Analysis of Stress in *C. elegans* Neurons

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1. Abstract

The stress conditions challenging neurons are manifold, and thus neuronal response to stress must be dynamic and efficient. The nervous system depends on efficient neuronal communication to respond to an ever-changing environment and the stress it produces. Well-formed and well-functioning neuronal networks are, therefore, vital to maintaining health, learning, and movement. If function is impaired, such as in cases of neurodegeneration, the resulting neurological condition can have devastating impact on the organism's ability to survive. The study of how neuronal damage can be prevented or even reversed is thus of great importance.

The Rongo Lab is studying the importance of protein homeostasis in neuroprotective mechanisms. Effective protein homeostasis is essential for limiting protein misfolding, aggregation, and proteotoxic stress, which are components of many age-related neurodegenerative diseases, such as Parkinson's, Huntington's, and Alzheimer's Disease (AD). Protein homeostasis is regulated by stress sensors, such as heat shock response and unfolded protein response mechanisms. It also includes other stress signaling pathways that prevent misfolding and aggregation, and facilitate the turnover of damaged proteins (van Oosten-Hawle and Morimoto).

The Rongo Lab is particularly interested in understanding the neuronal and mitochondrial response to hypoxia (low oxygen) levels, as well as understanding pathways involved in protein homeostasis as potential therapeutic targets for neurological conditions. To this end, we focus on the hypoxia response and Hexosamine Pathways in the *C. elegans* model system.

The hypoxia response pathway is activated under low-oxygen conditions by the master transcriptional regulator of hypoxia response, HIF-1. If HIF-1 levels are elevated, animals exhibit improved survival phenotypes as well as neuronal mitochondrial hyperfusion, which is believed to be neuroprotective (Ghose et al.). We explored whether CHN-1, an ortholog of mammalian Carboxyl-terminus of Hsc70 Interacting Protein (CHIP), through its actions in the hypoxia response pathway, regulates HIF-1. CHIP/CHN-1 mediates ubiquitin-mediated turnover of numerous proteins, and thus plays an important role in protein homeostasis. Our preliminary results indicate that CHN-1 negatively regulates HIF-1 by reestablishing protein levels following hypoxia response. Further study of the hypoxia response pathway is ongoing.

To explore protein homeostasis within the context of neurodegeneration, The Rongo lab is studying strains of *C. elegans* which contain mutated human Tau (hTauV337M) protein associated with AD (Fatouros et al.). This hTauV337M strain has an uncoordinated phenotype and significantly lower neuronal mitochondrial number compared to wild-type. We studied the therapeutic potential of a protein homeostatic pathway metabolite, N-Acetyl Glucosamine, in our AD *C. elegans* model. The Hexosamine Pathway is a well-known branch of glycolysis which is partly responsible for regulating stress resistance, metabolism, and quality control mechanisms, among other downstream mechanisms. Studies have found that increasing the synthesis of precursor N-acetyl glucosamine can slow down aging, and thus reduce the effects of age-related diseases (Denzel et al.). We found that mutant animals treated with N-acetyl Glucosamine showed improved mobility and increased neuronal mitochondrial number compared to the untreated group.

These studies emphasize the importance of proper protein homeostasis in the maintenance of neuronal health. Improved understanding of the mechanisms behind neuronal

stress response will aid in the development of therapies and preventative measures for neurological disorders.

2. Introduction

2.1 Neuronal Structure and Function

The nervous system is the control center of the body, formed by networks of neurons and other cells to regulate both voluntary and involuntary actions. Neurons are the functional units of the nervous system and are of essential importance as transmitters of electrochemical signals to other cells in the body. These specialized cells are composed primarily of three parts: the soma, axons, and dendrites (Figure 1).

The soma, also known as the cell body, is home to the nucleus, smooth and rough endoplasmic reticulum, mitochondria, and other cellular components. Axons are the structures that extend electrical impulses from the soma of one neuron to the dendrites of its neighbor, which is responsible for receiving messages and sending them to the soma.

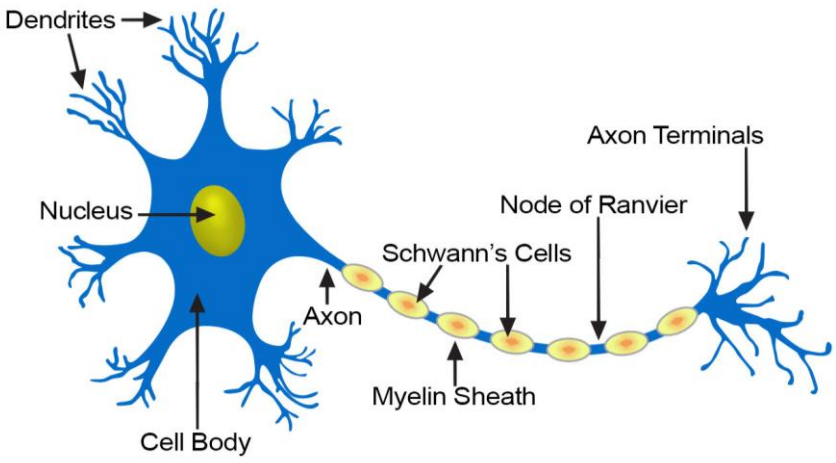

Figure 1. Structure of a Neuron (National Institute of Health)

Communication between ensembles of neurons underlies the brain's ability to mediate sensation, movement, and behavior. Neurons transmit information as an array of regulated signals embedded within a transport complex comprised of synaptic proteins and neurotransmitters, a process known as neurotransmission. Neurotransmission occurs at synapses, which are specialized junctions between the axons and dendrites. Synapses are comprised of a presynaptic region, which releases neurotransmitters from synaptic vesicles in the upstream neuron, and a postsynaptic region, which contains receptors that bind neurotransmitters and propagate the signal to the downstream neuron.

Neuronal structure very much informs its function. As the body learns and adapts to new experiences, neuronal connections will strengthen, weaken, or otherwise modify the neuronal network to effectively respond to environmental changes. The dynamic nature of these axon-dendrite connections allow communication between cells along long distances and in response to various stimuli, allowing ensembles of neurons to mediate movement, sensation, and behavior. This ability to change is known as neuronal (or synaptic) plasticity, and it occurs during processes such as learning and memory formation (Citri and Malenka).

However, this signal propagation does not occur in a vacuum. Necessary proteins and organelles required for the maintenance of cell-to-cell signaling are synthesized in the cell body and transported along microtubules running across the length of the axon to repair, renew, or energize as needed via anterograde transport. Microtubules are also the tracks upon which damaged organelles can also move back to the cell body to be degraded—a process known as retrograde transport. Without this responsive transport mechanism, neurons could not maintain the level of activity and plasticity that they are known for. Defects in the mechanisms that

underlie synaptic transmission and plasticity might be an underlying cause of diseases of the nervous system.

2.2 Neuronal Function in Disease States

2.2.1 Disease States and Neuronal Communication

The nervous system depends on efficient neuronal communication to respond to an ever-changing environment and the stress it produces. Stress to the body can be broadly characterized as anything that contributes to a disruption in homeostasis or otherwise threatens one's well-being. Through interoceptive and exteroceptive cues, information is sent to the central nervous system, which activates the necessary response mechanisms to minimize the negative effect the stressor has on the body. Well-formed and well-functioning neuronal networks are thus vital to maintaining health in the presence of stress conditions.

Disorders of the nervous system often involve either changes in communication between neurons or neuronal degeneration, and lead to widespread effects to the rest of the body. Ineffective neuronal communication is seen in cases of epilepsy and multiple sclerosis (Centonze et al.; Maglóczky and Freund). These diseases have differing disease profiles and pathologies, further emphasizing the importance of neuronal communication in a wide array of processes.

Although these diseases are etiologically linked to problems in synaptic transmission, there is often a close association between defective neuronal communication and neurodegeneration. Neurodegeneration is characterized by a progressive deterioration of the neurons both in structure and function. The underlying mechanism of such degeneration is still under investigation, though synaptic loss and neuronal signaling defects do play a role. Proper synaptic circuitry formation is essential for sustaining survival in the developing nervous system (Linden). Whereas the adult brain is better equipped to deal with some loss of afferent sensory

input, it is still vulnerable to neurodegeneration, as seen in diseases such as Alzheimer's, Huntington's and Parkinson's disease.

While much attention in the study of neuronal communication is given to the processes regulating synaptic function, the focal point of neurodegeneration studies has not been so clear. Some themes touched upon in the field include intracellular mechanisms (i.e. protein degradation and mitochondrial dysfunction), protein misfolding, and programmed cell death, to name a few. Complicating matters is the ever-present need of complex organisms to maintain their neurological function to properly send messages to the rest of the body. Shortcomings in the nervous system will thus have widespread effects, making it difficult to pinpoint the exact cause when diseases manifest.

2.2.2 Neuronal Mitochondria

Because of their essential role in maintaining health, neurons have high energy demands; although the brain contributes to only 2% of total body mass, it consumes 20% of the oxygen and 25% of the glucose consumed during normal metabolism (Silver and Erecińska). With no glycolytic reserves, neurons must rely heavily on the oxidative phosphorylation of mitochondria to maintain ion gradients, efficient neurotransmission and plasticity (Kann and Kovacs). We briefly discussed the important role of microtubules in facilitating the transport of proteins and organelles necessary for sustaining neuronal health. Mitochondria are a particularly important transported organelle because they are so extensively used to provide energy required for proper neuronal function.

Mitochondria are as dynamic as the neurons that they energize. Most cells contain multiple mitochondria constantly moving around the cell and undergoing division (fission) and

joining (fusion) to form mitochondrial networks; mitochondria also play a role in multiple cellular signaling pathways, including those that result in apoptotic or necrotic cell death, as well as in the regulation of cytosolic calcium levels.

Mitochondrial dysfunction is implicated in several other human neurological and neurodegenerative disorders, such as Amyotrophic Lateral Sclerosis (ALS), which is characterized with the disruption in calcium homeostasis (Tadic et al.). Under conditions of stress (e.g. ischemic stroke, traumatic brain injury, spinal cord injury), mitochondria can also be a threat to the cell, releasing reactive oxygen species and potentially triggering inappropriate cell death. Quality control mechanisms are thought to survey mitochondrial health and remove damaged mitochondria, with one such mechanism being a specialized form of autophagy called mitophagy. This process is thought to be a cellular defense mechanism for removing damaged mitochondria before they cause additional damage to the cell. However, the detailed mechanisms that mediate this vital process remain unclear.

Many of the factors that mediate mitochondrial fission, fusion, transport, and mitophagy – mitochondrial dynamics – are not well understood. Through studies in yeast, several factors have been identified as being required for fission (e.g., Drp1) and fusion (Opa1 and the Mitofusins) (Imoto, Tachibana and Urrutia; Olichon et al.). Mutants for these genes in the nematode *C. elegans* have similar fission, fusion, and transport phenotypes, and the simple anatomy, transparency, and powerful forward genetics of *C. elegans* have allowed researchers to study mutants with defects in mitochondrial dynamics in live animals. There are likely additional factors that either were missed in the studies in yeast or are unique to multicellular organisms, perhaps tailored to individual cell types.

The Rongo lab has begun to explore mitochondrial dynamics by generating a strain of *C. elegans* nematodes that express MitoGFP, a green fluorescent protein reporter that contains a mitochondrial matrix localization signal, solely in neurons (Ghose et al.). Individual mitochondria, appearing as MitoGFP puncta distributed along command interneurons, can be directly observed in living nematodes by standard epi-fluorescence microscopy (Figure 2). We have introduced the transgene into known mutants with defects in mitochondrial motility, fission, and fusion, and we have observed the expected phenotypes, which are easy to score by eye.

Figure 2 MitoGFP in C. elegans Model

MitoGFP is expressed in command interneurons under the glr-1 promoter.

With a visual representation, we can work toward answering the plethora of questions regarding the mechanism of mitochondrial dynamics. It is not yet clear what the precise payoff of mitochondrial fission or fusion is, both in stress and non-stress conditions. For example, in cases of mitochondrial damage, fission may make the resulting smaller mitochondria easier to break down through mitophagy, while fusing with a healthier mitochondrion may improve efficiency more effectively. This competing functionality muddles the assumed dichotomy typically associated with fission and fusion, which makes grasping neuronal mitochondrial dynamics as challenging as the neurons we study them in.

2.3 Proteotoxicity

2.3.1 Protein Homeostasis

Just as mitochondrial dysfunction is often associated with neurological disorders, so too is proteostatic imbalance—especially in neurodegenerative diseases. Proteostasis requires a highly-responsive network of macromolecules to maintain a tight regulation of mechanisms important for protein folding and turnover. Proteostasis is regulated by stress sensors, such as heat shock response and unfolded protein response mechanisms. It also includes other stress signaling pathways that prevent misfolding and aggregation, and facilitate the turnover of damaged proteins (van Oosten-Hawle and Morimoto). Proteins undergo clearance by either the ubiquitin-proteasome system or through autophagy.

The ubiquitin proteasome system involves breaking down proteins labeled with multiple ubiquitin monomers. Protein turnover by the ubiquitin proteasome system involves activation of the ubiquitin molecule, conjugation of ubiquitin to the damaged protein (monoubiquitination), the addition or subsequent ubiquitin monomers to form a chain (polyubiquitination), and finally degradation by the proteasome (Sorokin, Kim and Ovchinnikov).

Protein turnover also occurs by autophagy, which can be executed in three ways: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Macroautophagy involves the formation of an autophagosome, a membrane-bound vesicle around the protein, that is transported to a lysosome to be degraded by lysosomal hydrolases (Mizushima, Ohsumi and Yoshimori). Microautophagy occurs when the lysosomal membrane engulfs the protein directly (Cesen et al.). CMA is the most selective form of autophagy, as proteins degraded through this pathway must have a recognition site for the hsc70 complex (heat shock cognate protein 70). The

substrate-chaperone complex is recognized by a lysosomal protein, which allows the complex to enter through the CMA receptor (Kaushik and Cuervo).

2.3.2 Disease States and Proteotoxicity

Neurons are post-mitotic cells, which makes them vulnerable to homeostatic imbalance, particularly as it affects maintaining cell cycle arrest (Herrup and Yang). Disruption of homeostatic processes can lead to proteotoxicity and disease, as improper protein folding can be deleterious to the body. If proteins, misfolded or otherwise, are not turned over properly, they make the cell incapable of efficient response. For example, mutated forms of the Tau and Huntington proteins can aggregate and lead to neurodegeneration in Alzheimer's disease (AD) and Huntington's disease, respectively. An increase in proteasome activity or autophagic-lysosomal potential increases lifespan and protects organisms against symptoms associated with proteotoxic disorders, particularly age-related disorders (Vilchez, Saez and Dillin). Additional studies have found that improving protein quality control promote improved lifespan and health outcomes (Denzel et al.; Ladiges).

2.3.3 Alzheimer's Disease: Tauopathy

The disease profile of Alzheimer's disease illustrates the effects of neuronal dysfunction and protein homeostatic imbalance. Alzheimer's Disease is a progressive neurodegenerative disease which affects a patient's memory, language skills, and coordination. The average life expectancy following diagnosis is typically around 3 to 10 years (Zanetti, Solerte and Cantoni). According to the Alzheimer's Association, an estimated 5.4 million Americans have the disease, affecting one in nine adults over the age of 65 . As the aging population increases in number, identifying an effective treatment method will be pivotal to combat the exponential rise in disease prevalence that is expected in the coming years.

