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Research Article

The Effect of Temperature-Induced Stress at Different Developmental Periods on Short-Term Memory of *C. elegans*

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Abstract

As the incidence of neurodegenerative diseases continues to increase, it is essential to evaluate the causal factors that lead to neurodegeneration and resultant memory loss. In this study, *Caenorhabditis elegans* were used as a model organism to explore the effects of developmental stress on learning and short-term memory. Half of the population were placed into elevated temperatures, to invoke heat stress, while the other half was kept at their optimal functioning temperature of 22℃. The worms were then taught a simple chemosensory learning task. Results show that 25℃ produced a small reduction in learning, while 28℃ produced a substantial reduction. In a follow-up study, *C. elegans* were exposed to 28℃ at different life stages Day 0 (embryo), Day 1, Day 2, and Day 3 stress each reduced learning when compared to controls, with the greatest deficit being stress experienced during Day 2. Insights into neurodevelopmental time periods of vulnerability to stress and the potential mechanisms affected by early-life stress can help in the prevention of neurodegenerative diseases and their associated cognitive decline.

Keywords: *C. elegans*; Stress; Neurodevelopment; Learning; Memory; Neurodegenerative disease.

INTRODUCTION

Improvements in healthcare and quality of life over the past several decades have led to a worldwide growth in the elderly population. With this increase in the aged population, there has been a concurrent increase in cases of dementia, a common condition closely associated with age. There are nearly 10 million new cases of dementia every year, which is the equivalent of a new case every three to four seconds [1]. "As the elderly population grows in the decades ahead, a better understanding of the life course factors that increase or decrease the risk for cognitive decline and dementia will be increasingly important, given the wide-ranging effects that cognitive decline has on older individuals, their families, and public expenditures" [2]. There are many life-course factors that are targets for dementia research, such as inflammation and melatonin [3-5]. Stress, both chronic and

early in life, is another potential pathway for research in dementia and possibly a necessary factor to understanding the causes of Alzheimer's Disease.

Stress and Neurodegeneration

In examining factors that contribute to neurodegenerative disease, it is necessary to understand that the majority of the evolutionary history of humans has involved working in small, nomadic hunter-gatherer groups. Sapolsky argues that our stress-response system, which evolved in very different contexts than our current lifestyles, presents an evolutionary mismatch in which humans experience chronically high glucocorticoid levels [6]. The chronic stress of our environments causes deleterious effects on the body, and more notable in its relation to dementia, cognitive function [7]. Consistently high glucocorticoid levels have been linked to neuronal death in the hippocampus, an area responsible for cognitive processes such as memory [8]. Additionally, early-life stress has been found to alter development of the stress response system to increase sensitivity, which persists throughout one's life [9].

The developmental role of stress has been established in many mammalian models, previous research using mice and rats has found that both prenatal and chronic stress lead to dysregulation of the stress response pathway leading to enhanced stress response and reduced learning as an adult [10-11]. Schneider et al. found prenatal stress in rhesus macaques was related to heightened stress post birth and impaired performance on learning tasks [12]. Non-experimental clinical research on humans parallels the non-human animal research, with early-life stressors being associated with reduced hippocampal volume and memory deficits [13]. Gee and Casey suggest that sensitive periods of neural development are uniquely vulnerable to the effects of stressors, and acute stressors at these sensitive time points can permanently alter the course of neural development [14]. These previous studies suggest that extreme stressors during development lead to dysregulation of the stress response and cognitive deficits.

The developmental role of stress in neurodegeneration and sensitivity in the hippocampus makes it a prime area of research as a potential cause for a range of neurodegenerative diseases and mental disorders [15]. Chronic stress in mice is related to an increase in amyloid-β protein release and aggregation [16]. Although not completely understood, amyloid-β protein accumulation and aggregation is a known component in the progression of Alzheimer's Disease [17].

C. elegans as a Model for Stress

One model organism to explore the relationship between stress and Alzheimer's Disease is the nematode *Caenorhabditis elegans* (*C. elegans*). *C. elegans* have been used in the study of aging due to their short lifespan, entirely sequenced genome, and straightforward lifespan assays [18]. Researchers have utilized *C. elegans* to identify and study genes related to Tau and amyloid-β protein production as potential causes for Alzheimer's Disease [19, 20].

