensities to an average signal intensity for each experiment. Where applicable, genes showing significant differences in intensity from baseline were identified by Significance Analysis of Micro-array (SAM). For the GABA dataset, genes showing 1.7 fold change or better at a 1% false discovery rate, were kept as up or down-regulated. The resulting lists were annotated and compared with previously described PERL scripts using wormbase 170 (wb170) (Von Stetina et al., 2007a). To develop a list of genes with known GABA expression patterns, we searched WormMart under the term 'cell group' for genes whose expression are associated with the terms VD, DD, RME, AVL, DVB, or RIS. We also searched WormBase for gene descriptions, listing expression in GABA neurons, all neurons, or ventral nerve cord. This list was then hand

annotated to confirm GABA-specific expression for these genes. The 'stress gene' list was created by searching WormMart for genes connected with the gene ontology (GO) terms 'response to oxidative stress', 'DNA repair', 'chaperone', 'autophagy', or 'unfolded protein response.' This list was then supplemented with orthologs of known stress-response genes in mammals, such as mitochondrial biogenesis regulators, GSTs, and stress-response transcription factors.

Brood size determination. Single, L4 hermaphrodites were placed on NGM agar plates (1 worm per plate) and incubated at 20ºC. Each day for 5 days, worms were transferred to new plates, and the offspring were allowed to hatch and grow overnight, and counted the following day.

Male mating behavior assay. Young, *alr-1* or N2, reproductively mature males were placed in a drop of egg salt on an agar pad, along with a young, adult N2 hermaphrodite. Upon evaporation of the buffer, worms were observed for 3 minutes, and any tracking of the male along the hermaphrodite including at least 1 turn was scored as positive for mating behavior. Pairs that did not make contact were discarded (Dan Ruley and David Greenstein, personal communication).

Microscopy. Transgenic animals and cultured cells were visualized by differential interference contrast (DIC), or epifluorescence microscopy using either a Zeiss Axioplan or compound microscope. Images were recorded with CCD cameras (ORCA I, ORCA ER, Hamamatsu Corporation, Bridgewater, NJ).

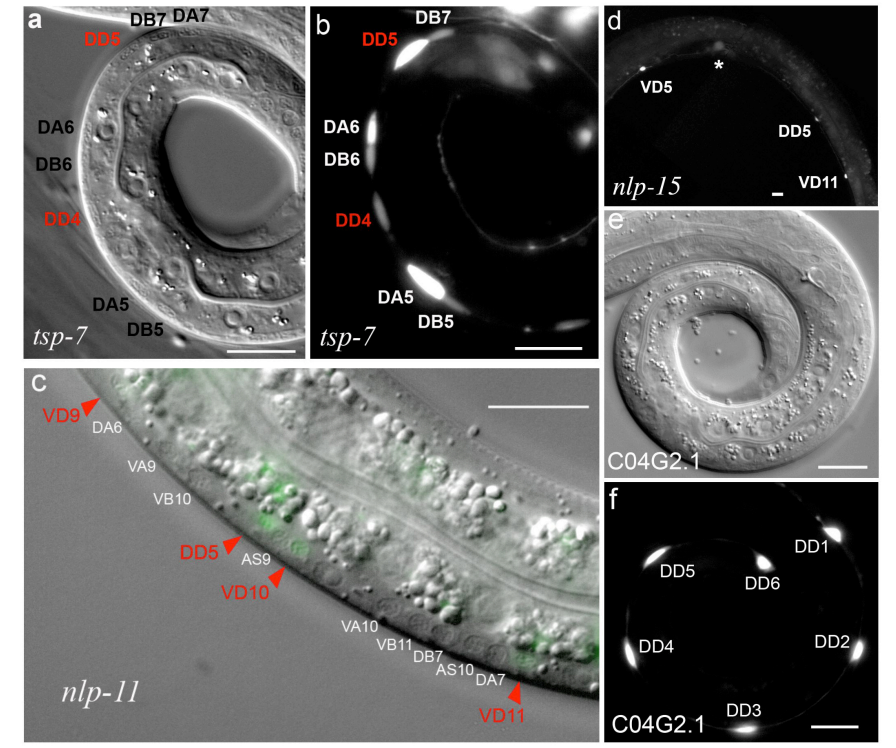
Results

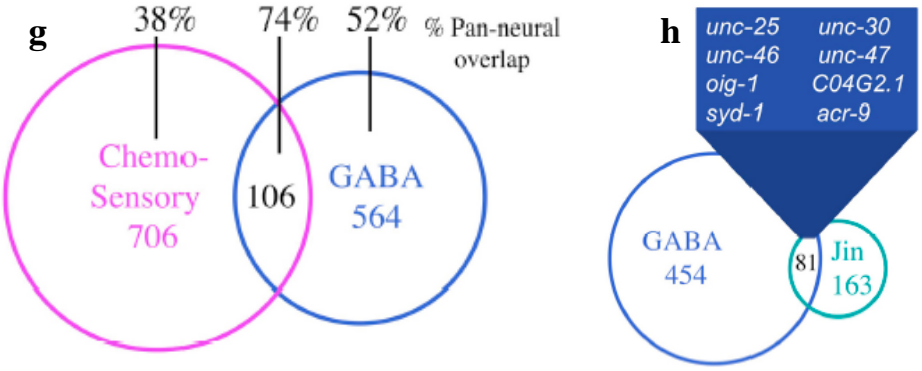
Bioinformatic strategies to validate the GABA micro-array list and discover trends in the data.

In collaboration with Dr. Joseph Watson, I used bioinformatics both to validate the GABA-enriched dataset generated by Dr. Barlow, as well as to search for interesting trends in the data. We first used WormBase to generate a list of 161 genes known to be expressed in GABA neurons in *C. elegans* (see Methods). We found 86% of these genes to be expressed in our GABA dataset, with 20% of them listed as enriched. In addition to surveying published results, the lab created a series of GFP reporter constructs using promoters of genes identified as enriched in each dataset. The expression patterns of selected reporters can be seen in figure 2.1. GFP expression for promoters from the GABA dataset ranged from complete specificity for only the VD and DD neurons, as with *C04G2.1*::GFP (Fig2.1e-f), to expression in every neuron along the ventral cord, as in the case of *tsp-7::*GFP (Fig 2.1a-b). Eighty percent of selected transcripts from the GABAergic dataset were present in GABA neurons by GFP reporter analysis. This result demonstrates that the GABA dataset contains novel GABA-enriched transcripts that may be important for GABA neuron function.

Comparison of GABA dataset with published micro-array profiles

With an ever-growing number of cell-specific profiles, we are afforded the opportunity of comparing different cell types, as they are defined by their profile of expressed or enriched genes. Genes enriched or expressed in our GABA dataset were





i

Dataset	All genes	Panneural	Acr-2	DA	GABA
%genes Expect<e^{-10}	48%	55%	60%	53%	68%

Figure 2.1 Validation of GABA enriched microarray dataset **(a-f)** GFP reporters for genes predicted to be enriched in GABA neurons by micro-array. VD and DD GABA neurons are marked in red, while cholinergic neurons are labeled in white **(g)** Overlap between GABA neuron enriched and Chemosensory neuron enriched genes shows high percentage of pan-neuronal genes. **(h)** Comparison of GABA dataset with published dataset shows known GABA genes in common. **(i)** GABA genes are highly conserved between *C. elegans* and humans

compared with numerous published datasets to identify any trends among the lists (data not shown). Venn diagrams summarizing some of these results are shown in figure 2.1g-h. Comparison of our GABA profile with published profiles for other neuronal subtypes showed that the overlap in gene expression for any two neuron types is highly enriched for pan-neural genes (Von Stetina et al., 2007a) (Fig 2.1g). For example, synaptic vesicle proteins, such as ric-4/SNAP25 and snt-1/synaptotagmin were often found in the intersection of two neuron-specific profiles. This is expected, as genes important for basic neuronal function would be shared among neuronal subtypes. We also compared our GABA gene list with one previously published by the Jin lab (Cinar et al., 2005) (Fig2.1h). The overlapping list of 81 transcripts included many key GABA genes, such as *unc-30* and *unc-25*. However, our experiments detected 454 genes that were not included in the Jin dataset. This may be the result of the high enrichment for GFP-positive cells that Dr. Barlow obtained. It also may be due to variations in experimental technique. Interestingly, 91.4% of genes from the Barlow dataset shown in chapter III to have effects on movement were from the 'Barlow only' subset of genes, rather than the shared genes. We believe that this is an indication that the 454 'Barlow only' genes contain many previously unknown factors relevant to neuronal function.

Antioxidants are expressed at reduced levels in GABA neurons

An alternative approach to searching micro-array data for trends is to use these data to answer a specific question about GABA neurons. As mentioned in chapter I, relatively selective death of medium spiny GABA neurons in the striatum occurs in Huntington's disease (Martin and Gusella, 1986), whereas GABAergic purkinje cells are

significant targets for degeneration in cerebellar ataxias. The source of this sensitivity of GABA neurons remains unexplained in these diseases. One possibility is that GABA neurons express lower levels of genes involved in protection against stressors, rendering them sensitive to degeneration upon certain insults.

To test this hypothesis, we created a list of 299 known defense genes, including antioxidants, chaperones, DNA repair enzymes, and autophagy genes (table 2.1). We then identified genes with altered expression in the GABA profile relative to a published embryonic Pan-neuronal profile. A comparison of these genes to the protective gene list identified 42 protective genes that are differentially expressed in GABA neurons (Fig 2.2). 64% of these genes were reduced, suggesting that GABA neurons may have a generalized reduction in cellular defenses. Some notable anti-oxidants are diminished in GABA neurons, including transcripts encoding the superoxide dismutase *sod-4* and the catalase *ctl-3*. The most striking reduction, however, was that of glutathione-S-transferase (GST) genes. GSTs are a large family of enzymes, which are well known for their role in detoxifying both intra-cellular (e.g. Radical Oxygen Species (ROS)) and environmental toxins. 13 of the known GST genes were reduced in GABA neurons compared to all neurons. The finding that so many GST transcripts are reduced in GABA neurons may suggest a role for GSTs in the sensitivity of GABA neurons to degeneration in diseases such as Huntington's disease and cerebellar ataxias. A role for GSTs in susceptibility to degeneration is not unprecedented. Pharmacological inhibition or RNAi knockdown of GSTpi has been shown to sensitize dopaminergic neurons to degeneration in the MPTP model of Parkinson's disease (Smeyne et al., 2007). Similarly, the basal

Stress Response Genes	
Gene Class	**Examples**
Autophagy genes	TOR, unc-51, bec-1, sir2.1, pqn-5,29,35,54,74,76,78,91,95,abu-2-11
Oxidative stress transcription factors	jnk-1, cep-1,hsf-1
anti-oxidant enzymes	superoxide dismutases(sod-1-5), catalses(ctl-1-3)
glutathione-S-transferases	gst-1-44
Glycolysis	F14B4.2,C50F4.2,fbp-1,tpi-1,pgk-1
Mitochondrial Biogenesis regulators	Daf-16,kin-29,aak-1,aak-2,pmk-1,pmk-3,sir2.1-2,3,cmk-1,unc-43
Peroxiredoxin/peroxidase	prdx-2,3,6,mlt-7
Unfolded Protein Response	ire-1,xbp-1,tor-1,tor-2
GO-term response to oxidative stress	axl-1,bli-3,che-11,ctl-1-3,mlt-7,pmr-1,pxn-1-2,skn-1,smk-1,srd-71-72,sto-1…
Heat-shock proteins	hsp-1-70 (~30 genes)
DNA repair	ubc-1,smc-3,smc-4lig-1,lig-4,mix-1,mrt-2,msh-6,nth-1,polh-1,polk-1,hpr-9,17

Table 2.1 Stress response genes. A search for Gene Ontology terms (GO) related to stress response in *C. elegans*, and a search for gene classes that are known to be important for stress response yielded a list of 299 genes, many of which are listed above. Relative depletion of genes in this list was used as a way of determining vulnerability to stress in GABA neurons.

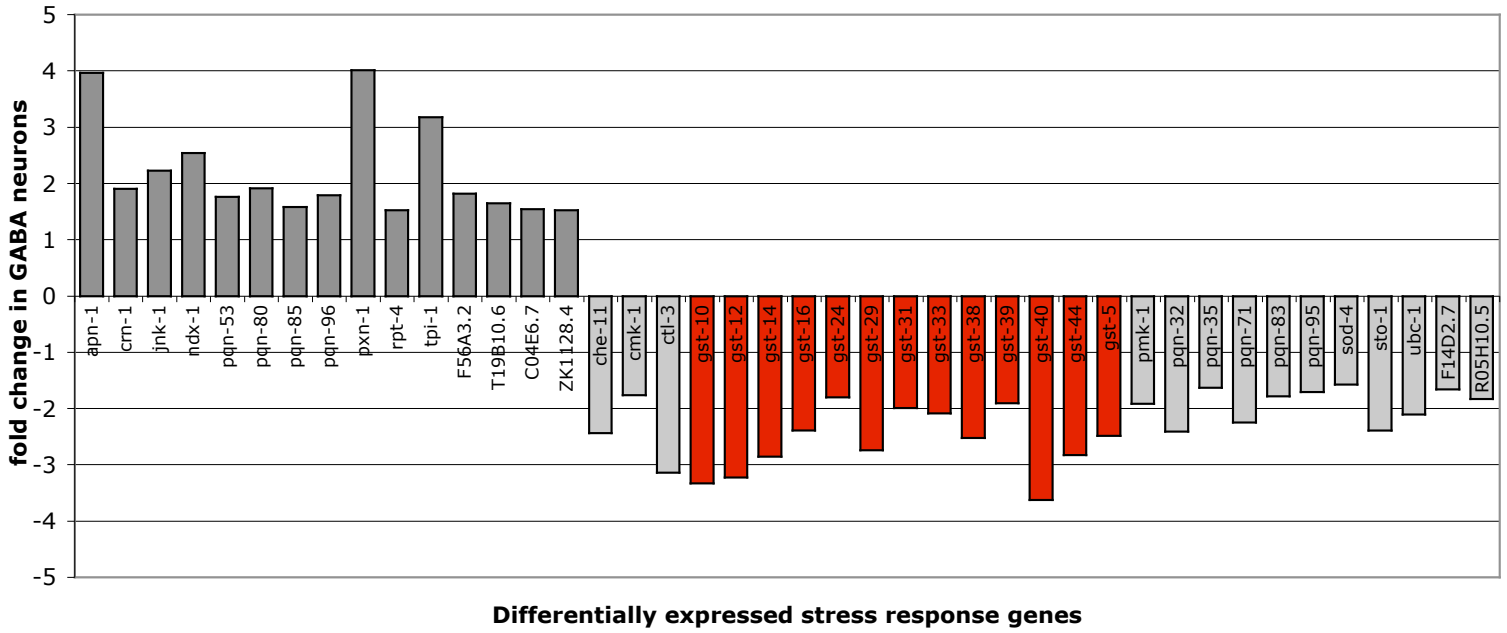


Figure 2.2 Comparison of stress-response gene expression between GABA and Pan-neural datasets. Embryonic GABA neuron array was compared to embryonic pan-neural dataset, to determine genes that were up/down-regulated in GABA neurons versus all neurons. These genes were then compared to a list of stress-response genes (Table 2.1), to determine differential expression of these genes in GABA neurons. Many genes for GSTs, highlighted in red, were relatively reduced in GABA neurons.

reduction of GSTs in GABA neurons may weaken defenses against mitochondrial stress, causing selective loss of these neurons in HD and SCA.

Additional phenotypic characterization of *alr-1* mutants

Axon outgrowth defects in VD-class GABA neurons

As mentioned, previous work in the lab had shown that *alr-1* mutants display defects in axon outgrowth of the embryonically-derived DD subclass of GABA neurons. To follow up on this we first repeated the experiment labeling GABA neurons with *C04G2.1::GFP* to show that the axonal outgrowth effect was not an artifact of the *unc-25::GFP* transgene. We saw the same axon outgrowth defect in DD neurons with this reporter. Furthermore, because *C04G2.1::GFP* is not integrated, we took advantage of mosaicism in the strain to analyze the post-embryonically derived VD neurons for the defect. Because VD and DD neuronal processed overlap, it is currently impossible to separate these processes in an integrated strain. We found animals in which *C04G2.1*::GFP expression in some DD neurons was off, so that the VD neuron processes in that region could be viewed. As seen in Figure 2.4d, the axon outgrowth defect was also found in VD neurons, indicating that *alr-1* is important for the development of both embryonic and larval-born GABA neurons.

