Characterization of Neurotransmitter Involvement in *C. elegans* Hypoxia-Induced Longevity Pathway

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University of Michigan

Mira Bhandari

Sponsor: Dr. Scott Leiser, Associate Professor of Molecular & Integrative Physiology, Internal Medicine, and Geriatric and Palliative Medicine

Co-Sponsor and Reader: Dr. Anuj Kumar, Professor of Molecular, Cellular, and Developmental Biology

Reader: Dr. Randy Stockbridge, Associate Professor of Molecular, Cellular, and Developmental Biology

Abstract

The physiological effects of advanced age predispose older individuals to a range of chronic health conditions and cognitive challenges. As such, understanding the mechanisms of aging and its symptoms provides targets for the development of therapies that could enhance human longevity and health in later life. Previous studies in *Caenorhabditis elegans* have established that hypoxia, or low oxygen availability, is among a set of environmental factors that can elongate lifespan through the induction of flavin-containing monooxygenase-2 (*fmo-2*). However, the genes and signals that link hypoxic conditions to the pro-longevity phenotype they yield is not well understood. In this thesis, lifespan assays identify the neurotransmitter tyramine as a chemical messenger involved in the hypoxia-induced lifespan extension pathway. Through similar methods, it is also shown that stimulating individual adenylyl cyclase enzymes is partially required for hypoxic conditions to extend lifespan, suggesting these enzymes operate as downstream effectors of the serotonin-binding event known to be involved in this pathway. Additionally, fluorescent imaging of *fmo-2* transcription levels in tyramine-deficient strains revealed that this neurotransmitter extends lifespan under hypoxia through an alternative pathway that is independent of *fmo-2* induction. Together, this project advances our understanding of neurotransmitter involvement in the mechanisms underlying hypoxia-driven lifespan extension and identifies *fmo*-2-independent pro-longevity pathways. Through these findings, this research provides insights that can inform future studies aiming to develop targeted therapies to promote longevity and health in aging populations.

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Introduction

1.1 An Aging Population

In the next half-century, the current global population of individuals aged 60 years and older is projected to grow from 1 billion to nearly 1.4 billion people. Today's declining fertility rates coupled with longer life expectancies are key phenomena driving aging on a population level (Wan et al., 2016). For countries like the United States, this demographic turning point implies an almost two-fold increase in the proportion of the nation's older individuals from 12% to 23% by the year 2060 (Vespa et al., 2020). As the aged population continues to increasingly represent more of the globe, it behooves researchers to study the biological and physiological implications of aging in order to develop methodologies of healthcare to better promote healthy aging.

1.2 Aging is a Risk Factor

From ischemic heart failure to Alzheimer's disease and cancer, various fatal illnesses are more likely to appear in individuals of an advanced age than in their younger counterparts. The risk of neurodegeneration rises with age as the efficiency of protein homeostasis declines. Diminished effectiveness in regulating processes such as gene expression, protein folding, and degradation increases the body's susceptibility to the accumulation of aberrant protein aggregates, a hallmark of several neurodegenerative diseases (Douglas et al., 2010). Similarly, vulnerability to accumulating genetic mutations in tumor suppressor and proto-oncogenes over time makes aging a critical risk factor contributing to the onset of cancer (Berben et al., 2021). Heart failure is also made more likely by aging due to progressive stiffening and deterioration of cardiac tissue structures (Li et al., 2020). Because the physiological symptoms of advanced age commonly predispose older individuals to developing these diseases, studying aging provides a singular approach through which this collection of illnesses can be targeted and understood at once.

1.3 Known Effectors of Human Aging

Genetic and environmental variables compound to influence lifespan in humans. A longitudinal twin study carried out in Scandinavia on over 20,000 individuals suggested that genetic factors may account for a notable amount of human lifespan diversity. Among 10,500+ pairs of monozygotic and dizygotic twins, the researchers found a significant difference between

the correlation a monozygotic twin's lifespan had on that of their co-twin compared to dizygotic twins. For each year a male, monozygotic twin survived beyond the age of 60, the average lifespan of their co-twin was projected to increase by 0.39 [95% CI (0.28, 0.50)] years while for dizygotic twins, this rate of influence was 0.21 [95% CI (0.11, 0.30)] years. The increased similarity in lifespan among twins with a larger amount of genetic similarity – a trend that was also reflected in the corresponding rates for female twins – implies the existence of human genes that impact longevity (Hjelmborg et. al., 2006).

External conditions also exert influence on human lifespan, often exacerbating the symptoms of aging. Cigarette smoking or prolonged exposure to secondhand smoke is a widely documented accelerator of aging and its effects. The free radicals present in cigarette smoke can lead to the onset of oxidative stress-related damage and inflammatory diseases otherwise associated with aging (Nicita-Mauro et. al., 2008). While other comparatively innocuous environmental factors such as diet and sleep have been proposed to modulate aging mechanisms as well, their involvement is supported by only a small body of evidence. Diets excluding red meat and ultra-processed foods are thought to promote beneficial metabolic responses and deter premature shortening of leukocyte telomere length – another potential indicator of biological aging (Leitão et. al., 2022). Additionally, chronic insomnia and other sleep disorders can lead to fragmentation of sleep patterns. This disruption dysregulates normal metabolic and endocrine processes in a manner thought to result in the release of reactive oxygen species that can also affect telomere length (Carroll et. al., 2021). However, insufficient support for these models precludes them from offering any significant insights into mechanisms of aging (Lescinsky, et. al., 2022).

2.1 *Caenorhabditis elegans* as a Biological Model

The soil nematode, *Caenorhabditis elegans,* is a simple yet advantageous system in which to study diverse biological mechanisms. These worms were first used as model organisms in 1949 by Victor M. Nigon, who established foundational guidelines for working with *C. elegans* in laboratories by elucidating key features of their reproductive cycles and optimizing culture conditions (Nigon, 1943). However, it was not until 1974, when early molecular biologist Sydney Brenner published his seminal work detailing the first genetic mapping experiments conducted on *C. elegans* mutants, that the free-living nematodes became lucrative model systems

for many molecular and developmental biologists (Brenner, 1974). In fact, in the following decades, *C. elegans* would become the first animal to have its genome fully sequenced (Meneely, et. al., 2019).

2.1.1 Laboratory Advantages of *C. elegans*

Upwards of 83% of protein-coding genes in *C. elegans* have homologous counterparts in humans (Lai, et. al., 2000). This caliber of similarity confers potential for findings from biochemical and genetic experimentation done on the nematodes to be extrapolated to human biology and therapeutics as well. With their rapid life cycles and prolific reproductive patterns, *C. elegans* research allows for the high-throughput analysis of *in vitro* systems while maintaining an *in vivo* experimental environment. Silencing genes of interest in the worms, for example, can occur on a large scale due to the availability of RNA interference (RNAi) plasmid libraries (Kaletta & Hengartner, 2006). Primarily existing as self-fertilizing hermaphrodites, the worms are also amenable to genetic modification as consistent crossing is not required to maintain their genotypes. The advantages of crossing, however, are not lost when working with *C. elegans* as they can also exist as males with the ability to mate with hermaphrodites and yield more genetically diverse progeny. In addition to the animal's advantages from a genetic perspective, the small, transparent anatomy of *C. elegans* makes it amenable to fluorescent tagging for *in vivo* protein visualization.

2.1.2 The *C. elegans* Nervous System

Locomotion, sensation, and behavior are among many aspects of the nervous system and its impacts that are investigated widely in *C. elegans*. These studies are made possible by two key features of the model organism. First, nematodes exhibit distinct, observable phenotypes upon mutation of genes involved in the nervous system. Loss of function in *unc-13,* a gene responsible for synaptic vesicle fusion, for example, yields an uncoordinated motility phenotype that visibly differs from the sinusoidal movements of wildtype worms.

Additionally, mutations in genes encoding olfactory receptors result in a behavioral phenotype characterized by loss of sensitivity to the receptors' target odorants (Sengupta, 1996). With nervous systems containing on the order of 10^5 and 10^8 neurons respectively, other common model organisms such as fruit flies and mice pose a challenge to neuroscientific

research due to their size and complexity (White, 2016). Consisting of exactly 302 neurons, however, the simplicity of the hermaphroditic *C. elegans* nervous system is the second feature of the organism that optimizes it for study of neuronal pathways. As such, the lineage of all *C. elegans* neurons as well as the animal's connectome have been well characterized, providing an accessible foundation for research involving neuronal circuits in *C. elegans.*

2.2 Modeling Aging in *C. elegans*

Since environmental cues are known to influence lifespan and the stimuli generated by those cues are interpreted by an organism's nervous system, the benefits of utilizing *C. elegans* as a neuroscientific model also contribute to the animal's advantages as a model of biological aging. In addition to these characteristics, the rapid progression of the nematode life cycle also primes it for experimentation investigating longevity. Upon hatching, *C. elegans* will proceed through four larval stages (L1-L4) that can be visually characterized by changes in size as well as physical features that are unique to specific stages. Within 2.5 to 4 days, wildtype hermaphrodites develop into mature adults that can lay between 250 and 300 eggs during their fertile stage. Their lifespans also extend post-reproductively, ranging from 18 to 20 days on average when cultured at 20°C. The timeframe of their life cycle allows for the effects of manipulating genetic or environmental factors experimentally to be observed within approximately a month, a timeline that would otherwise not be feasible in longer-lived, mammalian models.