Relevant to the current research, *C. elegans*, although evolutionarily distant from mammals, demonstrate a significant conservation in signal transduction pathways related

to stress, including protein targets and regulatory mechanisms [21]. Generating stress in *C. elegans* is a well-established research model, with previous research establishing the role of stress in metabolic disorders and even neurodegenerative diseases, such as Parkinson's Disease [22, 23]. There are many methods to generate a stress response in *C. elegans*, one such way is to maintain the nematodes at a higher temperature than they are typically grown. Temperatures such as 30-35°C generate a stress response, including the upregulation of heat-shock proteins, a conserved response to high temperatures. Temperature-induced stress response, including the production of heat-shock proteins, has been linked to altered behaviours, such as feeding behaviours [24].

Heat stress is a particular method that may prove useful in the study of stress and aging. Heat-shock proteins in neurodegenerative conditions are related to the progression of the degeneration, heat-shock protein levels are higher in longer-lived species, and stress alters heat-shock protein expression [25]. In *C. Elegans*, both chronic and early-life heat shock have been found to alter the aging process [26]. Specific to neurodegenerative diseases, such as Alzheimer's Disease, heat-shock proteins may be up-regulated in response to protein misfolding and aggregation [27]. In their review, Rodriguez et al. conclude that the *C. Elegans* stress response to heat shock involves the same genes and pathways that underly Alzheimer's Disease and can serve as a useful model in better understanding the neurodegenerative disease [28].

Learning and Memory in C. Elegans

A primary symptom of Alzheimer's Disease and other forms of dementia is cognitive decline. In addition to serving as a model for stress and aging, *C. elegans* also demonstrate learning and memory. Although *C. elegans* have a much simpler nervous system, they also utilize glutamate as a means of memory formation. The glr-1 (glutamate receptor 1) is a homolog of the mammalian non-NMDA glutamate receptors and is key to forming long-term memory for habituation [29]. There are many paradigms that demonstrate learning and memory in the nematode, including no-associative learning, associative learning, and imprinting. Additionally, *C. elegans* have shown the ability to learn from a range of environmental stimuli, including taste, temperature, oxygen level, and smell [30].

Chemoreception is a primary sensory system for *C. elegans*, with 7% of their genome dedicated to chemoreception [31]. *C. elegans* have a small number of olfactory neurons with each neuron having many different odorant receptors. Additionally, different odorants elicit different behaviours in *C. elegans*, including attraction, avoidance, feeding, and mating [32]. Kauffman et al. introduce a modified aversive olfactory learning paradigm to produce a positive response in which both short- and long-term associative memories are demonstrated [33].

The current study utilized Kauffman's method to test the effects of heat stress at various time points of the *C. elegans* lifespan on learning and memory. Previous studies in *C. elegans* using this method have shown the efficacy in measuring learning and memory in various experimental conditions, such as following sleep disturbances and with

alterations to insulin signalling pathways [34, 35]. Ultimately, this approach can be used to test the effects of chronic and early-life stress on the pathways that underly neurodegeneration in diseases such as Alzheimer's Disease.

METHODS

C. elegans strains and culture conditions

Wildtype strain was obtained from *Caenorhabditis* Genetics Center (CGC, University of Minnesota), and worms were cultivated on nematode growth medium plates (NGM) seeded with *E. coli* OP50 at 22°C.

Age-Match Bleaching Technique

Worms were collected using Distilled H2O (1mL) swirled around the plate and then pipetted into a centrifugation tube (1.5mL). Two washes of each plate were performed. A bleach solution (2:1 bleach to NaOH) was added to each centrifugation tube with worms (0.4mL), and vigorously shaken every two minutes for a total of ten minutes. After the ten minutes, the centrifugation tubes were centrifuged for 30 seconds in 22°C at 16.0 RPM. All liquid was removed without disturbing the pellet and 0.4mL DI was added to each tube before repeating centrifugation. The pellets were washed and centrifuged a total of three times. Pellets were combined and then plated by pipetting onto corresponding plates.