Thrashing

The effect of *alr-1* mutation on outgrowth of DD GABA axons was ~40% penetrant. Because only a portion of GABA neurons was affected, we could not predict

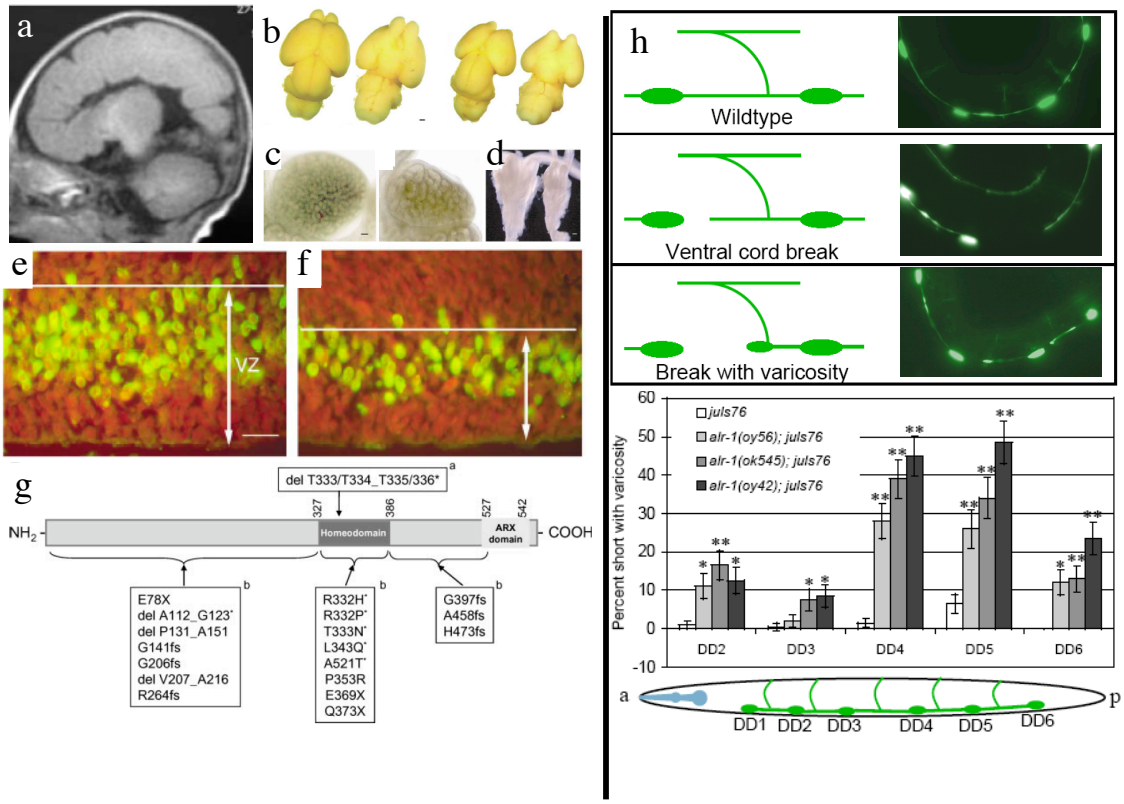


Figure 2.3 Aristaless/ARX/ALR-1 functions in GABA neuron development in mammals and in *C. elegans*. **(a-g)** Mammalian ARX phenotypes. **(a)** lissencephaly in a child with ARX mutation, **(a)** and **(g)** from Shambhu *et al.*, 2005. **(b-f)** ARX mutant mice (right) compared to wildtype littermates (left) reveal smaller-sized brains (b), testes **(c)**, and seminiferous tubules **(d)**, and decreased proliferation in the ventricular zone at E14.5, from Kitamura *et al.*, 2002. **(g)** Mutations in ARX that have been linked to mental retardation or seizure disorders in humans. **(h)** DD GABA neurons in *alr-1* mutant worms display axon guidance defects, from Barlow *et al.*, unpublished.

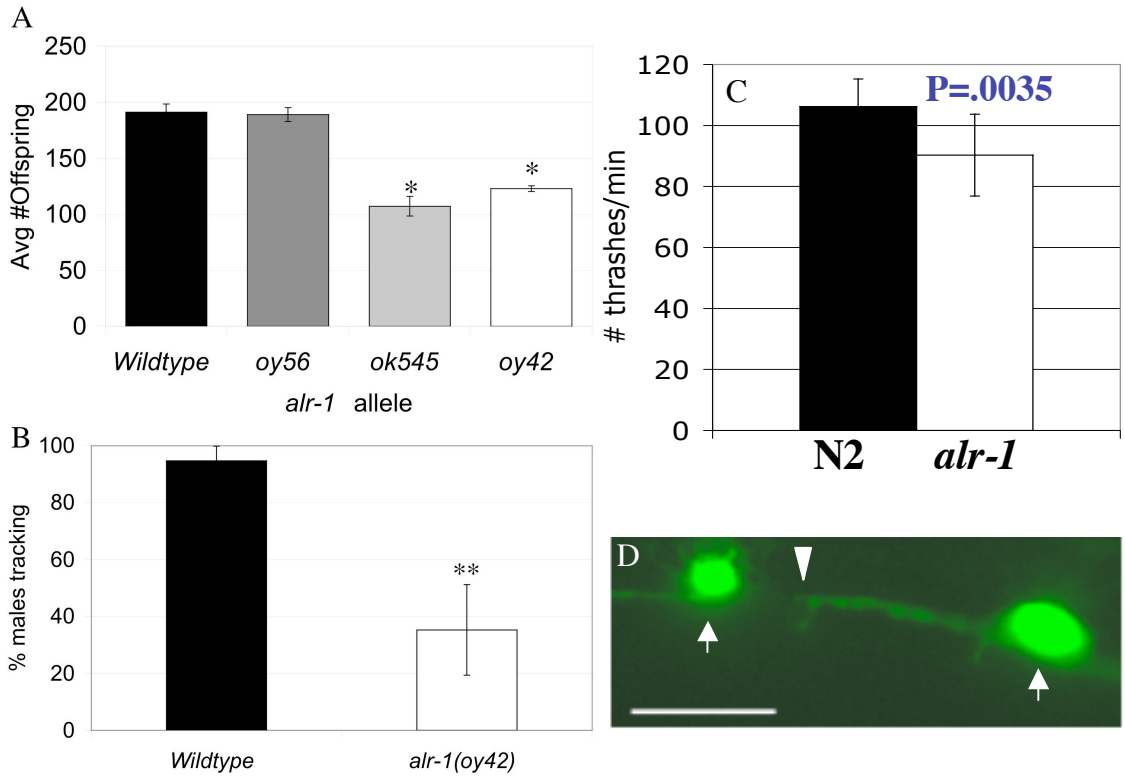


Figure 2.4 Further phenotypic studies of *alr-1* mutants (**A-B**) *alr-1* reproductive defects: *alr-1* hermaphrodites produce smaller broodsizes **(A)** and *alr-1* males display a lower percentage of tracking/mating behavior **(B)**. **(C)** Slight movement defect is detectable in *alr-1* adults by thrashing assay. **(D)** Mosaic analysis of *alr-1;C04G2.1*::GFP reveals defects in axons of VD neurons. Arrowhead points to break in the VD axon. Arrows indicate VD cell somas

whether the *alr-1* mutation would affect normal movement. Forward and backward movement appeared normal in these animals, and they did not display the 'shrinker' phenotype normally associated with GABA neuron ablation. To determine whether subtle defects were present, we tested *alr-1(oy42)* mutants in a thrashing assay, and measured a slight, but statistically significant, decrease in thrashing in these mutants (figure 2.4c).

Aristaless mutants display reproductive defects

Like mouse and human ARX mutants, aristaless mutants in *C. elegans* expressed reproductive deficits. *alr-1* mutant hermaphrodites laid fewer eggs than wildtype counterparts (Fig 2.4a). We also noticed that *alr-1* male animals did not produce progeny in genetic crosses. Male worms undergo a series of specific behavioral steps in order to mate with hermaphrodites. One of these behaviors involves the male's tail tracking along the hermaphrodite body to locate the vulva (Liu and Sternberg, 1995). We found that wildtype males placed with hermaphrodites perform this behavior 95% of the time, if contact is made with the hermaphrodite. *alr-1* mutant males, however, showed a ~66% decrease in tracking behavior when contacting hermaphrodites (fig2.4b), which may explain their inability to mate. Though the nematode reproductive system differs greatly from that of mammals, the effects of *alr-1* mutation on reproduction may provide clues to its function in the mammalian reproductive system.

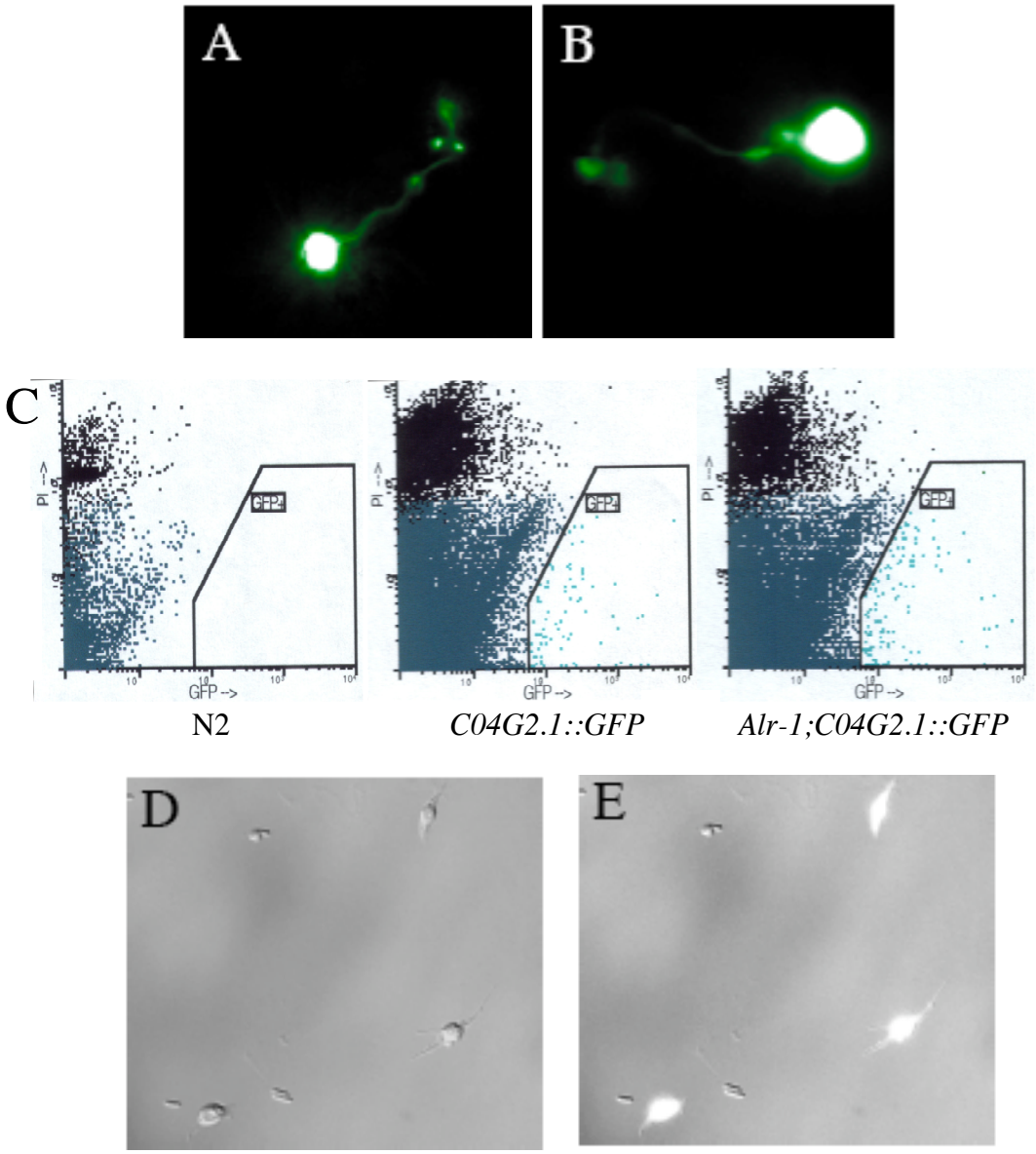


Figure 2.5 Strategy 1 to identify targets of the *alr-1* transcription factor **(A-B)** GABA neurons in culture **(A)** *C04G2.1::GFP* and **(B)** *alr-1;C04G2.1::GFP*-expressing GABA neurons cultured for 72 hours **(C)** FACS sorting of GFP-positive cells cultured from wildtype (N2-background), *C04G2.1::GFP*, or *alr-1;C04G2.1*::GFP animals. **(D-E)** Post-FACS sort *C04G2.1::GFP* expressing GABA neurons DIC **(D)** and GFP overlay**(E)**, showing enrichment of GFP-positive cells after sorting

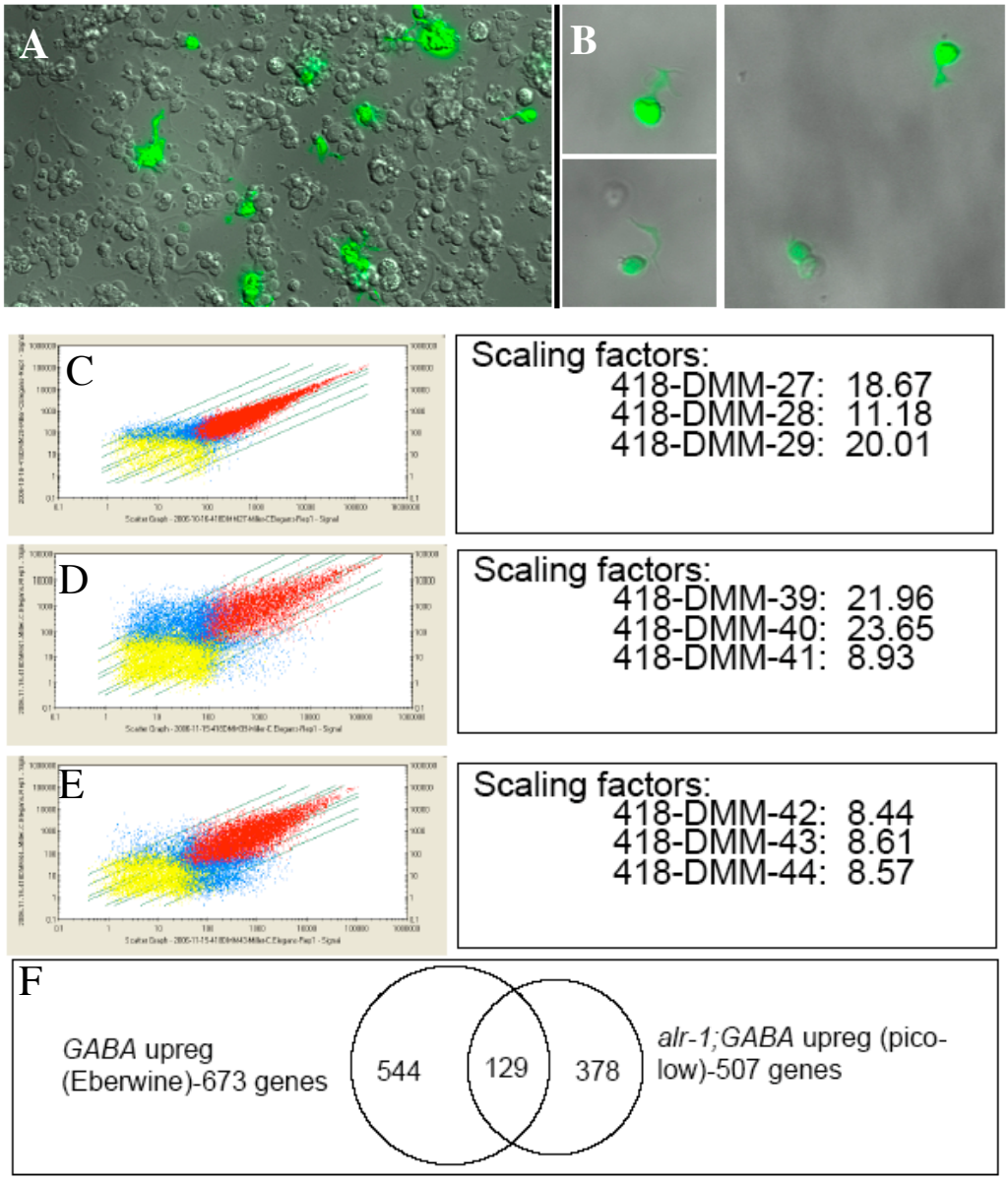


Figure 2.6 Strategy 2 for identification of *alr-1* target genes (A-B) *alr-1;unc-25*::GFP cells in culture before **(A)** and after **(B)** sorting to show enrichment of GFP-positive cells **(C-E)** Scatter plots of fluorescence intensities of genes from 2 different data points for N2 **(C)**, *unc-25*::GFP-labeled GABA neurons **(D)**, or *alr-1;unc-25*::GFP-labeled GABA neurons **(E)**. Scaling factors (background) for each micro-array experiment are listed on the right side. **(F)** Venn diagram of overlap between previously developed GABA enriched dataset with new *alr-1;unc-25*::GFP dataset shows some genes in common.

Search for targets of *alr-1* using micro-array profiling

The determination of *alr-1* transcriptional targets is central to understanding its role in GABA neuron development and disease. To do this, we designed experiments to sort GABA neurons, labeled with GFP, cultured from wildtype and *alr-1* mutant embryos as described above. We then attempted to isolate RNA from the sorted cells, amplify and determine differential gene expression levels using micro-array.

Because the observed phenotype occurs in ventral cord GABA neurons, we first attempted a culture strategy that utilized the *C04G2.1*::GFP reporter, which shows expression only in those neurons. As shown in figure 2.5, cells from *C04G2.1*::GFP embryos can be cultured and have been profiled through FACS, showing that the GFP-positive population of cells can be sorted from other cells and debris. We sorted and isolated RNA from cells of both the C04G2.1::GFP, and *alr-1*;C04G2.1::GFP lines. However, we were unable to significantly amplify this RNA for hybridization.

Our second approach used the *unc-25*::GFP reporter that had previously been used in the lab to generate the GABA-enriched micro-array list. We were able to obtain RNA from three independent background (N2, all cells) and *alr-1;unc-25*::GFP samples. However, we were unable to obtain enough wildtype *unc-25*::GFP RNA samples for comparison with the mutant cell samples. Scatter plots in figure 2.6 show reasonable agreement between the mutant and background samples obtained. However, the wildtype *unc-25*::GFP RNA samples collected consistently gave high scaling factors, and showed little similarity between samples. After multiple tries, we were only able to obtain a single wildtype *unc-25::*GFP sample with a low scaling factor. This is not enough to make the statistical comparisons necessary to predict *alr-1* target genes.

Discussion

The identification of targets of *alr-1* remains an incomplete endeavor. We are not sure why we were unable to get good RNA from sorted *C04G2.1::GFP*-labeled neurons. Based on other projects in the lab that involve profiling rare populations of cells by the sorting method, it seems that the RNA isolated from such sorts tends to contain a contaminant that leads to over-estimation of RNA concentration. *C04G2.1*::GFP labels only 6 cells per embryo, and, since it is not integrated, even that is an over-estimation.