Despite these myriad benefits, there also exist limitations when studying *C. elegans* aging from a translational lens. The lack of more complex features that have been implicated in human biological aging like a blood-brain barrier and the ability to directly innervate peripheral tissues are not present in nematodes (Zhang, et. al., 2020). However, the ability to map signaling circuits in a simple system like the worm provides an invaluable foundation that studies on higher organisms can leverage.

2.3 Environmental Effects on *C. elegans* Lifespan

Interactions with environmental modulators at various instances in the *C. elegans* life cycle are integral to determining their lifespan. Exposing worms to pharmacological compounds can accelerate or decelerate their aging processes, unveiling potential avenues for the discovery of anti-

aging drugs (Zhang, et. al., 2023). Several non-chemical external factors ranging from incubation temperature to the availability of nutrients can also significantly influence the longevity of the animals. The most rigorously studied among these conditions include dietary restriction, mitochondrial stress, and hypoxia (Miller, et. al., 2020).

2.3.1 Dietary Restriction

Studies conducted on a range of organisms including mice and humans as well as nematodes reveal that constrained yet adequate nutrition can defer the onset of aging and its symptoms. In mammalian models, the effects of dietary restriction (DR) are seen across several organ systems, working in tandem to increase health and lifespan. In the excretory system, mammals on restricted diets saw lowered risk of diabetic nephropathy and renal fibrosis. Etiologies like amyloid aggregation and adiposity were also reduced, exemplifying the effects of DR conditions on the nervous and endocrine systems as well (Green, et. al., 2022).

Epidemiological investigations on geographically diverse human populations collectively indicate a positive impact on lifespan due to dietary or caloric restriction. In Okinawa, Japan, centenarians represent 50 out of every 100,000 people, approximately five times the average number of individuals aged 100 years and older observed in mainland Japan. Analysis of serum samples from Okinawans as compared to Americans of the same age revealed higher levels of superoxide dismutase, an enzyme involved in oxidative stress responses, and hydroxyproline, a marker of bone resorption, in the former (Suzuki, et. al., 2001). These protective phenotypes are thought to be linked to the antioxidant-rich, low-calorie diets reported in school children in the 1970s (Redmann & Ravussin, 2011). A two-year, randomized controlled study investigating the effects of caloric restriction (CR) on nonobese American adults corroborated the observations made in Okinawans. As a result of 25% CR, participants experienced a reduction in the fasting levels of insulin and core body temperature, two biomarkers of longevity (Das, et. al., 2007).

Molecular pathways involved in exerting DR's influence on lifespan have been elucidated in *C. elegans* by observing changes in lifespan length along with induction of enzymes that are markers for longevity. These studies first implicated the neurotransmitters serotonin and dopamine in the DR-induced lifespan extension pathway by way of food odor perception (Miller, et. al., 2022). The availability of extensive mapping data for the *C. elegans*

nervous system subsequently led the researchers to link classes of serotonergic and dopaminergic neurons to the odor signaling and DR circuit as well.

2.3.2 Mitochondrial Stress

Hormesis, a phenomenon in which exposure to stressors at a low level exerts beneficial impacts on an organism, is exemplified by the lifespan extension observed in *C. elegans* subjected to small amounts of mitochondrial stress. While substantial defects in the mitochondria put nematodes at risk of stunted development and accelerated biological aging, minor suppression of genes regulating the mitochondrial respiratory chain have been linked to longevity. Mitochondrial (Mit) mutant strains including *clk-1* and *isp-1* were found to be longer lived despite interference in the electron transport chain caused by the mutations (Maglioni, et. al., 2014).

These findings have also been corroborated in other model organisms. Targeted RNA interference depleting two genes of the mitochondrial respiratory complex I was sufficient to extend lifespan in *Drosophila melanogaster* without consistently diminishing the health of the fruit flies (Copeland, et. al., 2009). This pattern was also reflected in experiments on mammalian models. The SURF-1 gene in mice encodes an assembly factor associated with cytochrome c oxidase, a necessary enzyme complex for the terminal step in the electron transport chain. Despite its integral function, longer lived phenotypes were observed in mice deficient in SURF-1 along with a reduction in spontaneous neurodegeneration (Dell'agnello, et. al., 2007).

2.3.3 Hypoxia

Exposing *C. elegans* to low environmental oxygen conditions beginning at their fourth larval stage was also found to significantly increase nematode lifespan. Moreover, placing the worms in hypoxia chambers at fractional oxygen levels for 24 hours revealed that the positive effect of low oxygen conditions on lifespan persists even when exposure to hypoxia is transient (Mehta, et. al., 2009). This causality has recently been substantiated in mammals through experiments utilizing *Ercc1 Δ/-* mice, which model accelerated aging. Introducing restrictive oxygen levels to mice at four weeks resulted in a 50% increase in lifespan along with delayed onset of neurodegeneration (Rogers, et. al., 2023). The conservation of these phenotypes across

species suggests that findings in the *C. elegans* hypoxia-induced lifespan extension pathway could inform future studies in mammals.

However, complications arise when attempting to extrapolate this mechanism to humans, as hypoxia has been implicated in several human diseases including cardiac and kidney disease, tumor formation, as well as preeclampsia and endometriosis (Chen, et. al., 2020). Because of the broad physiological impacts of low oxygen on humans, elucidating downstream effectors of the hypoxia-induced longevity pathway in simpler organisms can uncover targets with narrower influence that could promote its beneficial phenotypes in isolation.

3.1 The Hypoxia Response Pathway

Across species, hypoxic conditions trigger a highly conserved molecular circuit known as the hypoxia response pathway (HRP). This response operates through the hypoxic stabilization of the hypoxia-inducible factor (HIF-1), a transcription factor that upregulates the expression of genes containing the hypoxia response element (HRE) motif. HRE-containing genes are involved in metabolic processes including glycolysis, gluconeogenesis, and glucose transport. Other HIF-1-induced genes stimulate vascularization by positively regulating pro-angiogenic pathways to meet metabolic oxygen needs with limited supply (Wong, et. al., 2017). Since the disparity between oxygen supply and demand is not as pronounced at normal oxygen levels, the enhanced expression of HRE genes no longer becomes necessary for the organism. Thus, normoxia leads to the proteasomal degradation of HIF-1 through a pathway mediated by a collection of key enzymes (Fig. 1).

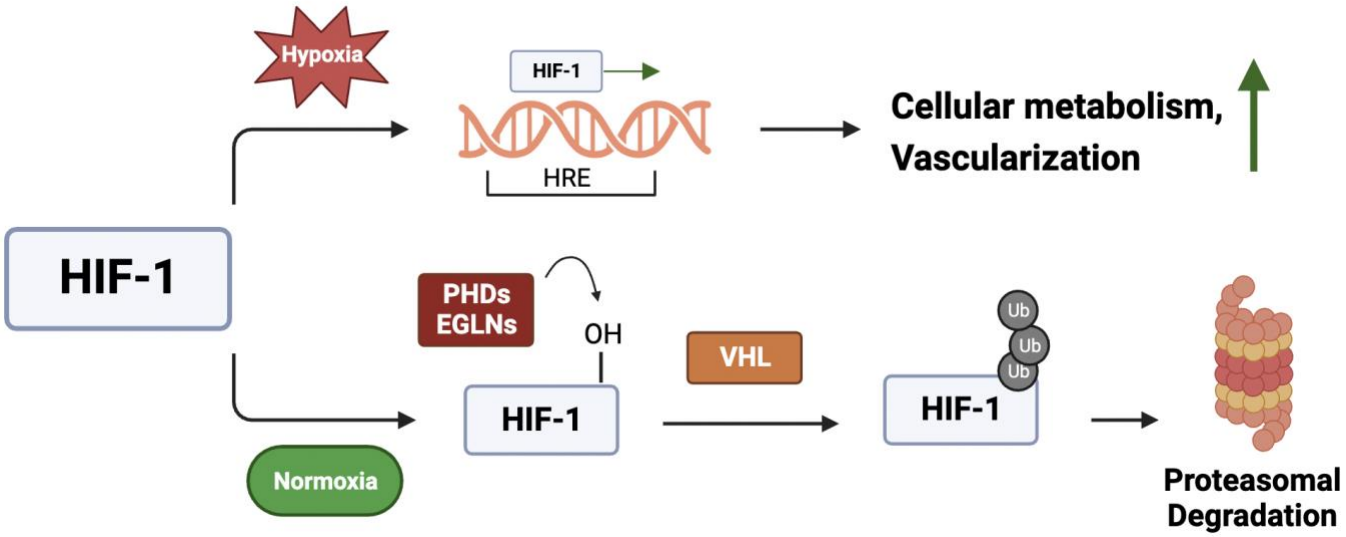

Fig. 1 | HIF-1 is a transcription factor that is central to the highly conserved hypoxia response pathway (HRP). Low oxygen conditions necessitate the upregulation of angiogenic and metabolic genes. This enhancement is driven by stable HIF-1 entering the nucleus and recognizing these hypoxia response element-containing genes. Under normoxia, HIF-1 is hydroxylated by the *C. elegans* prolyl hydroxylase enzyme, EgL-9, or the orthologous PHD enzymes in humans. The hydroxyl motif is recognized by the E3 ligase, VHL-1, which polyubiquitinates HIF-1, effectively targeting it for proteasomal degradation in the cytosol. *Created with BioRender.com.*

The first modification HIF-1 is subjected to in normoxic conditions is the O_2-dependent conversion of proline residues into 4-hydroxyproline (Semenza, 2004). The hydroxyl moiety on the altered amino acid then becomes a target for von Hippel-Landau 1 (VHL-1), an E3 ligase that forms a protein complex with the ability to conjugate ubiquitin molecules onto HIF-1 and target it for degradation. The necessity of VHL-1 in the normoxic branch of the HRP implies that its absence will force the stabilization of HIF-1, thereby prompting the hypoxic response regardless of physiological oxygen levels. This genetic approach toward simulating conditions of hypoxia was confirmed by comparing the longevity of wildtype *C. elegans* to that of *vhl-1* knockout worms, resulting in a 30-50% lifespan increase in the latter (Leiser, et. al., 2013). The findings from this experiment thus nominated *vhl-1* as an efficient genetic mimetic of hypoxia, creating

opportunities for future investigations to explore effects of low oxygen levels without the use of hypoxia chambers.

