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Caenorhabditis elegans As a Model System to Study Aging of Learning and Memory

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Abstract

The nematode *Caenorhabditis elegans* is an excellent model organism to study biological processes relevant to a wide variety of human and rodent disease systems. Previous studies have suggested that mutants of the insulin/insulin-like growth factor-1 pathway show life extension and increased stress resistance in various species, including *C. elegans,* the fruit fly, and the mouse. It has recently been shown that the life-extending mutants, including the *age-1* phosphatidylinositol-3 OH kinase mutants and the *daf-2* insulin-like receptor mutants, display improvement in a type of associative learning behavior called thermotaxis learning behavior. The *age-1* mutant shows a dramatic threefold extension of the health-span that ensures thermotaxis learning behavior, suggesting strong neuroprotective actions during aging. The *age-1* and *daf-2* mutants show resistance to multiple forms of stress and upregulates the genes involved in reactive oxygen species scavenging, heat shock, and P450 drug-detoxification. The life-extending mutants may confer resistance to various stress and diseases in neurons. Therefore, *C. elegans* provides an emerging system for the prevention of age-related deficits in the nervous system and in learning behaviors. This article discusses the aging of learning and memory and the neuroprotection effects of life-extending mutants on learning behaviors.

Index Entries: Aging; age-related memory impairment; learning and memory; Insulin/IGF-1 signal; stress resistance; cognitive behavior; neuroprotection; *C. elegans*.

Thermotaxis Learning Behavior in *Caenorhabditis elegans*

Learning and memory is an important behavioral function that is observed in various animal species. Associative learning behavior is a type of behavioral plasticity that associates a stimulus or behavior with another stimulus *(1)*. After conditioning at a given temperature in the presence of food, *Caenorhabditis elegans* learns to use temperature as a clue to look for food. This classical conditioning is a type of associative leaning called thermotaxis learning behavior, which associates the paired stimuli of food and the

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given temperature (temperature-food association). This associative learning behavior can be assessed either in a single-animal assay (isothermal tracking) or in a thermotaxis population assay. *C. elegans* can also be conditioned by starvation (the absence of food) to avoid the temperature (temperature-starvation association). Two additional types of associative learning paradigms have been reported in *C. elegans*, including chemotaxis learning behavior and slowing response to food (reviewed in ref. 2). Thermotaxis learning behavior may share mechanisms similar to those in mammals, such as calcium signaling and low-density lipoprotein (LDL) signaling. A modulator of thermotaxis learning behavior, the *ncs-1* gene, encodes an EF hand, calcium sensor protein, which is well-conserved in various species *(3)*. Expression of a neuronal calcium sensor protein gene, *ncs-1*, in the AIY interneurons (*see* next section) can increase isothermal tracking performance, whereas an *ncs-1* knockout reduces performance *(3)*. Another modulator, *hen-1*, encodes a secretory protein with an LDL receptor motif gene and is expressed in AIY and a chemosensory neuron ASE *(4)*. Mutations in *hen-1* abolish thermotaxis and chemotaxis learning behavior. Note that serotonin mediates food responses associated with thermotaxis and chemosensory learning behavior *(5,6)*.

