Journal of High School Science

Reaction time of $A\beta_{1–42}$ expressing nematode mutants to a specific auditory frequency may serve as a demonstrative surrogate of decreased neurodegeneration in dementia

Chen C

Submitted: November 14, 2024, Revised: version 1, February 7, 2025, versions 2 and 3, February 25, 2025
Accepted: March 12, 2025

Abstract

Alzheimer's Disease and other related disorders have steadily increased as the population ages. The conventional wisdom is that misfolding and accumulation of proteins such as Amyloid-β (Aβ) leads to neurodegeneration in these diseases. Available treatments are limited by marginal efficacy and side effects, raising the need to develop alternative therapies. The present study investigated the differing reactions of the nematode *Caenorhabditis elegans* mutant strain smg-1 CL2355, containing a human Aβ transgene engineered into its pan-neuronal cells, compared to its counterpart N2 wildtype control when exposed to different airborne auditory frequencies, contributing to the investigation of frequency treatment as a less expensive and non-invasive potential treatment for patients with dementia. The mutant strain *smg-1 CL2355* contains a human Aβ transgene engineered into its pan-neuronal cells, resulting in an accumulation of Aβ plaques in its cerebral cortex, causing impairments in cognitive function. The *smg-1*, which exhibits deficits in thermotaxis memory (using the promoter of the *C. elegans* synaptobrevin ortholog (*snb-1*) to stimulate the pan-neuronal expression of $A\beta_{1–42}$), was used as a model of neurodegeneration. This study observed the time for the organisms (*N2* and *smg-1* hermaphrodites) to react, in the form of backward sinusoidal locomotion, as well as the time taken for the organism to recover and return to the normal movement (forward-moving sinusoidal undulations), when exposed to the frequency spectrum (2s; 100Hz, 450Hz, 1-5kHz; 80dB SPL). The N2 wildtype strain reacted faster than the *smg-1* across all frequencies except those at 4000Hz, where the *smg-1* reacted and recovered faster than the *N2* strain. The finding that exposure to a specific frequency may prevent neurodegeneration in this mutant nematode model may have implications for using auditory stimuli for treating dementia.

Keywords

Alzheimer's Disease, *C. Elegans*, Airborne frequencies, Aβ plaques, Dementia, Amyloid-β, Neurodegeneration, Auditory stimulus, Cognitive function, Nematode model

Chloe Chen, Brentwood School, 100 S Barrington Pl, Los Angeles, CA 90049, USA. chloe@dreamisland.io

Introduction

~ 55 million people have dementia, and the number of patients is projected to grow to 78 million by 2030 (1). Dementia is the loss of cognition that affects social or occupational functions (2). Donepezil based medications, such as Aricept® and Namzaric® are approved pharmaceuticals to treat dementia but they have side effects, such as increased frequency of bowel movement and nausea (3). Alternatively, the other option available is surgery, an extreme case for patients in intense pain, such as deep brain stimulation surgery, which is invasive and has a high risk of side effects, such as heart problems and nausea (4). This study used mutant nematodes, *Caenorhabditis elegans* (*C. elegans*) as a model for dementia, and observed their reaction time to different auditory frequencies as a surrogate for a decrease in neurodegenerative symptoms. The objective was to slow the progression of dementia, particularly Alzheimer's, through the deposition of Amyloid-$\beta_{1\text{-}42}$ in the cerebrum by using C. elegans as a model organism due to the similarity of its nervous system components to mammals. The mutant strain's (*smg-1 CL2355)* human Aβ transgene, engineered into its pan-neuronal cells, accumulated Aβ plaques in its cerebral cortex, engendering impairments in cognitive function and deficits in thermotaxis memory (using the promoter of the *C. elegans* synaptobrevin ortholog (*snb-1*) to stimulate the pan-neuronal expression of $A\beta_{1\text{–}42}$) (5-9) . The presence of Aβ plaques is linked to neurotoxicity, inflammation, synaptic dysfunction, and failure to produce serotonin (10). The present investigation evaluated the time for the organisms to react, in the form of backward sinusoidal locomotion, and the time taken for the organism to recover by returning to normal movement (forward-moving sinusoidal undulations) (11,12). The backward movement is a direct response when given a sensory stimulus and can convey neuroplasticity in response to stimuli (13). The present study was inspired by McDermott et al. (14), who investigated dementia patients' external behavioral response when exposed to different types of music and determined that music relates to an individual's sense of self and may correspond to an individual's abstract appreciation of life. This research aimed to contribute to the investigation of music and dementia by identifying which auditory frequencies (airborne sounds) reduce the behavioral differences between nematodes with dementia and those without, to explore frequency treatment as a potential dementia treatment option with reduced side effects and lower costs. *C. elegans* serves as a model for investigating neurodegenerative diseases and the organism can sense airborne sounds to a maximum of 5kHz (7,12,15-18). The *C. Elegans'* reaction to sound is attributed to the airborne frequencies that vibrate the *C. Elegans*' skin, which is a pressure-to-displacement transducer similar to an eardrum. This leads to the sound-sensitive neurons attached to the skin being activated with a display of phonotaxis behavior in the form of backward locomotion (12). This study took advantage of the model organism's vibration sensitivity and its ability to exhibit a direct response to backward locomotion (Figure 2b, 2c, 2d) (13,19).