There are two prominent proteins associated with Alzheimer's Disease currently under investigation: A-beta and Tau. The A-beta model links a difference in the processing of the Amyloid Precursor Protein (APP) to the production of A-beta plaques. Enzymes alpha- and gamma-secretase cleave APP to produce peptides of yet-unclear function; however, in AD, beta-secretase cleaves APP instead of alpha-secretase, leading to the formation of A-beta (Chow et al.). These A-beta peptides aggregate as plaques outside of the neurons and lead to synaptic dysfunction and impaired neuronal connectivity (Reddy and Beal). The study of A-beta and its role in AD is complicated, however, because A-beta deposit density shows weak correlation with the disease expression in humans (Terry et al.). However, A-beta peptides form soluble oligomers before forming the aggregates typically associated with the disease, and many studies indicate that these oligomers may play a role in the observed pathology (Haass and Selkoe). Recent clinical trials driven by the amyloid theory have proved unsuccessful. For example, pharmaceutical company Merck recently announced that it would be halting further clinical trials of a small molecule inhibitor of an A-beta-forming cleavage site that was ineffective in improving the disease outcome (Hawkes). The clinical trials studying these drugs are completed in patients already very advanced in their disease progression, which may be the reason why they are ineffective in lessening deterioration despite targeting A-beta cleavage.

Both the A-beta and Tau models of Alzheimer's Disease posit the insoluble protein aggregates as highly associated with the disease. However, compared to A-beta, Tau accumulation shows a better correlation to disease severity (Treusch, Cyr and Lindquist). Tau is a microtubule-stabilizing protein that is abundant in neurons in six different isoforms transcribed from a single gene on chromosome 17. The Tau protein has two main functional domains, the projection domain and the microtubule-binding domain. The projection domain determines the

distance between Tau proteins on the microtubules, while the microtubule-binding domain is the region that actively binds to the microtubule (Gustke et al.). The isoforms are differentiated by the presence or absence of a fourth 31 amino-acid repeat in the microtubule-binding domain on exon 10 as well as either 29 or 58 amino-acids inserts in the amino terminal end of the sequences (Figure 3). In normal functioning adult brains, there is an equal ratio of three-repeat and four-repeat sequences in the microtubule-binding domain, but the relative levels of these isoforms are different in cases of degeneration (Hutton et al.).

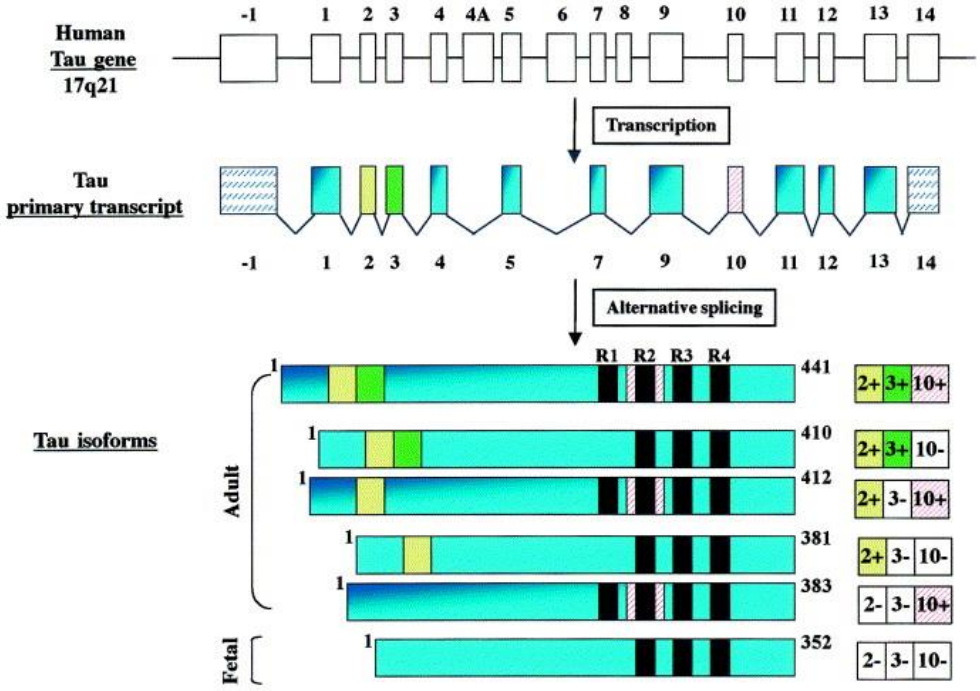

Figure 3 Tau Isoforms (Buée et al.)

A schematic representation of Tau isoforms produced as a result of alternative splicing. Exons 1, 4, 5, 7, 9, 11, 12, 13 are constitutive, while the expression of exons 2, 3, and 10 are variable. The yellow and green boxes represent the presence of exons 2 and 3 in the projection domain, respectively. The black boxes represent the number of repeats in the microtubule-binding domain.

Tau proteins interact with microtubules and are important for remodeling the microtubule cytoskeleton (Samsonov et al.). Tau's involvement in microtubule stability, as well as evidence

of its interaction with cytoplasmic organelles (such as mitochondria), highlights its significant role in neuronal plasticity and axonal transport and maintenance (Rendon, Jung and Jancsik).

Tau's genetic link to neurodegeneration can be traced back to the study of thirteen families with frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), which is an autosomal dominant neurodegenerative disorder caused by mutations in the *tau* gene. (Foster et al.). The groups of individuals with advanced-stage FTDP-17 typically experience personality disturbances, cognitive impairments, and motor dysfunction. A majority of FTDP-17 cases exhibit mutations that affect Tau through two mechanisms. The first involve mutations in non-coding regions that increase the proportion of 4R *tau* isoforms through the inclusion of exon 10 via alternative splicing. The second is characterized by mutations in the microtubule-binding domain of the *tau* gene that weaken tau association to microtubules and accelerate Tau aggregation into neurofibrillary tangles (NFTs) (Wszolek et al.).

The formation of neurofibrillary tangles comprised of aggregated Tau is a shared feature in many neurodegenerative diseases known as Tauopathies. Because mutations in Tau have been directly associated with familial frontotemporal dementia, Tau is a protein of interest in the study of neurodegenerative diseases such as Alzheimer's. Although no known mutation in the *tau* gene is associated with the Alzheimer's Disease, NFTs are a prominent hallmark of the AD brain. The strong correlation of NFTs with AD disease severity and FTDP-17 makes the study of tauopathy critical for elucidating the particularly complex mechanisms underlying pathogenesis of neurodegenerative disorders such as Alzheimer's Disease.

2.3.4 *C. elegans* Tauopathy Model

Investigating Tau pathology in *C. elegans* began with Brian Kraemer of the Schellenberg lab. While *C. elegans* may seem like an unintuitive choice of organism for modeling AD and tauopathy, there are many benefits to studying Tau in nematodes. Nematodes have a relatively short life span, which facilitates the study of age-related disorders such as AD. The transparent nature of *C. elegans* makes them excellent for analyzing physiological processes *in vivo* with fluorescent markers, and with a simple nerve system, the *C. elegans* model allows us to study Tauopathy in a well-defined system. It was previously reasoned that a crucial advantage to studying Tau in *C. elegans* is that we could avoid problematic interferences with endogenous Tau, as the nematode Tau homologue, PTL-1, was believed to be expressed in a subset of cells (Goedert et al.). However, our results indicate that PTL-1 is likely expressed in other neurons, as well (Figure 13).

Tau aggregation has been examined by the Baumeister and Schellenberg labs. Expression of an essentially wild-type version of Tau (Human 4R1N-isoform Tau) from the pan-neuronal rab-3 promoter resulted in only subtle effects on nematode neurons compared to the mutant Tau strain. These effects included mild accumulation of insoluble Tau, partial cholinergic neurotransmission impairment, and mild locomotion defects (Kraemer et al.) Tau strains with FTDP-17 mutations were also generated, resulting in a transgene that expresses full length Tau with the V337M mutation from the pan-neuronal rab-3 promoter (FLTauV337M). The FLTauV337M transgenic strain exhibited significantly decreased lifespan, uncoordinated movement, among other defects (Kraemer et al.). The Baumeister group enhanced the tau aggregation phenotype of the FLTauV337M strain by introducing a fragment (amino acids 258–360) of the FTDP-17-associated ΔK280 Tau mutation (F3ΔK280) (Fatouros et al.). This

F3ΔK280 corresponds to a cleaved fragment of the ΔK280 mutation and has been found to nucleate Tau aggregation by interfering with lysosomal function (Wang et al.). Evidence from the Baumeister lab also suggests that mitochondrial transport is altered by Tau expression in this system.

In order to study the effects of mutant Tau on mitochondria in more detail, we have generated nematodes containing a combination of transgenes that expresses MitoGFP, FLTauV337M mutated Tau, and the F3ΔK280 fragment associated with proteotoxicity studied by the Baumeister lab (this combination of will be hereafter referred to as mAgg-Tau). We observed mitochondria transport and dynamics through preliminary microscopy, while keeping record of the effects of mAgg-Tau on feeding, mobility, and sterility. For each genotype, we measured the size, number, morphology, and subcellular localization of MitoGFP-labeled mitochondria in neurons using epi-fluorescence microscopy and morphometric quantification using scripts written in ImageJ. These experiments tell us how Tau expression alter mitochondrial dynamics in this simple genetic system. This system also allows us to visualize potential improvements in the prevention or alleviation of protein aggregation to treat tauopathies following various treatments or genetic crosses. One potential drug target of particular interest to us is the Hexosamine Pathway, which appears to modulate proteostasis.

2.3.5 The Hexosamine Pathway

The Hexosamine Pathway is a well-known branch of glycolysis that is partly responsible for regulating stress resistance, metabolism, and quality control mechanisms, among other downstream mechanisms (Figure 3). Studies have found that increasing the synthesis of the N-glycan precursor N-acetyl glucosamine can slow down aging, and thus reduce the effects of age-related diseases (Denzel et al.). Because ER proteins require glycosylation via N-glycan

oligosaccharide addition for proper folding, increasing the levels of N-acetyl glucosamine is believed to improve ER protein homeostasis by preventing misfolding due to decreased glycosylation (Denzel et al.). Increasing N-acetyl glucosamine levels also induces autophagy, ER-associated degradation, and proteasome activity, although the specific molecular mechanism is not understood. Because this one pathway impacts both aging and proteostasis, this effect supports the quality control model of aging: improved quality control reduces aggregation by enhancing clearance mechanisms and is thus protective against proteotoxicity (Ladiges). In the current study, we examined the effects of enhanced quality control on our Tauopathy model.

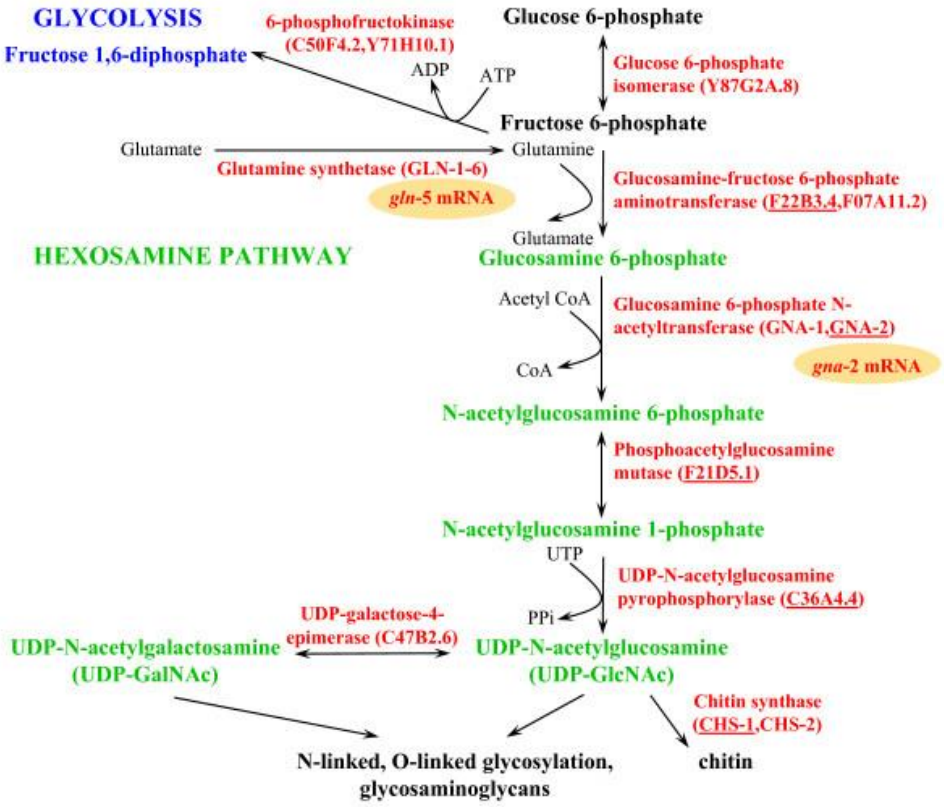

Figure 4. The Hexosamine Pathway

2.4 Hypoxia

2.4.1 Neuronal Oxygen Dependence

As described so far, many neurological disorders have an underlying etiology of impaired proteostasis or mitochondrial quality control. A third common factor in neurological disorders and disease is oxygen deprivation. Neurons are especially sensitive to low oxygen environments, such as are observed during ischemic strokes, traumatic brain injury, and spinal cord injury (Hinkle and Guanci). As neurotransmission is an energy-intensive process that relies heavily on oxidative phosphorylation in mitochondria, neurons are particularly sensitive to changes in oxygen levels in the environment.

2.4.2 Disease States and Hypoxia

Hypoxia has been linked to many nervous system disorders such as ischemic stroke, traumatic brain injury, spinal cord injury, and cerebral palsy. Oxygen deprivation during stroke results in the rapid depletion of energy stores, resulting in membrane depolarization, massive release of neurotransmitters like glutamate, overactivation of glutamate receptors, and excitotoxic neurodegeneration. Ischemic stroke induces neurodegeneration through over-activation of the glutamate receptors, a process known as excitotoxicity (Baron et al.). Stress-induced changes in glutamate receptor expression or trafficking are mechanisms by which some organisms protect their neurons from stroke-induced damage (Lai, Zhang and Wang). Mitochondrial damage within these neurons is also associated with neurodegeneration (Sims and Muyderman). Studying neuronal mitochondrial response under hypoxic conditions such as ischemic stroke might provide an understanding of the brain's various neuroprotective mechanisms. We explored this relationship between mitochondria and stress-induced neuropathologies specifically in neurons expressing a glutamate receptor GLR-1 with our MitoGFP reporter.

2.4.3 Hypoxia Response Pathway

The Rongo lab has shown that glutamatergic signaling is depressed and recycling of the glutamate receptor GLR-1 to synapses is blocked under hypoxia. Under normal oxygen conditions (normoxia), an isoform of EGL-9, a prolyl hydroxylase and oxygen sensor, works with LIN-10, a PDZ scaffolding protein, to promote glutamate receptor recycling (Park et al.). Without LIN-10, GLR-1 accumulates in elongated endosomes, which highlights the role of LIN-10/EGL-9 in recycling (Rongo et al.). This recycling mechanism is important because it allows for proper maintenance of homeostatic plasticity among neurons, and is a neuroprotective mechanism that somewhat mitigates the damage caused by hypoxia in neurons.

EGL-9 regulates the transcription factor HIF-1. In normal oxygen conditions, EGL-9 associates with HIF-1, resulting in hydroxylation of HIF-1 proline side chains, ubiquitination of HIF-1, and ultimately turnover of the HIF-1 protein. By contrast, under hypoxic conditions, EGL-9 is inactivated, and so HIF-1 does not undergo turnover and transcribes its target genes. Multiple transcriptional targets of HIF-1 are known in mammals and in *C. elegans*, and two prominent target genes in *C. elegans* that are used as markers are the genes CYSL-2 and NHR-57. The Rongo Lab is currently identifying more target genes as well as studying their function; however, just as important as the target genes themselves is the mechanism that regulates their expression.

2.4.4 CHN-1/CHIP and the Hypoxia Response Pathway

There are likely to be additional regulators of the hypoxia response (Liu et al.). In *C. elegans*, one candidate is CHN-1, which is the ortholog to the mammalian gene CHIP, a chaperone protein with a highly-conserved protein sequence that is expressed in cardiac muscle and the brain. CHIP interacts with heat shock proteins Hsc70-Hsp70 and Hsp90 to induce

substrate degradation through the ubiquitin-proteasome pathway (Ballinger et al.). Heat shock proteins themselves facilitate protein folding and transport (Craig, Gambill and Nelson). In cases of prolonged hypoxia, CHIP is recruited by heat shock proteins to promote HIF-1 proteasomal degradation (Luo et al.). This function has made CHIP a strong potential therapeutic target that might enhance HIF-1 activity and encourage the development of new blood vessels in cases of Critical Limb Ischemia (Zhou et al.).