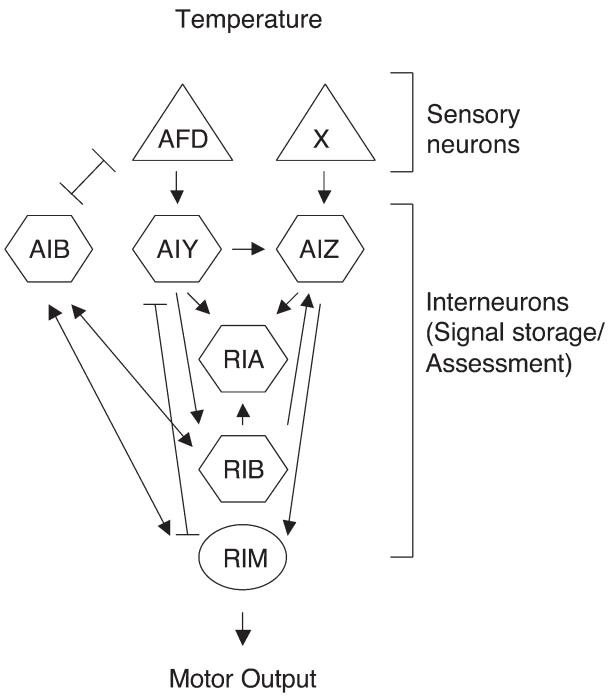

Fig. 1. Thermosensory neurons. The AIY and AIZ interneurons, which are downstream of the AFD sensory neurons, are important for sensory signal processing and integration *(57)*. The processed information leads to changes in behavioral outputs through the motor neurons such as RIM. Symbols indicate synaptic neurons (arrows), gap junctions ("H" shaped arrows), sensory neurons (triangles), interneurons (hexagons), and motor neurons (circles). (Adopted from ref. *57* and modified.)

Simple Thermotaxis Circuits

C. elegans has a simple nervous system with a small number of neurons compared to other model animals *(7)*, providing an ideal system to study the aging of the nervous system. There are only 302 neurons (out of 959 somatic cells) in adult hermaphrodites, and the majority of neurons are classified as sensory, interneuron, or motor neurons. Thermotaxis neuronal circuits consist of several types of neurons (Fig. 1). A pair of interneurons, AIY and AIZ, mediate thermal inputs from the thermosensory neuron AFD. These AIY and AIZ interneurons (Fig. 1) may be core sites for the functions of this associative learning. Interestingly, the thermosensory circuits in *C. elegans* show conservation with the visual sensory circuits in vertebrates *(8)*. Therefore, *C. elegans* may have evolved a thermal sensory system instead of a visual sensory system.

Oxidative Stress and Thermotaxis Learning Behavior

Deleterious oxidative stress increases during aging and is a major risk factor for age-related cognitive declines in mammals *(9–11)*. Investi-

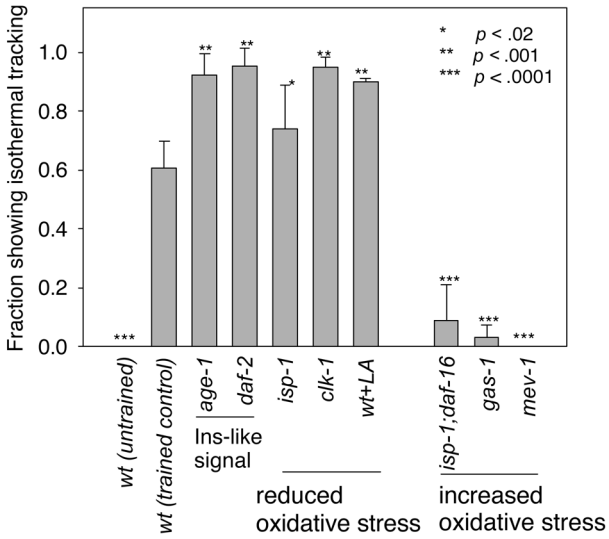

Fig. 2. Improved learning in *age-1, daf-2,* and oxidative-stress reducing conditions. The mutants in the insulin/IGF-1 pathway (*age-1* and *daf-2*), in the mutants with reduced oxidative metabolism (*isp-1* and *clk-1*), in the animals treated with an antioxidant Lipoic acid (LA), and the mutants with increased oxidative stress (*isp-1;daf-16, gas-1* and *mev-1*) are shown. The published data were used *(20,21)*. The level of significance was calculated by using ANOVA.

gation of the effects of oxidative stress on learning and memory could reveal mechanisms that cause the vulnerability of the aging neurons to stress insults. Mutations that increase oxidative stress (*gas-1* and *mev-1*) have been shown to impair isothermal tracking (Fig. 2) and slightly reduce the locomotion rate. The *mev-1* and *gas-1* mutants have defects in components of mitochondrial respiratory complexes II and I, respectively *(12,13)*. These mutants cause hypersensitivity to oxidative stress.

The *clk-1* and *isp-1* mutants have been shown to improve learning behavior (Fig. 2). The mutants lower oxygen metabolism and have been proposed to have decreased oxidative stress in mitochondria and cytoplasm, respectively *(14,15)*. Therefore, oxidative stress significantly affects the ability to learn and remember. The results suggest that the learning assay is a sensitive system to determine levels of physiological oxidative stress.