Counterintuitively, the beneficial lifespan extension effect observed in *C. elegans* in response to *vhl-1* function loss is met with a detrimental impact in humans. Von Hippel-Landau syndrome is an autosomal dominant disease driven by inherited mutations in the *vhl-1* gene. The formation of benign tumors consisting of proliferating blood vessels, or hemangioblastomas, in the central nervous system is characteristic of the disease. These growths commonly lead to vision loss and ataxia in humans, but do not show analogous effects in *C. elegans* due to their post-mitotic somatic cells and lack of a circulatory system (Leiser et. al., 2015). Since loss of function in relatively upstream molecular players in the HRP like *vhl-1* leads to the onset of unwanted diseases in humans, identifying downstream effectors of this pathway could pave the way for more targeted therapies against aging and its symptoms.

3.2 Cell Nonautonomous Targets of HIF-1

To elucidate the effects of the HRP's branches downstream of HIF-1, the exact tissues in which the transcription factor acts to extend lifespan were determined. In particular, expression of stabilized HIF-1 under a serotonergic neuron-specific promoter was sufficient to enhance longevity in *C. elegans* (Leiser, et. al., 2015). A subsequent high throughput screen was conducted to identify targets further downstream of HIF-1 by assaying for the appearance of age-associated phenotypes upon knockdown of candidate genes using RNAi. Among the targets identified through this experiment was *fmo-2,* an enzyme expressed in the intestine of *C. elegans*. Along with restoring age-related autofluorescence in *vhl-1* knockout worms when depleted, *fmo-2* was also sufficient to increase nematode lifespan when overexpressed (Leiser, et. al., 2015). The simplest model for this link would rely on intestinal HIF-1 directly binding the promoter of *fmo-2* to upregulate its expression. However, this proposed mechanism was dismissed by observing the effects of intestine-specific HIF-1 through a constitutively active transgene. Unlike neuronal HIF-1, intestinal HIF-1 did not yield an effect on longevity of the tested worms despite being localized to the same tissue as endogenous *fmo-2.* This finding implies that hypoxia-induced lifespan extension is a cell-nonautonomous mechanism that must rely on chemical

messengers and molecular intermediates to propagate the pro-longevity signal from neurons to the intestine.

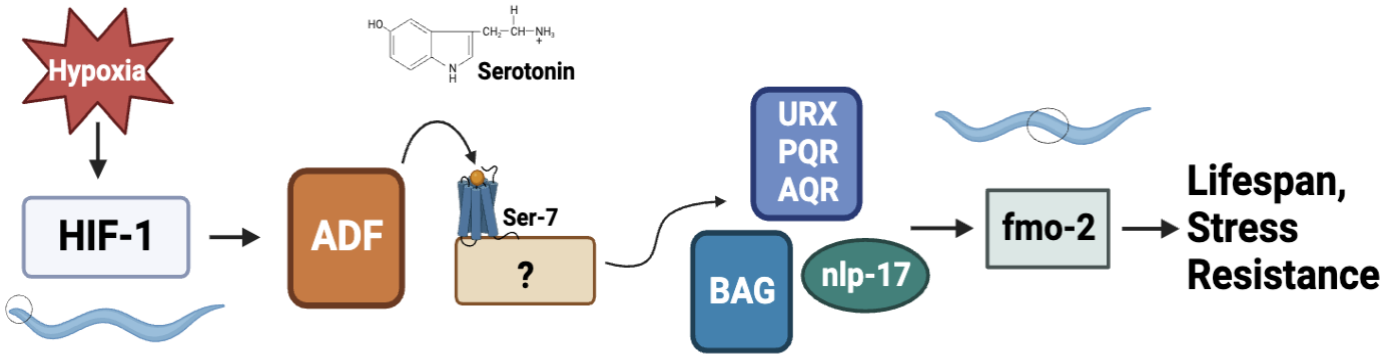

Fig. 2 | Hypoxia-induced lifespan extension is a cell-nonautonomous pathway that relies on several signaling intermediates downstream of the HRP. The stabilization of HIF-1 in response to hypoxic conditions in serotonergic ADF neurons leads to the release of serotonin. The neurotransmitter then binds to receptors encoded by *ser-7* on currently unidentified downstream target cells. Additionally, low oxygen sensing BAG neurons and high oxygen sensing URX, PQR, and AQR neurons are necessary elements of this signaling circuit. Along with the neuropeptide *nlp-17,* these effectors work in tandem to enhance *C. elegans* lifespan (Huang, Unpublished). *Created with BioRender.com.*

The discovery of serotonin's involvement in the hypoxia-induced lifespan extension pathway preceded the identification of the class of neurons responsible for the neurotransmitter's release. The four candidate types of serotonergic neurons in *C. elegans* include amphid chemosensory ADF neurons, neurosecretory motor neurons (NSM) neurons, and HSN neurons that are implicated in egg laying (Schwartz et. al., 2021). Of these classes, ADF neurons were able drive lifespan extension in response to localized HIF-1 stabilization. Additionally, the ablation of low oxygen sensing BAG neurons via upregulation of apoptotic genes resulted in a loss of lifespan extension phenotypes under hypoxic conditions, suggesting that these neurons are necessary elements of the pathway. Similarly, high oxygen sensing URX, PQR, and AQR neurons were also implicated through loss of lifespan extension in ablated strains. Additionally, a large RNAi screen revealed the necessity of the neuropeptide, *nlp-17,* in the *C. elegans* hypoxia-induced pro-longevity pathway.

4.1 FMO Induction Enables Lifespan Extension

The DR and hypoxia-induced lifespan extension pathways in *C. elegans* converge at the intestinal enzyme, flavin-containing monooxygenase-2 (FMO-2). Induction of *fmo-2* is necessary and sufficient for a pro-longevity effect to be observed in nematodes through a pathway that is currently unclear. However, characteristics of the FMO family of enzymes implicate two mechanisms through which *fmo-2* activity could yield lifespan enhancement. FMOs can function as detoxifying enzymes that help organisms clear unwanted foreign compounds known as xenobiotics. Insoluble xenobiotics in particular challenge renal excretory pathways that rely on the solubility of the nonnative compound to facilitate clearance. To this end, FMOs attach molecular oxygen to the nitrogen or sulfur atoms on xenobiotic compounds, increasing their solubility at physiological pH and making their excretion more efficient as a result (Eswaramoorthy, et. al., 2006).

In addition to facilitating the clearance of insoluble, nonnative compounds, FMOs have also been linked to one carbon metabolism (OCM) as a means of extending lifespan in *C. elegans*. Suppression of the methionine cycle, an OCM-associated pathway, was found to extend lifespan in wildtype worms without enhancing longevity in animals under genetically induced DR conditions. This finding made OCM a candidate downstream pathway through which *fmo-2* induction could enhance lifespan. Metabolomic analysis subsequently revealed that overexpression of *fmo-2* results in significant enrichment of glycine, serine, and threonine metabolism. Since glycine supplementation is known to remodel the methionine cycle, this insight provided a connection between *fmo-2* induction, OCM, and lifespan extension (Choi, et. al., 2023).

4.2 Mammalian Homologs of Nematode FMOs

From yeast to humans, flavin-containing monooxygenase family enzymes are highly conserved. The *C. elegans* genome consists of five arbitrarily numbered FMO-encoding genes, *fmo-1 - fmo-5,* that are expressed in either the intestine or hypodermis of the animal. Mouse and human FMOs are also found in analogous tissues -- namely the liver, kidneys, and adipose tissue. Additionally, mammalian *FMO5* is most genetically comparable to nematode *fmo* genes due to its large degree of sequence identity.

Despite their common existence in all studied phyla, however, FMOs vary between organisms in their tissue-specific expression and paralog numbers. While nematodes express five types of FMOs, mammalian genomes contain six *Fmo*-encoding genes. Moreover, *fmo-2* in *C. elegans* is localized to the intestine, whereas murine *Fmo-2* is found in the lungs and in most humans, functional *FMO2* is not expressed in any cell type (Huang, et. al., 2021). Thus, FMOs are most effectively compared between organisms based on common expression patterns and phenotypes.