The *C. elegans* CHN-1 protein is a ubiquitously expressed protein that interacts with the E4 ligase UFD-2 (Hoppe et al.). However, CHN-1 also has E3 activity, which allows it to add one or more ubiquitin molecules to a heat shock protein (Hsp90) co-chaperone UNC-45, as well as to Hsp70 (Hoppe et al.). Because CHN-1, like CHIP, interacts with heat shock proteins, investigating whether or not CHN-1 plays a role in HIF-1 degradation and whether it acts though a HSF-dependent manner is worthwhile.

2.4.5 *C. elegans* Hypoxia Response Model

Recognizing this overall connection between CHIP/CHN-1 to the hypoxia response pathway, we studied the change in mitochondrial dynamics during normoxia and anoxia conditions of *chn-1* mutants compared to the wild type strain (Figure 4). We found that while *chn-1* mutants in normal oxygen conditions do not have a change in their mitochondrial morphology and dynamics, by contrast mitochondria following anoxia-reoxygenation undergo hyperfusion in a manner dependent on HIF-1 levels. Mutants for *chn-1* also have high survival rates compared to that in wild-type (N2) animals. Because these results are similar to our studies of *egl-9* mutant mitochondrial dynamics, we continued to investigate CHN-1's potential role in the hypoxia response pathway as similar or associated with EGL-9's role as a HIF-1 regulator.

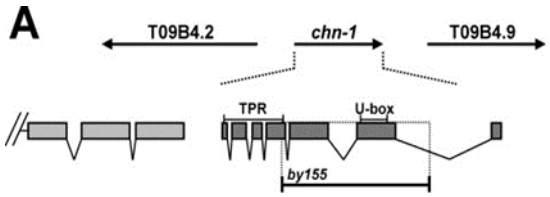

Figure 5. Deletion of chn-1.

The *chn-1* gene is on chromosome I, and encoding regions for TPR motifs and U-box domains are shown. Deletion region (by155) is also indicated. (Hoppe, T. et. al.)

2.5 Project Design

2.5.1 Tauopathy Project

Changes in mitochondrial dynamics are associated with many neurodegenerative disorders, and these changes are possibly etiological. The most compelling data for abnormalities in mitochondrial function, transport, and dynamics contributing to disease is in Parkinson's Disease (PD), where PD-causing mutations occur in proteins like Parkin, which mediates the removal of damaged mitochondria (Pickrell and Youle, 2015). Altered mitochondrial dynamics have also been observed in Amyotrophic Lateral Sclerosis (ALS), Huntington's Disease (HD), and Alzheimer's Disease (AD), although their role here is not as clear (Su et al.). The large number of neurological disorders with a mitochondrial component likely reflects the fact that the regulation of mitochondrial movement and dynamics is particularly important in neurons, which use large amounts of ATP that have to be supplied primarily through oxidative phosphorylation in their mitochondria. The Rongo lab has recently shown that mitochondria undergo fission when placed under anoxia, a condition that mimics some aspects of ischemic stroke and traumatic brain injury in humans. Upon reoxygenation, the mitochondria can rapidly re-fuse. Interestingly, mutants for the prolyl hydroxylase *egl-9* show an excessive and rapid re-fusion following reoxygenation, and these *C. elegans* mutants are

particularly resistant to anoxic stress, suggesting that enhanced mitochondrial fusion might play a protective role during stress.

In this current study, we examine whether Tau, when expressed under pathological conditions, can alter mitochondrial dynamics, turnover, and/or transport in neurons. Several other labs have modeled Tau in *C. elegans* by generating transgenes that express these proteins (Fatouros et al.; Brandt et al.; Alexander, Marfil and Li). As mitochondrial transport is altered under Tau expression, we introduced these transgenes into *C. elegans* strains that also harbor mitochondrial reporters to observe the effects of these transgenes on mitochondrial dynamics.

We follow this analysis with an examination of whether improved protein quality control, through N-Acetyl Glucosamine treatment, alleviates the tauopathy phenotype observed in our *C. elegans* model through behavioral, physiological, and biochemical study.

2.5.2 Hypoxia Response Project: CHN-1

In this study, we examine whether CHN-1, through its actions on the hypoxia response pathway, regulates mitochondrial dynamics following anoxia-reoxygenation. In mutants lacking the prolyl hydroxylase EGL-9, the transcription factor HIF-1 is over-activated, which results in mitochondrial hyperfusion and rapid neuromuscular recovery following anoxia-reoxygenation, suggesting that HIF-1 regulates the expression of one or more genes involved in mitochondrial dynamics and function. Our preliminary tests show that *chn-1* mutants also undergo mitochondrial hyperfusion following anoxia-reoxygenation, a phenotype reminiscent of *egl-9* mutants. We hypothesize that CHN-1 is a key component of the hypoxia response pathway, and that it acts as a ubiquitin ligase to negatively regulate HIF-1 by promoting its proteolysis under normal oxygen conditions. Under hypoxic and anoxic conditions, EGL-9 and CHN-1 are

inactivated, resulting in HIF-1 stabilization and changes in downstream target gene expression that favor anaerobic metabolism and altered mitochondrial dynamics. An improved understanding of *chn-1* mutants will uncover more of the mechanism underlying the role of CHN-1 in mitochondrial dynamics and neuronal recovery from low-oxygen stress.

3. The Hexosamine Pathway Metabolite N-Acetyl Glucosamine Alleviates Tauopathy in *C. elegans*

3.1 Materials and Methods

3.1.1 Strain List

Animals were grown at 20 °C on standard NGM plates seeded with OP50, unless otherwise mentioned. Several strains were provided by the Baumeister Lab. The following strains were used:

odIs70[Pglr-1::MitoGFP, Cbunc-119], *odIs71[Pglr-1::MitoGFP, Cbunc-119]*, *byIs161[Prab-3::F3 ΔK280, Pmyo::mCherry], bkIs10[Paex-2::hTau V337M, Pmyo-2::gfp]*, *byIs162[Prab-3::F3 ΔK280 I227P I303P, Pmyo::mCherry]*, *bkIs193[Prab-3::F3 ΔK280, Pmyo-2::mCherry]*, *bkIs194[Prab-3::F3 ΔK280-PP, Pmyo-1mCherry]*, *baIs34[Peat-4::Abeta42, Pmyo-2::mCherry]*, *adIs1240[Peat-4::GFP]*, and *baEx134[Peat-4::mCherry, Pmyo-2::mCherry]*,mAgg-Tau(OR3584): *bkIs193[Prab-3::F3 DK280, Pmyo-2::mCherry]; bkIs10[Paex-2::hTau V337M, Pmyo-2::gfp]; odIs70[Pglr-1::MitoGFP, Cbunc-119]*,BR5706: *bkIs193[Prab-3::F3 DK280, Pmyo-2::mCherry]; bkIs10[Paex-2::hTau V337M, Pmyo-2::gfp], odIs1[Pglr-1::SNB-1::GFP],* OR3628: *bkIs193[Prab-3::F3 DK280, Pmyo-2::mCherry]; bkIs10[Paex-2::hTau V337M, Pmyo-2::gfp]*; *odIs1[Pglr-1::SNB-1::GFP], nuIs25[P glr-1::GLR-1::GFP],*OR3629: *bkIs193[Prab-3::F3 DK280, Pmyo-2::mCherry]; bkIs10[Paex-2::hTau V337M, Pmyo-2::gfp]*; *nuIs25[P glr-1::GLR-1::GFP],* OR3608:*odEx[Punc-47::Tom20::mCherry, Pttx-3::rfp],* OR3631: *bkIs193[Prab-3::F3 DK280, Pmyo-2::mCherry]; bkIs10[Paex-2::hTau V337M, Pmyo-2::gfp]; odEx[Punc-47::Tom20::mCherry, Pttx-3::rfp],* OR3630: *bkIs193[Prab-3::F3 DK280, Pmyo-2::mCherry];*

bkIs10[Paex-2::hTau V337M, Pmyo-2::gfp]; *odEx[Punc-47::UNC-116::TOMM-7, Punc-47::Tom20::mCherry, Pttx-3::rfp]*,OR3590: *odIs70;bkIs10[Paex-2::hTau V337M, Pmyo-2::gfp]*,OR3979: *bkIs193[Prab-3::F3 DK280, Pmyo-2::mCherry]; bkIs10[Paex-2::hTau V337M, Pmyo-2::gfp], odIs1[Pglr-1::SNB-1::GFP]*;*odIs70;ptl-1(ok621)*,OR3830: *odIs70;ptl-1(ok621)*,OR3911: *odEx[pglr-1::EBP-2::GFP];ptl-1(ok621*),OR3513: *odEx[pglr-1::EBP-2::GFP], bkIs193[Prab-3::F3 DK280, Pmyo-2::mCherry]; bkIs10[Paex-2::hTau V337M, Pmyo-2::gfp], odIs1[Pglr-1::SNB-1::GFP];gfat-1(dh784)*

3.1.2 Fluorescence Microscopy

GFP- and mCherry-tagged fluorescent proteins were visualized in nematodes by mounting L4 and young adult animals on 2% agarose pads with 20mM levamisole using an epifluorescent microscope, unless otherwise mentioned.

3.1.3 Time-Lapse Imaging

Day 2 adult animals were mounted on 7% agarose pad with 0.05µm polystyrene beads and serial images were captured (80 frames over a period of 120 seconds for a frame rate of 0.66 frames per second). Time-lapse images were converted to kymographs and contributing velocities of mitochondria were studied. Contributing velocity was determined by calculating the velocity with only the distances and associated time intervals that contributed to the mitochondrion's overall displacement along the neurite, such that the velocity of only anterograde movement in a mitochondrion that moved in that direction would be studied. This limited noise in velocity data caused by oscillating mitochondria or mitochondria that began moving later in the 2-minute experiment. Mobile mitochondria were determined as those moving at velocities above a threshold of 0.9 microns/second.

3.1.4 Behavioral Assay

Behavioral activity was observed using WormLab4.0 (MBF Bioscience, Williston, VT USA). Three minutes of adult Day 2 nematode activity were recorded. Scale was set at 12.89 microns/pixel. Worm track length (total forward and reverse motion) and straight line distance (displacement) were determined via WormLab. n=40-60.

3.1.5 N-Acetyl Glucosamine and D-Arginine Treatment

L4 animals were treated with 10mM N-Acetyl Glucosamine or D-Arginine for 6 hours for 2 consecutive days. Animals were returned to standard NGM plates and observed 24 hours later as Day 2 adult nematodes.

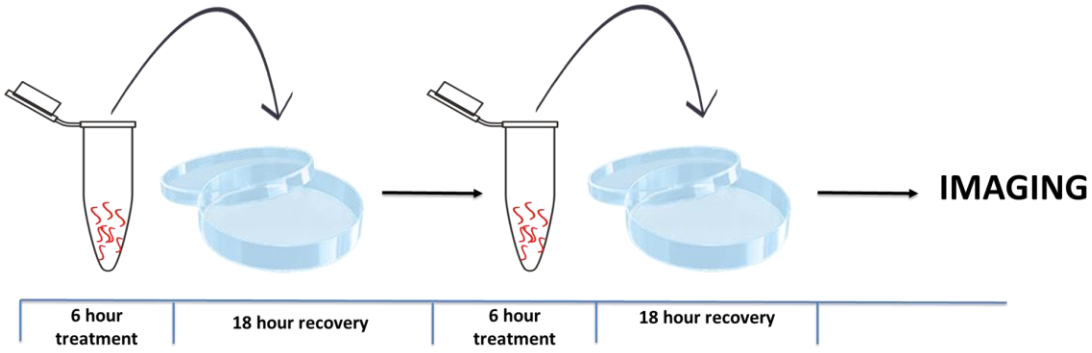

Figure 6 N-Acetyl Glucosamine and D-Arginine Treatment Protocol

3.1.6 Aldicarb Assay

Day 2 adult animals were placed on Aldicarb plates or 70% ethanol control plates (1mM concentration) and percentage of paralyzed animals counted at 30-minute intervals.

3.2 Results

3.2.1 FTDP-17-associated Tau Mutation Negatively Affects Animal Behavior

C. elegans expressing the FLTauV337M with F3ΔK280 (mAgg-Tau) mutations in all neurons were smaller, grew slower and laid fewer eggs as compared to controls. We used WormLab4.0 (MBF Bioscience, Williston, VT USA) to record and analyze worm activity, and found mAgg-Tau animals to be less active compared to our wild type N2 (Figure 7). Animals exhibited uncoordinated movement. As animals aged, these behavioral phenotypes worsened (data not shown).

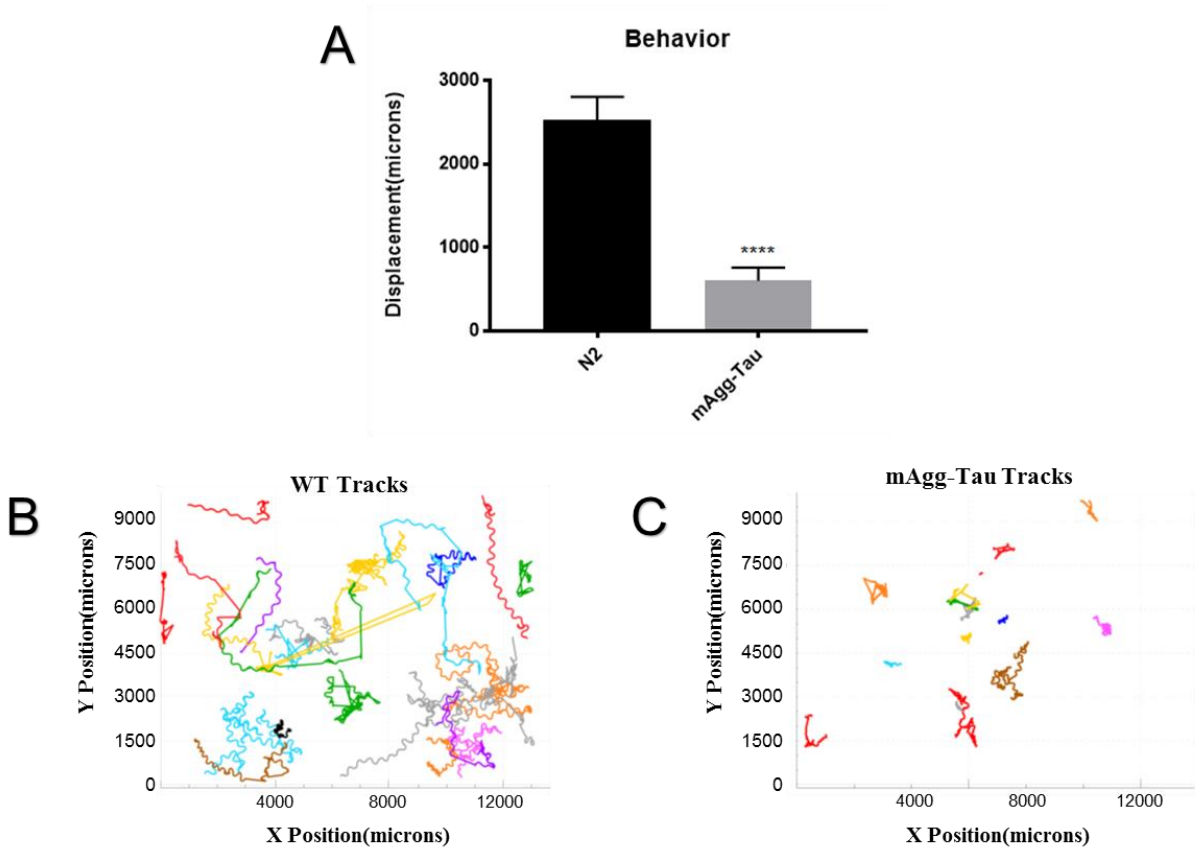

Figure 7. *C. elegans activity decreased under mAgg-Tau mutant background.*

[A] Activity measured as displacement of animals on non-seeded NGM plates in microns. n = 29-30. [B, C] Trace of animal movements. Subset of Adult day 2 animals observed shown with overlay of trace images. Error bars indicate SEM. Asterisks represent level of significance ($p<0.0001$).