Interestingly, the treatment with an antioxidant called lipoic acid (LA) improves learning (Fig. 2) and partially rescues learning impairment caused by the *mev-1* mutant. LA is a metabolic antioxidant that is readily incorporated into the cells *(16)*. LA is reduced to a potent antioxidant (dihydrolipoic acid) that can recycle other antioxidants such as vitamins C and E *(17)*. LA can decrease oxidative damage in the brains of older rats and partially restore age-related declines in nervous functions *(16)*. In mammals, LA along with statin (inhibitors of HMG-CoA reductase) can reduce oxidation of LDL, which is a risk factor for development of neurodegenerative and cardiovascular diseases *(18)*.

Aging of Learning and Memory in *C. elegans*

Only three studies have investigated aging of nonassociative learning *(19)* and associative learning *(20,21)* in *C. elegans*. Similarly to mammals, not all functions of learning and memory decline with increasing age. After a repeated mechanical stimulus, animals show reduced response to the stimulus. This nonassociative learning is called habituation. Beck and Rankin *(19)* suggested that habituation showed an increase in old animals, whereas recovery from habituation was slower in the old animals compared to the young animals. It has been shown that simple chemotaxis to benzaldehyde and chemotaxis avoidance to octanol declines during aging *(22)*. However, additional experiments are needed to conclude whether the declines were caused by reduced motor activity or by reduced chemosensory functions.

There is a general trend toward early declines in associative learning behavior (referred to as early changes) that occurs during the mid- to late reproduction periods, compared to declines in sensory and motor activity (referred to as late

changes) that occur postreproductively. Late changes in behaviors include declines in motor activity and simple thermotaxis (refs. *20* and *21;* Murakami H., et al., unpublished). A decline in temperature-food thermotaxis learning behavior occurs between the mid- and late reproduction periods, *(21)*. Similar trends have been observed in chemotaxis learning behavior (Murakami H. et al., unpublished). Thermotaxis learning behavior declines from 80 to 40% with increasing age *(20)*. Because motor declines of up to 40% did not affect thermotaxis learning behavior (temperature-food; ref. *20*), the effects of motor activity should be relatively minor until the postreproduction period.

Life-Extending Mutants of the Insulin-Like Pathway

More than a hundred life-extending genes have been identified in *C. elegans.* The genes mediate various biological processes, including larval development, stress resistance, oxidative metabolism, and reproduction. The insulin-like signal transduction pathway includes *daf-2* (insulin-like receptor gene) and *age-1* (phosphatidylinositol-3 OH kinase gene), which negatively regulates the Forkhead transcription factor, encoded by *daf-16* (Fig. 3). Mutations in the *age-1* and *daf-2* genes cause increased longevity, stress resistance, increased innate immunity, increased formation of diapause larvae (called dauer), and other phenotypes *(23–26)*. It has been shown that the *age-1/daf-2* insulin-like pathway functions in neurons to specify longevity *(27)*, although the effect may be relatively minor *(28)*.

Parallel to the insulin-like pathway, the transforming growth factor (TGF)-β pathway also regulates dauer formation *(23)*. Unlike the insulin-like pathway mutants, mutants in the TGF-β pathway do not show increased longevity and are not resistant to oxidative stress and UV irradiation. Interestingly, the insulin/ IGF-1 signal interlocks with a steroid pathway that involves the DAF-9 P450 and the DAF-12 steroid receptor *(29–31)*. P450 is an enzyme that metabolizes cholesterol, which may produce a ligand of DAF-12 steroid receptor.