Materials and Methods

This study compared separate aspects of the *smg-1 (cc546)* mutant and *N2* wildtype's kinesis reactions in response to a frequency spectrum. The objective was to decrease the difference in the nematode's reaction (reaction

distance, reaction time, reaction frequency, recovery time) to reduce the overlying differences between the mutant and wildtype behavioral reactions. The controlled variables, the method of frequency emission, exposure directly towards the anterior of the worm, and the age of the nematode (L4) were constant.

The presence of a reaction was determined by a change in the organism's normal movement, represented by slow, sinusoidal undulations (20), with a rapid head movement or, most commonly, backward locomotion in a sinusoidal movement (11). The backward movement represented a direct response when the organism was administered a sensory stimulus (13).

The reaction and recovery times were recorded by analyzing the recordings of all the reactions through the Tracker software (See Figure 2).

Design to deliver audio frequency

Earbuds were attached to the Helping Hands (Pro'sKit 900-015 Helping Hands Soldering Aid) to produce the frequency. ⅛ PVC tubing pipette tips were attached to the speaker ends of the earbuds with the aid of Parafilm laboratory tape (See Figure 1) to direct the frequency.

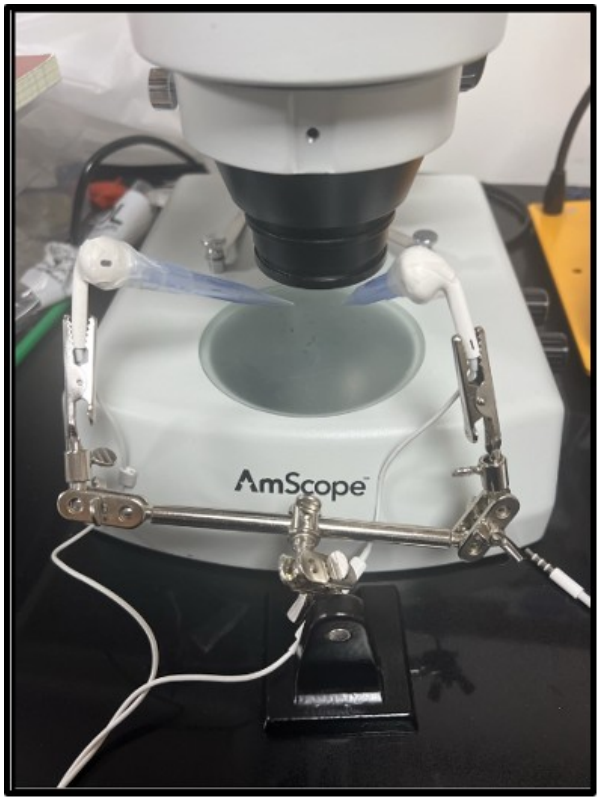


Figure 1. Speaker system that emitted frequencies with precise accuracy when directed at a worm, which allowed for the angle and distance from the plate to be adjusted.

Culture medium

The *E.coli* bacterial stock was grown on nematode growth medium (NGM) plates, cultured overnight at 37 ºC.