3.2.2 Tau Mutations Lead to Impaired Global Transport

Introducing FTDP-17-associated Tau mutations to our *C. elegans* model system not only impacted behavior, but also altered neuronal physiology. As mentioned before, mitochondrial dysfunction has been implicated in neurodegenerative disorders. We used our MitoGFP marker to visualize the distribution of mitochondria in the ventral cord interneurons in mAgg-Tau strains. We focused primarily on the posterior region of our animals to properly observe transport along the neurite, away from anterior cell bodies and the strong pharyngeal GFP marker, which was coinjected with the FLTauV337M mutation (*myo-2::GFP*).

The mAgg-Tau strain showed distinctly fewer mitochondria at the posterior region compared to wild type (Figure 8A, B). The average size of the mitochondria did not significantly change, so fission and fusion dynamics were most likely not impaired (Figure 8C). The Baumeister group also studied mitochondrial distribution and observed a mitochondrial mislocalization phenotype in the DA9 neuron in the posterior region of the animal (Fatouros et al.). These results imply that the mAgg-Tau mutations impair mitochondrial transport or otherwise lead to mitochondrial damage.

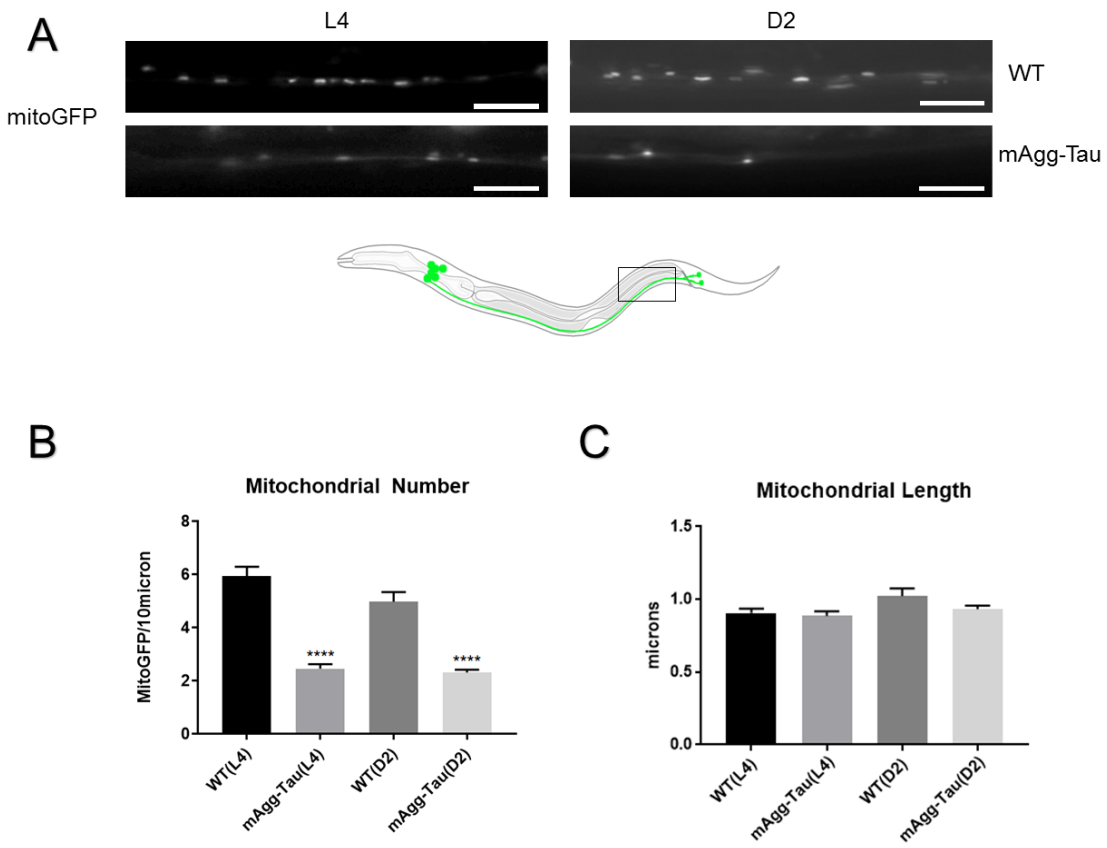

Figure 8 Mitochondrial number is significantly decreased in mAgg-Tau in both L4 and Adult Day 2 stage animals.

[A] Fluorescence was observed along the ventral cord neurite near the posterior of the animals as indicated. n = 10-35. [B, C] Mitochondrial number and length quantified using ImageJ. Error bars indicate SEM. Bar, 5µm. Asterisks represent level of significance ($p<0.0001$).

We wished to confirm that the phenotypes observed were the result of the aggregation, and not an artifact of the introduction of foreign proteins into the *C. elegans* model. To confirm that the aggregation is critical to this phenotype, we studied a strain expressing only human FLTauV337M mutation with the MitoGFP marker, without the ΔK280 mutation that promotes aggregation. While this strain did have comparatively fewer mitochondria, the extent of mutated Tau effect on mitochondrial number was milder (Figure 9). The severity of the phenotype is thus enhanced by aggregation-mediated toxicity.

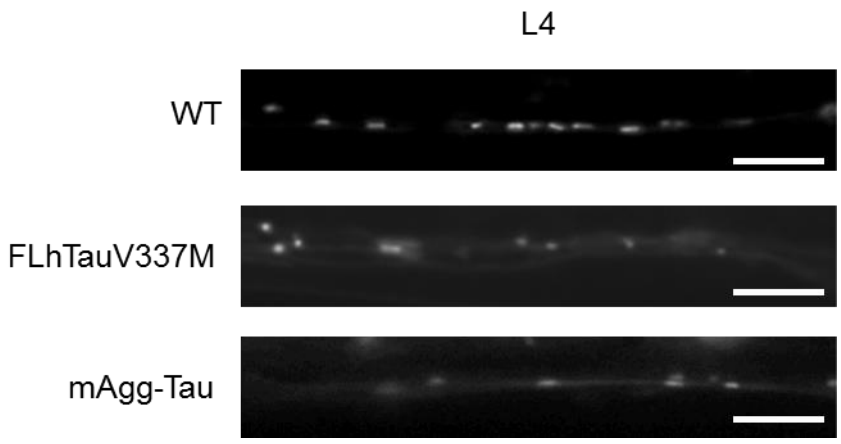

Figure 9 Mitochondrial dysfunction observed in the mAgg-Tau strain is aggregation-mediated.

Fluorescence was observed with the MitoGFP reporter along the ventral cord neurite near the posterior of the animals as indicated in Figure 7. mAgg-Tau indicates the combined expression of FLhTauV337M and ΔK280 mutations. Bar, 5μm.

As MitoGFP expression is driven by the *glr-1* promoter, the altered mitochondrial phenotype we observed may be symptomatic of impaired transport in the command interneurons. We therefore wanted to test whether Tau aggregation affects the localization of presynaptic and postsynaptic proteins as well, or if the aggregation specifically damaged mitochondria in this model. We visualized presynaptic terminals in our mAgg-Tau strains using a transgene that expresses GFP-tagged synaptobrevin-1 (SNB-1::GFP) from the *glr-1* promoter. SNB-1 plays an important role in synaptic transmission as a transport vesicle docking protein. We analyzed postsynaptic elements by studying GLR-1::GFP localization, as GLR-1 is an AMPA-type ionotropic glutamate receptor whose activity is vital for proper behavioral response, memory formation, and overall synaptic health.

Our analysis showed that distribution of presynaptic and postsynaptic components was significantly impaired in the mAgg-Tau mutant background. These defects in were observable in animals at the L4 stage, and the phenotype was more pronounced in the Day 2 adult stage, (Figure 10C, D). These results indicate that Tau aggregation may affect transport on a global

scale in a manner that progressively worsens with ageing. The aggregation-mediated toxicity is clearly impairing processes synaptic profiles, though it is not yet clear whether the cause is due to some damage to the microtubules or because of the aggregated protein tangles.

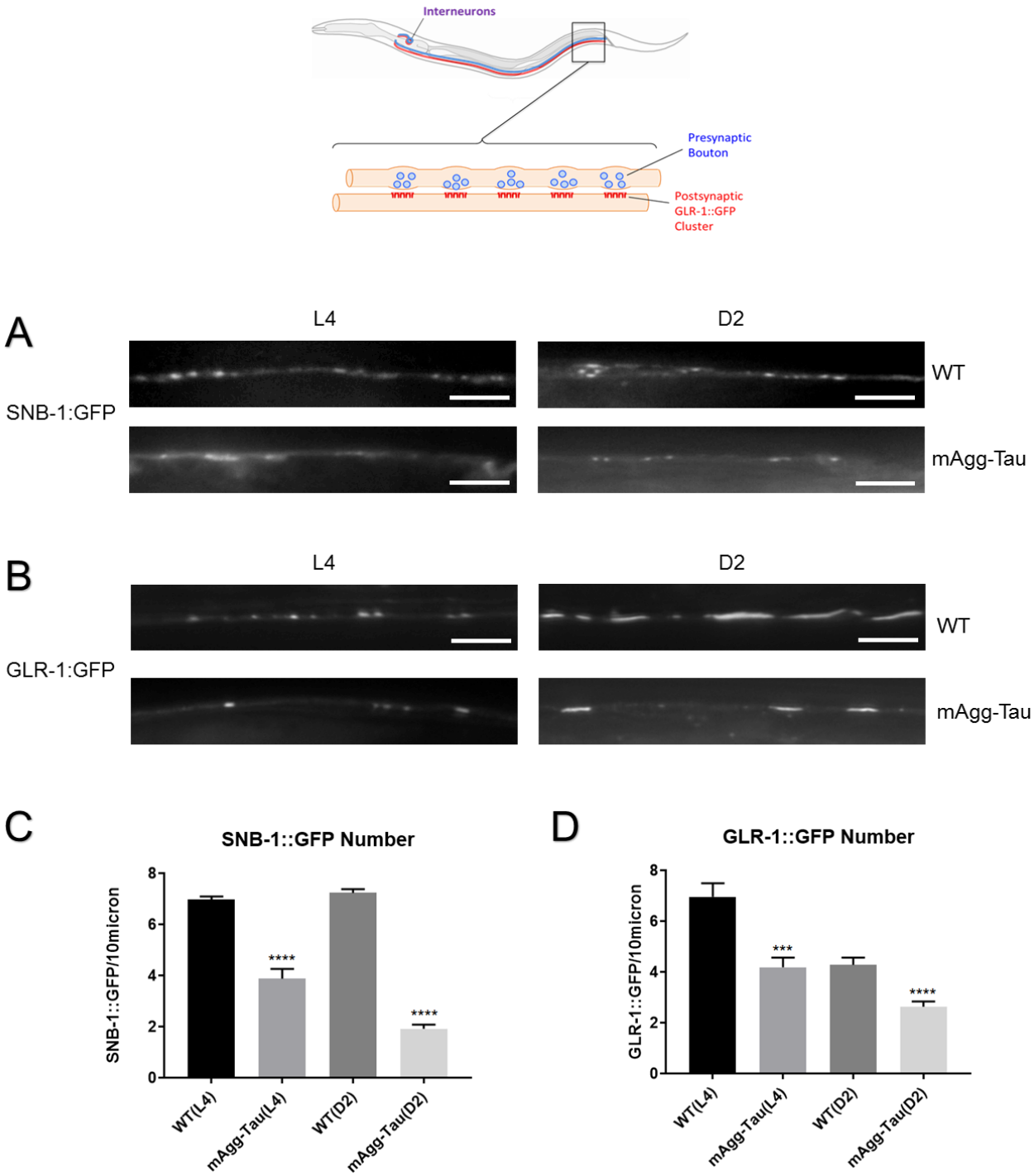

Figure 10 mAgg-Tau Strains Exhibit Impaired Transport of Presynaptic and Postsynaptic Components Necessary for Proper Synaptic Transmission.

[A, B] Fluorescence was observed along the ventral cord neurite near the posterior of the animals as indicated. [C, D] Mitochondrial number quantified using ImageJ. Error bars indicate SEM. Bar, 5µm. Asterisks represent level of significance ($p<0.0001$).

3.2.3 Mitochondrial Transport Impairment Is Not a Result of Microtubule Destabilization

Current models to explain the neurodegeneration observed in Tauopathies often emphasize the role of functional Tau in maintaining microtubule stability. It is thought that hyper-phosphorylated Tau dissociates from the microtubules to create neurofibrillary tangles, the subsequent microtubule destabilization may play a role in the observed neurodegeneration in Tauopathies. It is not yet clear, however, whether this destabilization is the cause of neurodegeneration, or if the aggregation is the primary culprit. Utilizing mitochondria as a readout of our established mAgg-Tau model, we sought to determine whether the transport defects we observed are a result of microtubule destabilization or the aggregates themselves.

We began by studying the extent of the potential microtubule damage by overexpressing a chimeric transport protein Kinesin-Tom7 (R. L. Rawson et al.). Kinesin-1 is a microtubule-based motor that is required for successful synaptic vesicle and organelle transport across microtubules (Figure 11). Tom7 is an essential mitochondrial import protein located in the outer membrane of the mitochondria which, in our model, will interact with another constructed fusion protein, Tom20::mCherry. The Tom7 will bind with the translocation of outer membrane complex (TOM) machinery, which includes the Tom20::mCherry we've introduced. Because Tom7 is linked to the Kinesin and is overexpressed, it will effectively force the bound mitochondria to traverse the microtubule. We overexpressed this chimeric protein and the Tom20::mCherry protein in GABA-type neurons under the *unc-47* promoter, and while Kinesin-Tom7 is also interacting with endogenous Tom20, we can observe its effect on transport with the Tom20::mCherry marker. GABA is an inhibitory neurotransmitter that mainly acts in neuromuscular synapses.

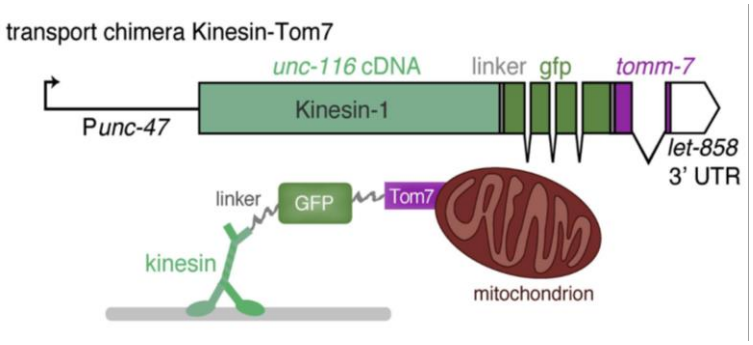

Figure 11 Kinesin-Tom7 Schematic. (Randi L. Rawson et al.).

If the microtubules have been damaged because of the mAgg-Tau mutations, then overexpression of the Kinesin-Tom7 motor should not improve the mitochondrial phenotype, as these are microtubule-based motors. However, we observed that overexpression of the Kinesin-Tom7 chimeric protein led to moderate recovery of the mitochondrial phenotype (Figure 12). The fact that the mitochondrial number increased under this forced-transport method indicates that damaged microtubules may not be the cause of the apparent dysfunctional transport we observed.