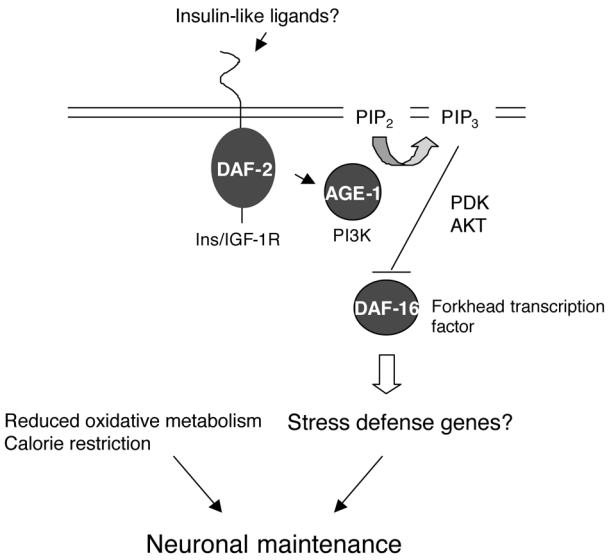

Fig. 3. A model of the regulation of learning and memory by insulin-like signal. The DAF-2 insulin/IGF-1 receptor and the AGE-1 PI3 Kinase negatively regulate the DAF-16 FOXO/Forkhead transcription factor. DAF-16 is known to regulate expression of various stress-defense genes. (For details, *see* text. The figure from ref. *21* was modified.)

Increased Learning and Delayed Age-Related Learning Decline in Life-Extending Mutants in *C. elegans*

Young *age-1* and *daf-2* mutants show significantly higher levels of thermotaxis learning behavior than the wild-type (Fig. 2; ref. *21*). The mutants showed an approx 35% increase. Conversely, the *daf-16* mutants showed reduced isothermal tracking, ranging from 22 to 62% reduction. The *daf-16* mutants show short lifespan and dauer-defective phenotypes, which are the effects opposite for the *age-1* and *daf-2* mutants. Therefore, mutations that reduce insulin/IGF-1 signal increase associative learning, whereas mutations that increase insulin/ IGF-1 signal are likely to decrease learning.

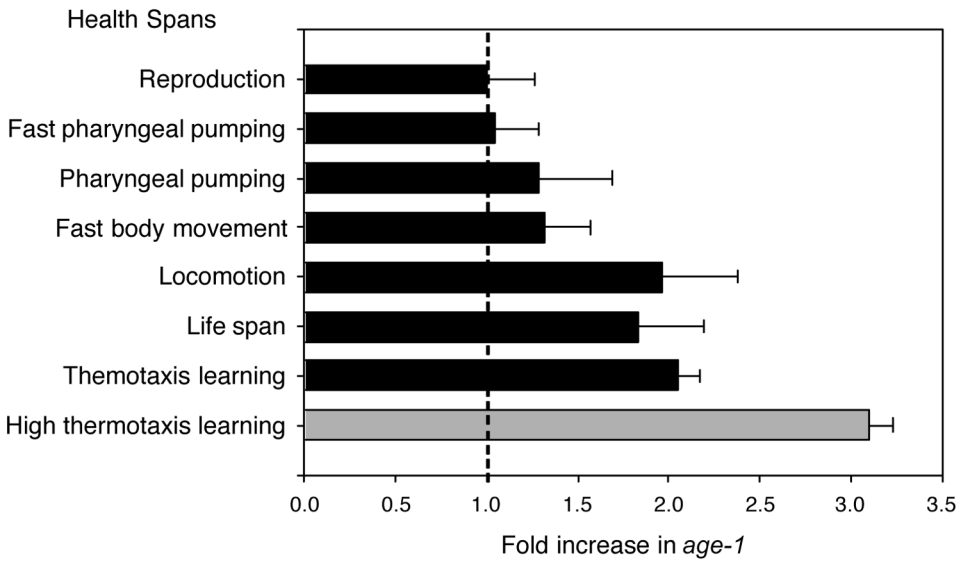

Fig. 4. Various health spans in the *age-1* mutant. Mean of each health span were used to calculate relative ratio against each health span in the wild-type. A gray bar shows the health span that ensures high thermotaxis learning. (The figure from ref. *21* was modified.)

The *age-1* mutant delays age-related decline of thermotaxis learning behavior. The *age-1* animals showed significantly higher isothermal tracking than the wild-type during aging *(21)*. The results suggest that the *age-1* mutation delays age-related declines of isothermal tracking. Additionally, the *age-1* mutant showed a significant increase early in life during physiological aging, which compares isothermal tracking to survivorship, an indicator of physiological aging *(32)*. Conversely, motor activities were similar in *age-1* and the wild-type during physiological aging (data not shown). The results suggest age-dependent and -independent modulation of thermotaxis learning behavior by the insulin/IGF-1 pathway.