5.1 Neurotransmitters

The cell-nonautonomous nature of lifespan extension pathways in *C. elegans* implies a reliance on chemical messengers that can propagate signals from one cell type to another. Determining the identity of these molecules is therefore crucial to understanding how external conditions are able to trigger signaling cascades that ultimately exert influence on lifespan. Neurotransmitters are endogenous signaling molecules that are released from vesicles at the end of a nerve fiber in response to action potential-generating stimuli. Upon release, the small chemicals diffuse across a synapse or junction and bind to specific receptors on target cells. This binding event can then elicit various responses or further propagation of the signal in the recipient cell. While neurons are responsible for the release of neurotransmitters, the target cells displaying receptors for these molecules could be of other cell types (Sheffler, et. al., 2023). These characteristics suggest that neurotransmitters could be the chemical messengers that permit neuronal responses to pro-longevity stimuli to manifest in the induction of intestinal FMOs to extend lifespan. This model was confirmed through loss of long-lived phenotypes in worms deficient in *unc-13,* a gene required for synaptic vesicle fusion (Huang, Unpublished).

Certain mammalian neurotransmitter types such as norepinephrine and histamine are not present in the *C. elegans* nervous system. However, six of the seven classes of neurotransmitters that are endogenous to nematodes are major human neurotransmitters as well. The robust mapping of cells expressing specific neurotransmitter receptors in *C. elegans* thus upholds nematodes as meaningful translational models of neurotransmitter involvement in pro-longevity pathways.

5.1.1 Dopamine, Acetylcholine, and Glutamate

Knockouts of *unc-17,* a necessary gene for acetylcholine loading, *eat-4,* which encodes a glutamate cotransporter, and *cat-2,* a gene involved in dopamine synthesis, did not result in a loss of long-lived phenotypes under hypoxic conditions (Huang, Unpublished). These neurotransmitters are therefore not likely in the direct pathway of hypoxia-induced longevity. However, this finding does not preclude their potential involvement in other pro-longevity pathways. The dopamine receptor, DRD2/DOP-3, for instance, is necessary for DR-induced lifespan extension, suggesting that dopamine acts as a chemical messenger in this longevity circuit.

5.1.2 Serotonin

The involvement of serotonin in various lifespan extension pathways has been elucidated by observing abrogation of long-lived phenotypes in *C. elegans* strains deficient in *tph-1,* the rate-limiting enzyme for serotonin synthesis. Following this foundational discovery, specific classes of serotonergic neurons and the types of stimuli they respond to were also determined through systematic ablation of these neurons. Through their involvement in food abundance sensing, NSM neurons were implicated in regulating DR-induced lifespan extension (Miller, et. al., 2022). While ADF neurons do not play a role in the DR pathway, HIF-1 stabilization in these serotonergic neurons is necessary and sufficient for lifespan extension as a response to hypoxia (Huang, Unpublished).

Looking further downstream of the HRP, the serotonin receptor, SER-7, was also found to be necessary for induction of *fmo-2*, suggesting that the serotonin molecules released by ADF neurons ultimately bind SER-7 receptors on currently unidentified target cells. Mammalian homologs of the SER-7 G-protein coupled receptor are known to stimulate adenylyl cyclase enzymes upon serotonin binding (Hobson, et. al., 2006). Whether this mechanism is reflected in SER-7 activity in the context of the hypoxia-induced lifespan extension is yet to be determined.

5.1.3 Octopamine, Tyramine, and GABA

While octopamine is a neurotransmitter endogenous to both *C. elegans* and humans, it is found only in trace amounts in mammals. In fact, the mammalian neurotransmitter, norepinephrine, is considered a more meaningful structural analog for nematode octopamine

instead. Octopamine is known to regulate several fundamental behaviors in nematodes, including egg laying, pharyngeal pumping, and locomotion (Mills, et. al., 2012). Tyramine, another neurotransmitter common to nematodes and mammals, is a precursor to octopamine and modulates various behavioral responses to environmental stimuli in *C. elegans*. In humans, tyramine has been implicated in blood pressure regulation. The role of both neurotransmitters in the hypoxia-driven longevity circuit is currently unknown.

γ-Aminobutyric acid (GABA) is also a major, conserved neurotransmitter with an unspecified role in nematode hypoxia-induced lifespan extension. GABA is a key messenger of inhibitory and excitatory signals at neuromuscular synapses that is crucial for maintaining normal locomotion patterns by facilitating muscle relaxation (Zhou & Bessereau, 2019). Accordingly, loss of function in the gene *unc-25*, which encodes an enzyme crucial for GABA biosynthesis, results in worms displaying uncoordinated locomotion.

5.2 Tyramine and Octopamine Biosynthesis

The neurotransmitters tyramine and octopamine are generated through the same biosynthetic pathway. The amino acid tyrosine is a common precursor for both neurotransmitters. The decarboxylase enzyme *tdc-1* converts tyrosine into tyramine, which can then be packaged into synaptic vesicles and propagate chemical signals through tyraminergic neurons. Alternatively, when processed by the enzyme *tbh-1,* tyramine is converted into octopamine, which can similarly carry out its function as a messenger via octopaminergic neurons.

Statement of Goals

This study aims to elucidate the involvement of neurotransmitters in the hypoxia-induced longevity pathway as a means of gaining a better understanding of the molecular mechanisms that govern aging patterns in response to low oxygen levels. These experiments will be conducted in the model organism, *C. elegans,* a species of nematode that is an advantageous model of biological aging due to its relatively short lifespan and amenability to genetic manipulation.

Of the six classes of neurotransmitters in *C. elegans,* three have yet to be studied in the context of the hypoxia-induced lifespan extension pathway. The hypothesized involvement of two of these neurotransmitters, octopamine and tyramine, will be investigated in this thesis. Lifespan assays have been robustly employed in the study of aging mechanisms and will likewise be applied in this study. Observing abrogation of lifespan extension in strains of worms deficient in the rate-limiting enzymes for octopamine or tyramine synthesis (*tbh-1, tdc-1,* respectively) would suggest that these neurotransmitters are necessary to obtain long-lived phenotypes in hypoxic conditions. If found necessary, these findings can be contextualized within a specific hypoxia pathway through imaging assays visualizing transcription levels of the intestinally expressed flavin monooxygenase-encoding gene, *fmo-2*.

While the necessity of serotonin in hypoxia-induced longevity is well-characterized, the collection of downstream effectors it signals through is not. Thus, this study will also detail lifespan assays conducted on three adenylyl cyclase mutants (*acy-1, acy-2,* and *acy-3*), which are involved in serotonin signaling through the SER-7 receptor.

Overall, the experiments described here aim to build upon the finding that hypoxia-induced lifespan extension is a cell-nonautonomous pathway by testing candidate chemical messengers for their potential role in propagating pro-longevity signals between cell types. Identification of these signaling molecules can inform future studies to ultimately promote healthy aging by mimicking the beneficial effects of hypoxia on lifespan.

Materials and Methods

1. *C. elegans* Maintenance

Standard nematode culture conditions were used for all described experiments. All animals were maintained on solid Nematode Growth Medium (NGM) seeded with *Escherichia coli* (OP50) as a food source. Lifespan scores and transfers were conducted using a platinum wire pick. Outside of handling, animals were cultured at 20°C in temperature-controlled incubators.

2. Experimental Strains and Backcrossing

The *C. elegans* strains utilized in these studies were ordered from the Caenorhabditis Genetics Center or generated in lab (Table 1). To normalize background mutations, *tbh-1* and *tdc-1* were backcrossed to our lab's wildtype strain four times. Crosses were conducted by transferring one hermaphrodite of the mutant phenotype and five wildtype males to 60 mm plates and vice versa for alternate generations.

Description	**Strain Details**
Wildtype	N2, CGC
Optimized for neuronal RNAi uptake	TU3311, CGC
fmo-2p::*mCherry*	[(pCF150) (fmo-2p::mCherry + H2B::GFP) + Cbr-unc-119(+)] II, Leiser Lab
fmo-2p::mCherry; tdc-1 #1	Cross conducted in Leiser Lab
fmo-2p::mCherry; tdc-1 #2	Cross conducted in Leiser Lab
tbh-1 (n3247)	MT9455, CGC
tdc-1 (n3419)	MT13113, CGC
vhl-1 (ok161)	CB5602, CGC

Table 1 | Complete names and sources for all experimental strains.

3. Plate Preparation

Maintenance of all utilized worm strains was carried out on NGM plates while lifespan and imaging TELs were performed on plates with RNAi media followed by transfers and scoring on RNAi + fluorodeoxyuridine (FUdR) plates. Plates were filled with 9 mL of media poured by hand or PourBoy Sterile Media Dispenser and left to dry at room temperature. RNAi media

contained 25 μg/mL of the antibiotic carbenicillin as a selective marker and 1 mM β-D-isothiogalactopyranoside (IPTG) as a synthetic inducer of gene expression along with NGM components. RNAi + FUdR media included 330 μL of 150 mM FUdR/L, an inhibitor of cell division, as well. All RNAi plates were seeded with HT115 *Escherichia coli* bacteria cultured from the Ahringer or Vidal *C. elegans* RNAi feeding libraries.

4. Lifespan Assays

To synchronize the ages of the tested animals, ten gravid worms were transferred onto RNAi plates for a 4-6 hour timed egg lay (TEL) before removal. Once their progeny reached day one adulthood, a second TEL was conducted to enhance RNAi penetrance. Upon reaching late L4 stage, 60-80 animals from each plate were transferred onto RNAi + FUdR plates to inhibit larval development of eggs laid post-transfer. To further combat the proliferation of new progeny, the transferring process was repeated thrice on alternate days for a total of four transfers onto freshly seeded RNAi + FUdR plates. The lifespan plates were also treated with 80 μL of 10mM palmitic acid (Sigma-Aldrich) dissolved in 100% EtOH poured along the outer rim to prevent fleeing and desiccation of worms.