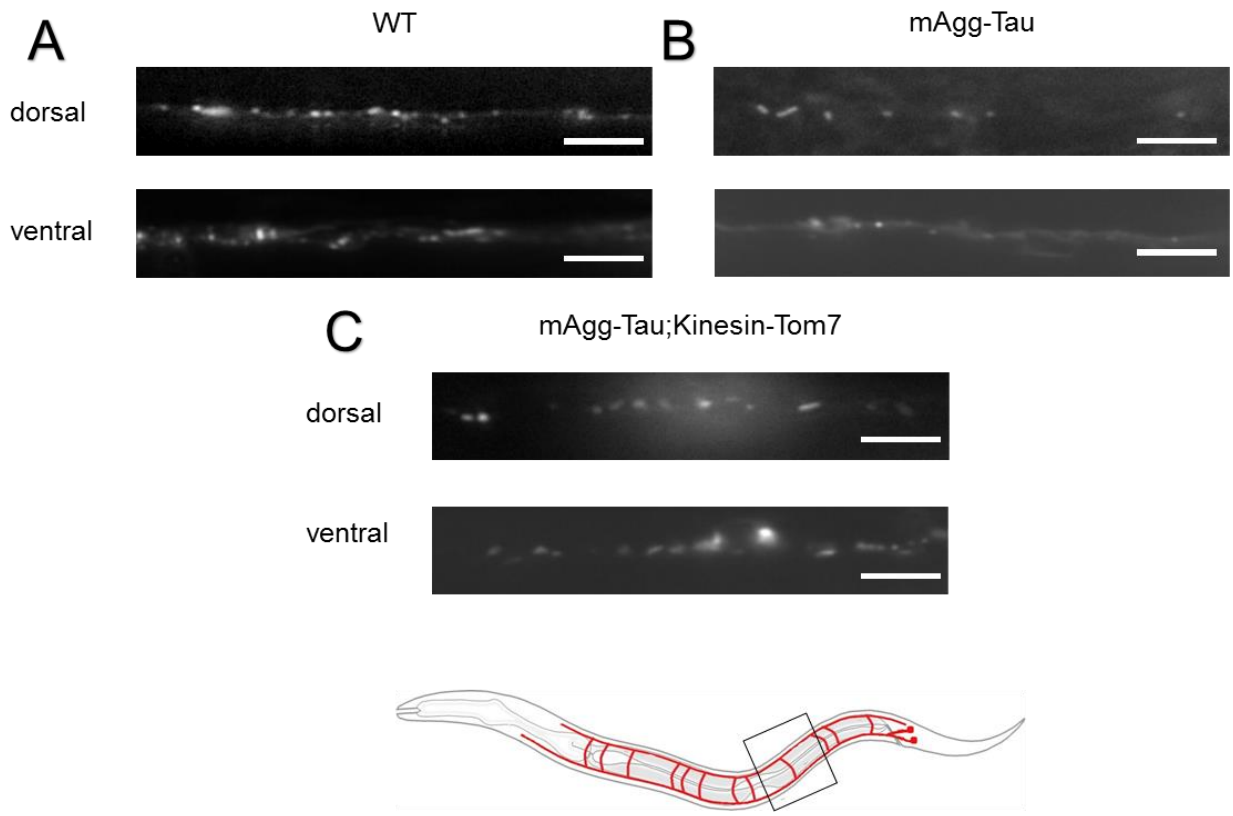

Figure 12 Overexpression of Kinesin-Tom7 Motor Protein Increases Mitochondrial Number.

[A, B, C] Fluorescence was observed along the ventral cord neurite near the posterior of the animals as indicated. Bar, 5μm

There is a possibility that microtubule damage does occur, and that the mitochondrial number increase we observe is a result of the presence of endogenous *C. elegans* PTL-1, named for protein for tau-like repeats, which is a homologue of the human Tau protein (McDermott, Aamodt and Aamodt). PTL-1 is also a microtubule-associated protein and has been found to regulate lifespan, though its exact role in microtubule stabilization is still under investigation. If PTL-1 stabilizes the microtubule through a similar mechanism as Tau, then we cannot rule out microtubule damage as a possible cause for the transport defects. This is because the endogenous PTL-1 may be interacting with the microtubules or mAgg-Tau proteins. Alternatively, mAgg-

Tau aggregates could be sequestering functional PTL-1 away from the microtubules, resulting in destabilization.

We wanted to confirm whether or not such an interaction occurred by analyzing the mitochondrial distribution in both *ptl-1* single mutants and *ptl-1;*mAgg-Tau triple mutants. If PTL-1 functioned in a similar manner to Tau in its role in microtubule stability in neurons, then we would expect to see an impaired mitochondrial transport in when it is missing. Instead, we found that the absence of PTL-1 does not alter the mitochondrial distribution compared to the wild-type strain (Figure 13B). This result rules out a model in which mAgg-Tau impairs mitochondrial transport by sequestering (and thus impairing) endogenous PTL-1.

Under normal conditions, PTL-1 is not required for mitochondrial transport. In contrast, PTL-1's role in regulating transport along microtubules might be as a kind of regulatory sign post that is present on the microtubules in gradients, opposing the retrograde movement of kinesins and the anterograde movement of dyneins during axonal transport (Tien et al.). PTL-1 supports microtubule stability by regulating transport, and because the anterograde movement of kinesins is not opposed, the presence or absence of PTL-1 will not show a strong phenotypic change in the unipolar command interneurons as they require mostly anterograde movement.

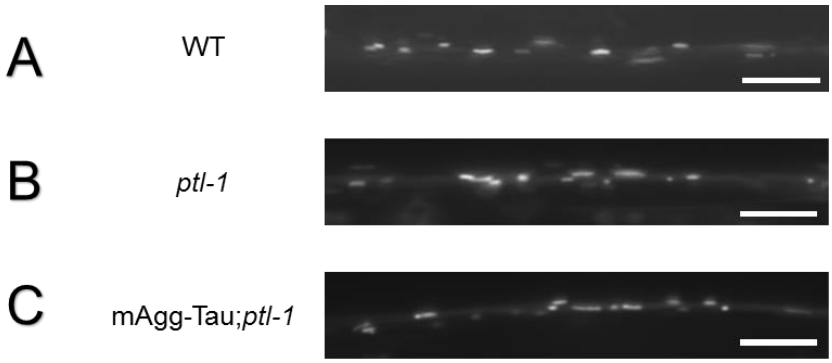

Figure 13 PTL-1 depletion in both mAgg-Tau and control strains does not exhibit mitochondrial transport impairment.

[A, B, C] Fluorescence was observed along the ventral cord neurite near the posterior of the animals as indicated. Bar, 5µm

Interestingly, PTL-1 depletion reverses the mitochondrial phenotype in the mAgg-Tau strain (Figure 13C). This further affirms that PTL-1 is unlikely to be stabilizing the microtubule in the same manner as human Tau, as we would not expect an improved mitochondrial transport phenotype if that were the case. Intuitively, the removal of an obstacle hindering the anterograde and/or retrograde movements of kinesins and dyneins would likely improve transport, particularly if the Tau aggregation itself is the cause of the transport defect. The presence of PTL-1 acts as a roadblock for motor proteins, and while this is useful for maintaining a balance in vesicular transport, it would likely be an additional burden on vesicular transport in strains dealing with Tau aggregation under the mAgg-Tau background. The lack of this roadblock in *ptl-1* mutants may help mitigate the transport effect observed in neurons with aggregated Tau. Further study is required to quantify the intensity of the phenotypic rescue by the *ptl-1* knockout, as well as to address whether endogenous PTL-1 is required to observe the mAgg-Tau pathology.

To more directly observe the relationship between PTL-1 and the microtubule network itself, we utilized the well-established EBP-2::GFP reporter to visualize the growing plus ends of

microtubules in *ptl-1* mutant backgrounds. When microtubules are stabilized, EBP-2::GFP is diffuse along the neurite. However, if microtubules become damaged (severed) and/or begin regenerating following insult, EBP-2::GFP assumes a punctate appearance (Figure 14).

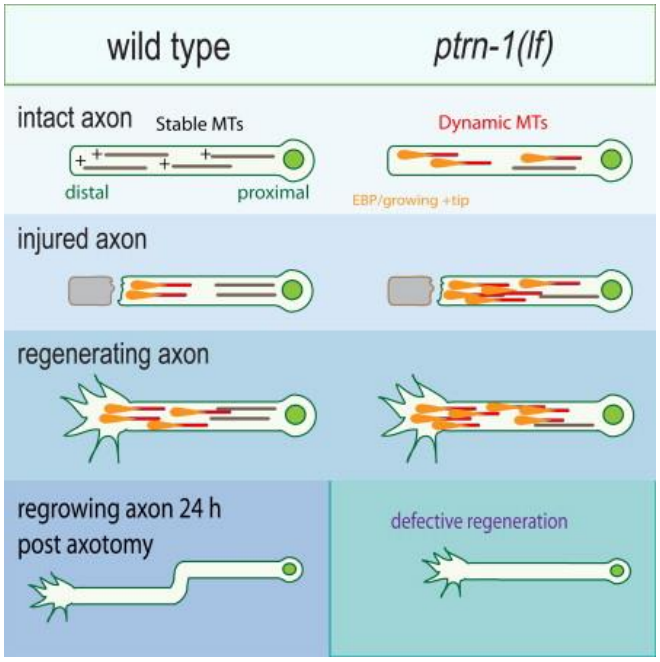

Figure 14 Graphical Representation of EBP::GFP localization (orange) in injured or regenerating microtubules (Chuang et al.).

Under normal conditions, EBP-2::GFP was mostly diffuse in both the wild-type and *ptl-1* single mutant, indicating that the microtubules were stabilized in both strains (data not shown). However, following 30 minutes on an agarose pad for microscopy (a condition which results in hypoxic stress), EBP-2::GFP became more punctate in the *ptl-1* mutant as compared to wild type (Figure 15). This could either mean that the absence of PTL-1 allowed for more immediate, less-regulated movement of the EBP-2 protein, or that PTL-1 depletion itself led the microtubules to be more susceptible to damage, thus resulting in the punctate phenotype. Further study is required to identify this distinction; however, from the phenotypes of EBP-2::GFP under normal conditions, we can conclude PTL-1 is not stabilizing the microtubule in the same manner as Tau.

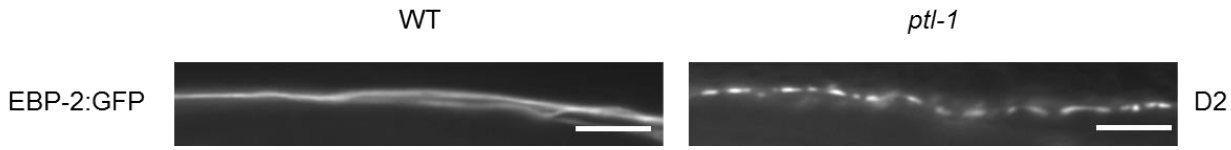

Figure 15 EBP-2::GFP is more punctate in ptl-1 mutant in stress conditions of extended time on a mounting pad.

3.2.4 N-Acetyl Glucosamine Treatment Improves Global Transport in mAgg-Tau Strain

We have been using mitochondria and other synaptic transport vesicles as read-outs for the neuronal dysfunction that is associated with Tauopathies. With evidence to support that the impairment is unlikely to be caused by microtubule destabilization caused by Tau dissociation, we can address whether removing the Tau aggregation itself will alleviate the Tau pathology. To decrease the mutant Tau levels, we must consider the protein homeostatic processes that are involved in the removal of misfolded, damaged, or aggregated proteins. The Hexosamine Pathway is a particularly relevant pathway that has been linked to the suppression of proteotoxic proteins, such as PolyQ repeats (of Huntington's Disease) and α-synuclein (Parkinson's disease). A study found that N-Acetyl Glucosamine (NAG) supplementation improved motor function and reduced toxicity in *C. elegans* animals in both models, and has also been found to improve protein homeostasis in the Endoplasmic Reticulum (ER) (Denzel et al.). It is believed that increasing the endogenous levels of the metabolite UDP-GlcNAc not only led to improved ER function via glycosylation, ER-associated protein degradation (ERAD) function, and proteasome signaling, but also to broad-spectrum enhancements in protein quality control in general.

We wanted to see if NAG supplementation in our mAgg-Tau model would also lead to improved behavioral and neuronal transport phenotypes. The expectation was that if increasing the levels of NAG would really enhance protein quality control mechanisms and lead to the removal of the Tau aggregation, then we would be able to observe this improvement *in vivo*.

Indeed, we saw significant improvement in presynaptic, postsynaptic, and mitochondrial distribution following treatment compared to our D-Arginine-treated osmolarity controls (Figure 16).

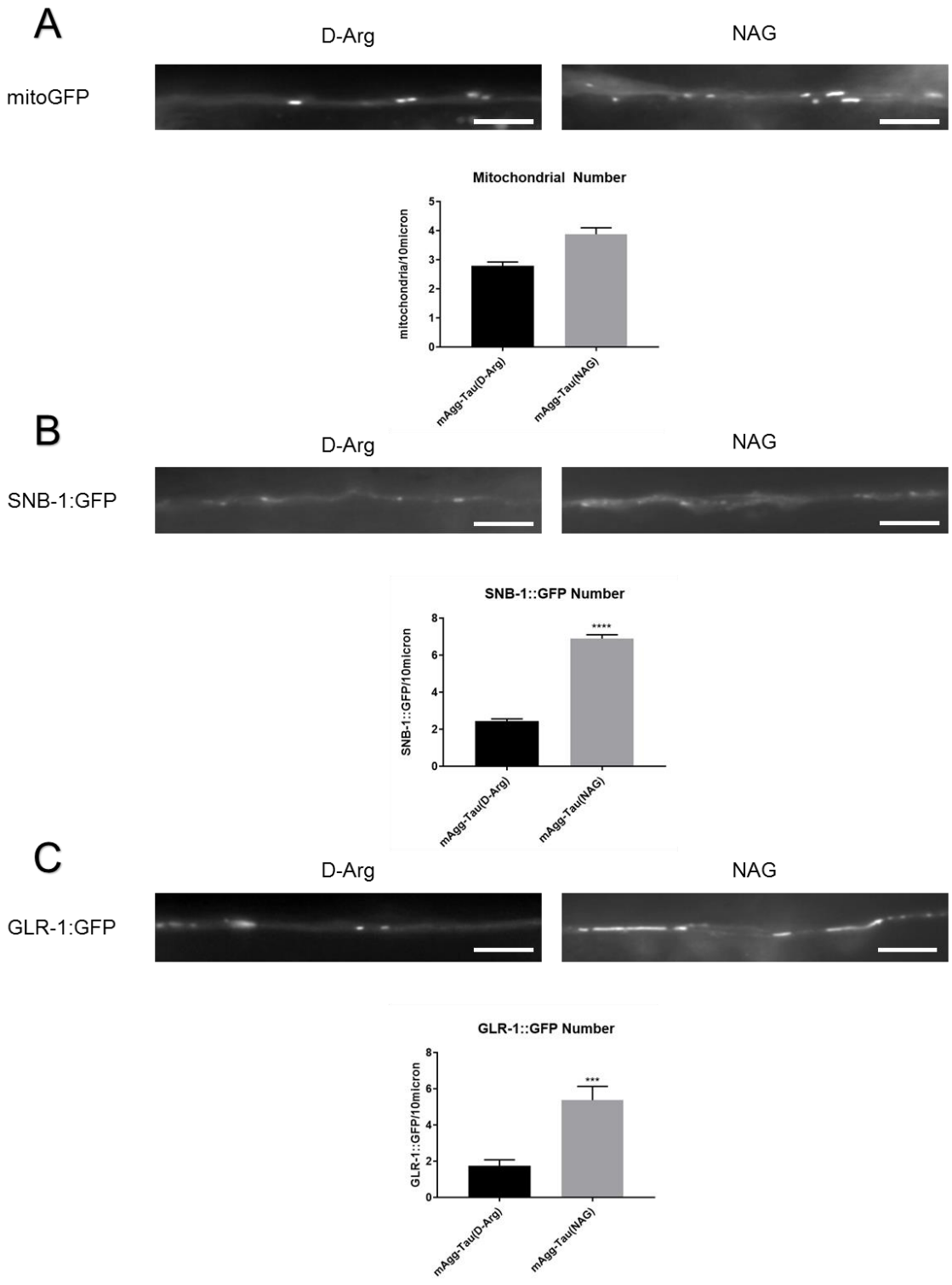

Figure 16 Global Transport is Significantly Improved in mAgg-Tau Strains following NAG Supplementation.