Threefold Extension of Young Period With High Learning Ability in the *age-1* Mutant

Surprisingly, *age-1(hx546)* shows a 210% extension of the period that ensures a high thermotaxis learning (high thermotaxis learning span; Fig. 4). The high learning span was estimated based on the criteria that more than 75% of animals demonstrate isothermal tracking. The *age-1* effect (high thermotaxis learning span) is greater than the thermotaxis learning span (thermotaxis learning span), suggesting that *age-1* is effective in extending the health span with a high learning. The effect was also dramatic compared to a 65% life extension by the *age-1* mutant *(33)* and compared to the extension of other physiological health spans, including the reproduction period, fast body movement, fast pharyngeal pumping span, and pharyngeal pumping span (ranging from 0 to 29% extension) *(21,34)*.

A Key Neuron for Increased Learning Behavior in *age-1*

The AIY interneuron is essential for increased thermotaxis learning behavior in the *age-1* mutant *(21)*. Moreover, expression of *age-1* in the AIY neuron suppressed the increase of isothermal tracking in the *age-1* mutant, whereas

expression in 10 neurons (mostly touch neurons) did not suppress it. Therefore, AIY interneurons may be a key site of the aging defense mechanism by life-extending mutants of the insulin/IGF-1 pathway. Additionally, expression of *age-1* in all neurons restored increased isothermal tracking of *age-1,* whereas it did not affect the wild-type isothermal tracking. Therefore, it is unlikely that nonspecific reduction of isothermal tracking causes the suppression phenotype by the expression of *age-1*.

The Effects of *age-1* and *daf-2* on Chemotaxis Learning Behavior

Naïve animals show chemotaxis to an attractant, such as benzaldehyde. When animals are conditioned with benzaldehyde in the absence of food, they are no longer attracted by the odor and exhibit avoidance behaviors. This chemotaxis avoidance is shown to be a function of associative learning but not exhaustion of sensory or motor functions *(6)*. Naïve *age-1* and *daf-2* animals show an increase in chemotaxis index toward benzldehyde *(21)*. After being conditioned with benzaldehyde and starvation, the *age-1* and *daf-2* animals showed increased chemotaxis learning. Therefore, it is likely that the *age-1* and *daf-2* mutants increase either chemosensory ability or chemosensory attraction, leading to increased learning behavior.

Models and Controls

Figure 3 summarizes a model for the modulation of learning behavior. Increased temperature-food association was observed in various life-extending mutants, including mutants in the insulin/IGF-1 pathway (*age-1* and *daf-2*), mutants with reduced oxidative metabolism (*clk-1*), and caloric restricted mutants (*eat-2*). Therefore, it is likely that the effects of life extension, or improved neuronal maintenance, confer the increase in temperature-food association. The *age-1* and *daf-2* mutants were the only life-extending mutants that showed an increase in temperature-starvation association. Therefore, increased temperature-starvation association is specific to the insulin/IGF-1 pathway mutants.

It is unlikely that the life-extending effects of the mutants cause relative increases in motor activity or sensory functions, leading to increased learning behavior *(21)*. Typically, the assays have used young animals comparable to day 2 after hatching (onset of reproduction period). During the ages ranging from days 2 to 4 (onset to midreproduction period), there are little or minor changes in sensory and motor activity *(20,21)*. Therefore, life-extending effects during that period should be minimal. Additionally, the mutants (*age-1, daf-2, clk-1, isp-1, mev-1,* and *gas-1*) and the LA treatment showed a modest decline in locomotion rate, ranging from 60 to 80 % of the wild-type level *(20)*. The level of the motor decline did not affect thermotaxis learning. Therefore, the alternative model of increased motor activity is unlikely to be useful *(21)*. The model also does not explain the modest extension of the health span that ensures motor activity (Fig. 4).

Similarly to the wild-type (data not shown), the life-extending mutants (*age-1, daf-2, clk-1*, and *isp-1*) showed simple thermotaxis to the growth temperature (25°C), which rules out the alternative model of increased sensory function. Interestingly, the *mev-1* animals showed reduced, but not impaired, thermotaxis to the growth temperature (26% of total animals), suggesting that the mutant exhibits a deficit in thermosensory and a more severe deficit in thermotaxis learning.