The *unc-25*::GFP line may have worked better for sorting because it is integrated and labels more cells per worm. In addition, our previous success with this sorting and profiling this strain was promising for this project. However, we still were unable to obtain a complete GABA profile with this reporter strain, due to high background in the affymetrix chip data. Again, we have no good explanation for this. Both isolation and amplification protocols were changed prior to beginning this study. Either change may have introduced the difficulties faced with this attempt at sorting from this strain. Also, working with an amplification protocol that is still under development may have caused unforeseen issues, though the same version of the kit was supposed to be used throughout. Since we are so close to a complete dataset, solutions to these issues could bring about useful data rather quickly. If these issues cannot be worked out, one alternative would be to amplify *alr-1;unc-25*::GFP RNA by the previous method, and compare the resulting micro-array profile with the existing, validated GABA dataset.

Bioinformatic studies of the GABA enriched geneset have shown that the GABA transcriptome of *C. elegans* contains a high percentage of conserved genes. Analysis of

the dataset led us to studies of the *alr-1* transcription factor in GABA neuron development. Scrutiny of the geneset also led to the hypothesis that the relative depletion of defense genes, especially GSTs, may contribute to the susceptibility of GABA neurons to degenerative disease. The use of the model system *C. elegans* will allow for ready pursuit of such hypotheses. The nematode is also ideal for the systematic study of genes in this list, and may reveal unexpected insights into GABA neuron function. An example of such a study is described in the next chapter.

CHAPTER III

RNAi SCREEN TO IDENTIFY GENES IMPORTANT FOR GABA NEURON FUNCTION AND MOVEMENT IN *C. ELEGANS*

Introduction

In chapter II, I described informatic approaches to search for trends in genomic data. Such manipulations of large datasets are undertaken with the aim of developing hypotheses for future research. For example, we chose the candidate gene aristaless for follow-up, based on its well-known function in mammalian GABA neuron fate. In addition, we used bioinformatic comparison to generate the hypothesis that GABA neurons may be vulnerable to cell stresses, based on their relative reduction of glutathione-S-transferase enzymes. Although choosing noticeable trends in datasets is useful for generating hypotheses, the interests and expertise of the researcher naturally bias the genes chosen for further study. For example, we would not have discovered the relative depletion of GSTs in GABA neurons had we not been interested in stress-response genes. In addition, many genes that are differentially expressed in GABA neurons have no known function, and thus would not likely be chosen in a candidate approach. An unbiased strategy to test genes in these datasets for functional importance can therefore complement a candidate gene approach. One advantage to model systems such as *C. elegans*, is that entire datasets, such as the GABA-enriched list discussed

above, can be screened for function using the available RNA interference (RNAi) technology.

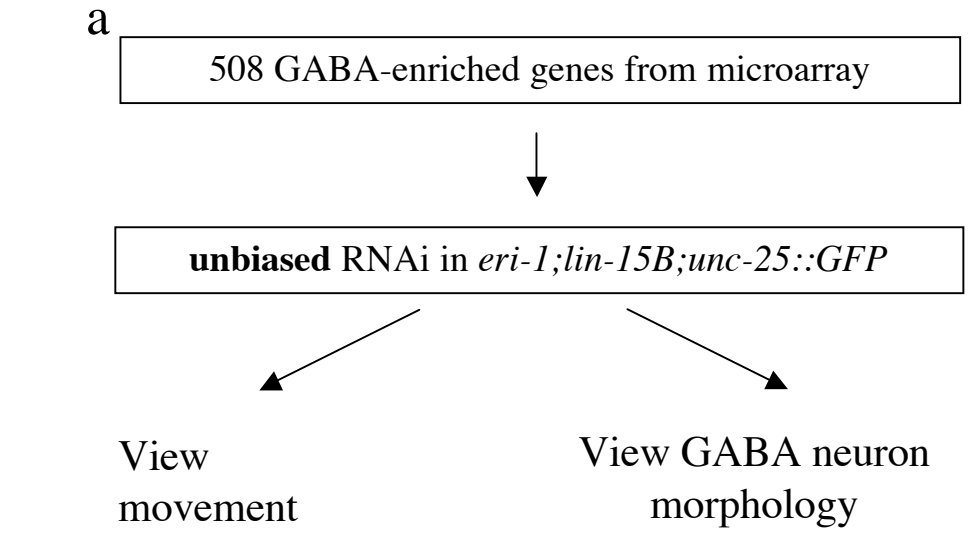
In 1998, Craig Mello and Andrew Fire published their Nobel Prize-winning discovery in *C. elegans* that injection of double-stranded RNA specific to a given gene's transcript sequence can interfere with the expression of the subsequent protein (Fire et al., 1998). Although gene mutation and knockout are powerful tools for understanding the function of specific genes, only ~25% of the *C. elegans* genome is associated with knockouts or mutants to date (Mark Edgley, personal communication). In contrast, RNAi constructs have been generated for nearly all of *C. elegans* genes (Kamath et al., 2001). Knockdown can now be easily accomplished by feeding worms bacteria, which contain plasmids encoding double-stranded RNAs specific for a given target gene. RNAi has the additional advantage that knockdown may reduce gene levels only partially, and can therefore be used to study the function of genes whose ablation is lethal. This can also be a disadvantage, however, as knockdown of a gene may be insufficient to produce a phenotype.

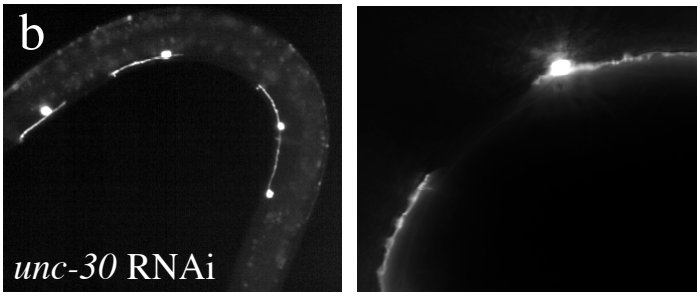
Over the past ten years, the RNAi pathway has been studied in depth, and several discoveries have been made that optimize the potential of this methodology. For example, some tissues are more sensitive to RNAi knockdown than others. Neurons have been shown to be refractory to RNAi in wildtype *C. elegans*. This has been an obstacle for scientists who wish to use RNAi to study neuronal gene function. A recent genetic screen revealed that the *eri-1;lin-15B* double mutant enhances sensitivity to RNAi, even in neurons (Kennedy et al., 2004; Wang et al., 2005). We have exploited this mutant

strain to conduct an RNAi screen of 508 of the GABA-enriched genes from the microarray list discussed in Chapter II.

Materials and Methods

The general strategy for the screen is outlined in Fig 3.1a. The *unc-25* gene is the ortholog of mammalian glutamate decarboxylase (GAD) (Jin et al., 1999). GAD is required for synthesis of GABA, and thus expression of GFP under the control of the *unc-25* promoter specifically marks GABA neurons. We crossed unc-25::GFP into the *eri-1;lin-15B* RNAi-hyper-sensitive background. We then fed *unc-25::GFP;eri-1;lin-15B* worms with bacteria expressing dsRNA for 508 genes that had previously been shown by micro-array to be enriched in GABA neurons (chapter II). RNAi assays were performed by feeding as previously described (Fire et al., 1998; Timmons and Fire, 1998), using clones from the Ahringer library (Fraser et al., 2000; Kamath et al., 2003). After 5 days of feeding, we scored worms for movement phenotypes by tapping on the head and tail and observing the resultant forward and backward movement. Putative phenotypes were verified by at least 2 independent researchers. Gene identity was always unknown to the experimenter. *unc-25::GFP* fluorescence was then observed through a compound microscope to detect any effects of RNAi treatment on GABA neuron morphology.





GFP	Percentage
Normal	15
Dim	40
Off	45

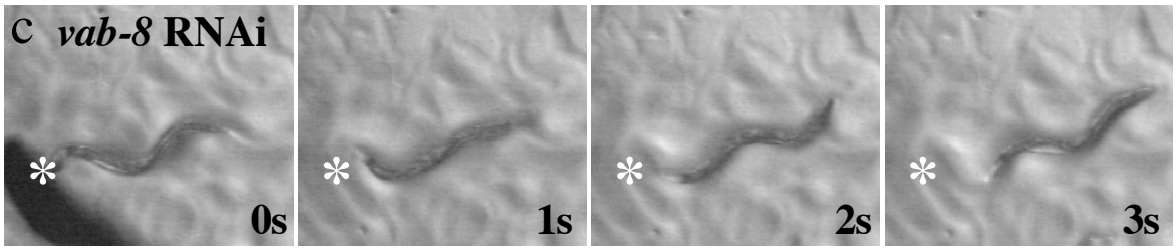


Figure 3.1 RNAi strategy to identify genes important for GABA neuron function and movement in *C. elegans*. **(a)** Strategy for RNAi screen **(b)** Suppression of *unc-25*::GFP by *unc-30* RNAi images show cleargaps in unc-25::GFP labeled neurons. Graph shows quantification in 18 worms of *%unc-25::GFP* neurons in which GFP was observed to be normal, dimmed, or off in response to *unc-30* RNAi **(c)** Unc phenotype of *vab-8* knockdown animals validates sensitivity of neurons to RNAi in the *eri-1;lin-15B* strain. * indicates point of touch, and animal's position at 1, 2, and 3 seconds is shown.

Results

RNAi screen detects novel genes required in neurons for locomotion

Mutation of *unc-30*, the pitx-2 transcription factor ortholog (Westmoreland et al., 2001), which controls *unc-25* expression, causes both an uncoordinated phenotype and a reduction in *unc-25::GFP* expression (Eastman et al., 1999). RNAi of *unc-30* in wildtype animals does not produce a phenotype, presumably due to neuronal resistance to RNAi. However, we observed both an Unc phenotype and loss of *unc-25::GFP* expression with knockdown of *unc-30* in the *unc-25::GFP;eri-1;lin-15B* animal (Fig 3.1b). This result validates the neuronal sensitivity of the screen, and *unc-30* was subsequently used as our positive control. Like *unc-30*, genes such as *vab-8 (Manser and Wood, 1990)* and *unc-11 (Brenner, 1974; Hosono and Kamiya, 1991)* had not previously displayed movement phenotypes by RNAi, but knockout of these genes results in an Unc phenotype. Furthermore, the function of these genes specifically in neurons is important for the Unc phenotype in both cases. Knockdown of *vab-8* and *unc-11* also produced movement defects in our screen (fig3.1c), further confirming the increased sensitivity to RNAi in neurons.

We also validated the screen by calculating the percent detection of certain known RNAi phenotypes among our dataset. We identified 81% of genes that had previously been shown to have movement phenotypes by RNAi. We also identified 87% of genes whose knockdown produces sterile or lethal phenotypes. This allows us to estimate the sensitivity of our screen at ~80-87%.

We detected ~30 genes whose knockdown resulted in various movement phenotypes (Table 3.1). We tested 28 of these genes for quantifiable movement deficits

Table 3.1 Results of RNAi Screen		
Gene	**Predicted Function**	**Observed RNAi Phenotype**
Receptor Trafficking		
unc-11	Clathrin Adaptor Protein	unc
aps-2	Clathrin Adaptor Protein	unc
apt-10	Regulator of clathrin adaptor complexes	unc
F57B10.5	Membrane trafficking protein	slow
odr-4	7TM receptor localizing protein	fwd unc
Signaling		
unc-108	Small G protein	fainter/fwd unc
C26F1.7	Protein Kinase C Inhibitor-like	~slo
lin-18	Rik/Derailed family of RPTKs	poss axon gdnce
tyra-3	tyramine receptor	poss axon gdnce
C56A3.6	EF Hand Ca^{2+} binding protein	unc/slogro~sick)
Metabolism		
T03F1.3	Phosphoglycerete Kinase 1 Ortholog	slow
coq-1	CoQ Synthesis	unc-cell death
gpd-2	GAPDH	unc/slo/inc defasciculation
dhs-17	short chain dehydrogenase/reductase	slo/slogro
M01F1.7	Phosphatodylinositol transfer protein	fwd slo
ubc-20	Ubiquitin protein ligase	long/pale
M04B2.4	Possible Oxidoreductase	unc/lpy
Mitochondrial		
F23C8.5	Electron Transfer Flavoprotein β Subunit	unc/slo
F43E2.7	Mitochondrial carrier protein	ex amp/ste
Transporters		
F10E7.9	Na+/K+ Symporter	goofy/lpy mvmt
vha-14	Vacuolar proton-translocating ATPase	long/ex amp/fig 8/hyper
C53B4.6	UDP-N-acetylglucosamine transporter	~slo
Other		
F25H9.6	Halotolerance protein HAL3	~sick/examp/spdy/some unc
C56C10.11	DnaJ Family Molecular Chaperone	unc
vab-8	Cell migration/axon pathfinding	lpy/coiler
F43D2.1	None	slogro/long/snaky/examp/ste
C14A11.6	None	poss cell fate
F46B6.2	None	~slo/ste/sick/fwd unc
T22F7.4	None	slo(2XNP)
W03F9.2	None	~sick/examp/spdy/pvul
R10H10.4	None	fwd unc/jerky
C35D10.1	None	sick/fwd unc/let
R10E4.1	None	fwd slo
T04A8.13	None	unc
K02B12.5	None	~slo
R05D7.3	None	bk unc

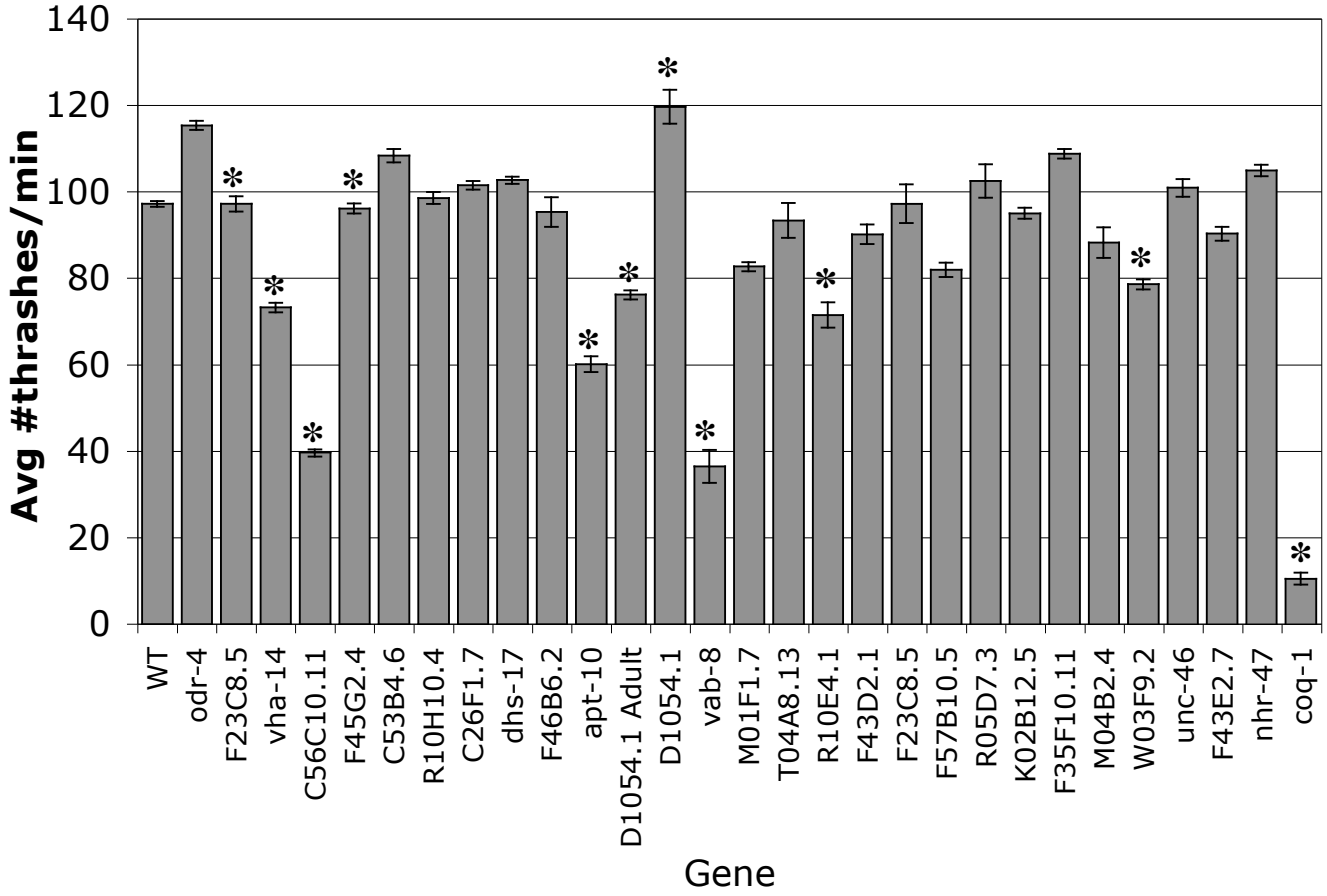


Figure 3.2 Quantification of movement defects for RNAi Hits. Movement defects discovered in the RNAi screen were quantified by thrashing assay. Bars represent avg +/- S.E.M., * $P < 0.05$, Students T test, n=10.

by a thrashing assay (Miller et al., 1996), of which 11 showed statistically significant ($P<.05$, Student's T) changes in thrashing (fig 3.2). One explanation for why the other 17 hits were not measurably altered in the thrashing assay is that RNAi shows variable penetrance. Knockdown sufficient to affect a high percentage of animals on the plate may not have been achieved for these genes, though deficits in some small portion might have been readily apparent. Additionally, not all movement abnormalities on the plate may translate to swimming deficiencies.

Due to the presence of cross-contamination in the RNAi library, it was necessary to sequence clones of all hits, to be sure that phenotypes produced were the result of treatment with the expected dsRNA-expressing bacteria. Although the identity of most hits were confirmed in this process, we discovered that the gene that produced the most severe Unc phenotype was not the expected clone, C32F10.8, but a contaminating colony. The actual clone producing the phenotype in this case was specific to the *coq-1* gene. Whereas *coq-1* was not among the enriched genes in the GABA microarray list, we decided to follow up on it anyway, as it produced such a prominent phenotype. The ensuing experiments are discussed in chapter IV.

RNAi hits were divided into categories, depending on their known function, or the function of putative orthologs. A brief discussion of the importance of selected categories is followed by descriptions of specific hits. Some of these may be interesting candidate genes for future studies.