Stress Induction Technique

3-day stress: Worms were placed into either 25°C or 28°C incubator for 72 hours post bleaching before being subjected to Learning and Memory Assay.

Day 0 stress: Worms were placed into 28°C incubator for 12 hours prior to bleaching and then 22°C for 72 hours post bleaching before being subjected to Learning and Memory Assay.

Day 1 stress: Worms were placed into 28°C for 24 hours post bleaching and then moved to 22°C for 48 hours before being subjected to Learning and Memory Assay.

Day 2 stress: Worms were placed into 22°C for 24 hours post bleaching, and then moved to 28°C for 24 hours, and then moved to 22°C for 24 hours before being subjected to Learning and Memory Assay.

Day 3 stress: Worms were placed into 22°C for 48 hours post bleaching and then moved to 28°C for 24 hours before being subjected to Learning and Memory Assay.

Learning and Memory Assay

Adapted from the protocol from Kauffman et al. [35], a chemotaxis assay with training for scent association (butanone) learning and memory is utilized for short term learning and memory assay. *C. elegans* are exposed to the scent (butanone) associated with food following periods of starvation. Following a 30-minute holding period to allow for examination of short term memory, *C. elegans* are placed on plates with the trained scent and control scent (ethanol) and movement to the trained scent is used to obtain a Learning

Index. Given the magnitude of the effect sizes, we can conclude that the sample size used for the study was more than sufficient.

RESULTS

Initial experiments aimed to determine if temperature stress, and at what extremes of stress, would lead to impacts on learning and memory. To examine this, worms were exposed to temperatures greater than the normal 22°C that is ideal for survival. Following age matching, worms were exposed to 25°C or 28°C for 72 hours (the normal life span of a *C. elegans*). At the end of 72 hours a Learning and Memory Assay with a chemotaxis assay, based off the previously described procedure from Kauffman et al. [35], was used to obtain a Learning Index. Statistical analyses were performed in IBM SPSS Statistics 28.

We hypothesized that worms exposed to elevated temperatures would demonstrate reduced learning. Worms exposed 22°C, 25°C, or 28°C showed a significant difference in Learning Index, $F(2,35)=21.42$, $p<.001$, $\eta^2=.55$. Exposure to extreme temperature stress of 28°C ($M=.06$, $SD=.04$) led to a significant reduction in learning and memory compared to 22°C ($M=.33$, $SD=.16$) but mild temperature stress of 25°C ($M=.28$, $SD=.12$) did not, as shown in Figure 1. Thus, exposure to 28°C reduced learning with an effect size that is considered very large [36].

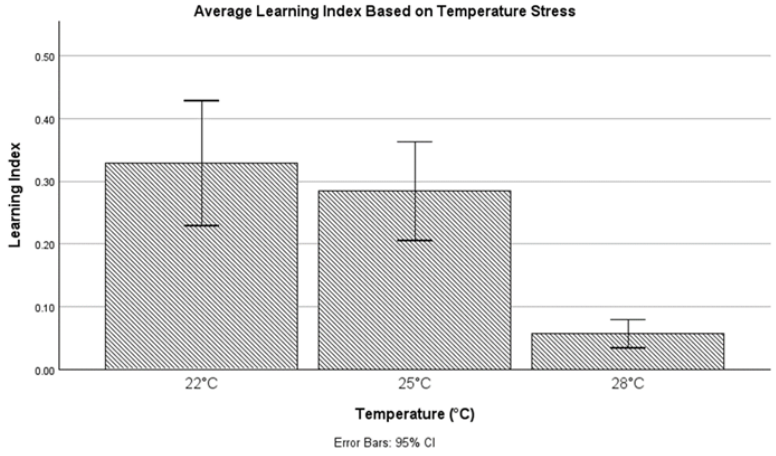

Figure 1. Average learning index based on temperature stress

To further explore the impact of temperature stress on learning and memory, learning and memory assays with temperature stress were repeated at individual time points to represent corresponding time periods in the human lifespan.