Despite the extensive controls, there are a few other possibilities. First, the *clk-1* animals show high variation in population because of the asynchronous delay of the developmental rate. Synchronized *clk-1* animals may cause unintended selection of population that may show altered thermotaxis learning. Second, the insulin/IGF-1 pathway modulates a starvation response that may affect increased response to food. Our current model in Figure 3 supports the hypotheses: (a) neurons relevant to associative learning undergo oxidative damage, lead-

ing to reduced associative learning; and (b) the life-extending mutants protect from oxidative damage, leading to increased associative learning. To further confirm the hypotheses, one should examine oxidative damage in the relevant neurons.

Multiplex Stress Resistance: A Potential Mechanism of Increased Learning

Resistance to environmental stress may be a key factor to ensure health during aging *(35)*. The insulin/IGF-1 pathway regulates stress resistance to various environmental stress, including UV light *(36)*, oxidative stress *(37,38)*, heat *(39,40)*, and cadmium *(41).* The *age-1* mutants show resistance to polyglutamine (polyQ) similar to that observed in Hungtinton's disease *(42)* and resistance to paraquat that generates oxidative stress and toxicity in sensory neurons *(38,43)*.

It has been shown that the DAF-16 forkhead transcription factor, a downstream component of the insulin/IGF-1 pathway, shows a rapid nuclear localization in response to oxidative stress, starvation, and heat *(44)*. DAF-16 regulates transcription of several reactive oxygen species enzyme genes (*sod-3, ctl-1, ctl-2, gst-4,* etc.; refs. *45* and *46*), chaperons (*hsp-16* genes; refs. *46* and *47*), and 18 genes out of 77 cytochrome P450 genes that are involved in phase 1 and phase 2 drug detoxification *(48)*. Therefore, the insulin/IGF-1 pathway regulates multiplex stress resistance, which may play a role in neuronal protection against various stressors and diseases.

Age-Related Memory Impairment in the Fruit Fly

Similarly to *C. elegans,* there may be a trend toward early declines in associative learning (i.e., olfactory conditioning) compared to declines in exploratory activity and sensory functions, such as geotaxis, phototaxis, and olfaction (reviewed in ref. *49*). The olfactory conditioning paradigm typically shows decline at age 10 d, whereas exploratory and sensory function decline at 14 to 28 d; notably, the fly's maximum lifespan is 50 to 80 d *(49)*. Tamura et al. *(50)* made an interesting observation that aging causes deficits in middle-term memory (MTM). They used an olfactory conditioning paradigm with an electric shock that involved three memory phases: short-term memory, MTM, and longer lasting anesthesia-resistant memory (reviewed in ref. *51*). Aging specifically impairs MTM at age 20 d and after *(50)*. The age-related MTM impairment is identical to the deficits found in the MTM mutation in *amnesiac (amn)*, which are restored by expressing the *amn* gene in the mutant background *(50).* The *amn* gene encodes a putative pituitary adenylyl cyclase-activating polypeptide-like neuropeptide (PACAP), which triggers the effects through the adenylylcyclase (AC) pathway *(52)*. Therefore, the PACAP-AC pathway may be sensitive to aging.

Conclusion

Oxidative stress is a major risk factor for age-related cognitive declines during normal aging. Recent studies suggest that oxidative stress is involved in the relatively early phase of brain aging in humans *(53)*. Consistently, physiological oxidative stress plays a role in thermotaxis learning behavior early in the *C. elegans* lifespan *(20)*. Investigation of the neurons that show an age-related increase in oxidative stress could provide a useful model system to study the vulnerability of the aging neurons to stress insults. Additionally, thermotaxis learning behavior may share mechanisms similar to those in mammals, such as calcium signaling and LDL signaling. In mammals, the initial phase of neurodegenerative diseases involves oxidative stress and other factors, including altered glutamate signaling, altered calcium homeostasis, decreased growth factors, and genetic mutations *(54)*. Notably, *C. elegans* has been used as

model systems to study various neurodegenerative diseases, including Alzheimer's disease, Huntington's disease, Spinocerebellar ataxia, and Parkinson's disease (reviewed in refs. *55* and *56*). Together, *C. elegans* provides a useful system to study genetic mechanisms to explore a link to aging, age-related learning impairment, and neural oxidative stress.

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