C. elegans strains and cultivation

This study used Wild type *N2,* and CL2355: *Caenorhabditis elegans smg-1(cc546)dvls50.* (dvls50 [pCL45 (snb-1.:Aβ $_{1-42}$:.3' UTR(long) + mtl-2: GFP] I.) nematodes fed *E. coli* OP50 (SMG-1 [gene]). The CL2355 strain was cultivated at 16 ºC to reduce selection against the transgene, while the wildtype N2 strain was cultivated at 20 ºC. The nematodes were sourced from the Caenorhabditis Genetics Center (CGC), University of Minnesota. This study selected specifically L3 strains (third larval stage of *C. elegans*); nematodes from each strain were picked, wrapped in Parafilm, and cultivated in a 100 x 50mm Petri dish (21).

The CL2335 strain was heat activated once received from the CGC to 25 ºC for 24 hours in an incubator (22). Once the $A\beta_{1-42}$ gene was heat-activated, the gene was fully activated for the concurrent and following nematode generations. The experiments were then performed at 20 ºC.

Exposure to frequency

Seven frequencies, each 2s, 80dB Sound Pressure Level (SPL), corresponding to 100Hz, 450Hz, 1000Hz, 2000Hz, 3000Hz, 4000Hz, and 5000Hz, were emitted to the nematodes through the earbuds. Each of the two strains were exposed to an airborne frequency between 10-minute intervals so as to provide time for the nematode to recover to its normal behavior. This process of exposing each strain to every frequency was performed 4 times. The auditory frequencies played were in the form of a single tone at 80dB SPL for 2 seconds.

Data collection

Each video of the organisms was imported into the open-sourced Tracker software (23), and the reaction times were calculated by subtracting the time for the first sign of a reaction from the time the sound was emitted, as seen in Figure 2. The reaction distance was calculated by creating a calibration line on the diameter of the pipette tip in the corner of the video of 1.25mm. By doing so, the program then knew the scale of the recording. To deduct the distance traveled by the worm during its backward locomotion, a calibration point was placed on the organism's tail during the first frame of reaction, and another calibration point was placed at the furthest point the tail reached during the reaction. Keeping in mind the pixels are calibrated to scale, the program accurately calculated the distance traveled by the worm during the reaction.

Data was collected by isolating a single L3 organism from the culture plate and recording its reaction and response to each frequency on the frequency spectrum, then repeated for the *smg-1* strain. The trials were repeated four times.

Statistical analysis

This study used Microsoft Excel to calculate the averages of each strain's reaction to a specific frequency across all trials. Then, a two-tailed t-test was performed using Microsoft Excel on the averages of each strain at a specific frequency. Results were considered significant at $p < 0.05$.

Results

As seen in Figures 3a and 3b, when emitting

the frequency to the anterior, the *N2* wildtypes responded with backward locomotion ~ 2.05s after the sound was produced. The *smg-1* strains reacted with delayed backward locomotion ~ 6.24s after exposure to the frequency. The *N2* wildtype in Figures 3a and 3b maintained a significantly faster reaction time of 2.05 seconds than the *smg-1* mutant's reaction time of 6.24 seconds at 1000Hz, correlating with the trend observed in Figure 5a where the N2 wildtype had a significantly faster reaction time than the *smg-1* ($p < 0.05$).

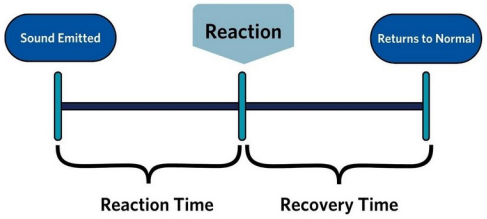


Figure 2. The division and definition of reaction and recovery time throughout the recording represented by a diagram.