Day 0 temperature stress was performed for 12 hours prior to age matching, when the worms would still be in the embryo state, representing the pre-natal period. We hypothesized that worms exposed to elevated temperature of 28°C as an embryo would demonstrate reduced learning as an adult. Exposure to temperature stress of 28°C ($M=.14$, $SD=.11$) during the embryo state did lead to a significant decrease in Learning Index in C. elegans when compared to the 22°C condition ($M=.33$, $SD=.16$), as show in Figure 2,

$t(29)=3.99$, $p<.001$, $d=1.47$. Thus, exposure to 28°C as an embryo reduced learning later in life with an effect size that is considered very large [36].

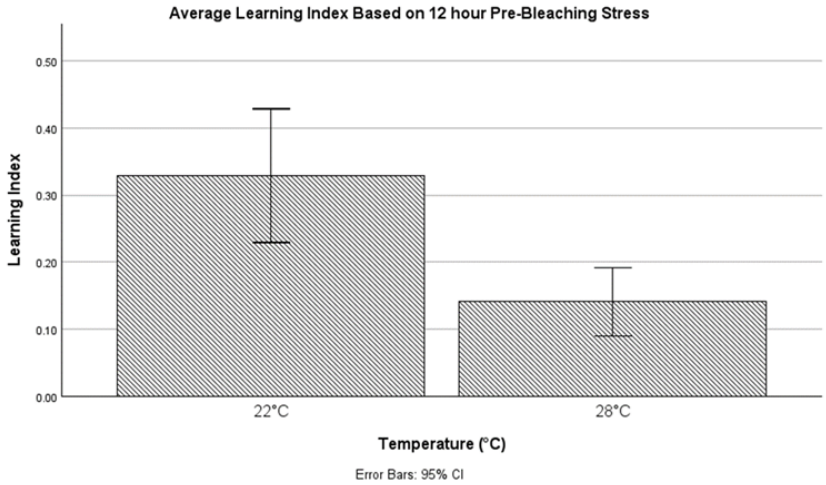

Figure 2. Average learning index based on 12 hour Pre-Bleaching Stress

To explore "early childhood" stress, Day 1 temperature stress was performed where worms were exposed to temperature stress (28°C) for 24 hours post age matching, and then they were moved to normal 22°C for 48 hours before performing the Learning and Memory Assay. We hypothesized that worms exposed to elevated temperature of 28°C in their first 24 hours would demonstrate reduced learning as an adult. Day 1 stress ($M=.11$, $SD=.15$) additionally led to significant reductions in Learning Index when compared to the no stress condition ($M=.33$, $SD=.16$), as shown in Figure 3, $t(37)=4.00$, $p<.001$, $d=1.39$. Thus, exposure to 28°C in "early childhood" reduced learning later in life with an effect size that is considered very large [36].

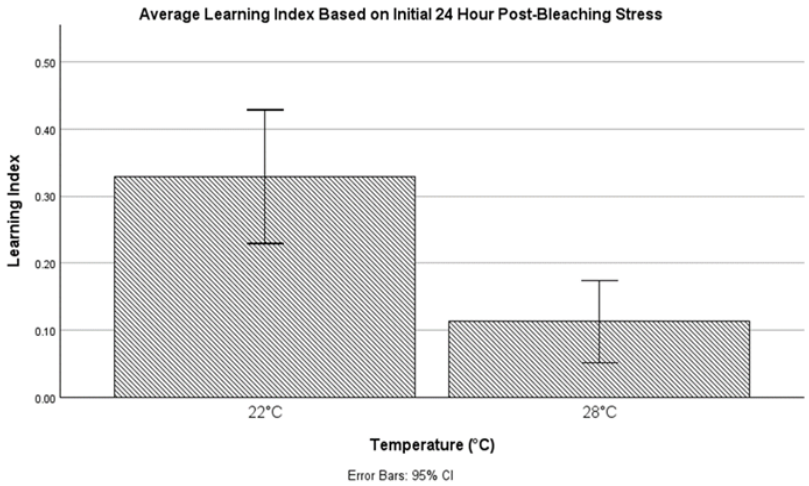

Figure 3. Average learning index based on initial 24 hour Post-Bleaching Stress

Adolescent-like stress was examined using Day 2 temperature stress, where worms were exposed to temperature stress (28°C) 24 hours following age matching for 24 hours