Trafficking/endocytosis/exocytosis genes

The expression and clustering of specific receptors is necessary for synapse function. Endocytosis and exocytosis are necessary both at the presynapse for vescicle release and at post-synaptic sites for receptor internalization and sorting as part of the adaptive responses to neurotransmitter signaling (Kandel and Schwartz, 2000). Disruption of any of these processes could affect motor circuit function. Therefore, it is not surprising that RNAi for genes involved in endo/exocytosis and receptor trafficking resulted in Unc phenotypes in this screen.

Adaptin complex proteins

Adaptins mediate the formation of clathrin-coated vesicles at cellular membrane and contribute to sorting of vesicles once separated from the membrane surface (fig3.3b) (Ross et al., 1995). This process contributes to synaptic vesicle function, receptor trafficking, and vesicle budding from organelles, such as the golgi apparatus. Adaptin proteins combine to form adaptor protein (AP) complexes, which target specific membrane proteins, and assemble the machinery necessary for vesicle formation (fig3.3b). There are 4 such complexes in mammals (AP-1 through AP-4). AP-2 is involved in rapid endocytosis at the plasma membrane (Boehm and Bonifacino, 2001). One of the genes that produced an Unc phenotype in our screen was *aps-2*, the *C. elegans* ortholog of the adaptinσ2 component of AP-2. *aps-2* was recently identified in an RNAi screen in the *eri-1;lin-15B* background for genes that confer resistance to aldicarb, indicating that knockdown of *aps-2* results in decreased ACh release or decreased

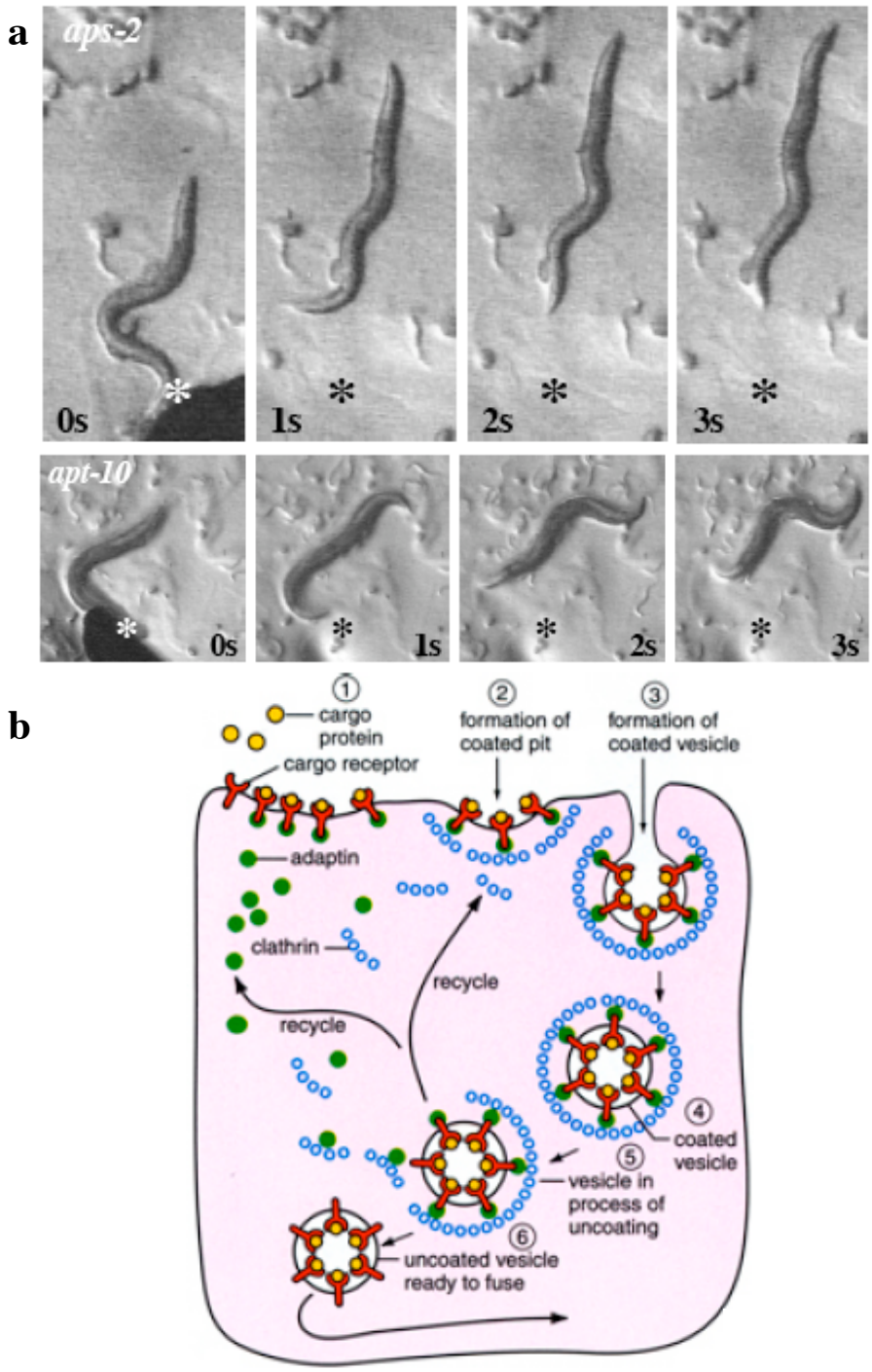


Figure 3.3: Adaptins in movement and neuronal function (a) Uncoordinated movement in *C. elegans* treated with RNAi to adaptin AP-2 complex member *aps-2*/AP-2σ or adaptin complex AP-1 and AP-2 regulator *apt-10*/Stonin. * indicates point of touch, and animal's position at 1, 2, and 3 seconds is shown. (b) Diagram of adaptin complex function in vesicle endocytosis, adapted from Ross et al, 1995.

response to ACh (Sieburth et al., 2005). This validates a role for this gene in neuronal communication in *C. elegans*.

A closely–related family of proteins, known as stonins, act as regulators of AP complexes. A member of this family, *apt-10*, was also found to display an Unc phenotype by RNAi. *C. elegans apt-10* shares 41% homology with mammalian stonin 2 and 45% homology with drosophila stoned B. Both stoned B and stonin 2 have been shown to be important at presynaptic sites for recycling and sorting of the synaptic vesicle fusion protein synaptotagmin. Stonins do this through their interaction with and regulation of the AP-2 complex. (Fergestad et al., 1999; Walther et al., 2004; Diril et al., 2006). Both *apt-10* and *aps-2* are expressed throughout the nervous system in *C. elegans*. Therefore, the Unc phenotype produced by RNAi of these genes may be due to defects in many neuronal subtypes.

Metabolic and Mitochondrial genes

Nine genes important for metabolic and mitochondrial function were found by this screen to produce movement phenotypes. Genes in this category are interesting because, whereas null alleles of such vital genes are likely lethal, knockdown may not be. As emphasized in chapter I, metabolic genes are precious to neurons, because of their unique energy needs. Therefore, it is not surprising that such genes were found to have roles in movement.

T03F1.3/Phosphoglycerate Kinase-1

T03F1.3 encodes a gene that shares 69% identity with human phosphoglycerate kinase-1 (*pgk-1*) (fig3.4d). PGK-1 catalyzes the transfer of phosphate from 1,2-diphosphoglycerate to ADP, yielding ATP and 3-phosphoglycerate as part of glycolysis (fig3.4a). Therefore, PGK-1 is important for energy production in all cells. In addition, PGK-1 has been shown to be associated with synaptic vesicles in glutamatergic neurons, where the ATP that it produces is necessary for the uptake of transmitter into the vesicle (Ikemoto et al., 2003)(fig 3.4b). This finding suggests an additional, neuron-specific, role for PGK-1. Whereas null alleles of such an important metabolic gene are likely lethal, several alleles of PGK that reduce its function have been identified in humans. The three major manifestations of PGK deficiency are myopathy, anemia, and brain disorders, specifically seizure and mental retardation syndromes (Tsujino et al., 1995). Because the improper development of GABA neurons in the brain has long been associated with mental retardation and seizures, the development of such conditions in PGK deficiency may signify an important role for PGK in the development, function, or survival of GABA neurons in humans.

Studies in drosophila have discovered a temperature sensitive pgk-1 mutation that causes inducible seizures at the restrictive temperature. Subsequent experiments have shown that reducing PGK-1 activity results in reduced resting membrane potentials and decreased endocytosis in the fly brain. The authors hypothesized that GABA neurons have a higher need for glycolytic ATP for vesicle endocytosis, as GABA neurons have smaller vesicle cycling pools. They believe that that pgk-1 mutation produces seizures because of this preferential affect on GABA neurons (Wang et al., 2004). This work

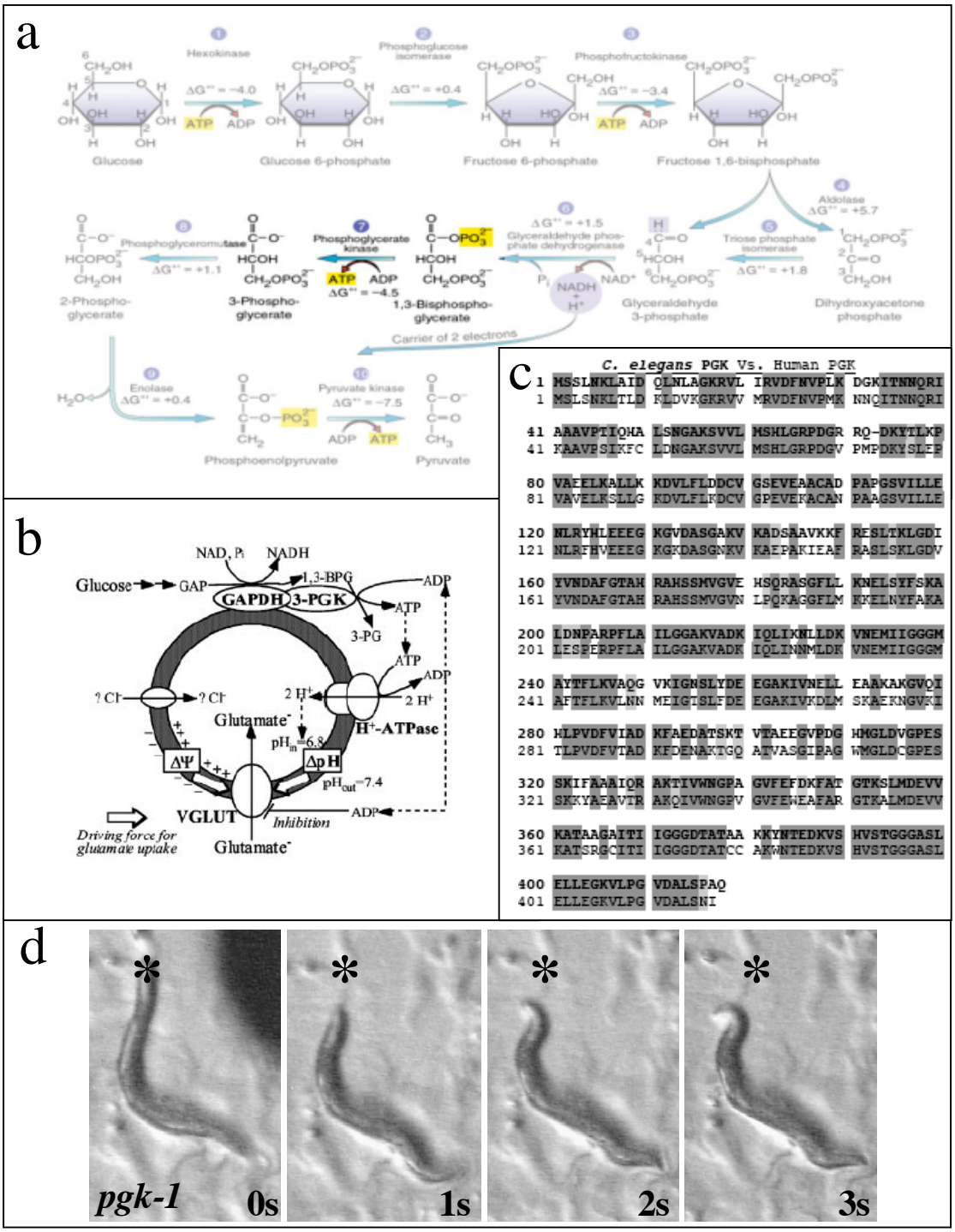


Figure 3.4: Phosphoglycerete kinase-1 (pgk-1) (a-b) pgk-1's role in glycolysis and ATP production (a), adapted from http://fig.cox.miami.edu/~cmallery/ and vescicular neurotransmitter loading (b), adapted from Ikemoto et al, 2003. (c) alignment of *C. elegans* PGK-1 with human reveals 69% identity. (d) movement defects in worms fed RNAi for *pgk-1*. * indicates point of touch, and animal's position at 1, 2, and 3 seconds is shown.

demonstrates not only a highly conserved neuronal function for PGK-1, but also suggests a critical role in GABA neurons.

Consistent with an important and conserved role for PGK-1 in GABA neurons, this transcript was 2.5-fold enriched in our *C. elegans* GABA microarray dataset. Movement of animals treated with RNAi for PGK-1 was slow, especially backward movement (fig3.4c). The knockdown of PGK in *C. elegans*, like the ts allele in drosophila, could be used to better understand the molecular function of this gene in neurons, and may be an excellent model for the developmental abnormalities in the human PGK-1 deficient brain.

Unclassified Genes

~30% of the hits from the screen were genes whose functions have not been studied in *C. elegans*. Some of these genes are homologous with disease-relevant genes in humans, including some of the degenerative diseases previously discussed. The phenotypes produced by RNAi knockdown of these genes may make them useful tools for studying the molecular mechanisms of their involvement in disease.

C56C10.11/DNAJ

Knockdown of *C56C10.11* produced one of the most severe and highly penetrant Unc phenotypes of all genes screened (Fig 3.2b). This gene encodes a protein with similarity to the DNAJ/HSP40 class of chaperone proteins, which are preferentially expressed in neurons (Cheetham et al., 1992). DnaJ family proteins recognize and bind misfolded proteins. Interestingly, DNAJ is mis-expressed in the degenerating neurons of

the wobbler mouse (Boillee et al., 2002), and over-expression of DNAJ family members has been shown to protect against degeneration in drosophila models of polyglutamine toxicity (Fayazi et al., 2006), in a cell culture model of *huntingtin*-induced degeneration (Chuang et al., 2002) and in cell culture and mouse models of SCA1 (Cummings et al., 1998). This emphasizes a possible role for DNAJ in the protein folding checkpoint for cell survival discussed in chapter I.

C56C10.11 was ~2.5 fold up-regulated in the GABA neuron microarray profile, indicating a possible role for this chaperone in *C. elegans* GABA neurons. Because of the severity of its RNAi phenotype, we studied the morphology of GABAergic, cholinergic, and dopaminergic neurons and muscle in *C56C10.11* knockdown animals throughout the life-cycle. Whereas animals were severely uncoordinated (Fig 3.5a), we observed no major morphological disturbances in any of these tissues. Only a subtle difference in the placement of the ADE dopaminergic neuron cell bodies was detected. Measurement of the distance between the ADE neuronal cell bodies and the RME head neurons showed a statistically significant increase in this distance in *C56C10.11* knockdown animals (fig 3.5b-c). Dopamine is known to be important for normal movement in the worm, as application of Dopamine to worms causes a flaccid paralysis similar to that produced by treatment with the GABA agonist muscimol (Schafer and Kenyon, 1995; McDonald et al., 2006). While ADE neurons are important for movement, these studies do not resolve whether this slight difference in ADE placement is the root of the Unc phenotype associated with *C56C10.11* knockdown, nor whether it is symptomatic of some greater defect in the development of dopaminergic neurons or other tissue types. These are interesting questions for future studies.

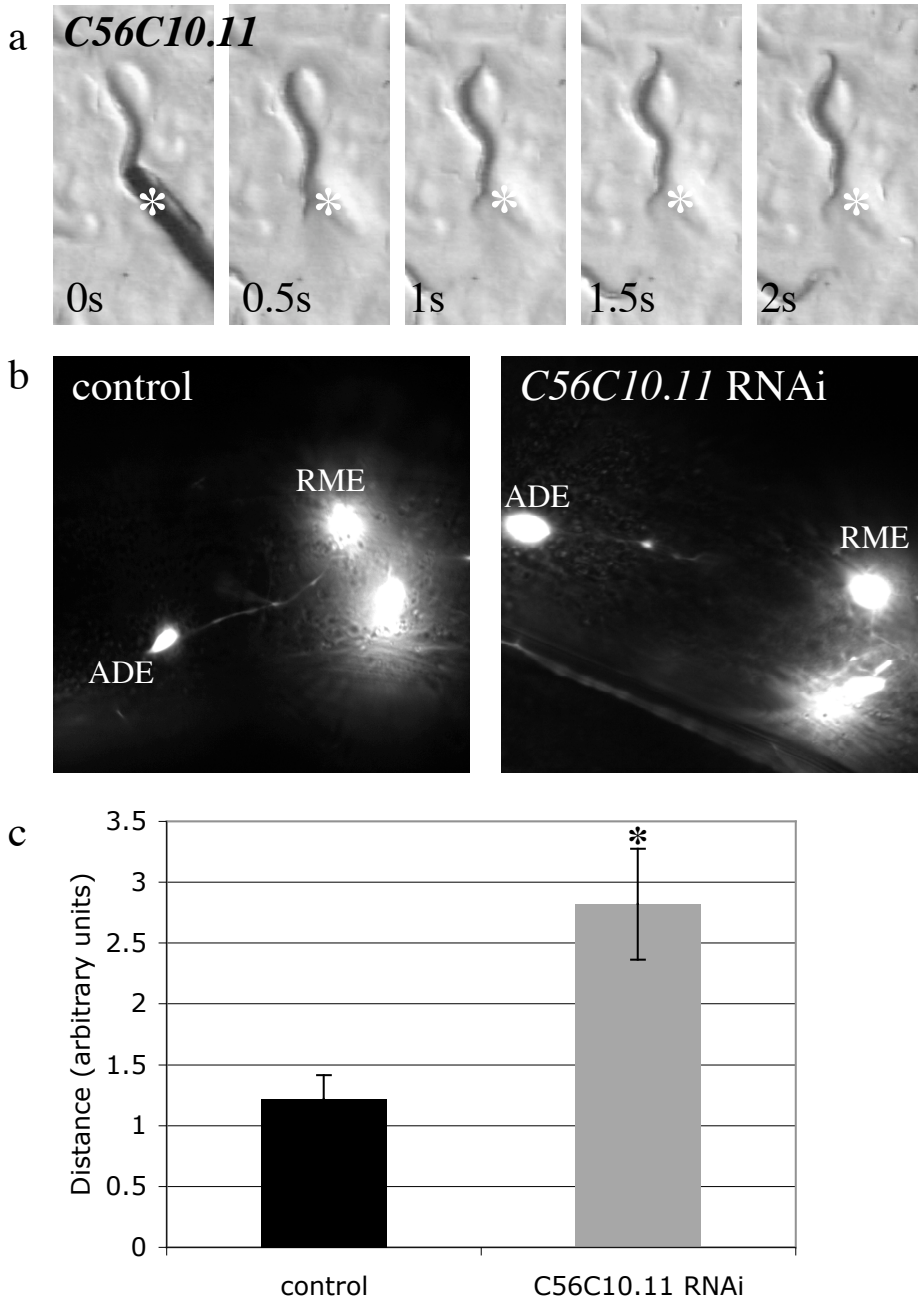


Figure 3.5 C56C10.11/DNAJ in movement and dopaminergic neuron fate. (a) RNAi knockdown of *C56C10.11* causes severe Unc phenotype. (b) Position of ADE dopaminergic neuron in reference to RME GABAergic neuron in control and *C56C10.11* knockdown animals. (c) Quantification of ADE to RME distance. Bars represent average +/- std dev, *P < 0.01, Student's T test, n=16.