[A, B, C] C. elegans were treated for 6 hours/day with 10mM NAG for a total of two days. Fluorescence was observed along the ventral cord neurite near the posterior of the animals the following day at the Day 2 stage. GFP number was quantified using ImageJ. Error bars indicate SEM. Bar, 5µm. Asterisks represent level of significance ($p<0.0001$).

To confirm that the effects of NAG were not limited to command interneurons, we tested the effects of NAG in our GABA-type neurons to similar results (Figure 17). However, due to worm-to-worm variability and the fact that the Kinesin-Tom7 and Tom20::mCherry proteins free-arrays, the effect proved difficult to score.

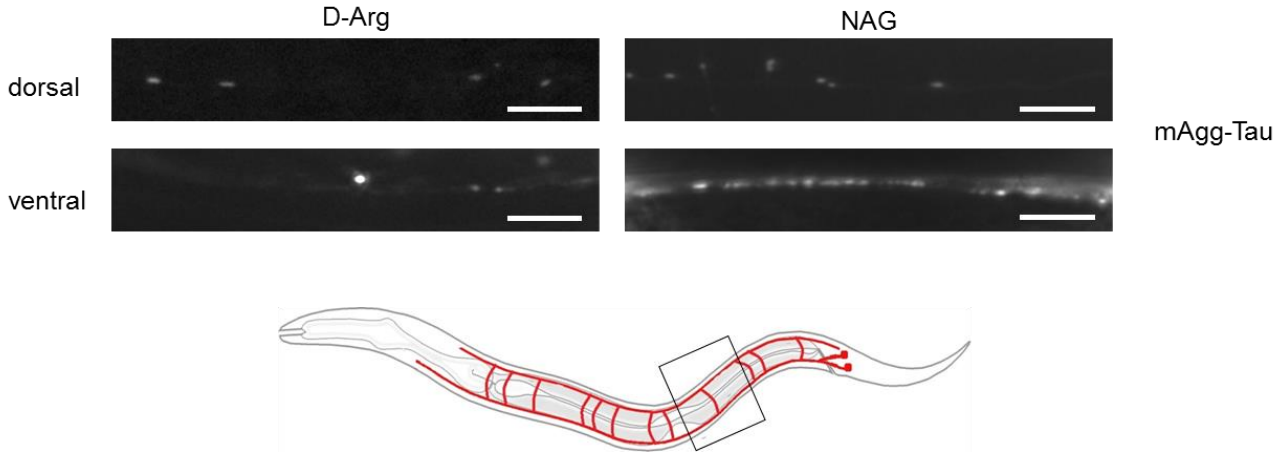

Figure 17 NAG Supplementation Also Improves Mitochondria Transport in GABA-Type Neurons.

C. elegans were treated for 6 hours/day with 10mM NAG for a total of two days. Fluorescence was observed along the ventral cord neurite near the posterior of the animals the following day at the Day 2 stage. Bar, 5µm.

This improvement in mitochondrial transport was further confirmed through kymograph analysis of the wild-type, D-arginine- and N-Acetyl Glucosamine-treated animals. Active mitochondria, identified as any mitochondria that traveled at contributing velocities faster than the threshold of 0.9µm/second, were more abundant in wild-type and NAG-treated animals (Figure 18 B, C). Contributing velocities were measured as the average distance traveled over the span of time that contributed to the forward or reverse displacement of the mitochondrion. This method of calculating mitochondria velocity does not take into account times when the

mitochondria are immobile or moving in the opposite direction of its original movement, reducing noise. There was also a distinct difference in the range of contributing velocities measured in the D-Arginine animals (between 1.07 μm/second to 1.85 μm/second), and the wild-type (1.20 μm/second to 5.00 μm/second) and N-Acetyl Glucosamine animals (0.90 μm/second to 5.98 μm/second) (Figure 17D). As D-Arginine supplementation is a negative control for any effect that a change in osmolarity may have on *C. elegans* mAgg-Tau phenotype following N-Acetyl Glucosamine treatment, the marked differences between these two groups indicates the improved mitochondrial phenotype is a benefit derived from NAG supplementation itself. These results support the idea that transport can be improved if protein quality control mechanisms are more active.

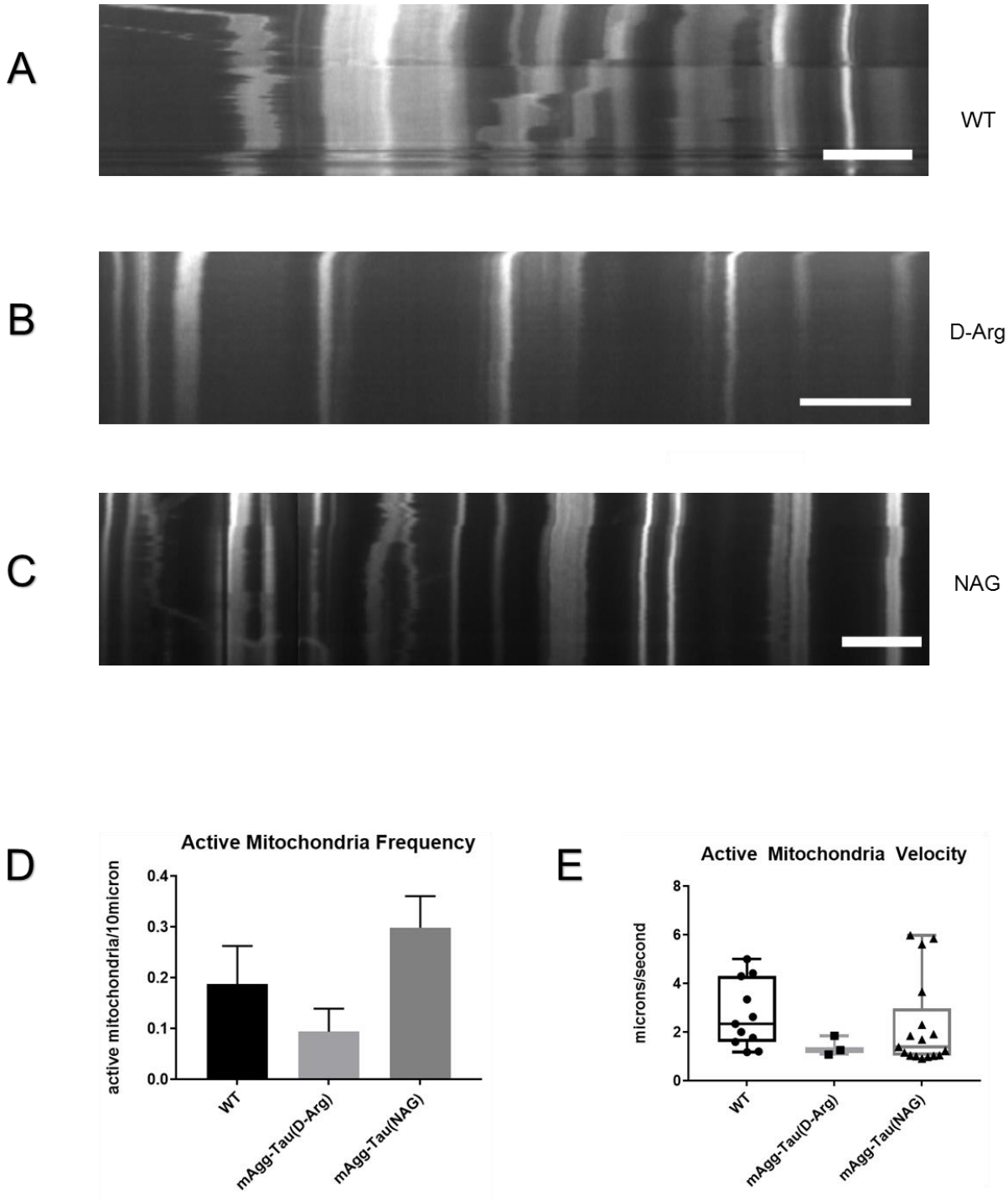

Figure 18 Increased Mitochondrial Activity Observed in NAG-treated mAgg-Tau Animals.

[A, B, C] Kymograph constructed using ImageJ macros to convert and quantify Time-Lapse Images taken with iVision program (80 frames, 120 seconds). Error bars indicate SEM. Bar, 5μm.

We also studied the behavior of these NAG-treated mAgg-Tau animals, as well as mAgg-Tau animals expressing a *gfat-1* gain-of-function mutation. GFAT-1 is an upstream regulator of the Hexosamine Pathway. Increasing GFAT-1 levels should increase endogenous NAG levels, resulting in improved neuronal proteostatic activity. Of course, we have been using transport as a read-out for degeneration, but if these improvements to the Tau pathology also restore neuronal function, then we should observe an improvement in the animals' behavior as well. Indeed, we find that both NAG-supplemented and *gfat-1* gain of function animals exhibit active behavioral phenotype and less uncoordinated behavior (Figure 19).

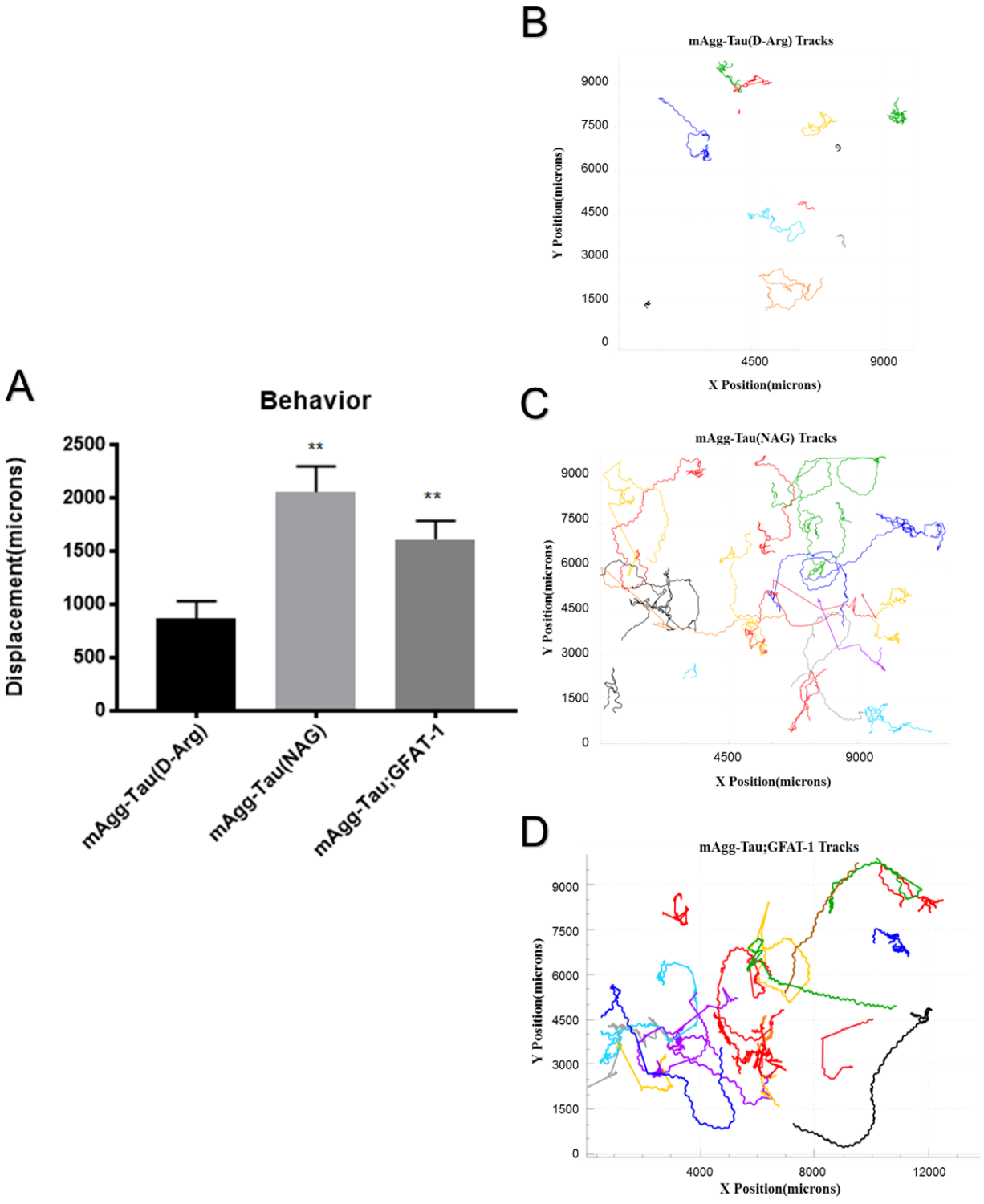

Figure 19 Increase in Hexosamine Pathway Metabolite, N-Acetyl Glucosamine, improves mAgg-Tau C. elegans Behavioral Activity.

[A] Activity observed using WormLab program, and quantified as total displacement. [B, C, D] Tracks summarize three minutes Day-2 nematode activity. [B] mAgg-Tau animals treated with 10mM D-Arginine, [C] treated with 10mM N-Acetyl Glucosamine, and [D] expressing GFAT-1 gain-of-function mutation. Error bars indicate SEM. Asterisks represent level of significance ($p<0.0001$).

While the exact mechanism is not yet clear, our results indicate that NAG supplementation likely improves neuronal transport through Tau aggregation clearance. This clearance allows proteins and important organelles such as mitochondria to travel more freely along microtubules, and should improve synaptic activity. We tested synaptic activity using an established aldicarb assay which studies resistance to aldicarb-induced paralysis (Mahoney, Luo and Nonet). Aldicarb is an acetylcholinesterase inhibitor which prevents the hydrolysis of acetylcholine. The subsequent buildup of acetylcholine in the synapse leads to hyper-contraction as a result of overactive cholinergic receptors, which eventually causes paralysis and death in nematodes. Animals that release less acetylcholine will experience paralysis at a slower rate than wild-type animals. Through this assay, we confirmed that NAG treatment improves synaptic activity through an aldicarb assay, which shows that NAG-treated mAgg-Tau animals paralyze similarly to wild-type animals (Figure 20).