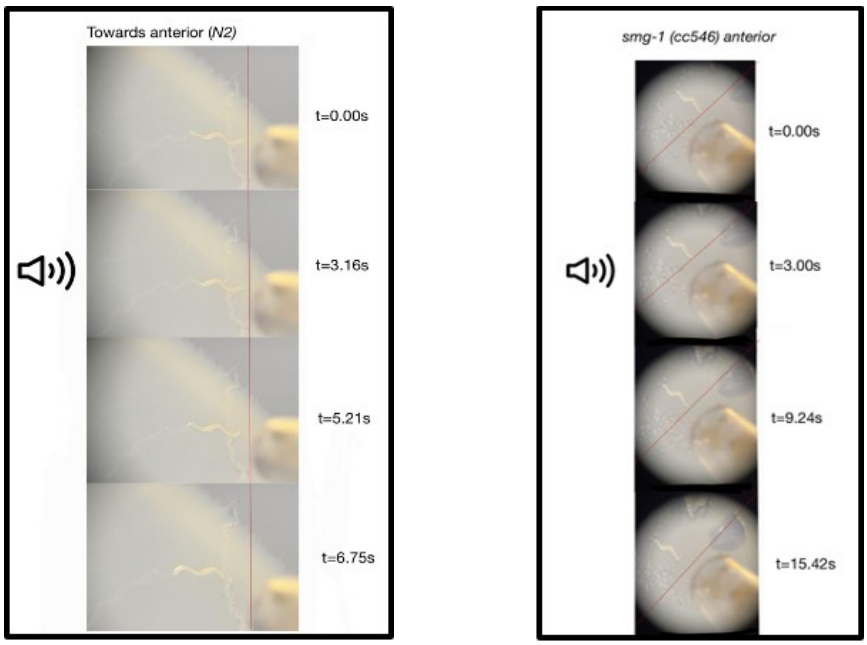


Figure 3. a. Left panel, Reaction time and body movement of the *N2* toward the anterior. An emission of 1kHz for 2 seconds is delivered to the head of the worm. The red line indicates the position of the worm in the field. The second to last frame demonstrates the worm initiating its reaction. The last frame indicates the final position of the worm during backward locomotion before its recovery. **b. Right panel,** Reaction time and body movement of the *smg-1 (cc546)* toward the anterior. A brief pulse of sound (2 s, 1 kHz, 80 dB SPL) was delivered to the head of the worm. The red line indicates the position of the worm in the field. The second to last frame demonstrates the worm initiating its reaction. The last frame indicates the final position of the worm during backward locomotion before its recovery.

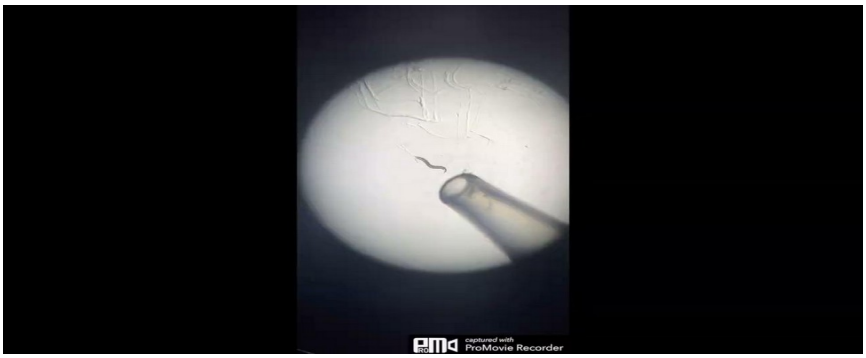
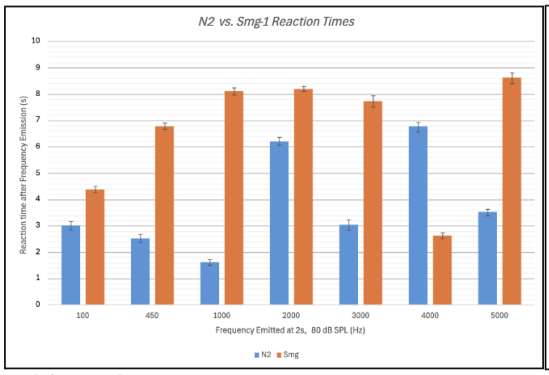
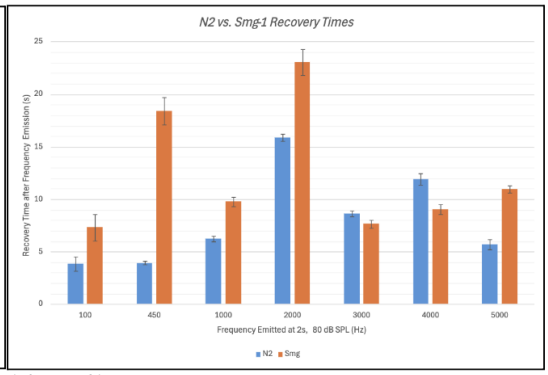