R10E4.1/kelch-like protein

Knockdown of *R10E4.1* produced an Unc phenotype that was characterized as 'forward slow' in our screen. This phenotype was highly reproducible, appearing in three independent trials. RNAi of this gene also produced a statistically significant decrease in thrashing (fig 3.2b). This phenotype is presumably neuronal in nature, as it has not previously been identified by RNAi studies in the wildtype background. *R10E4.1* is predicted to encode a kelch-like protein, a member of a family of proteins defined by repeats of 'kelch' motifs, named for their founding member in drosphila. Expanded gultamine repeats in a non-coding RNA for a kelch-like gene, *klhl1,* are responsible for Spinocerebellar ataxia type 8 (Nemes et al., 2000). Mouse knockouts of the *klhl1* locus either in all tissues or specifically in Purkinje cells produces a nearly identical phenotype: progressive ataxia with Purkinje cell dysfunction *without* cell loss (Friocourt et al., 2006). These studies have shown that the non-coding *klhll1* RNA is important for normal PC function, leading to the hypothesis that CTG expansion in this RNA may cause decreases in KLHL1 expression, resulting in the disease state. RNAi knockdown of *R10E4.1* in *C. elegans* may act similarly to the *klhl1* antisense RNA in mammals, to produce GABA neuronal dysfunction. Studies of this phenomenon in the worm may shed light on the molecular function of both the KLHL protein and the non-coding RNA in humans.

R05D7.3/KIAA1319

R05D7.3 encodes a putative protein of unknown function, which is worth mentioning, as knockdown of this gene was the only one in the screen to produce an exclusively backward Unc phenotype (Fig 3.6a). Because the ablation of GABA neurons

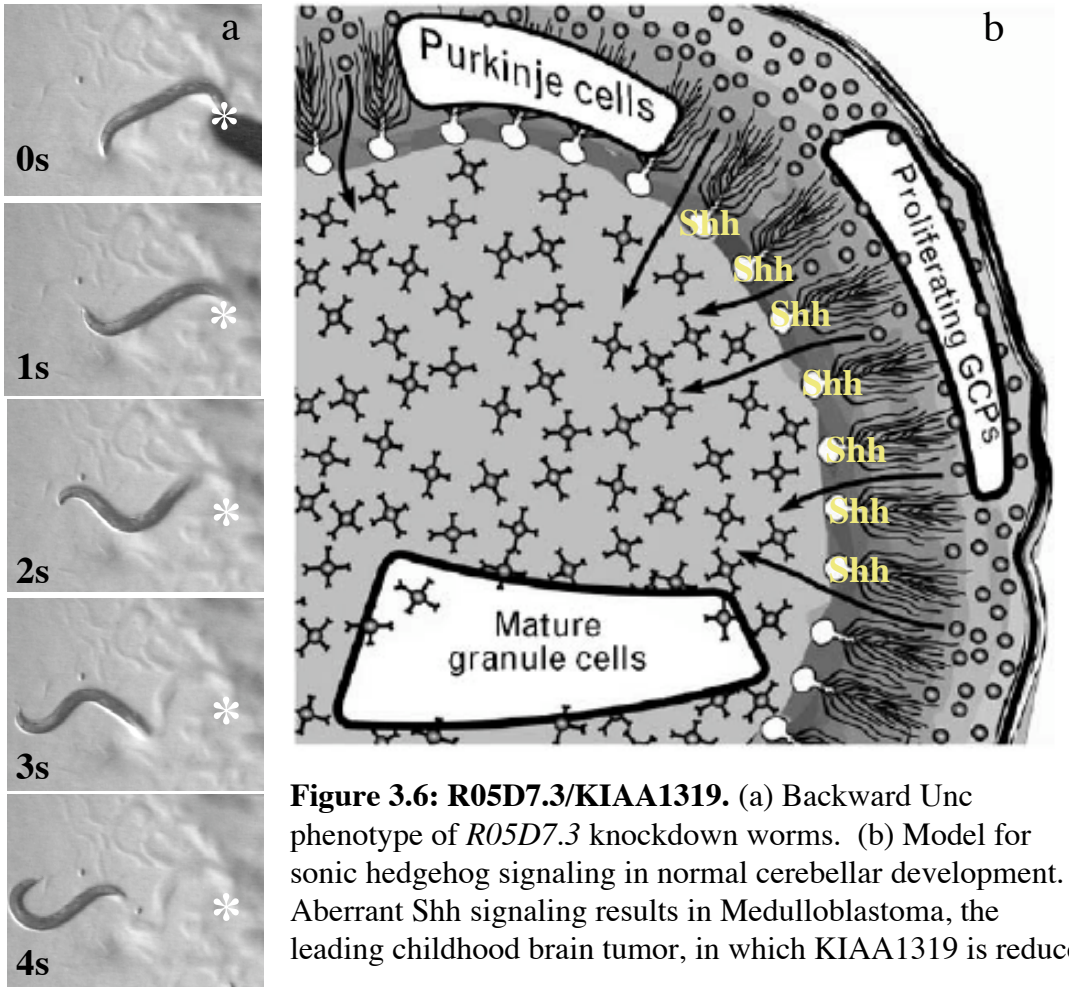


Figure 3.6: R05D7.3/KIAA1319. (a) Backward Unc phenotype of *R05D7.3* knockdown worms. (b) Model for sonic hedgehog signaling in normal cerebellar development. Aberrant Shh signaling results in Medulloblastoma, the leading childhood brain tumor, in which KIAA1319 is reduced

is associated with loss of backward, but not forward locomotion, the backward Unc phenotype potentially indicates a specific role for this gene in GABAergic function. *R05D7.3* is homologous with a gene known only as KIAA1913 in mammals. This gene is of interest, as its expression has been shown to be commonly down-regulated in primary medulloblastomas (Hui et al., 2005). Medulloblastomas are the most common childhood brain tumor, and arise in the cerebellum due to abnormal signaling between Purkinje cells and granular cells. Normally, Purkinje cells secrete sonic hedgehog (Shh), which signals to granular precursors in the external granular layer to proliferate and migrate to the internal germinal layer, where these precursors differentiate into terminal granular cells (Wallace, 1999; Wechsler-Reya and Scott, 1999; Lewis et al., 2004)(fig3.6b). Aberrant Shh signals lead to transformation of these granular cells, and results in tumorogenesis (Grimmer and Weiss, 2006). Whether the down-regulation of KIAA1913 is cause or result of tumor formation is unknown. It is also unknown whether the Shh signal that affects tumorigenesis also modulates KIAA1913 expression. *C. elegans* could be used to study the effects of mutants of conserved hedgehog pathway components on *R05D7.3*-mediated movement defects. Furthermore, because nothing is known of the function of KIAA1913, its worm ortholog may be useful for understanding its role in normal GABA neuron function. These studies could provide insight into the molecular role of KIAA1913 in cerebellar development and tumor formation.

Discussion

RNAi screens can complement bioinformatics in the generation of hypotheses from large datasets obtained by genomic approaches. This RNAi screen has provided

several avenues for hypothesis-driven research. However, there are potential caveats to pursuing any of these hits. For example, although sequencing confirmed the intended target of each RNAi construct, dsRNAs may have unintended effects on off-target genes. Therefore, careful follow-up to rule out off-target effects is necessary when studying gene function using RNAi. Such follow-up might include rescue by over-expressing the candidate gene, particularly an allele that is resistant to the RNAi construct. In addition, we can request that mutants be isolated by the *C. elegans* consortia to validate the RNAi results.

Several genes produced movement phenotypes when knocked down, and are interesting candidates for future research. Some genes, like *pgk-1* and *R05D7.3* are appealing because their putative orthologs have known roles in disease. The conservation of gene function will be a critical first step in choosing genes to examine further. If expression of proposed mammalian orthologs rescues the Unc phenotypes associated with RNAi, these candidates may be studied as models for human disease. Such studies might include genetic screens to discover additional pathway components. This type of study is readily performed in the worm, and candidates can then be tested in more complex organisms for relevance to the disease process. RNAi screening in cultured mammalian neurons has recently become feasible (Paradis et al., 2007). Knockdown of the mammalian orthologs of these hits in neurons may also be a useful strategy to study conservation of function.

As mentioned, few hits in the screen produced a purely backward Unc phenotype, indicating probable effects in other neurons or muscle. One drawback of this screen is that movement phenotypes found cannot be directly attributed to the gene's function

specifically in GABA neurons. Although roles in GABA neurons are hypothesized above, additional studies with each gene are needed to determine in which cells these genes actually function to affect movement. Such questions can be answered using neuron subtype-specific promoters to drive expression of rescue constructs.

CHAPTER IV

THE COENZYME Q SYNTHESIS GENE *COQ-1* PROTECTS *C. ELEGANS* GABA NEURONS FROM CALCIUM-DEPENDENT APOPTOSIS

Introduction

Coenzyme Q (CoQ) transfers electrons from complexes I and II to complex III in mitochondrial electron transport (Crane et al., 1957). Human CoQ deficiency is a rare genetic disease, caused by mutation in one of the CoQ synthetic enzymes (COQ1-10), and results in cerebellar ataxia with atrophy (Musumeci et al., 2001; Lamperti et al., 2003; Artuch et al., 2006) and/or myopathy (Ogasahara et al., 1989; Lalani et al., 2005; Horvath et al., 2006). Cerebellar ataxia can also arise from other genetic causes, including mutations in genes important for protein folding, calcium homeostasis, mitochondrial function and apoptosis (Duenas et al., 2006). Hallmarks of this disease include the age-dependent dysfunction and/or loss of the GABAergic cerebellar Purkinje cells (PCs), as well as degeneration in other brain regions and muscle. The occurrence of cerebellar ataxia and myopathy in CoQ deficiency provides evidence that cells with high metabolic needs, such as neurons and muscle, are especially sensitive to the reduction of CoQ. Furthermore, the symptoms associated with CoQ deficiency reveal that some brain regions and neuronal subtypes are selectively sensitive to metabolic compromise.

Because of its short life span, anatomic simplicity and genetic tractability, the nematode *C. elegans* is a useful model for studying phenomena associated with aging.

We used RNA interference (RNAi) to knock down the first gene in the CoQ synthesis pathway, *coq-1,* in order to mimic CoQ deficiency. We observed that reduction of *coq-1* levels in *C. elegans* results in an age-dependent loss of motor coordination that is correlated with progressive degeneration of GABA neurons. Exogenous CoQ_{10} rescues neurodegeneration. CoQ deficiency-associated GABA neuron death is executed through an apoptotic program, and depends on Ca^{2+} release from ER stores. This model of CoQ deficiency in nematodes may be useful for delineating the mechanism of GABA neuron degeneration in CoQ deficient humans and in related neurodegenerative diseases.

Materials and Methods

Strains and Maintenance. *C. elegans* strains were maintained at 20° C according to standard methods (Brenner, 1974). *glr-1(n2461), crt-1(bz30), eat-4(ky-5), coq-1(VC479), ced-3(n717), and ced-4(n1162)* strains were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis). All GFP-reporters were crossed into the RNAi-hypersensitive strain *eri-1(mg366);lin-15B(n744).* GFP reporter strains were *dat-1::GFP(pRN2003), tph-1::GFP(GR1366), eat-4::GFP(adIs1240), acr-2::GFP(CZ631), and myo-3::myo-3-GFP(stEx30), unc-25::GFP(juIs76).*

RNAi. RNAi assays were performed by feeding (Fire et al., 1998; Timmons and Fire, 1998), using clones from the Ahringer library (Fraser et al., 2000; Kamath et al., 2003). Briefly, 3mL LB/ampicillin (50μg/mL) was inoculated with 30μL overnight culture. Culture was grown in a 37°C shaker incubator to OD600 ~0.800, then diluted to 6mL

with LB/amp + IPTG (40mM final concentration) and incubated at 37°C for another 4 hours. Bacteria were pelleted, brought up in 250µl M9/IPTG, and spread onto NGM plates. L4 larvae were added to plates and incubated at 20°C for 5 days before scoring progeny. Cultures for Co-RNAi experiments (i.e. *coq-1* + *cep-1* and *coq-1* + *drp-1*) were grown separately, and then mixed just prior to plating.

Degeneration Assay. Animals were anesthetized in a drop of 0.1% tricaine/tetramisole on 2% agar pads (McCarter et al., 1997). The number of either axon intervals or commissures showing signs of degeneration was counted and divided by the total number of visible intervals or commissures (the number of visible processes varies slightly depending on the position of a given animal) to yield the percent degeneration. Scoring was done at 63X magnification. The experimenter was blinded to experimental versus control samples to avoid bias. Neighboring cholinergic neurons *acr-2::GFP;eri-1;lin-15B* animals were quantified in the same manner, and no significant degeneration was scored for these neurons (data not shown). Additional neuronal classes were studied, but no differences between control and knockdown animals were observed in these neurons.

Microscopy. Animals were visualized by Differential Interference Contrast (DIC) and epifluorescence microscopy using a Zeiss Axioplan compound microscope or a Zeiss LSM 510 confocal microscope. Images in the Zeiss Axioplan were recorded with CCD cameras (ORCA I, ORCA ER, Hamamatsu Corporation, Bridgewater, NJ). Confocal optical slices (40X) were 1µm. For GFP knockdown in reporter strains, quantification of

fluorescence in Z series of confocal optically sectioned images was done using histogram analysis in ImageJ (Rasband, 1997-2007).

Plasmid Construction. pC04G2.1::EGL-36 was made by PCR of the C04G2.1 promoter, using the primers pC04G21_3-5'atgatttttgttttaac and pC04G21_5-5'attattatttctatcggct. PCR product was ligated backward into TOPO® TA pcr2.1 vector (Invitrogen). *pmyo-3::egl-36(gf)* and *(lf)* plasmids were a kind gift from Michael Nonet. *egl-36* was cut from these plasmids using BamHI/ApaI sites, and ligated into pSL1180 with the *C04G2.1* promoter (cut w/NotI/XbaI).

Transgenic Strains. Transgenic animals were obtained by injecting 25-75ng/µl of plasmid and [dpy-20(pMH86)] into *unc-25::GFP;eri-1;dpy-20;lin-15B* animals using standard techniques (Mello and Fire, 1995).

Results

Knockdown of *coq-1* results in age-dependent loss of coordinated movement

COQ-1 catalyzes the first step in CoQ synthesis, the assembly of the lipophillic polyisoprenoid tail (Tran and Clarke, 2007). RNAi knockdown of *coq-1* is reported to induce uncoordinated (Unc) and Egg-laying defective (Egl) phenotypes, but the mechanism of these effects has not been studied (Simmer et al., 2003). We replicated this experiment, using the RNAi "feeding" method to expose an RNAi hyper-sensitive strain (Wang et al., 2005) to bacteria expressing *coq-1* double-stranded RNA (dsRNA). When treated with RNAi to *coq-1*, animals in the first three larval stages showed no

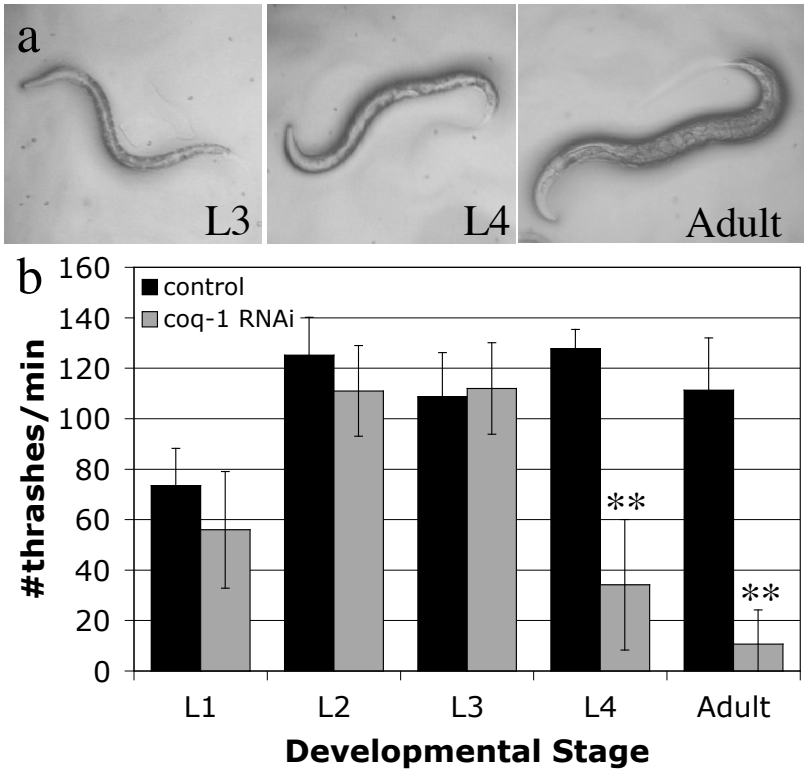


Figure 4.1 RNAi knockdown of *C. elegans coq-1* results in progressive loss of motor coordination. **(a)** Abnormalities in normal sinusoidal movement in L4 larvae and adults treated with RNAi to *coq-1*. **(b)** thrashing assay quantifying movement defects (Avg +/-s.d., **P<0.005, n=10)

apparent defects. A loss of motor coordination first appeared at the L4 larval stage as a kink in the normal sinusoidal wave that drives locomotion. Movement then gradually declined in adults, often culminating in paralysis (Fig 4.1a). Quantification of movement loss with a thrashing assay (Miller et al., 1996) verified the developmental progression of the Unc phenotype (Fig 4.1b).