Lifespan scoring was conducted approximately every two days and plates were wrapped in Parafilm and maintained at 20°C between scores. The number of deaths were evaluated under a dissection microscope based on movement of the animals in response to gentle prodding with a platinum pick. When contaminated regions appeared on the plates, a small razor was used to remove the agar in the affected areas. Instances of widespread contamination were handled by transferring animals to new plates with the corresponding conditions. Loss of vulval integrity due to advanced age was observed and noted but did not preclude the ruptured worms from being scored. However, animals that fled their plate or were removed during extraction of contaminated agar were censored.

For the *tbh-1* and *tdc-1* lifespans, N2 and *tdc-1/tbh-1* worms were separately placed on plates seeded with either empty vector (EV) or *vhl-1* RNAi. Lifespan assays for *acy-1, acy-2,* and *acy-3* were performed by placing TU3311 or TU3311; *vhl-1* animals on plates seeded with EV or RNAi for the three genes of interest. Two technical replicates were scored for each condition tested.

5. Fluorescence Microscopy

Two timed egg lays were first conducted to synchronize the ages of the analyzed worms and expose them to empty vector or *vhl-1* RNAi. 30-50 animals were transferred onto RNAi + FUdR plates approximately 24 hours prior to imaging. Microscope slides were prepared with 3% agarose pads trimmed to the width of a slide. Thirty worms for each condition were immobilized on the agarose mounts for two minutes using 10 μL of 0.5 M sodium azide. Images were obtained using a Leica microscope and Leica Application Suite X software at 32X magnification.

Two *tdc-1* backcross siblings were crossed into the *fmo-2p::mCherry* strain to detect *fmo-2* transcription levels. *fmo-2p::mCherry* animals were imaged as a control.

6. Quantification and Statistical Analyses

All data was entered in Microsoft Excel 365 and plotted and analyzed in R Studio. Significant survival trends were quantified using P-values obtained via log rank tests or Cox regression analysis. P values are *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. Fluorescent mean comparisons were then quantified using custom R code to identify individual worms and measure fluorescent intensity. The mean fluorescent intensity of the background (all non-worm pixels) was subtracted from the mean fluorescence of each worm. All outputs from this R code were manually quality controlled to eliminate any non-worm objects that were misidentified. Bar plots show data points representing individual worms, with the height of the bar indicating the mean fluorescence in a given condition.

Results

1. Octopamine is not required for lifespan extension under hypoxic conditions.

Because hypoxic conditions drive changes in the nervous system that modulate longevity, we asked whether the *C. elegans* neurotransmitters octopamine and tyramine were required for hypoxia to extend lifespan. To examine the role of octopamine in hypoxia, we crossed a strain carrying a deletion in *tbh-1,* the gene encoding a hydroxylase enzyme required for octopamine synthesis, with our lab's wildtype N2 strain four times. Three mutants deficient in octopamine from this backcross were placed on empty vector (EV control) and *vhl-1* (hypoxia mimetic) RNAi to determine whether octopamine synthesis is required for hypoxia-mediated longevity.

As expected, all *tbh-1* backcross sibling strains behaved similarly overall, with shorter lifespans in basal conditions compared to wildtype animals and no significant blunting of lifespan extension after RNAi-mediated *vhl-1* knockdown.

If, as hypothesized, octopamine were involved in this circuit, animals from strains deficient in the neurotransmitter would not be expected to live longer under hypoxic conditions. Across all tested *tbh-1* strains, however, significant lifespan extension was still observed in response to *vhl-1* RNAi. Additionally, Cox regression analysis revealed that lack of octopamine synthesis did not interact with the degree of lifespan elongation for *tbh-1* backcross #3 ($p = 0.064$), *tbh-1* backcross #4, ($p = 0.147$), and *tbh-1* backcross #7 ($p = 0.318$) in hypoxic conditions. Collectively, these results suggest that octopamine is not necessary for hypoxia-induced lifespan extension, and therefore does not act as a necessary chemical messenger in this pathway.

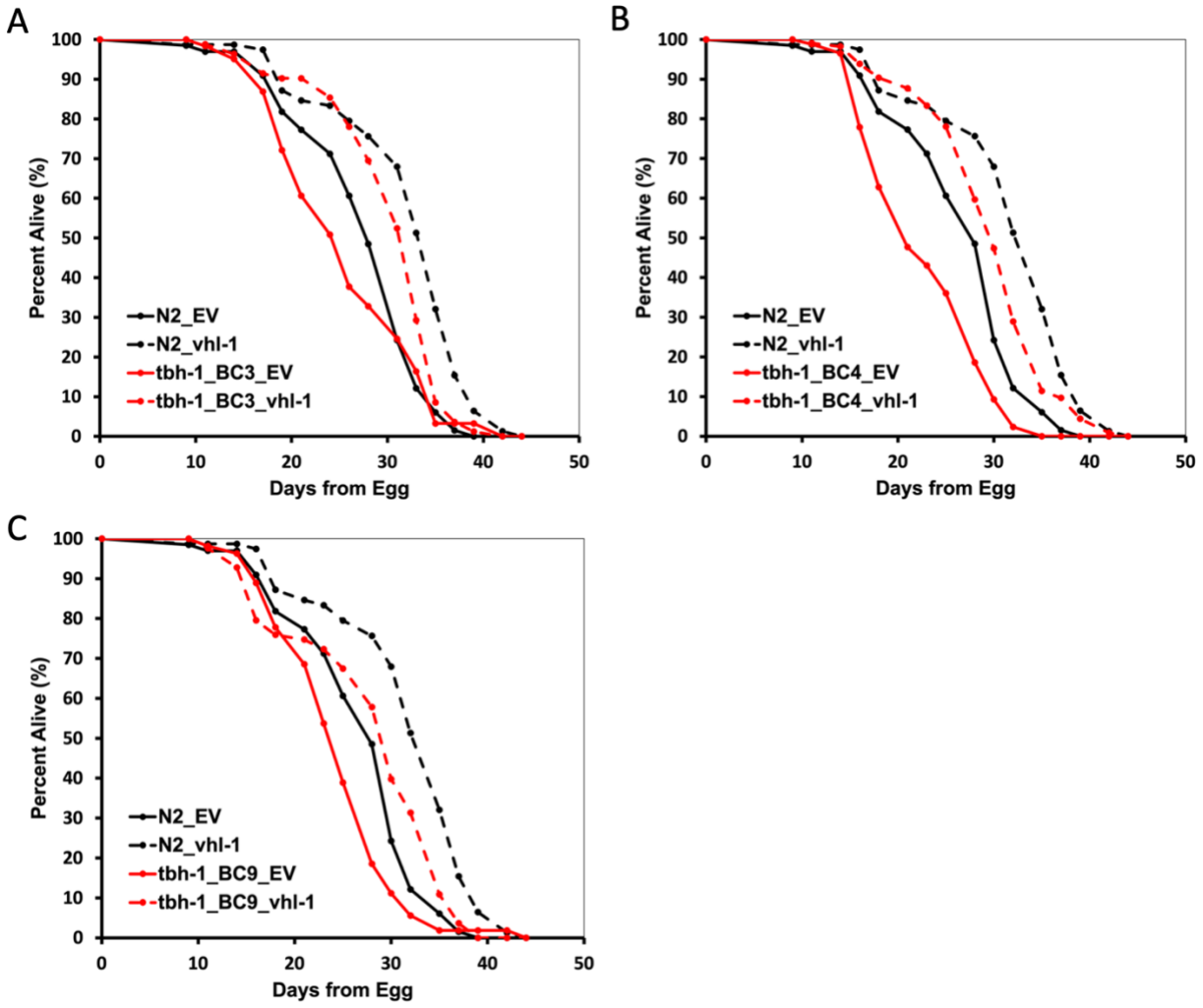

Fig. 3 | *tbh-1* mutation does not blunt lifespan extension under hypoxic conditions across three sibling strains. A. Survivorship curves shown for wildtype *C. elegans* (black) and *tbh-1* backcross sibling #3 (red). n = 66 (N2 EV), n = 78 (N2 *vhl-1* RNAi), n = 61 (*tbh-1* backcross #3 EV), n = 82 (*tbh-1* backcross #3 *vhl-1* RNAi). Significant lifespan extension between empty vector (solid lines) and *vhl-1* (dashed lines) was observed for both N2 ($p = 1.6 \times 10^{-5}$) and *tbh-1* animals ($p = 2.0 \times 10^{-4}$). **B.** Lifespan data for wildtype *C. elegans* and *tbh-1* backcross sibling #4. n = 86 (*tbh-1* backcross #4 EV), n = 114 (*tbh-1* backcross #4 *vhl-1* RNAi). Both strains displayed lifespan extension in response to hypoxia (*tbh-1* backcross #4, $p < 2.0 \times 10^{-16}$). **C.** Survival curves shown for wildtype *C. elegans* and *tbh-1* backcross sibling #9. n = 54 (*tbh-1* backcross #9 EV), n = 83 (*tbh-1* backcross #9 *vhl-1* RNAi). The longevity phenotype was exhibited by animals from both strains (*tbh-1* backcross #9, $p = 3.2 \times 10^{-2}$). Log-rank tests were employed to obtain all described P-values for Fig. 3.