before returning to normal temperature for the remaining 24 hours before undergoing the learning and memory assay. We hypothesized that worms exposed to elevated temperature of 28°C in their second day would demonstrate reduced learning as an adult. This group (M=-.02, SD=.21) did show significant reductions in Learning Index when compared to the no stress condition (M=.18, SD=.31), as shown in Figure 4, $t(40)$=2.51, p=.008, d=.80. Thus, exposure to 28°C in "adolescence" reduced learning later in life with an effect size that is considered large [36].

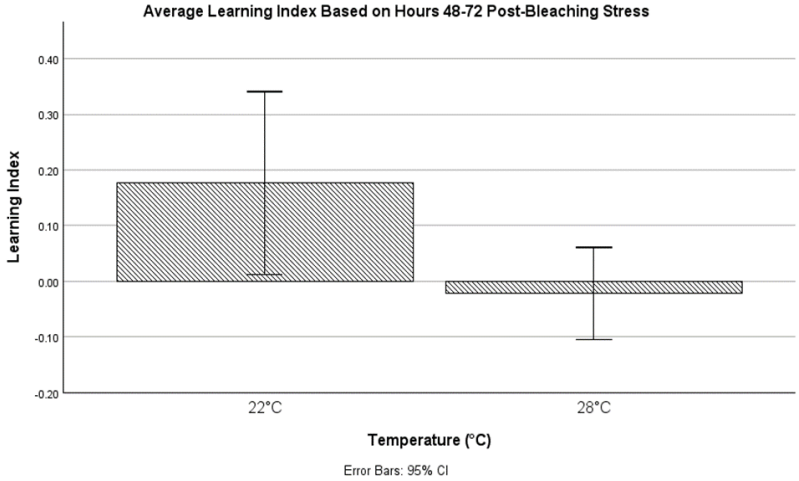

Figure 4. Average learning index based on hours 48-72 Post-Bleaching Stress

Adult stress was modelled via Day 3 stress, where this group was exposed to temperature stress (28°C) 48 hours following age matching for 24 hours. We hypothesized that worms exposed to elevated temperature of 28°C in their third day would demonstrate reduced learning a day later. After undergoing the learning and memory assay, it was determined that they (M=.08, SD=.17) did have a significant reduction in Learning Index when compared to the no stress condition (M=.21, SD=.28), as shown in Figure 5, $t(38)$=1.74, p=.045, d=.55. Thus, exposure to 28°C in as an "adult" reduced learning later in life with an effect size that is considered moderate [36].