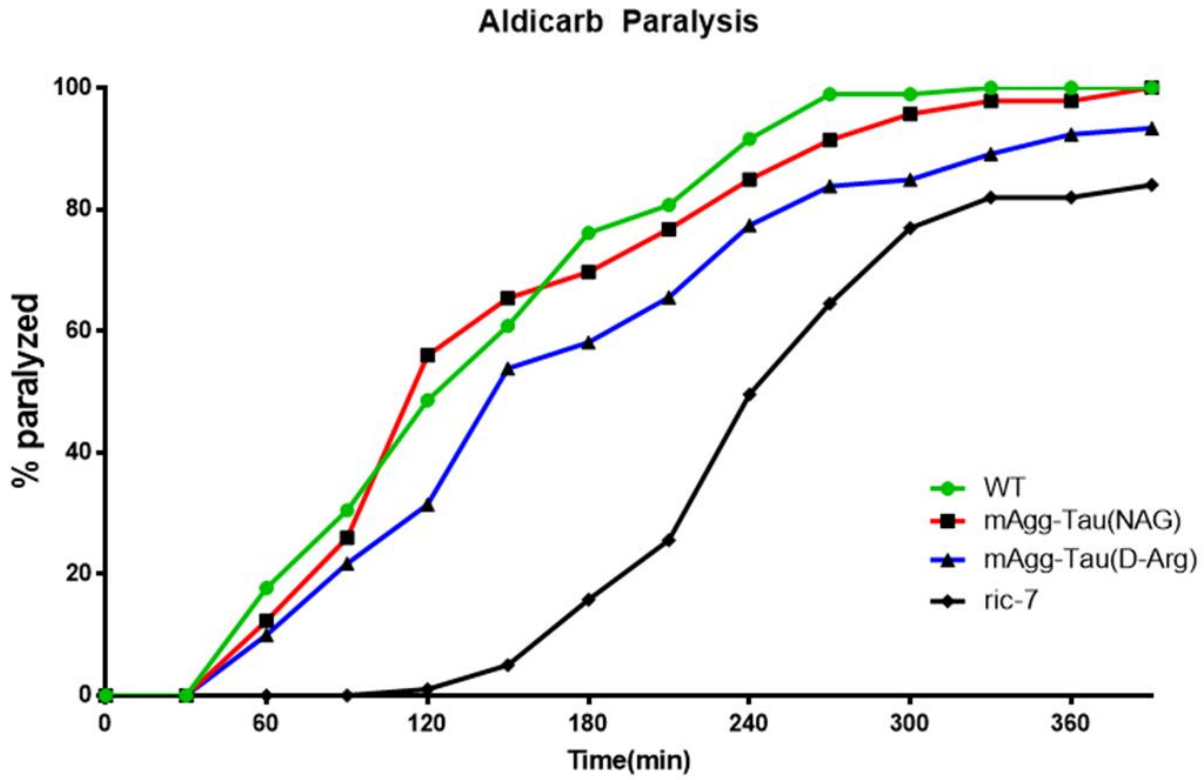

Figure 20 NAG-Supplemented mAgg-Tau Animals Exhibit Improved Synaptic Activity.

Animals were exposed to 100mM aldicarb in NGM OP50 plates. Observations were completed at 30-minute intervals.

3.3 Discussion

Here we have shown the potential of the Hexosamine Pathway and its metabolite, N-Acetyl Glucosamine, as a possible drug target for improved Tau aggregation clearance. With the ability to study neuronal transport *in vivo*, as well as the ability to observe Tau aggregation levels, this *C. elegans* model has a lot of utility for preliminary screening to identifying potential therapeutic mechanisms. With the benefits of this model, future analysis of whether the Hexosamine Pathway is preventing further nucleation or actively removing Tau aggregates can be studied. To strengthen the model, particularly for a genetic screen, an important subsequent

step would be to create a new transgenic mAgg-Tau line which has both mutations co-injected into the same chromosome, so that more cross possibilities can be allowed.

Establishing this model for future genetic analysis allows us to study Tauopathy and its detailed mechanistic effects—such as the transport defects—*in vivo*. While Tauopathies exhibit both microtubule destabilization and Tau aggregation, it is not clear which is involved in the toxicity observed. Our model, however, reveals that microtubule destabilization is not likely to be the main cause of the neurodegeneration, as neuronal communication and transport was impaired (Figure 8,9,10) despite microtubule stability in our model (Figure 12). These observations must be qualified with further studies in the role of endogenous PTL-1 in both normal and mAgg-Tau mutant conditions. Kymograph analysis of mitochondrial mobility in the *ptl-1* mutant and EBP-2::GFP strains expressing mAgg-Tau will be particularly insightful.

To confirm our findings, we will also be quantifying the levels of Tau aggregation with a Thioflavin-S staining protocol as utilized by the Baumeister group (Fatouros et al.). This will allow us to directly observe whether or not NAG treatment decreases the level of Tau aggregation to improve transport and the overall Tauopathy phenotype. This staining protocol will also be useful to determine whether Tau aggregation is seen in *ptl-1* mutants expressing the mAgg-Tau proteins. Clarification on these points will improve our understanding of the source of neurodegeneration in Tauopathies, and give us a more established model through which we can search for improved therapies for Tauopathies such as Alzheimer's disease.

4. The Role of CHN-1 in the Hypoxia Response Pathway

4.1 Materials and Methods

4.1.1 Strain List

Animals were grown at 20 °C on standard NGM plates seeded with OP50, unless otherwise mentioned. The strains constructed for the study were:

odIs70[Pglr-1::MitoGFP, Cbunc-119], N2/wild-type, *chn-1(by155),* OR3588: *odIs70; chn-1(by155), egl-9(sa307),hif-1*(*ia3),*OR2975: *odIs70*; *hif-1(ia3),* OR3847: *chn-1(by155);egl-9(sa307),*OR3848: *odIs70*; *chn-1(by155); egl-9(sa307),*OR3690: *chn-1(by155)1;hif-1(ia3),*OR3688: *odIs70*; *chn-1(by155)1;hif-1(ia3),*OR3693: *odIs130[hif-1-2xTY1-GFP-3xFlag, Cbunc119(+), nat, cat*];*chn-1(by155),*OR3336: *odIs130*[*hif-1-2xTY1-GFP-3xFlag, Cbunc119(+), nat, cat*], MT20483: nIs470, OR3850: *nIs470*; *chn-1(by155),* OR3689*: chn-1 (by155); odIs130,* OR3215*: nIs470[cysl-2/PK10H10.2::Venus; Pmyo-2::mCherry]IV; egl-9(sa307)V,* OR3248*: nIs470[cysl-2/PK10H10.2::Venus; Pmyo-2::mCherry]IV; hif-1(ia4) V, odIs130[TransgeneOme Fosmid clone 38576411703852465 A04, identifier WBGene00001851(hif-1-2xTY1-GFP-3xFlag, Cbunc119(+), nat, cat)]*

4.1.2 Fluorescence Microscopy

GFP- and mCherry-tagged fluorescent proteins were visualized in nematodes by mounting L4 and young adult animals on 2% agarose pads with 20mM levamisole using an epifluorescent microscope, unless otherwise mentioned.

4.1.3 Anoxia Survival Assay

Approximately 40 nematodes per strain per plate were placed in transparent AnaeroPack-Anaero sachets. To confirm anoxic conditions within each sachet, a methylene blue test was used. To normalize each experimental replicate, all genotypes were placed into the same sachet for the

times indicated, and control genotypes were carefully evaluated for suspended animation behavior to verify that anoxic conditions were generated. Plates were removed from the sachets after 72 hours and 96 hours, and then nematodes which showed movement 24 hours later were counted as having survived.

4.1.4 24-hour Emergence Assay

Approximate 30 animals were picked to NGM plates seeded with OP50, which were placed in AnaeroPack-Anaero sachets. A wild-type control was included along with the mutant strains. Following 24 hours of anoxia treatment, the plates were removed and simultaneously quantified for the number of animals in suspended animation versus moving (emerged) after five and after ten minutes. Additionally, nematodes were checked after 15 minutes of opening the bag via gently prodding to confirm the final number of *C. elegans* that emerged after 24 hours of anoxia treatment.

4.1.5 Anoxia-Reoxygenation Assay

Approximate 30 animals were picked to NGM plates seeded with OP50, which were placed in AnaeroPack-Anaero sachets. A wild-type control was included along with the mutant strains. Following 24 hours of anoxia treatment, the nematodes were immediately observed under an epifluorescent microscope for any differences in mitochondrial dynamics under anoxia. Nematodes from the same plates were checked again after three hours for changes in mitochondrial dynamics following reoxygenation. Mitochondrial lengths were quantified.

4.1.6 Hypoxia Exposure

Animals were incubated in a hypoxia chamber (C-174 chamber, Biospherix) at a controlled oxygen level (ProOx P110, Biospherix) for either 4 hours or 24 hours at 20°C.

4.1.7 RNA Preparation

Total RNAs to be extracted with Trizol (Invitrogen Co., Carlsbad, CA). L4 stage worms (20-30 animals each) were resuspended in 250 µl of Trizol and 20µl of M9 Buffer, and subsequently frozen in liquid nitrogen. 3 replicates for each of the N2, *chn-1, hif-1,* and *chn-1;hif-1* strains were prepared for L4 and D1 adults for normoxia and hypoxia treated animals.

4.1.8 Real-Time qRT-PCR

RNA samples were thawed with vigorous vortexing in 4°C for 30 min. PCR was performed utilizing an Eco real-time qPCR system (Illumina, San Diego, CA) using iScriptTM One-Step RT-PCR Kit with SYBR Green (Bio-Rad Laboratories Inc., Hercules, CA). For *nhr-57*, we used as forward (5′-CGTGATTGCAGACTTGAAAGC-3′) and reverse (5′-GCGTTTGACTTCCATCGTTTG-3′) primers. For *act-1*, we used as forward (5′-ACCATGTACCCAGGAATTGC-3′) and reverse (5′-TGGAAGGTGGAGAGGGAAG-3′) primers. Samples were measured 2 to 3 times and average values were used for the calculation of relative fold changes. The relative levels of *nhr-57* mRNA were normalized to the levels of *act-1* mRNA in each preparation. For each experiment, the value for wild type was set to 1 and other values were normalized accordingly.

4.2 Results

As in the previous study, mitochondria can be seen to play a prominent role in neuronal stress responses. In this study, we examine whether CHN-1, through its actions on the hypoxia response pathway, regulates mitochondrial dynamics following anoxia-reoxygenation. In order to test mitochondrial dynamics, we introduced a mitochondrial reporter (a transgene containing the *glr-1* promoter fused to a mitochondrial matrix localization signal MitoGFP) into *chn-1* mutants. We observed animals under normoxic conditions, immediately following anoxia

treatment, and 3 hours after anoxia treatment (the reoxygenation phase). All strains had a mitochondrial fission phenotype immediately following anoxia. We found that *chn-1* mutant mitochondria underwent mitochondrial hyperfusion upon anoxia-reoxygenation treatment (Figure 21A), and also observed that there was a window during which hyperfusion could be best observed, which is approximately 2 to 4 hours of reoxygenation (Figure 21B).

The mitochondrial phenotype of *chn-1* mutants following anoxia treatment closely mimics the phenotype seen in *egl-9* mutants: both strains show mitochondrial hyperfusion following anoxia-reoxygenation (Figure 21C). We are now looking into how exactly CHN-1 fits into the hypoxia response pathway. Because *chn-1* mutants showed similarities in mitochondrial dynamics to *egl-9* mutants, we will be completing a comparative study of the roles these two genes play in the hypoxia-response pathway. Although the data collection stage is not complete, ongoing experiments will be outlined below.

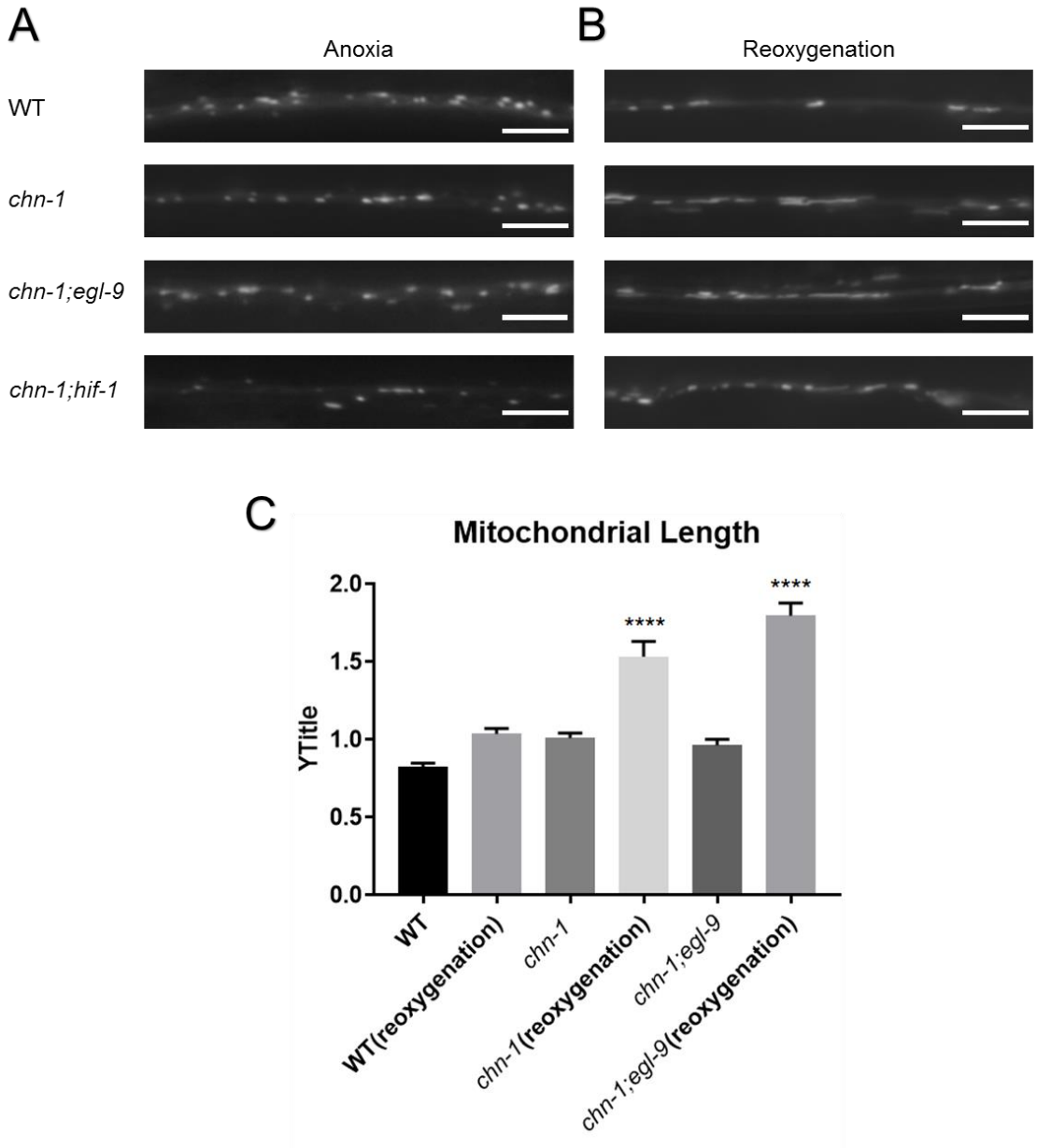

Figure 21 Mutant for chn-1undergoes egl-9-like mitochondrial hyperfusion upon anoxia recovery in HIF-1-dependent manner.

[A, B] Fluorescence was observed along the ventral cord neurite near the posterior of the animals the following day at the Day 2 stage. [C] Mitochondrial number quantified using ImageJ. Error bars indicate SEM. Bar, 5µm. Asterisks represent level of significance ($p<0.0001$).

In order to more directly observe the effect of CHN-1 on HIF-1 target genes, we constructed a strain with an integrated Venus-tagged HIF-1 transcriptional reporter CYSL-2 (*nIs470*) under a *chn-1* mutant background. The Horvitz Lab generated this reporter and tested in an *egl-9* mutant background, finding that *egl-9* mutants showed elevated levels of GFP signal compared to both wild-type and *egl-9; hif-1* double mutants, which showed no GFP fluorescence (Ma et al.). Consistent with these results and the current proposed model, we found that the *chn-1* mutants with this reporter showed greater GFP fluorescence, particularly compared to the wild-type strain, though to a lesser extent than the *egl-9* mutant (Figure 22.)