2.1 Tyramine is required for hypoxia-induced lifespan extension.

We next asked whether the *C. elegans* neurotransmitter tyramine is required for hypoxia to extend lifespan. To answer this question, we performed a lifespan assay using a strain with a mutation to *tdc-1,* the rate-limiting enzyme necessary for synthesis of tyramine. Notably, tyramine is a precursor to octopamine, meaning that *tdc-1* mutant animals lack the ability to synthesize octopamine and tyramine. By conducting the lifespan assay with *tbh-1* strains first, we identified that octopamine is not required for hypoxia-mediated longevity. Consequently, if *tdc-1* were found to abrogate lifespan extension, this would indicate that a role for tyramine synthesis exists in this pathway.

The *tdc-1* lifespan assay was carried out using three sibling *tdc-1* deletion strains that were backcrossed to wildtype four times. With similar sample sizes before and after plate transfers, the trends observed in the assay were consistent across the three different backcross sibling strains. Interestingly, however, log-rank testing revealed that *tdc-1* backcross sibling #7 animals were slightly longer lived on empty vector RNAi than wildtype worms in the same conditions (3.5% difference, $p = 7.9 \times 10^{-3}$). As expected, the wildtype worms experienced significant extension of lifespan when fed on *vhl-1* RNAi as compared to empty vector (Log-rank test, $p = 1.5 \times 10^{-8}$). Additionally, Cox regression analysis indicated a significant interaction between genotype and *vhl-1* RNAi in *tdc-1* backcross #7 ($p = 2.0 \times 10^{-7}$), *tdc-1* backcross #8 ($p = 1.3 \times 10^{-3}$) and *tdc-1* backcross #12 ($p = 1.2 \times 10^{-3}$) strains. The data thus suggest that lifespan extension as a result of exposure to hypoxia is contingent on proper tyramine function.

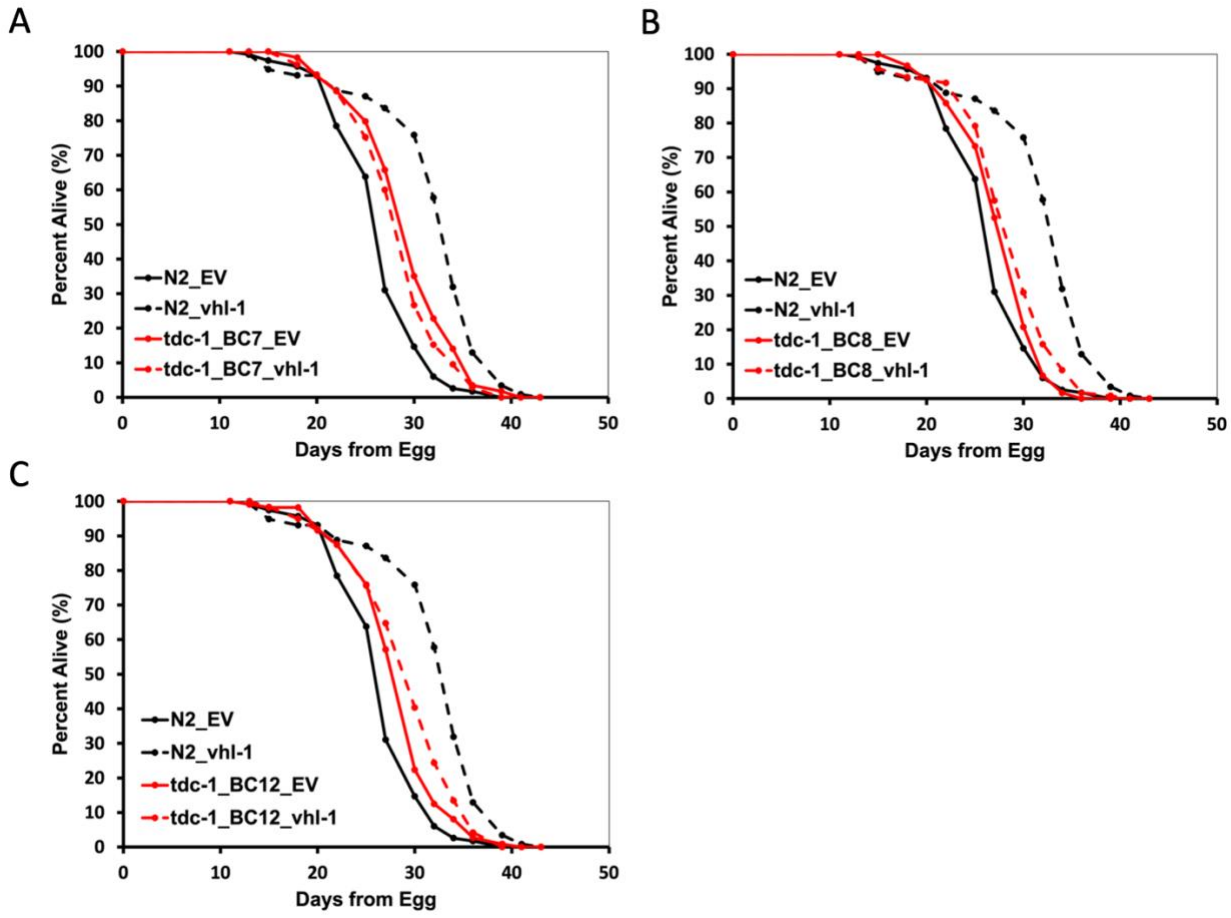

Fig. 4 | Loss of *tdc-1* function abrogates lifespan extension in conditions of hypoxia across three sibling strains. A. Lifespan curves shown for wildtype *C. elegans* (black) and *tdc-1* backcross sibling #7 (red). n = 116 (N2 EV), n = 116 (N2 *vhl-1* RNAi), n = 114 (*tdc-1* backcross #7 EV), n = 105 (*tdc-1* backcross #7 *vhl-1* RNAi). Wildtype worms exposed to *vhl-1* RNAi were longer lived than the animals fed on empty vector RNAi ($p = 1.5 \times 10^{-8}$). No such difference in lifespan was observed in the *tdc-1* backcross #7 mutant (p = 1.0). **B.** Survivorship data for wildtype *C. elegans* and *tdc-1* backcross sibling #8. n = 120 (*tdc-1* backcross #8 EV), n = 120 (*tdc-1* backcross #8 *vhl-1* RNAi). *tdc-1* mutation abrogated lifespan extension under hypoxic conditions for backcross sibling #8 (p = 1.0). **C.** Survival curves for wildtype *C. elegans* and *tdc-1* backcross sibling #12. n = 112 (*tdc-1* backcross #12 EV), n = 119 (*tdc-1* backcross #12 *vhl-1* RNAi). Loss of lifespan extension in response to hypoxia was also observed for *tdc-1* backcross sibling #12 (p = 1.0). Log-rank tests were employed to obtain all described P-values for Fig. 4.

2.2 Tyraminergic requirement for lifespan extension by hypoxic response is independent of *fmo-2* induction.

After determining that tyramine is necessary for hypoxia-induced longevity through a lifespan assay, it was then crucial to ascertain whether the neurotransmitter drives lifespan extension by contributing to *fmo-2* induction. To this end, two *tdc-1* sibling strains were crossed with a strain carrying a fluorescent reporter gene to enable detection of *fmo-2* transcription levels, *fmo-2p::mCherry.* If *fmo-2* induction were tyramine dependent, fluorescence levels in *fmo-2p::mCherry; tdc-1* #1 and *fmo-2p::mCherry; tdc-1* #2 animals on *vhl-1* RNAi would be significantly less than in *fmo-2p::mCherry* worms in an otherwise wildtype background.

The imaged worms were raised on either empty vector or *vhl-1* RNAi plates for two generations, transferred to RNAi + FUdR plates on day one of adulthood, and imaged on day two of adulthood. A second replicate of the imaging assay was conducted as minor contamination was observed on *fmo-2p::mCherry* and *fmo-2p::mCherry; tdc-1 #2* plates on the first day of imaging (data not shown).

The control *fmo-2p::mCherry* strain behaved as expected with a significant increase in *fmo-2* induction on *vhl-1* conditions as compared to empty vector. Surprisingly, these trends were also reflected in both *tdc-1* siblings. The marked induction of *fmo-2* in these strains suggests that tyramine is not necessary for *fmo-2* upregulation under hypoxic conditions. However, the lifespan data collected for tyramine-deficient strains indicates that the neurotransmitter does play a role in extending lifespan in worms exposed to hypoxia. Thus, these data collectively suggest that while tyramine is necessary for hypoxia-induced longevity, it likely operates through an alternate pathway that is not contingent on *fmo-2* induction.