RNAi or genetic depletion of CoQ induces age-dependent degeneration of GABA neurons

Observation of GABA motor neurons in the ventral nerve cord, labeled with the *unc-25::GFP* reporter, suggested a possible explanation for the loss in motor coordination. These neurons degenerate in *coq-1* knockdown animals, and cell bodies, viewed under DIC, demonstrated the raised "button-like" morphology characteristic of apoptotic cell death (Fig 4.2d). GABAergic axons in the ventral nerve cord, as well as circumferential commissures and dorsal cord processes appear discontinuous with apparent breaks (Fig 4.2b-c). These morphological defects first appeared in late larval development and progressed as animals aged (fig 4.2e-f) thereby mirroring the age-dependent pattern of the Unc phenotype.

We also tested RNAi clones for *coq-3, 4, 5, 7*, and *8* to determine whether knockdown of other CoQ synthetic pathway genes phenocopies *coq-1*. Animals treated with dsRNA specific to *coq-8* develop both the Unc and degenerative defects. Although penetrance is low (~10%), this phenotype is indistinguishable from that *coq-1* RNAi-treated animals. RNAi of other CoQ pathway genes produced no visible effects. This finding may be explained by earlier results showing that RNAi of *coq-1* reduces CoQ

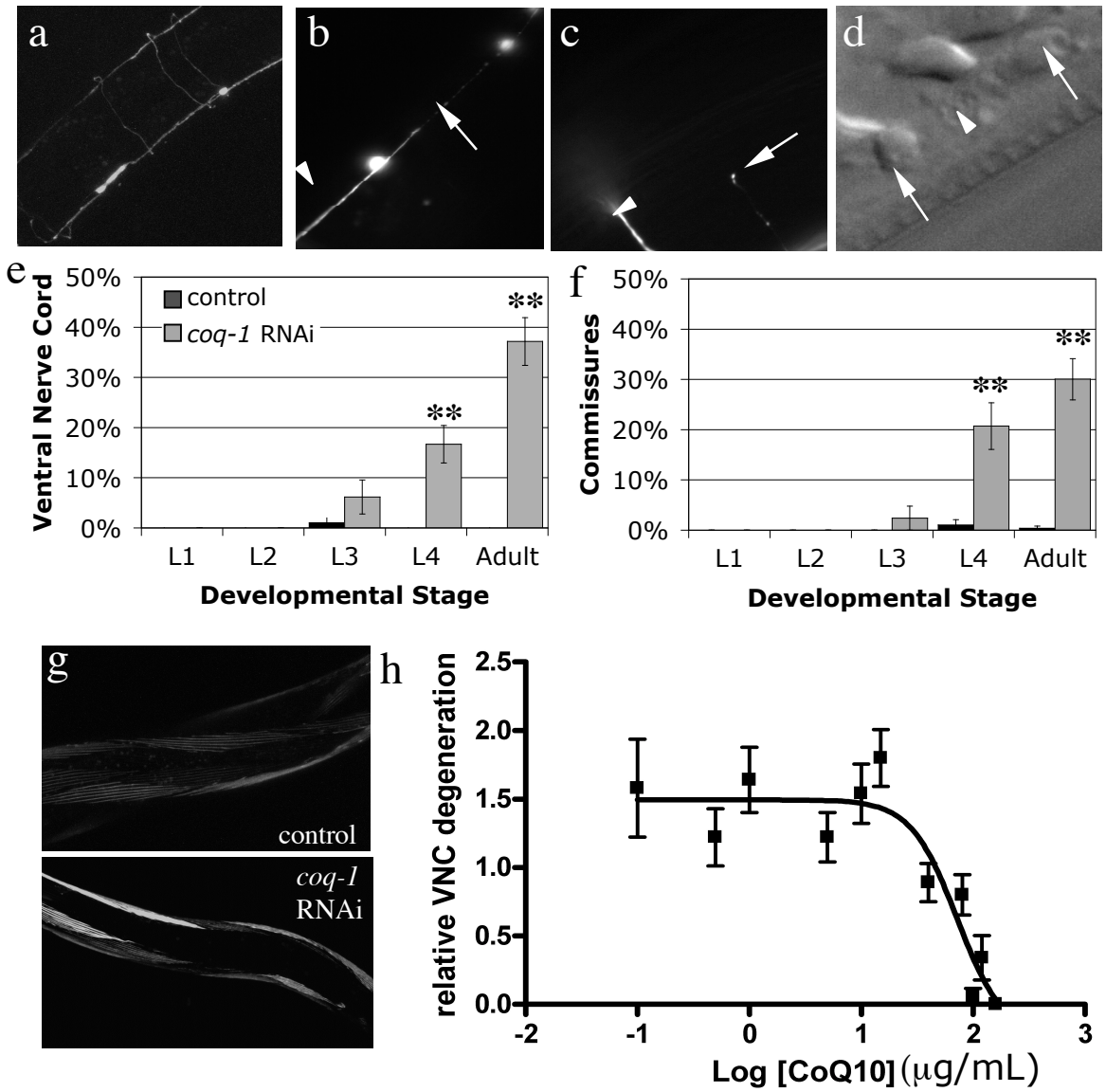


Figure 4.2 *coq-1* knockdown causes age-dependent, tissue-specific degeneration, which is rescued by exogenous CoQ_{10}. **(a)** Wildtype GABA neuron cell bodies and axons in ventral nerve cord, and dorsally projected commisures labeled with *unc-25*::GFP. **(b-d)** degeneration of GABAergic axon intervals in ventral nerve cord (b) and dorsally-projected commissures (c), and DIC images of swelling cell bodies (d) in coq-1 knockdown animals. Arrows indicate dying cells/processes, while arrowheads indicate healthy neurons. **(e-f)** Quantification of degeneration throughout development in ventral nerve cord (VNC) axons (e) and commissural axons (f) (Data shown are a composite of 3 or more experiments with error bars representing SEM. *$P < 0.01$, **$P < 0.001$). **(g)** myo-3::myo-3::GFP-labeled muscle structure in control and knockdown animals. **(h)** Dose-response curve showing rescue of neurodegeneration with exogenous CoQ_{10}

levels to a greater extent than does RNAi knockdown of other CoQ pathway genes (Asencio et al., 2003). The similar phenotypes displayed by RNAi of *coq-8* and *coq-1* substantiate the idea that the movement and degenerative defects result from loss of CoQ synthesis, rather than off-target effects or additional roles of these *coq-1* pathway enzymes.

We also validated our RNAi results by examining *coq-1(ok749)* mutant animals. The *ok749* allele is a deletion that removes the C-terminal region comprising approximately 70% of the coding sequence. Homozygous *coq-1(ok749)* progeny of heterozygous adults are viable but produce dead embryos. This finding indicates that the *coq-1(ok749)* mutation results in a maternal effect lethal phenotype in which the first generation of viable offspring progress through larval development with maternally provided CoQ. These homozygous *coq-1* mutants appear Unc at the L3 larval stage. Degeneration of GABA neurons is not seen until the adult stage (Fig 4.3a-c) and appears to affect GABA neurons preferentially, although neighboring cholinergic neurons were also missing on rare occasions. The similar degenerative pattern and age dependence shown by the *coq-1* knockout animal validated the specificity of the *coq-1* RNAi treatment. Prior studies of the *coq-1(ok749)* mutant reported paralysis and early larval lethality associated with destruction of vital tissues, such as pharynx and intestine (Gavilan et al., 2005). Although *coq-1(ok749)* animals were slow-growing, we did not observe early larval arrest and vital tissues were intact (Fig 4.3d)

Treatment with exogenous CoQ_{10} has successfully slowed disease progression of some cases of human CoQ deficiency (Salviati et al., 2005; Quinzii et al., 2006). To verify that CoQ deficiency was the cause of the uncoordinated phenotype in *C. elegans*,

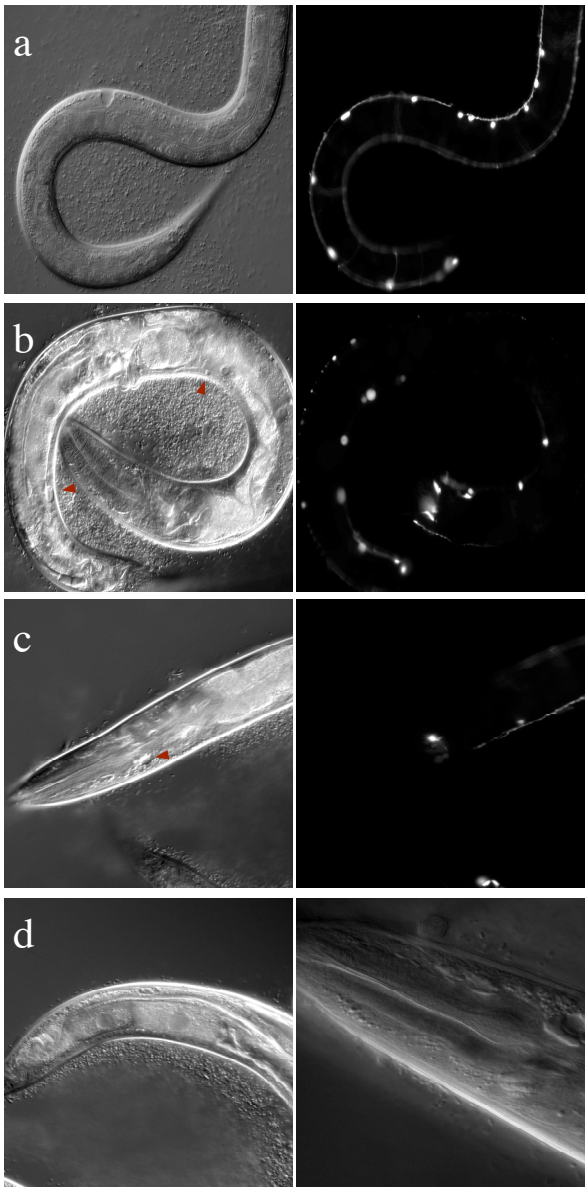


Figure 4.3 Analysis of *coq-1* knockout animals. **(a-c)** DIC and *unc-25::*GFP labeled GABA neurons in homozygous *coq-1* mutant at the L3 **(a)** and adult **(b-c)** stages, showing degeneration of VNC GABA neurons **(b)** and RME GABA neuron **(c)** in adult animals **(d)** DIC images of gut and pharynx of *coq-1* adult mutant verifying general health of animal.

we incubated coq-1 RNAi treated animals with different concentrations of CoQ_{10}. We found that supplemental Coenzyme Q_{10} rescued degeneration in a dose-dependent manner, with an EC50 of 72 μg/mL (Fig 4.2h). A requirement for high CoQ doses for efficacy has also been observed in human patients, and may reflect poor uptake of the drug by neurons (Ibrahim et al., 2000).

coq-1-dependent degeneration is specific to GABA neurons

Having observed that CoQ deficiency results in GABA neuron degeneration in *C. elegans*, we next utilized GFP reporters for other neuronal subtypes to evaluate their sensitivity to *coq-1* knockdown. Neuronal populations tested were, cholinergic (*acr-2::GFP*), serotonergic (*tph-1::GFP*), glutamatergic (*eat-4::GFP*), and dopaminergic (*dat-1::GFP*) neurons. Although all animals showed the age-dependent Unc phenotype, none of these neuron classes showed signs of degeneration comparable to that observed for GABA neurons (Fig 4.4). We considered the possibility that this differential effect could be due to the relative insensitivity of these neuron classes to RNAi. This does not appear to be the case, however, as all neuronal types were equally vulnerable to RNAi to GFP (Fig 4.4, graph).

Like neurons, muscle is also a highly metabolic tissue, and muscle degeneration is seen in some cases of CoQ deficiency (Lalani et al., 2005). Therefore, we used a GFP-labeled myosin heavy chain protein (MYO-3::GFP) to examine muscle structure in the *coq-1* knockdown animals. As seen in figure 4.2g, we detected no morphological abnormalities in body wall muscle, verifying that the Unc phenotype associated with this

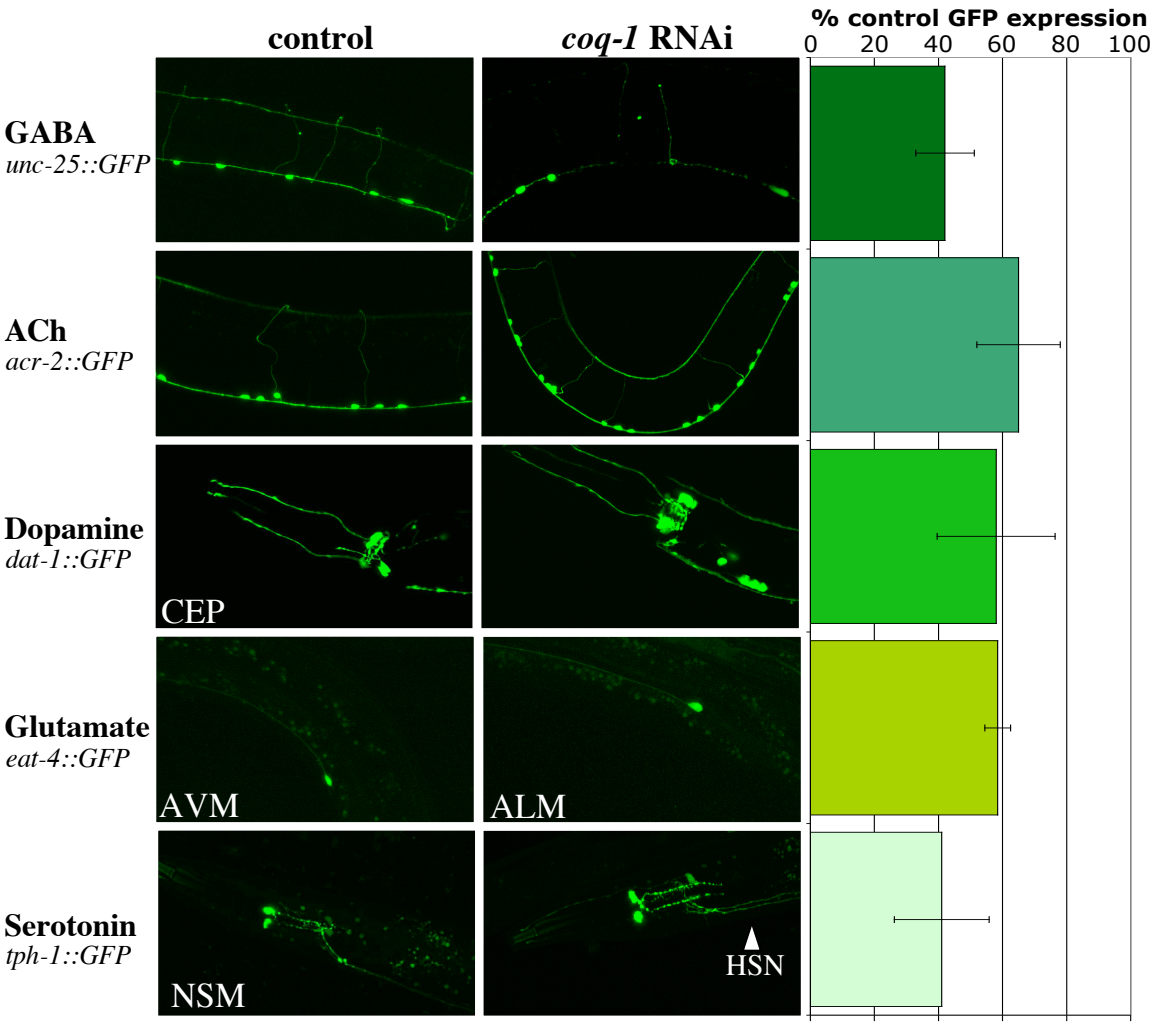


Figure 4.4 Neurodegeneration seen with *coq-1* knockdown is limited to GABA neurons. Confocal images of empty vector control-treated or *coq-1* RNAi-treated animals expressing GFP reporters specific for different classes of neurons are shown. The graph shows the relative sensitivity of each strain to RNAi, as determined by RNAi knockdown of the GFP transgene (n=6, avg +/- s. d.).

RNAi construct is not the result of muscle degeneration. We also examined vulval muscle, reasoning that defects in these muscles might explain the Egl phenotype. However, we observed no structural differences between vulval muscles in control versus RNAi-treated animals (data not shown).