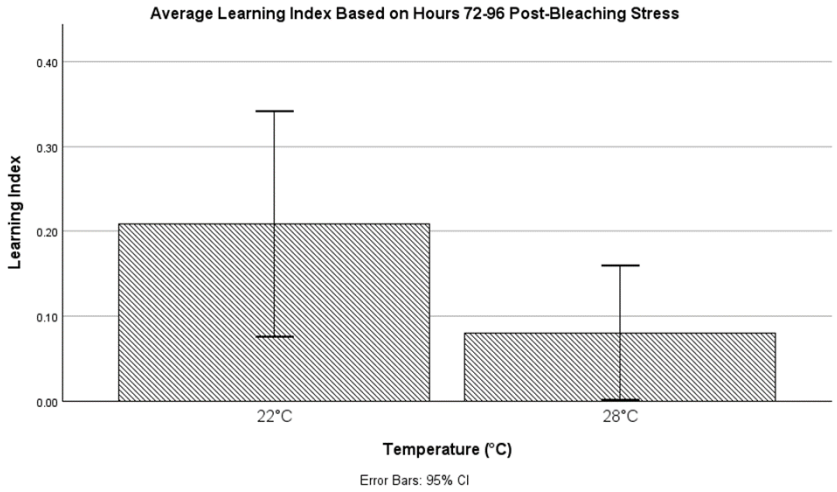

Figure 5. Average learning index based on hours 72-96 Post-Bleaching Stress

DISCUSSION

With decades of research, treatments for Alzheimer's Disease provide only minor reductions in symptoms while accomplishing little in slowing progression. For this reason, Viña and Sanz-Ros argue that our focus should be on prevention through lifestyle changes [37]. Previous literature suggests that one likely life-course factor in the development of Alzheimer's Disease, and a possible avenue for prevention, is lifelong and developmental stress. The current study sought to explore the role of developmental stress as a life-course factor related to the development of Alzheimer's using *C. elegans* as a model organism.

C. elegans are an established model organism in studying a range of diseases, including neurodegenerative diseases. Research using other animal models—such as mice, rats, and macaques—has indicated that there are periods in neural development where exposure to acute stressors can have life-long impact on developmental of the nervous system with effects found well into adulthood [10-12, 14]. The current study's primary strength and contribution to the literature is combining previous work on learning in *C. elegans* with stress models of *C. elegans* in a novel way to establish that *C. elegans* can be used as a model in studying the effects of stress during developmental time periods on learning and memory later in life.

The current study is not without limitation. In studying the developmental role of stress, it is essential to note that *C. elegans* development is very different than humans. *C. elegans* do not actually possess "early childhood" and "adolescent" time periods that are analogous to mammalian developmental windows. Additionally, *C. elegans* present a useful model for the study of Alzheimer's Disease only insofar as the underlying mechanism in which developmental stress reduces *C. elegans* learning later in life is the same mechanism by which developmental stress may contribute to Alzheimer's Disease susceptibility in humans. This has yet to be established. Previous research suggests that heat stress in *C. elegans* may be a model to explore the genetic and physiological pathway that underlies the neurodegeneration characteristic of Alzheimer's Disease [28]. The first step of this process, and the goal of this paper, is to establish that chronic and developmental stress reduces learning and memory.

CONCLUSION

In the first study, it was established that extreme, chronic heat stress of 25°C for 72 hours caused significant reduction in learning and memory in *C. elegans*. The following studies attempted to find if extreme heat stress at various developmental time points was associated with learning and memory deficits later in life. Extreme heat stress exposure to larvae before hatching caused significant reduction in learning and memory, as did extreme temperature stress during first day, second day, and third day of life. Although significance was found at all developmental timepoints, stress had the greatest impact 48-72 hours post bleaching. Although it is unclear why stress at this exact time point is related to the most deficit, previous research does support the finding that the timing of stress exposure is an important factor.

The next step of the research process is to establish the genetic and physiological dysregulation that underlies the reduction in learning and memory, with the hope that this dysregulation can provide insights into the pathway's characteristic of Alzheimer's Disease. Zhang et al. demonstrate that *C. elegans* are a good model organism to study these potential genetic and epigenetic mechanisms related to aging [38]. One potential avenue to explore is the Notch signalling pathway. This pathway is highly conserved, plays an important role in neural development, appears to play a role in learning and memory later in life, and may be linked to the pathophysiology of Alzheimer's Disease [39-41]. Another potential mechanism to explore is alterations to synaptic formation due to stress. Previous research shows chronic stress in *C. elegans* alters neuronal expression of SKN-1 and NLG-1 genes, both of which play a role in synaptic activity [42]. Finally, the glr-1 glutamatergic receptor should be explored as a potential mechanism by which stress alters learning and memory. Previous research has established this receptor is critical for both associative and non-associative learning in *C. elegans* [43]. The unfolded protein response (UPR) pathway is necessary for the movement of glr-1 subunits and has been found to upregulate in response to cellular stress. Although the glr-1 protein is not conserved, components of the UPR pathway are conserved across all eukaryotes [44].

Future research should explore these potential physiological mechanisms with the hope of better understanding the pathophysiological mechanism by which stress can contribute to the development of Alzheimer's Disease.

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CONFLICT OF INTERESTS

No potential competing interest was reported by the authors.

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