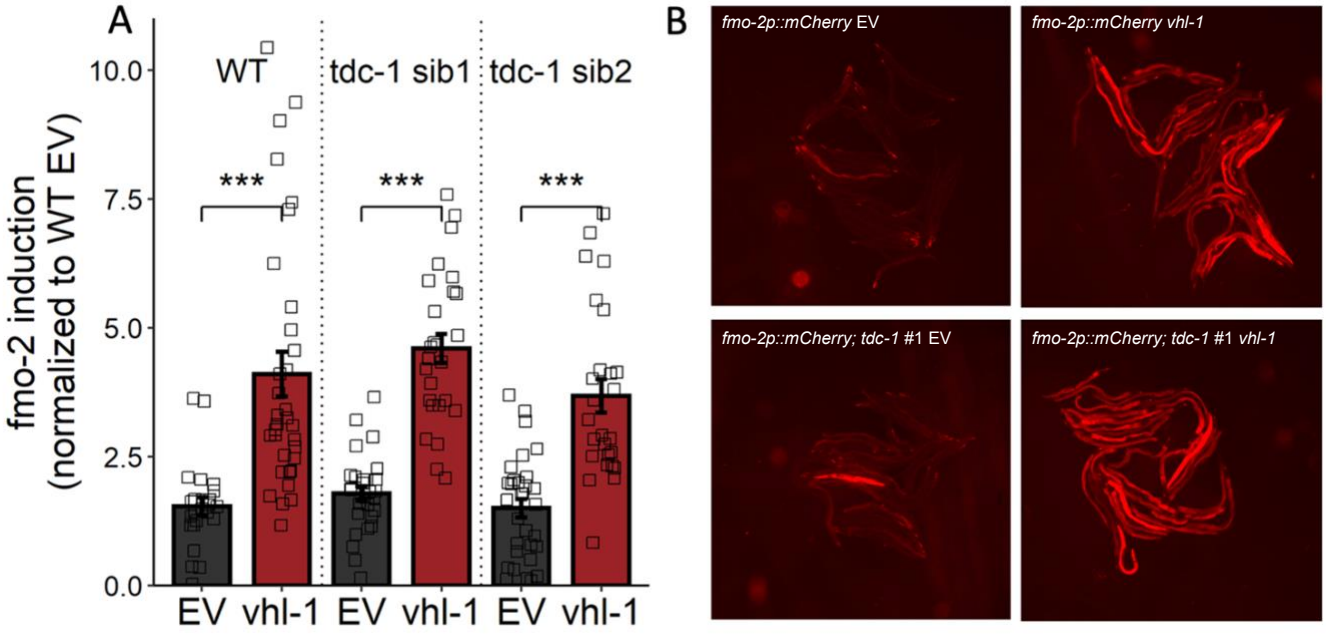

Fig. 5 | *fmo-2* is induced in hypoxic conditions in the presence or absence of functional tyramine. A. Quantified *fmo-2* fluorescence levels for wildtype, *tdc-1* sibling #1, and *tdc-1* sibling #2 strains on empty vector or *vhl-1* RNAi (n = 20 for all tested strains). For all conditions tested, *fmo-2* fluorescence was significantly increased on *vhl-1* RNAi as compared to EV ($p < 0.001$). **B.** Top two panels show induction of *fmo-2* in response to hypoxia in a control *fmo-2p::mCherry* fluorescent reporter strain. Bottom panels show *fmo-2* induction on EV (left) and *vhl-1* (right) for *tdc-1* sibling #1 but images are representative of trends seen for *tdc-1* sibling #2 (not pictured) as well.

3. *acy-1, acy-2,* and *acy-3* partially blunt lifespan extension under hypoxic conditions.

The serotonin-binding *C. elegans* G-protein coupled receptor, SER-7, is known to stimulate adenylyl cyclase enzymes, which are encoded by the genes *acy-1, acy-2,* and *acy-3,* in different nematode tissues. Thus, these enzymes are viable candidate downstream effectors of the serotonin binding event that is a known component of the hypoxia-induced lifespan extension pathway. A lifespan assay was conducted using TU3311, a strain optimized for neuronal RNAi uptake in an otherwise wildtype background and TU3311; *vhl-1* animals. Both strains were fed on RNAi corresponding to one of the adenylyl cyclase-encoding genes tested or empty vector RNAi. Two timed egg lays were performed to further ensure RNAi penetrance in the tested animals.

If knockdown of any of the three tested *acy* genes results in loss of lifespan extension in TU3311; *vhl-1* worms, this would suggest that the gene(s) are individually acting effectors of longevity under hypoxic conditions. However, no marked abrogation of lifespan extension was observed in any of the *acy* knockdown conditions tested in this lifespan assay. While collectively significant extension was observed based on log-rank testing, the magnitude of extension between the three knockdowns and the control was variable as indicated by Cox regression analysis. On *acy-1* ($p = 3.5 \times 10^{-9}$), *acy-2* ($p = 2.3 \times 10^{-4}$), and *acy-3* ($p = 1.8 \times 10^{-4}$) RNAi, there was a significant interaction between RNAi and genotype. In all *acy* knockdowns, the extent of lifespan elongation observed in hypoxic conditions was reduced as compared to EV RNAi. Notably, TU3311 plates on empty vector RNAi experienced mold contamination while the other plates did not. The potential confounding effects of this condition on the control data could impact the statistical analysis of this experiment and should thus be addressed by performing another replicate of the assay in consistently sterile conditions.

The observation of blunted lifespan extension in *vhl-1* animals on both empty vector and *acy* RNAi in this assay could suggest that individual adenylyl cyclase enzymes each partially contribute to lifespan extension for an overall additive effect. Thus, a cooperative mechanism between pairs or all three of the adenylyl cyclase enzymes tested may be necessary to obtain significantly long-lived phenotypes in response to hypoxia. This mutualistic model could be tested by generating double *acy* mutant animals and similarly assaying for lifespan extension or abrogation under hypoxic conditions.

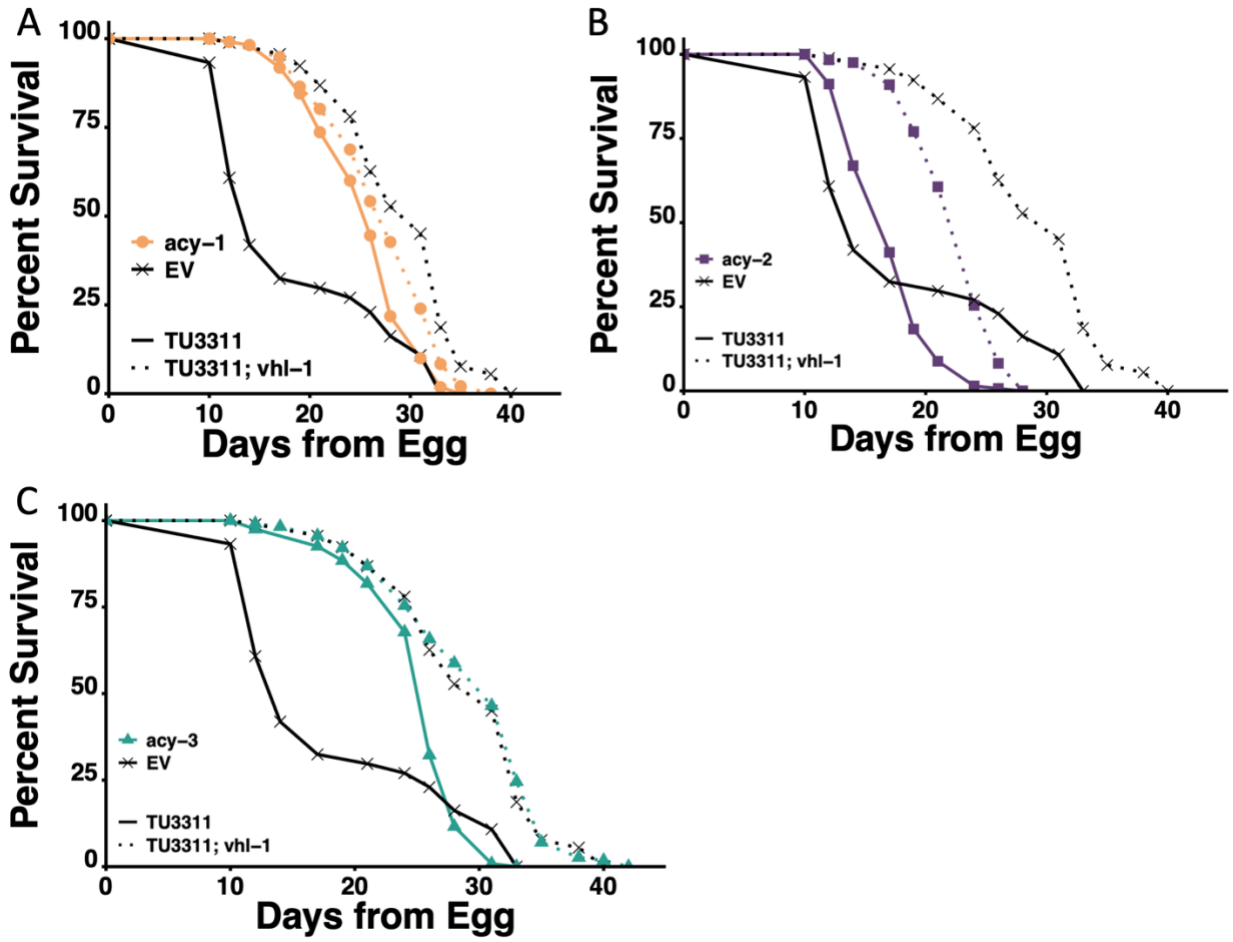

Fig. 6 | Longevity is blunted but not blocked in *vhl-1* animals on *acy-1*, *acy-2* or *acy-3* RNAi. **A.** Lifespan curves are shown for TU3311 *C. elegans* (black) and *vhl-1* animals (orange). n = 95 (TU3311 EV), n = 74 (TU3311 *acy-1* RNAi), n = 110 (TU3311; *vhl-1* EV), n = 96 (TU3311; *vhl-1 acy-1* RNAi). Significant extension of lifespan (Log-rank test, $p < 2.0 \times 10^{-16}$) was observed between control TU3311 strains on empty vector RNAi compared to TU3311; *vhl-1* animals (Log-rank test, $p < 2.0 \times 10^{-16}$). Similarly, lifespan extension was observed for TU3311; *vhl-1* animals on *acy-1* (Log-rank test, $p = 1.3 \times 10^{-4}$). However, there was a significant interaction between *acy-1* RNAi and genotype (Cox regression, $p = 3.5 \times 10^{-9}$). **B.** Survivorship data for TU3311 and TU3311; *vhl-1* animals on EV and *acy-1* RNAi (purple). n = 136 (*acy-2* EV), n = 122 (*acy-2* TU3311; *vhl-1* RNAi). Loss of functional *acy-2* partially abrogated longevity in hypoxic conditions (Cox regression, $p = 2.3 \times 10^{-4}$). **C.** Survival curves for TU3311 and worms on *acy-3* RNAi (teal). n = 121 (TU3311 *acy-3* RNAi) n = 114 (*vhl-1 acy-3* RNAi). While TU3311; *vhl-1* worms on *acy-3* RNAi were longer lived than TU3311 animals in the same

conditions (Log-rank test, $p = 7.9 \times 10^{-15}$), there was a significant interaction between *acy-3* RNAi and genotype, indicating *acy-3* is partially required for hypoxia-mediated longevity (Cox regression, $p = 1.8 \times 10^{-4}$).