While these results do not preclude the possibility that these neuronal types or muscle are functionally affected by *coq-1* knockdown, the degenerative phenotype was exclusively observed in GABA neurons. In fact, functional deficits in other neurons or muscle are likely as the movement defects of mutants in which GABA neurons are selectively disabled are less severe than the paralyzed adult phenotype that results from RNAi ablation of *coq-1* (McIntire et al., 1993b).

coq-1-mediated degeneration is Ca^{2+} dependent

Calcium is a key effector of neurodegenerative diseases involving mitochondrial dysfunction (Mattson, 2007). Calreticulin (*crt-1*) is a chaperone protein localized to the ER lumen where it maintains Ca^{2+} levels for ready release upon appropriate stimuli (Ostwald and MacLennan, 1974; Bastianutto et al., 1995; Mery et al., 1996; Corbett et al., 1999; Mesaeli et al., 1999; Michalak et al., 1999). Reducing ER [Ca^{2+}] by calreticulin knock-out is anti-apoptotic (Nakamura et al., 2000), whereas calreticulin over-expression promotes apoptotic cell death in mammalian systems (Arnaudeau et al., 2002). In *C. elegans*, mutants of *crt-1* block necrotic degeneration of motor neurons (Xu et al., 2001). We tested a *crt-1* null mutant (*bz30*) in our paradigm, and found that it prevented the progressive degeneration of GABA neurons in *coq-1* RNAi-treated animals (fig 4.5a). The calcium chelating agent, EGTA (0.5mM), was similarly protective (fig 4.5a). Taken

together, these results demonstrate that Ca^{2+} release from the ER is important for *coq-1* RNAi-mediated degeneration of GABA neurons.

Mutants of the apoptosis pathway suppress cell death in *coq-1* knockdown animals

Ca^{2+} release from the endoplasmic reticulum can result in necrotic (Driscoll and Gerstbrein, 2003) or apoptotic (Demaurex and Distelhorst, 2003) cell death. To distinguish between these possibilities, we tested mutants that block the *C. elegans* programmed cell death pathway for effects on GABA neuron degeneration. Mutants of *ced-4*, the worm ortholog of the caspase activator Apaf-1, and of the caspase gene, *ced-3,* blocked *coq-1*-dependent degeneration (fig 4.5a). These results indicate that loss of *coq-1* activity triggers an apoptotic pathway in GABA neurons.

Whereas mutants that disable the apoptotic machinery blocked degeneration, mutants of genes important for the necrotic glutamate excitotoxicity pathway (Driscoll and Gerstbrein, 2003) did not. Mutants of the *glr-1* glutamate receptor and the glutamate vesicular uptake transporter *eat-4* did not protect neurons from *coq-1* RNAi-induced death (fig 4.5a). These results are consistent with the idea that CoQ-deficient GABA neurons die through apoptosis rather than necrosis.

CoQ-dependent apoptosis depends on the mitochondrial fission gene *drp-1*

We found the calcium dependence of apoptosis in this model intriguing, as calcium is not known to play a role in developmental apoptosis in *C. elegans* (see discussion). Ca^{2+} has been shown, however, to stimulate mitochondrial fission (Breckenridge et al., 2003), which, in turn, has been linked to apoptosis in mammals

(Cipolat et al., 2006; Frezza et al., 2006) and in *C. elegans* (Jagasia et al., 2005). We reasoned that CoQ deficiency might sensitize mitochondria to Ca^{2+}-dependent fission related apoptosis. To test this idea, we performed co-RNAi knockdown of *coq-1* with *drp-1*, a gene that is necessary for fission-related apoptosis (Jagasia et al., 2005). RNAi of *drp-1* blocked degeneration of GABA neurons (fig 4.5b), thereby implicating the fission machinery in the pathology associated with *coq-1* knockdown in *C. elegans*.

Ca^{2+} uptake by mitochondria can also lead to apoptosis through oxidative damage signaling. This pathway involves the generation of reactive oxygen species (ROS), which normally occurs at a low level as a by-product of mitochondrial electron transport (Adam-Vizi and Chinopoulos, 2006). Loss of CoQ, a vital electron carrier, would be expected to reduce electron transport, which likely results in an increase in the alternative passage of electrons to molecular oxygen to create ROS. Transcriptional regulation by p53 plays a central role in oxidative stress-induced apoptosis in mammals (Culmsee and Mattson, 2005) and CEP-1, a primordial p53-like protein in *C. elegans* is required for DNA damage induced apoptosis in germ line cells (Derry et al., 2001; Schumacher et al., 2001). As shown in figure 4.5b, co-RNAi of *coq-1* with *cep-1*, inhibits GABA neuron degeneration. This result indicates that CEP-1/p53 functions in the soma as well as in the germ line to protect *C. elegans* cells from stress-induced apoptosis. The incomplete penetrance of *cep-1* knockdown on *coq-1* RNAi induced GABA neuron degeneration, however, indicates that additional pathways may function in parallel to CEP-1 to trigger the cell death cascade.

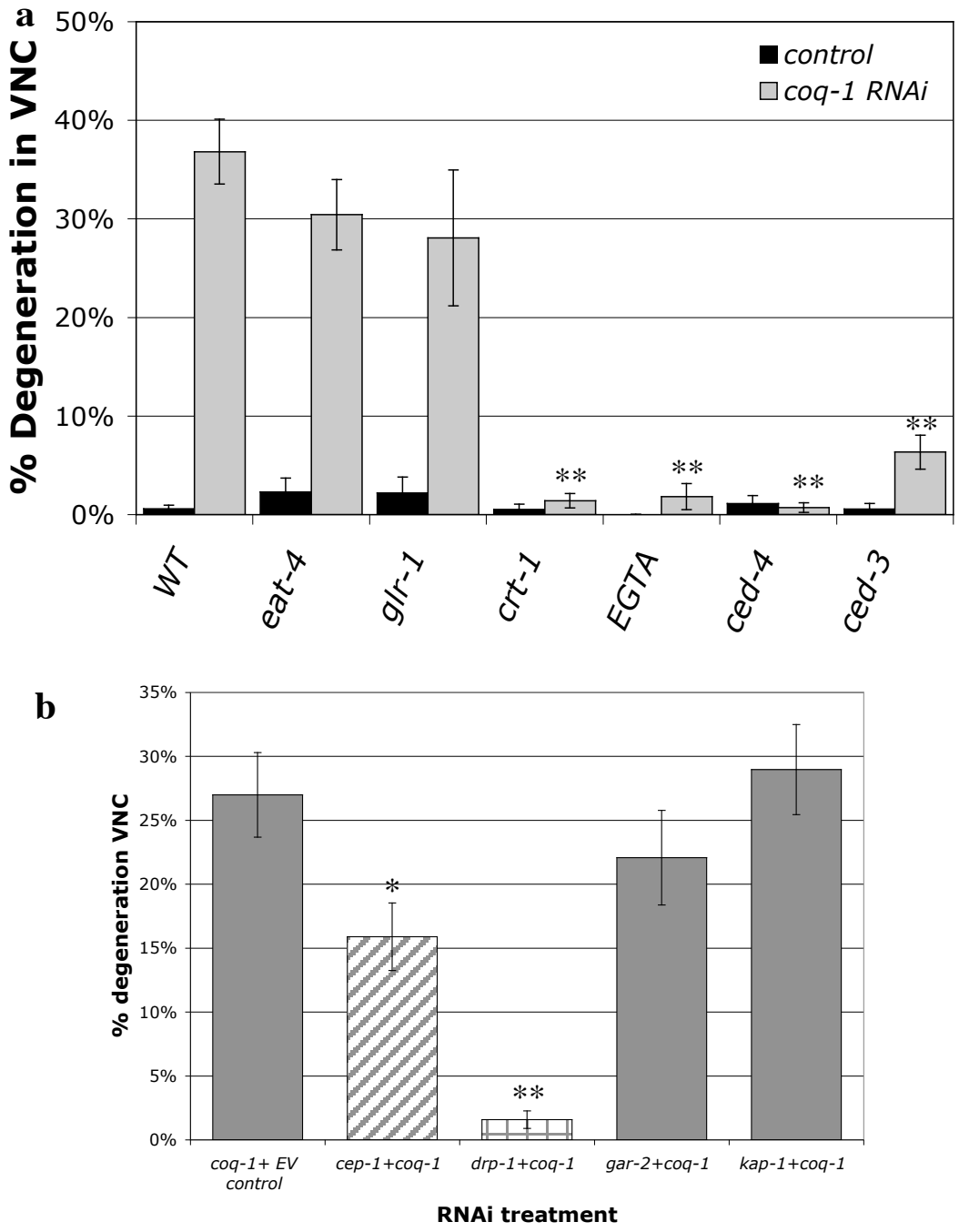


Figure 4.5 Mechanism of degeneration (a) Indicated mutants or EGTA were tested for their ability to block *coq-1*-knockdown-induced neurodegeneration. GABA neurons were scored in adults as in Fig1. **(b)** Co-RNAi of *coq-1* with *cep-1, drp-1* or controls (gray bars). Results are composites of 3 or more experiments, error bars representing SEM. *P<0.01,**P < 0.0001.

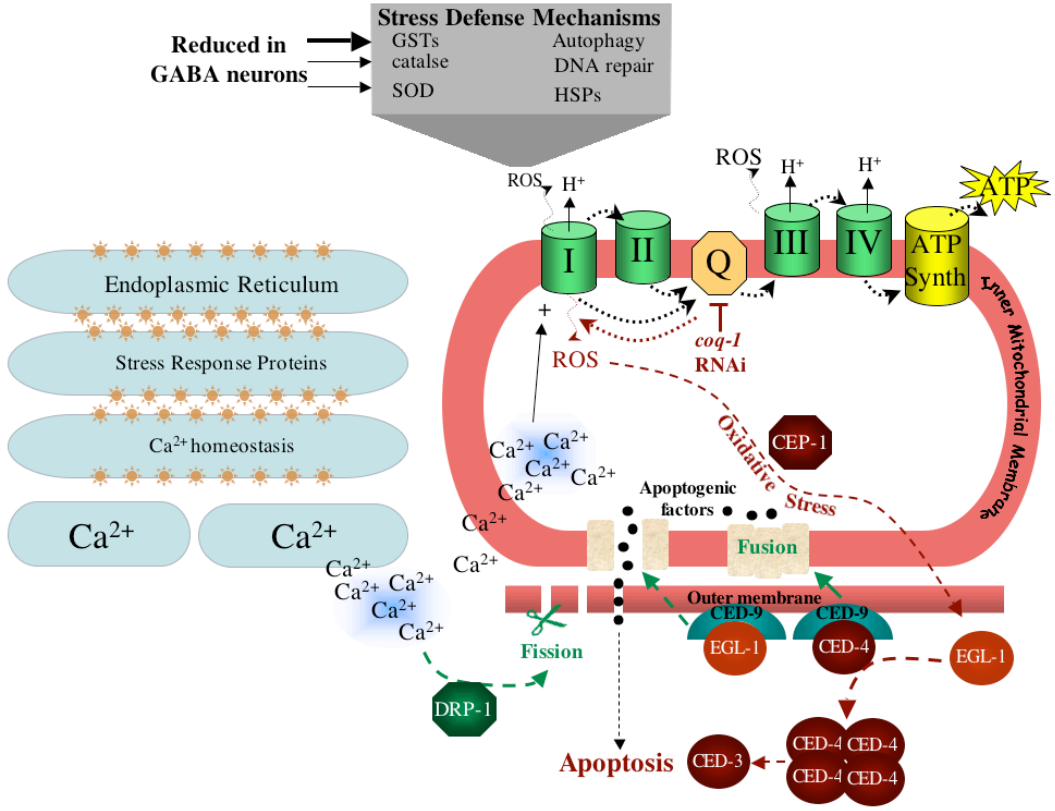


Figure 4.6 Model of COQ-1 involvement in degeneration of GABA neurons. *coq-1* knockdown reduces levels of CoQ, which backs up mitochondrial electron transport. This produces a higher sensitivity of the mitochondrion to Ca^{2+} released from the ER, and higher ROS release. Ca^{2+} may act through activation of *drp-1*-mediated mitochondrial fission, contributing to the permeability transition, and release of apoptogenic factors. ROS can also activate *cep-1*, which can transcriptionally activate *egl-1*, contributing to apoptosis. Factors such as high membrane activity or low defense mechanisms (chapter II) may contribute to the selective demise of GABA neurons.

Degeneration is independent of GABA neuron activity.

Since all cells would be affected by *coq-1* knockdown, the question of why GABA neurons selectively die remains. Some additional insult(s) must exist which makes these neurons more vulnerable to changes in CoQ levels. Although nothing is known about the elecrophysiological properties of GABA neurons in *C. elegans*, studies have shown that some GABA neurons in mammals have extremely high intrinsic firing rates. Cerebellar purkinje cells, for example, can discharge at rates of up to 125 Hz in the conscious monkey (Thach, 1968; Thach, 1970b; Thach, 1970a). Since membrane activity results in Ca^{2+} release from intracellular stores, firing of the cell could heighten the Ca^{2+} signaling which drives these neurons to perish. In order to test whether this is the case, we utilized a neuron-silencing mutant of the potassium channel *egl-36* (E142K).

EGL-36 is a Shaw-type voltage-gated K^+ channel, which is important for egg-laying behavior in *C. elegans (Johnstone et al., 1997)*. A gain of function mutation of glutamate 142 to lysine in this gene shifts the voltage dependence of activation, so that the channel activates at hyperpolarized membrane potentials. Premature activation leads to an influx of K+ ions, thwarting any would-be action potential, effectively silencing the neuron (Johnstone et al., 1997; Zhao and Nonet, 2000). We created a transgenic animal that expresses this gain-of-function channel under the control of a GABA motoneuron-specific promoter, *pC04G2.1*. These animals displayed a severe backward Unc phenotype (Figure 4.7a) that is reminiscent of the shrinker phenotype which occurs when GABA neurons are laser ablated (McIntire et al., 1993a). This phenotype is evidence that *pC04G2.1::egl-36*(E142K) is greatly reducing, if not silencing, the firing of GABA neurons. Over-expression of a loss-of-function channel, *egl-36*(E142K, G408E), a

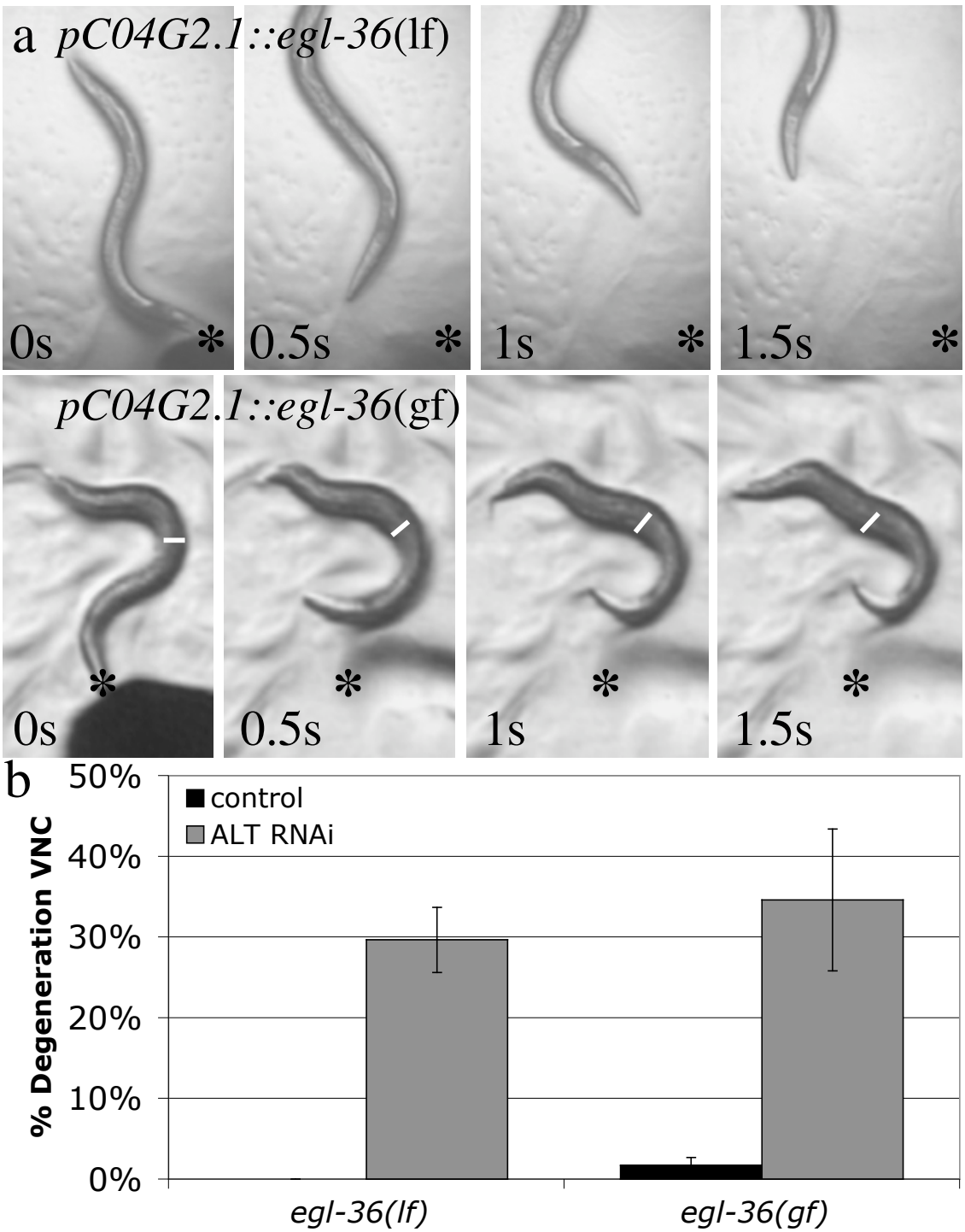


Figure 4.7 CoQ degeneration is independent of GABA neuron activity. (A) Phenotype of worms expressing gain of function or loss of function EGL-36 in GABA neurons. *lf* animals are wildtype, while *gf* animals are backward Unc and partially shrinker. White bar indicates thickness of midbody prior to tapping the worm on the nose. *Position of nose prior to tap (B) Quantification of degeneration in *pC04G2.1::egl-36* transgenic animals shows no effect of neuronal activity on degeneration. n=45.

mutant in which ion conductance is lost, resulted in wildtype movement. This result indicates that the blockage of GABA neuron activity by EGL-36(*gf*) is due to its K^+ conductance, rather than an artifact of expression of the channel itself.