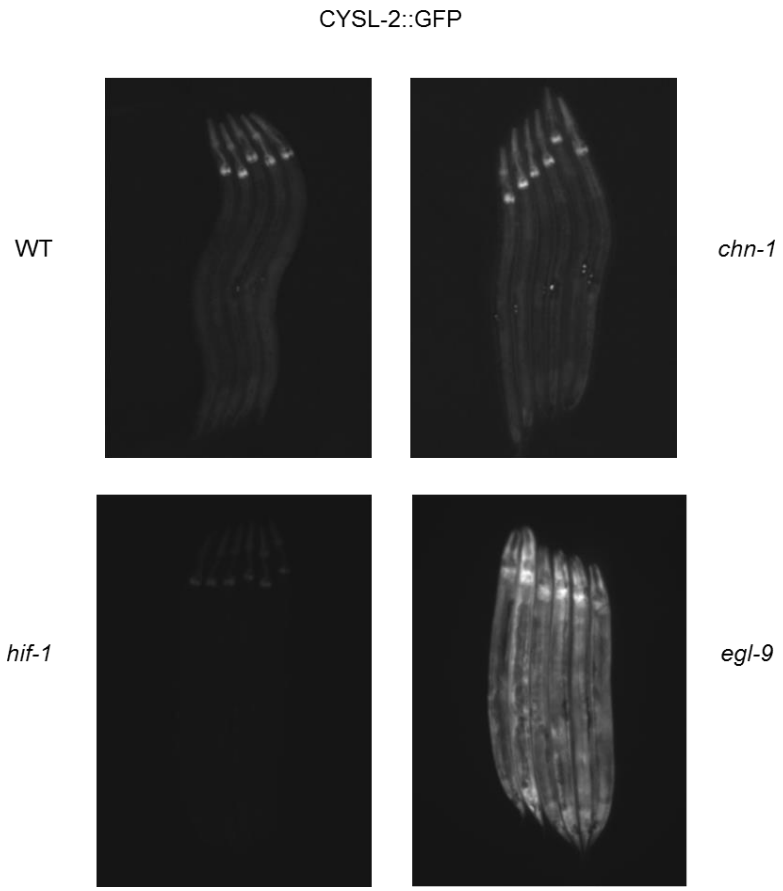

Figure 22 HIF-1 target gene CYSL-2 is expressed at higher levels under chn-1 mutant background.

Late-stage L4 animals were observed at 5x magnitude under normal conditions, without any treatment. Exposure 400, gain 0.

Following anoxia, *C. elegans* undergo an immobile state known as suspended animation, which protects the animals against damage due to stress. In previous studies the Rongo Lab has found that *egl-9* mutants emerge from this suspended animation at a faster rate than the wild-type N2 strain. We found that *chn-1* mutants also have faster emergence rates than the N2 strains, though not to the same degree as the *egl-9* mutants (Figure 23). This finding indicates that the *chn-1* mutation does not enhance the *egl-9* mutant phenotype. Interestingly, *chn-1; hif-1* double mutants were more similar to *hif-1* and wild-type single mutants, which suggests that CHN-1 activity is HIF-1-dependent.

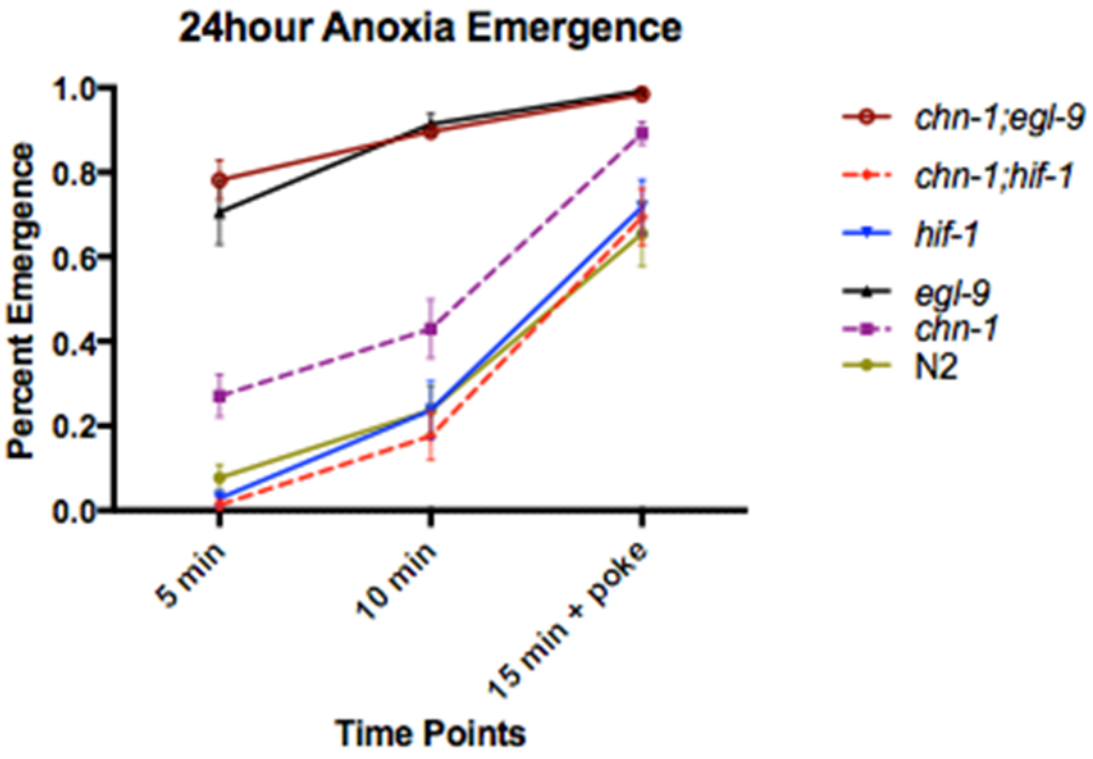

Figure 23 chn-1 mutants have faster rate of emergence than N2 animals, but not egl-9 single or double mutants.

hif-1 mutation recovers wild-type phenotype. Emergence was observed 5 minutes, 10 minutes, and 15 minutes following treatment. At 15 minutes, animals were gently prodded to confirm emergence phenotype. Error bars indicate SEM. n=30, 4-6 replicates.

To examine the effects of anoxia on *chn-1* mutants, we placed synchronized L4-stage animals in anaerobic bags at 20°C for 72 hours and 96 hours. After the allotted time, we removed the animals from the bags back into normoxic conditions and waited 24 hours for recovery. If *chn-1; hif-1* double mutants survive better than *chn-1* single mutants, then it would suggest that CHN-1 regulates survival through its inhibition of HIF-1. We can also compare *chn-1* single mutants with *egl-9* single and *chn-1; egl-9* double mutants to identify whether CHN-1 is working in the same or a separate pathway as EGL-9 and HIF-1. If CHN-1 acts in the same pathway, then the *chn-1; egl-9* double mutants would have a similar phenotype as either *chn-1* or *egl-9* single mutant. If CHN-1 and EGL-9 inhibit anoxia survival by separate mechanisms, then we would expect to see an additive effect and thus see even greater survival rates in the double mutant. In preliminary tests between *chn-1* mutants and wild-type animals, both expressing MitoGFP, we found an increase in survival rates in *chn-1* mutants (Figure 24).

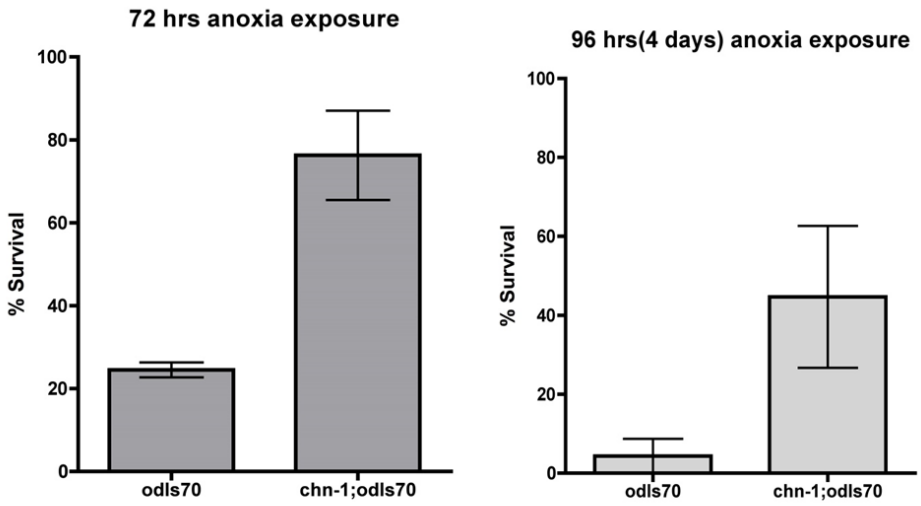

Figure 24 chn-1 mutants have a higher survival rate compared to wild-type under anoxia conditions.

Error bars indicate SEM. n=40, 3 replicates.

4.3 Discussion

Our recent findings are consistent with our hypothesis that CHN-1 plays a role in the hypoxia response pathway. Whether CHN-1 is interacting with EGL-9 directly to regulate HIF-1, or CHN-1 is a part of a parallel pathway, is a critical question to answer. The results from our anoxia emergence assay suggests that CHN-1 may be working in the same pathway as EGL-9.

Further analysis of CHN-1 and its interaction with EGL-9 will also be done with more thorough study of *egl-9; chn-1* double mutants in their survival, hypoxia emergence, and mitochondrial phenotype. If *egl-9;chn-1* double mutants show an additive effect—that is, improved survival rates, faster emergence times, and increased mitochondrial hyperfusion compared to either *egl-9* or *chn-1* single mutants—then the two proteins likely regulate HIF-1 expression in parallel pathways. Alternatively, if the double mutant phenotype is similar to the *egl-9* and *chn-1* single mutant phenotypes, then CHN-1 and EGL-9 are likely working in concert to regulate HIF-1.

Mutant survival rates are a useful endpoint for studying this pathway, so we have continued the survival assay, and included the *hif-1* and *egl-9* single and double mutant strains as mentioned in the results section. In anoxia conditions, *C. elegans* often move to the walls of the plates in search of oxygen, which leads to desiccation and subsequently lowers the number of animals considered in each experiment. Unfortunately, this escape behavior skews the proportion of surviving animals and has made gathering results from recent survival assays difficult. We have since tried smaller seeded plates for our other anoxia treatment assays and have been particularly successful in keeping animals from escaping. While this behavior did not have a dramatic impact on previous results depicted in Figure 24, we will be using plates with a smaller seeded area for future anoxia survival experiments to control for this as we move forward.

These tests have also shown that a three-day paradigm for testing mutant survival rates may provide more information than a four-day paradigm. This is because by four days, the wild-type strain has very low survival rates (Figure 23). Because of this, it would be difficult to compare mutants to the control N2 strain, particularly if they also have lower survival rates, with the four-day paradigm as opposed to the three-day paradigm.

A case may be made for CHN-1 regulating HIF-1 when HIF-1's transcriptional targets are over-activated, such as under prolonged hypoxia conditions. CHN-1 seems necessary for the regulation of HIF-1 transcriptional targets, as indicated by preliminary results shown in Figure 22. Our preliminary analysis of Venus-tagged CYSL-2 suggests that CHN-1 also plays a role in HIF-1 regulation under normal conditions as well, as there was more fluorescence under the *chn-1* mutant background than in the CHN-1-positive strain, even without any stress treatment (Figure 21). While this model for CHN-1's role in the hypoxia response pathway has merit, further validation is required.

The role of CHN-1 and the extent to which it interacts with HIF-1 in the hypoxia response pathway will be further validated in multiple subsequent quantitative real-time polymerase chain reaction experiments (qPCR). We will be analyzing *nhr-57*, a HIF-1 transcriptional target gene, and the experiments will further determine in greater detail HIF-1 transcriptional output under normoxia conditions. Additionally, we will observe the levels of HIF-1::GFP in *chn-1* mutants, under both normal and hypoxia conditions, to see if HIF-1 levels are elevated if CHN-1 is not present to regulate HIF-1 activity. This will confirm whether CHN-1 regulates HIF-1 in stress conditions exclusively, or if CHN-1 always regulates HIF-1 activity.

If CHN-1's role in HIF-1 regulation is confirmed, we can further study whether CHN-1 is directly involved in the degradation of HIF-1 through its actions as an E4 ligase. As CHN-1

plays a role in ubiquitin elongation, if it is involved in degrading HIF-1, *chn-1* mutants should still have levels of mono-ubiquitinated HIF-1, as CHN-1 would not be present for the polyubiquitination and subsequent degradation of HIF-1 to continue.

Our use of mitochondrial hyperfusion as a marker for the hypoxia response pathway certainly begs the question of why mitochondria hyperfuse in the first place. While it has been established the mitochondria hyperfuse under conditions of stress, such as UV-damage – a phenomenon known as stress-induced mitochondrial hyperfusion (SIMH) – we can only speculate as to what is the function of SIMH (Tondera et al.). One explanation posits that mitochondrial fusion is a part of a cellular defense mechanism. Following conditions of stress, mitochondria are damaged and undergo fission. Fission allows mitophagy machinery to more efficiently enclose the damaged mitochondria and selectively degrade it. If mitochondrial damage is not too extensive, and there is a chance of recovery, then healthy mitochondria may be able to fuse with the slightly damaged mitochondria and mix essential matrix proteins to effectively reverse the damage caused by stress. Mutants for *egl-9* and *chn-1*, and thus increased HIF-1 activity, may thus allow this cellular defense mechanism to be more active, thereby increasing the number of damaged mitochondria that are recovered instead of degraded.

A more exact way to study this phenomenon would be to maintain a mitochondrial count both before and after stress treatment, perhaps through real-time observation, and determine if the mitochondrial length increases in these mutant strains without any significant change in number. This would give us a better picture of whether mitochondrial hyperfusion occurs instead of degradation. In our current model, we are limited by the glutamatergic circuit that we are using. Because we are observing the mitochondria of multiple, distinct neurons, which have different machinery and physiologies, we cannot do a reliable study of the mitochondrial

number. The bundle of neurons also makes it difficult to distinguish whether two mitochondria are abutting each other or spatially adjacent but on different neurons. To this end, we plan to construct strains with single-neuron models under these hypoxia response pathway-related mutant backgrounds.

Another potential explanation for stress-induced mitochondrial hyperfusion in the context of the hypoxia response pathway relies on the knowledge that, on a cellular level, the reoxygenation stage is the most critical. Due to these stress conditions, the mitochondria release excess amount of reactive oxygen species, which are damaging to the cell. Ironically, it can also be damaging to the mitochondria itself. In this case, it may be that the mitochondria hyperfuse to protect themselves from the reactive oxygen species, and HIF-1 activation might facilitate this mitochondrial hyperfusion.

Such theories beg a different kind of question: is fusion or fission better for mitochondrial efficiency? It would seem that fused mitochondria likely produce more ATP (Tondera et al.; Mitra et al.). Based on the results of our current studies thus far, it would make sense that mitochondrial fusion is more energy-efficient, as this phenotype is associated with higher survival rates and faster emergence rates following anoxia treatment. This energy efficiency in conditions of stress may not translate well in healthy cells, as the increase in ATP production may be wasteful to a cell that does not require such elevated ATP output. In a generally healthy cell, mechanisms involved in protein turnover and baseline levels of ATP are sufficient to maintain function. If mitochondria were always in a hyperfused state, the excess ATP production would be an unnecessary burden on the cell.

An understanding of precisely what happens to the mitochondria immediately after anoxia treatment and then during the reoxygenation stage will be important for elucidating some

key points about mitochondrial dynamics and the hypoxia response pathway. To this end, we hope to capture mitochondrial mobility at both these times points to capture the exact events which occur on a physiological level via kymograph analysis. There may be local (short) or distal (long) transport of mitochondria, or perhaps mitochondrial biosynthesis restarts within the first five minutes following anoxia as the animals recover from suspended animation, and new mitochondria fuse with the damaged mitochondria.

Clearly, mitochondria are dynamic organelles that must rapidly respond to the needs of their cells. *C. elegans* themselves are animals that adapt their oxygen consumption and neuromuscular activity to improve their chances of survival. Analyzing the mitochondria in this organism is thus very informative, particularly when observing the behavioral changes in conjunction with the mitochondrial changes.

A clear understanding of the hypoxia response pathway may lead to more informed translational research to develop treatments for those recovering from ischemic strokes, as components of the hypoxia response pathway are excellent therapeutic targets for diseases involving ischemia. Considering CHIP's role in mitochondrial health and hypoxia-response, understanding its contribution to the HIF-1-associated hypoxia response pathway will give more context to the role mitochondria play in the neuroprotective mechanisms induced under stress conditions. If our future plans are successful, we may also be able to use these models to understand mitochondrial dynamics and the benefits of hyperfusion, fission, and mitochondrial trafficking patterns.

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