Discussion

As an underlying risk factor for the most prevalent and lethal illnesses across the globe, aging provides a single biological phenomenon that can be studied to gain insights on preventing these diseases collectively. While the relatively extensive lifespans of mammals preclude them from being feasible models for large-scale, exploratory studies on factors that may influence longevity, the model nematode, *Caenorhabditis elegans,* affords researchers the ability to gather these findings in a matter of weeks. Along with their 20-day lifespans, the 83% homology in protein-encoding genes between *C. elegans* and humans makes it possible for discoveries made in nematodes to inform future studies and applications in higher order organisms. Hypoxia, one of several environmental factors found to extend lifespan in worms, operates through a cell nonautonomous pathway that hinges on the induction of the conserved, intestinally expressed enzyme, FMO-2. However, the identification of all molecular intermediates involved in this pathway remains incomplete. As cell nonautonomous mechanisms rely on communication between different cell types, the six classes of neurotransmitters in *C. elegans* are candidate chemical messengers that may influence longevity under hypoxic conditions. Elucidating the role of neurotransmitters in the *C. elegans* hypoxia-induced lifespan extension pathway is therefore a productive next step in mapping this mechanism and identifying more of its candidate downstream effectors.

Previous studies have effectively employed imaging of *fmo-2* induction and lifespan assays to evaluate the involvement of potential molecular players downstream of the conserved hypoxia response pathway. In this study, these methods were applied with the new objective of defining novel roles for neurotransmitters in hypoxia-induced lifespan extension. A *C. elegans* strain deficient in the rate-limiting enzyme for synthesis of the neurotransmitter octopamine was first crossed into a lab standard strain then assayed for longevity in normal and hypoxic conditions using a genetic mimetic of hypoxia, *vhl-1* RNAi. Across three sibling mutant strains, no abrogation of lifespan extension was observed, suggesting that hypoxia-induced lifespan extension does not rely on the presence of functional octopamine. Likewise, animals with mutations in *tdc-1,* a decarboxylase necessary for production of the neurotransmitter tyramine, were similarly backcrossed and assayed for blocked lifespan extension. These experiments revealed that loss of tyramine does abrogate longevity in hypoxic conditions, suggesting that the neurotransmitter functions as a chemical messenger in hypoxia-induced lifespan extension. To

determine whether tyramine extends lifespan in response to hypoxia through the *fmo-2* induction pathway, imaging assays were conducted to observe changes in expression of the enzyme in the presence or absence of functional tyramine in hypoxic conditions. Notably, two technical replicates of the assay revealed that hypoxia induces *fmo-2* in wildtype and *tdc-1* mutant strains. These results indicate that tyramine's pro-longevity function in response to hypoxia does not require *fmo-2* induction and therefore likely functions via an alternate hypoxia-induced pathway.

Future studies aiming to identify the specific pathway tyramine functions in to enhance lifespan in hypoxic conditions could first focus on the insulin/IGF-like signaling (IIS) pathway, a second circuit that increases *C. elegans* lifespan in response to multiple stressors. Lifespan extension via the IIS pathway is contingent on the activity of the transcription factor, DAF-16. Animals with mutations in *age-1,* an upstream effector of DAF-16, stimulate the transcription factor and extend lifespan in hypoxic conditions (Leiser, et. al., 2013). Thus, to determine whether tyramine promotes longevity through the IIS pathway, lifespan assays could be carried out in *tdc-1* mutants on *age-1* RNAi. Abrogation of lifespan extension in these conditions would provide evidence to support tyramine's involvement in IIS downstream of *age-1.*

The thoroughly mapped *C. elegans* connectome has identified the classes of neurons that rely on specific neurotransmitters to propagate signals. As such, another approach that could be taken to elucidate the role of tyramine in hypoxia-induced lifespan extension is through the ablation of tyraminergic neurons. For example, RIM motor neurons are responsible for reverse locomotion and communicate with other classes of forward locomotion neurons via tyramine signaling (Alkema, et. al., 2005). Since tyramine is required in signaling circuits with RIM neurons, ablating these neurons in *C. elegans* and assaying lifespan extension in these strains could reveal whether tyramine signaling through RIM neurons is an intermediate step of hypoxia-induced longevity.

The studies performed on octopamine and tyramine mutants in this study coupled with the elucidation of the roles of other neurotransmitters such as serotonin resulting from previous research leave one remaining type of neurotransmitter in *C. elegans* that has not been investigated in the context of hypoxia-induced lifespan extension. This neurotransmitter, γ-Aminobutyric acid (GABA), is a chemical messenger necessary for facilitating muscle contraction and relaxation to aid locomotion in worms. The methods applied to the experiments conducted to better understand tyramine and octopamine can likewise be used to ascertain

whether GABA plays a role in hypoxia-induced lifespan extension. This would involve first backcrossing a strain of worms that have mutations in a gene like *unc-25,* which encodes an enzyme necessary for GABA synthesis, to a lab standard strain. Next, lifespan assays could be carried out on empty vector or *vhl-1* RNAi to determine whether the *unc-25* mutants no longer exhibit long-lived phenotypes under hypoxic conditions. Additionally, if abrogation of lifespan is observed in these mutants, imaging assays could be performed to establish whether GABA is involved in extending lifespan under hypoxia through *fmo-2* induction.

Serotonin, a known intermediate of this circuit that is released upon stabilization of the HIF-1 transcription factor in response to hypoxia, was also investigated in this study. As proof of the neurotransmitter's necessity in the pathway had already been documented, potential downstream targets of serotonin became the focus of the lifespan assays performed here. To determine whether serotonin signaling operates through adenylyl cyclase enzymes expressed in various *C. elegans* tissues, *acy-1, acy-2,* and *acy-3* were individually knocked down using RNAi in wildtype worms and animals in hypoxic conditions. Partial blunting of lifespan extension was observed in all three knockdown conditions, suggesting that serotonin stimulating an individual adenylyl cyclase is not a component of the hypoxia-induced longevity pathway.

Since RNAi against *acy-1, acy-2,* and *acy-3* in isolation blunted but did not completely block longevity, the results from this study suggest that the adenylyl cyclase enzymes tested may work in tandem to promote lifespan extension in hypoxic conditions. Future studies could thus involve carrying out lifespan assays using double or triple *acy* mutants to determine whether depletion of these enzymes in combination can block lifespan through an additive mechanism. If longevity is found to be abrogated under any of these conditions, cooperative action of adenylyl cyclase enzymes is a likely step in the hypoxia-induced lifespan extension pathway.

An overarching limitation that may impact the generalizability of the present study surrounds the use of *vhl-1* as a genetic mimetic of hypoxia. The normoxic branch of the highly conserved hypoxia response pathway involves the actions of prolyl hydroxylase enzymes as well as the E3 ligase, VHL-1. While mutating *vhl-1* stabilizes HIF-1 by preventing its proteasomal degradation, the transcription factor still undergoes prolyl hydroxylation as this step precedes its polyubiquitination. For this reason, *vhl-1* mutation is not completely interchangeable with exposure to hypoxia, as the hypoxic branch of the HRP stabilizes HIF-1 but does not introduce

any modifications to it. Despite this, the ability of *vhl-1* mutation to reliably increase lifespan without leading to restrictive health defects maintains it as an informative experimental tool.

In this thesis project, lifespan and imaging assays identified the neurotransmitter tyramine as a chemical messenger necessary for longevity in response to hypoxia via a pathway that is not contingent on the induction of the flavin monooxygenase, *fmo-2*. Additionally, the involvement of serotonin in this pathway was determined to be partially reliant on individual adenylyl cyclase enzymes in isolation. Lifespan assays also revealed that the neurotransmitter octopamine is not necessary for extending lifespan in response to hypoxia.

Given the projected 50% increase in the population of individuals that are at least 65 years of age within the next five decades, studying aging pathways has become increasingly imperative to address the age-related physical and cognitive challenges that affect a rapidly growing global demographic. Thus, the experiments in this study were carried out with the goal of contributing to the effort to better understand biological aging to identify potential biomarkers or therapeutic targets of age-related illnesses and ultimately develop innovative strategies to promote healthy aging and extend human lifespan.

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