We tested the EGL-36 channel-expressing transgenics for GABA neurodegeneration upon *coq-1* knockdown. As shown in figure 4.7b, reducing GABA neuron activity had no effect on degeneration. This suggests that normal excitation of ventral cord GABA neurons in *C. elegans* does not predispose them to degeneration, as has been proposed in some human degenerative diseases. However, these experiments must be repeated in an integrated line, before we make a final conclusion. It is possible that mosaicism in the strain resulted in a false negative result.

Discussion

Model for CoQ deficiency-related neurodegeneration in *C. elegans*

The model in figure 4.6 summarizes our results. We have shown that genetically induced reduction of Coenzyme Q leads to progressive loss of motor coordination and preferential degeneration of GABA neurons. The mechanism of cell death depends on the apoptotic genes *ced-4* (Apaf-1) and *ced-3* (caspase). The GABA neuron pathology that accompanies *coq-1* knockdown also relies on Ca^{2+} release from the ER and the mitochondrial fission protein DRP-1. These results emphasize an important role for CoQ in neuron survival and demonstrate a mechanism for pathological apoptosis in *C. elegans* which depends on ER-to-mitochondrial Ca^{2+} signaling.

ER-to-mitochondrial Ca^{2+} signaling has been the focus of apoptosis research in mammalian systems (Szalai et al., 1999; Pacher and Hajnoczky, 2001; Hajnoczky et al., 2002; Rapizzi et al., 2002). Dysregulation of this pathway is linked to neurodegenerative disease. For example, Ca^{2+} homeostasis genes are down-regulated in GABAergic cerebellar Purkinje cells in both a mouse model of Spinocerebellar ataxia type1 and in human patients (Lin et al., 2000). Furthermore, mitochondria isolated from lymphoblasts of Huntington's disease (HD) patients and and from brains of a mouse model of HD show heightened sensitivity to Ca^{2+} (Panov et al., 2002). Although mitochondria are compromised in all cells in these diseases, only selected neuronal populations, especially GABAergic neurons, respond by activating death pathways. We observe similar selectivity in the *C. elegans* model of CoQ deficiency described in this work.

The calcium-apoptosis connection in *C. elegans*

The Ca^{2+}-dependence of apoptosis with CoQ deficiency in *C. elegans* brings to light a long-standing difference between apoptotic models in mammals versus nematodes. Whereas the role for Ca^{2+} in apoptosis is well-established in mammals, an apoptotic function for this key molecule in *C. elegans* has not been previously described. In mammals, ER Ca^{2+} levels are kept in check by the ER-localized pool of the anti-apoptotic proteins Bcl-2 (Foyouzi-Youssefi et al., 2000; Pinton et al., 2000; Palmer et al., 2004) or BAP-31 (Breckenridge et al., 2003) In certain pathological situations, excessive cytoplasmic Ca^{2+} from the ER triggers mitochondrial fragmentation and consequent release of cytochrome c from the mitochondrial inner membrane. Liberated cytochrome c interacts with Apaf-1, which in turn activates caspases, thereby triggering apoptosis.

A cytochrome c–independent pathway is hypothesized to drive apopotosis in nematode cells (Rolland and Conradt, 2006). In this case, CED-4/Apaf-1 is sequestered at the mitochondrial outer membrane by the Bcl-2 ortholog, CED-9. In cells fated to die, EGL-1, a BH3-only Bcl-family member, is up-regulated and binds to CED-9/Bcl-2, thereby freeing CED-4/Apaf-1 to activate the caspase CED-3. Our results indicate a requirement not only for CED-3 and CED-4, but also for Ca^{2+} in CoQ-dependent apoptosis.

Although cytochrome c may not activate CED-3/caspase function, recent work has confirmed that mitochondrial fragmentation can trigger apoptosis in *C. elegans* (Jagasia et al., 2005). These results suggest that mitochondrial fragmentation in the nematode must release other apoptotic components that activate the cell death pathway. Because Ca^{2+} is known to trigger mitochondrial fragmentation in mammals, we asked if the Ca^{2+}-dependent killing of GABA neurons that we observe in *coq-1* RNAi treated animals also involves mitochondrial fragmentation. The dynamin-related protein, Drp1, mediates normal mitochondrial fission and is also necessary for induced mitochondrial fragmentation in mammals (Breckenridge et al., 2003). Recent studies have confirmed that the *C. elegans* homolog, DRP-1, is both necessary and sufficient for mitochondrial fragmentation but a role for Ca^{2+} in this mechanism was not explored. Here we have shown that both ER Ca^{2+} release and DRP-1 are required for the death of GABA neurons in CoQ deficient animals. We therefore suggest that the mechanism of cell killing induced by CoQ deficiency in *C. elegans* is likely to employ an evolutionarily conserved pathway in which Ca^{2+} release from the ER activates mitochrondrial fragmentation. This finding adds to a growing number of examples of degenerative conditions, in which the

mitochondrial fission/fusion machinery has been implicated (Alexander et al., 2000; Delettre et al., 2000; Cipolat et al., 2006).

Because of the common association between mitochondrial dysfunction and oxidative stress, we also tested the p53 ortholog, CEP-1, for a role in degeneration. RNAi mediated knockdown of *cep-1*/p53 partially rescued the *coq-1* RNAi degenerative phenotype (fig 4.5b). This result could be indicative of a parallel pathway to *cep-1*/*p53* that is necessary for complete activation of apoptosis. Alternatively, cep-1/p53 could mediate some of the degenerative events, but is not involved in the final apoptotic decision. A similar role for p53 in GABA neuron degeneration has been reported in a mouse model of Spinocerebellar ataxia type 1 in which p53 mediates morphological changes associated with degeneration of Purkinje cells, such as dendritic arbor loss, without causing apoptosis (Shahbazian et al., 2001).

CoQ deficiency in *C. elegans* as a model for human disease

The selective and age-dependent death of GABA neurons and loss of coordinated movement seen in *coq-1* knockdown worms are shared features of CoQ deficiency in humans. The most common outcome of this deficiency is cerebellar ataxia (Quinzii et al., 2007). Thus far, only mutations in the COQ-2 gene have been positively identified as causes of CoQ deficiency, although COQ-1 dysfunction is suspected to underlie some cases (Rotig et al., 2000). CoQ deficiency can also participate in the pathology of diseases, in which it is not the primary feature. For example, patients carrying mutations of the apraxin gene (APTX) display secondary CoQ_{10} deficiency and cerebellar ataxia, which improves with CoQ_{10} supplementation (Quinzii et al., 2005).

Although CoQ deficiency is rare in humans, it shares important pathologies with prevalent glutamine repeat diseases. These include the autosomal dominant Spinocerebellar ataxias (SCAs) and Huntington's disease (HD). The age-related death of medium spiny GABAergic efferents of the striatum occurs in HD (Martin and Gusella, 1986) and GABAergic Purkinje cells are significant targets for degeneration in the cerebellar ataxias (Zoghbi and Orr, 1995). GABA neuron sensitivity, mitochondrial dysfunction, altered Ca^{2+} homeostasis, and apoptosis are all shared features of these and other degenerative diseases (Duenas et al., 2006; Kwong et al., 2006).

The *coq-1* knockdown model of neurodegeneration in *C. elegans* can be used to study the genetic and environmental influences which sensitize GABA neurons to disease. Genetic screening for enhancers or suppressors of this RNAi phenotype (or of a hypomorphic allele of *coq-1*) could be used to determine additional genes in the pathway. Activity of these candidate genes could then be studied in mouse models and humans with CoQ deficiency.

Additionally, supplementation with exogenous CoQ has been shown to slow the progression of degeneration in only a subset of CoQ deficiency cases in humans. Therefore, additional therapies which increase the efficacy of CoQ supplements, or which target alternative components of the degenerative pathway, are needed. To this end, this *C. elegans* model could be employed for pharmacologic screening for small molecules that inhibit degenerative pathology. Such agents may then be developed as therapeutics for CoQ deficiency, as well as for related neurodegenerative conditions.

CHAPTER V

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Chapter IV described a model of *coq-1* deficient GABA neuron degeneration, and preliminary characterization of the molecular pathways involved. However, many additional experiments could be done to further characterize the pathology involved in *coq-1* mediated degenerating GABA neurons. In addition, this model could be used to discover novel genes involved in degenerative pathology and neuroprotective therapies. Further descriptive studies and potential applications of the *coq-1* model are discussed below.

Characterization of mitochondria in *coq-1* knockdown animals

Because of the apoptotic nature of degeneration and the involvement of *drp-1*, we can predict that mitochondrial structure changes and permeability transition are part of the degenerative process. However, we have not yet directly studied these events. Several dyes, derived from rhodamine 123, are taken up into mitochondria, and fluoresce in a way that is dependent on mitochondrial potential, $\Delta\Psi m$. Since $\Delta\Psi m$ is directly related to electron transport and membrane permeability, such dyes can be used to measure mitochondrial dysfunction *in vivo* (Foster et al., 2006). In addition, mitochondria can be isolated either from whole worms or sorted GABA neurons, and

ETC function can be assayed directly in the CoQ deficient condition (Kayser et al., 2004). Showing direct effects on mitochondrial function would greatly strengthen the model, as mitochondria are thought to be central of the pathological process. Since CoQ plays many roles in the cell, as discussed in chapter I, it will also be important to distinguish its mitochondrial functions as important to disease.

Involvement of mitochondrial fission/fusion pathways in degeneration

In addition to studies of mitochondrial function, we must also further elucidate the specific molecular players in the apoptotic pathway. While we have information on the involvement of *ced-3* and *ced-4*, for example, we may also want to show roles for CED-9/Bcl, and BH3-only proteins, such as EGL-1 and CED-13. Similarly, while we believe the dynamin-related fission protein, DRP-1 to be essential for degeneration, we could confirm this result, by showing that FIS-1, which escorts DRP-1 to mitochondria, is also involved. We could also corroborate these results by over-expressing fusion proteins, such as OPA-1 and PARL, to see if they are protective, as in previous studies (Cipolat et al., 2006; Frezza et al., 2006). Finally, the co-RNAi experiments testing for *drp-1* involvement are preliminary. Repeating these results with *drp-1* mutants would be ideal. While a *drp-1* null mutation is lethal, several constructs encoding dominant interfering mutants of *drp-1* have been created (Labrousse et al., 1999). Expression of these mutants, which are deficient in GTP hydrolysis, should flood the cell with inactive *drp-1*, which will compete with endogenous *drp-1*. If our preliminary conclusions are correct, GABA-specific expression of these constructs would be predicted to confer resistance to *coq-1* knockdown.

While the involvement of fission/fusion proteins in mitochondrial permeability transition is an exciting finding, it is not thought to be the ultimate cause of cell death in apoptosis. Release of apoptogenic factors is still believed to act downstream of fission-related structural changes to the mitochondria. In *C. elegans*, the identity of such factors is a mystery. While cytochrome c is not thought to be the culprit, additional factors may still be involved. For example, the AIF ortholog WAH-1 has been shown to be released form mitochondria during apoptosis, although its release is thought to occur downstream of *ced-3* activation (Wang et al., 2002). One of the advantages of working with *C. elegans*, is that we can readily use genetic screens to identify these factors. Loss of function mutations in apoptogenic genes released from the mitochondria would be expected to rescue the degenerative phenotype. By employing such a screen, we may be able to use this system to discover novel genes involved in fission-related apoptosis.

CEP-1/p53 in neurodegeneration

In addition to the cell fission machinery, we also tested a role for p53-mediated DNA damage pathway in apoptosis. p53 has been shown to mediate *huntingtin* toxicity in cell culture and *Drosophila* and mouse models of Huntington's disease (Bae et al., 2005), and has also been shown to mediate some of the degenerative events in a mouse model of SCA (Shahbazian et al., 2001). Preliminary data using co-knockdown of *coq-1* with *cep-1*/p53 suggests a role for *cep-1* in degeneration. However, rescue by *cep-1* knockdown was incomplete, either because reduction of *cep-1*/p53 was insufficient, or because it is important for only some of the degenerative events, as discussed in chapter IV. We now have a *cep-1* mutant, so we can address the first issue. The mechanism by

which p53 modulates these degenerative events is an interesting problem, and could be a rich source of future research. The transcriptional targets of p53 have been extensively studied in mammals (Vogelstein et al., 2000), but non-transcriptional mechanisms of the p53 response to stress have also been identified (Erster and Moll, 2005). Follow-up on this arm of the degenerative pathway could include testing known transcriptional and non-transcriptional targets of *cep-1*/p53 to determine their involvement in degeneration. Also, non-biased approaches, such as mutagenic screening, could be employed to identify suppressors of the *cep-1*/p53 arm of the pathway.

Dysfunction in other neuronal populations

Genetic screening can also be used to identify additional genes, which are important for the movement phenotype in neuronal populations other than GABAergic neurons. One feature of the *coq-1* knockdown phenotype that makes it ideal for screening is the readily visible movement defect. Unfortunately, as mentioned in chapter IV, the Unc phenotype of these worms is not exclusively backward Unc. This means that, while GABA neurons seem to degenerate preferentially, other neuronal subtypes are likely affected functionally. We may be able to separate out GABA-specific effects with those of other neuronal subclasses through pharmacological analyses. For example, if treatment with the cholinesterase inhibitor aldicarb or the ACh agonist levamisole rescues forward movement, this would indicate that cholinergic neurons are dysfunctional in these animals. We could then determine whether the pathway for cholinergic dysfunction is separate from that of GABA neurodegeneration, by performing a mutagenic screen for genes that exclusively rescue the forward movement defect. Likewise, we could also

look for genes that only rescue the backward movement defect, and then determine whether those genes do so because of effects on GABA neurodegeneration. If effects on cholinergic or other neurons were not of interest, we could also likely separate out these effects by expressing a *coq-1* ds-RNA specifically in GABA neurons. This should recapitulate the degenerative phenotype, without affecting other neuronal subtypes (unless effects on other neurons is secondary to GABA degeneration).

Understanding the apparent age-dependence of phenotypes

This age-dependent degenerative paradigm in *C. elegans* joins many similar examples in this and other organisms, emphasizing that the susceptibility of neurons to degenerative disease appears to increase as animals age. This can be the result of both the build up environmental exposure and age-dependent alterations in gene expression. The question of how both factors change as we age is important to the ultimate understanding of these diseases. Due to its short lifespan and well-established methodologies, *C. elegans* is an excellent model system for studying lifespan-dependent genomic changes. Microarray profiling throughout development has been performed in the whole worm (Hill et al., 2000). In these experiments, *coq-1* levels appear to decrease steadily throughout life. This natural decline may combine with RNAi knockdown to deplete *coq-1* levels below a survival threshold by the L4 larval stage, resulting in cell loss that increases with advancing age.

A deeper understanding of this issue may be forthcoming with the generation of expression profiles for specific cell types taken throughout the aging process. This is a long-standing goal for the *C. elegans* community. Recently, a pan-neuronal profile has

been obtained for both the embryonic and L2 larval stages (Von Stetina et al., 2007b). Additional profiles of specific neuronal subtypes at different stages are currently being performed in our labs and others. The ability to obtain age-dependent profiles of specific neuron classes may help us determine what pathways change during aging to render these neuronal subtypes sensitive in degenerative diseases. For example, the stress gene analysis performed in chapter II could be repeated for GABA neurons at each stage of development. This analysis may identify factors which betray GABA neurons by abandoning their protective posts as the animal ages.

Since the *coq-1* mutant studied was maternal-effect sterile, it is likewise not possible to tell whether degeneration of GABA neurons with aging in the mutant is a consequence of the exhaustion of maternal protein stores, or an age-dependent phenomenon. To settle this issue, *coq-1* could be rescued in tissues other than GABA neurons in the knockout, to create a *coq-1* mutant animal that is viable through multiple generations. GABA neurodegeneration could then be studied in animals upon which no maternal COQ-1 protein has been bestowed.

Understanding the selective vulnerability of GABA neurons

As with the age-dependence issue, the question of why GABA neurons are selectively sensitive to degeneration in *coq-1* knockdown animals is an important topic for future research. We have proposed two potential explanations for this phenomenon. First, as discussed in chapter IV, GABA neurons may be selectively vulnerable due to a high activity-related metabolic load, which translates into sensitivity to metabolic loss. While decreasing GABA neuron activity with the *pC04G2.1::egl-36(gf)* construct did not

affect degeneration, these experiments need to be repeated in an integrated line. This will ensure that the lack of a result was not due to incomplete penetrance of expression of the silencing channel.

In addition to activity-dependent effects, we also hypothesized in chapter II that GABA neurons may be rendered more vulnerable due to a cell-specific genetically-programmed lack of protective measures. Specifically, we showed a reduction in expression of GST genes relative to other neurons. Future experiments may seek to answer this question, by over-expressing one or more GSTs in GABA neurons using the *C04G2.1* promoter to determine whether this protects against loss of *coq-1*.

Use for pharmacologic screening

It is important to note that CoQ treatment has been shown to delay or reduce, but not to rescue, symptoms in the many diseases for which it has been tried as a treatment, including CoQ deficiency (Beal et al., 1998; Huntington_Study_Group, 2001; Ferrante et al., 2002; Artuch et al., 2006). Therefore, the search for additional targets for therapy in these diseases could lead to the design of efficacy-boosting add-ons to CoQ therapy. The mutagenic screens described above could be very helpful in this search. However, we need not know the target to discover a useful drug. Therefore, this model could be used for pharmacologic screening for small molecules which inhibit the pathology. In comparison to such screens in mouse models, our model, with a readily visible movement phenotype, could be quickly and inexpensively screened using a large number of small molecules (Burns et al., 2006). Automated movement tracking software with high throughput formats has been developed (Feng et al., 2004; Cronin et al., 2005), which

would add to the feasibility of such a screen. The use of *C. elegans* for screening also has an advantage over cell culture models of degeneration in that it is an intact animal with networked neurons in their normal physiologic environment.

Much work still remains to fully understand the CoQ-dependent degeneration described herein. It is clear from this model, however, that the vulnerability of neurons to metabolic changes is an ancient feature of these cells. Likewise, we have learned in the course of these studies that the molecular mechanisms involved in degeneration may be more conserved than originally thought. Moving forward with this model may reveal important insights in the degenerative process, and may also be useful in determining treatments for such diseases in humans.

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