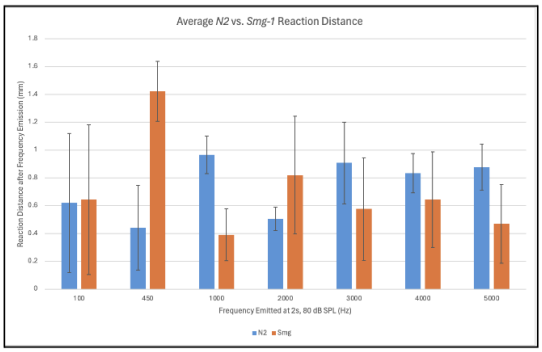
Figure 4. Recording still showing the reaction and body movement of the N2 strain reacting to a frequency of 4000Hz. The tube in the corner of the video indicates the pipette tip from which the frequency is emitted to the anterior. Video is in supplementary file.



(Figure 5a)



(Figure 5b)



(Figure 5c)

Figure 5. a. The average time of reaction across the frequency spectrum (100Hz-5000Hz) of the *N2* vs. the *smg-1(cc546)*. The error bars represent the standard deviation. **b.** The average time of recovery across the frequency spectrum (100Hz-5000Hz) of the *N2* vs. the *smg-1(cc546)*. The error bars represent the standard deviation. **c.** Reaction time and body movement of the *N2* toward the anterior. Through the use of the Tracker program, the average distance traveled during the reaction across the frequency spectrum (100Hz-5000Hz) of the *N2* vs. the *smg-1(cc546)* was calculated. The error bars represent the standard deviation.

Subsequently, the recovery times of the two strains (Figure 5b) relatively resembled their reaction times (Figure 5a), demonstrating how, throughout the length of the reaction, the *smg-1* strain had a slower reaction and recovery time in comparison to the N2 strain. However, the *smg-1* mutant demonstrated a faster reaction and recovery time than the *N2* wildtype when a frequency of 4000 Hz was emitted. When exposed to the frequency of 4000 Hz, the N2 strain reacted at an average of 6.77 seconds after the stimulus whereas the smg-1 strain reacted at an average of 2.62 seconds after the stimulus. Similarly, the *smg-1* strain recovered at an average of 9.07 seconds whereas the *N2* reacted slower at 11.91 seconds at this frequency. This frequency of 4000 Hz was the only tested frequency at which the *smg-1* organisms presented with a significantly faster reaction and recovery time. Excluding the *smg-1* and *N2* reaction times at 4000Hz, the average reaction times as well as the average recovery times for the rest of the tested frequencies were faster for the N2 strain than the *smg-1* strain. There was no significant statistical difference in reaction distances between each strain's reaction to any frequency ($p = 0.92$).

Discussion

Our study aimed to investigate the difference in reaction times of nematodes with genetically engineered amyloid-β plaques compared to an N2 wildtype control group when exposed to different airborne auditory frequencies. The *smg-1* strain presented with a significant decrease in reaction time in comparison to the N2 wildtype when exposed to a frequency of 4000Hz. At all other frequencies, this trend was reversed, with the N2 wildtype reacting significantly faster to the auditory stimulus than the *smg-1* strain. The frequency of 4000 Hz was significant as it was the only frequency in which the *smg-1* mutant had a significantly lesser average reaction and recovery time than the wild type.

The study suggested that frequencies have a varying impact on nematodes with the $A\beta_{1-42}$ transgene compared to nematodes without. The nematode's reaction distance did not vary significantly regardless of the frequency and/or strain. Lastly, the nematode retained its sinusoidal movement during and after its reaction of backward locomotion, despite the frequency emitted towards its anterior.

Holbrook et al. described his choice of 23Hz as biologically relevant for detecting the movements of predators. In addition, Iliff et al. concluded that the range of reaction had at a maximum of 5000Hz but could not verify the nematode's minimum response frequency due to mechanical limitations. As a result, seven optimally distributed frequencies up to 5000Hz were chosen by this study to cover the large range of frequencies the nematodes were sensitive to. Holbrook et al. found the N2 to exhibit a behavioral response to all tested frequencies and concluded that the wildtype underwent the least amount of reversals when exposed to 23Hz. In comparison, this experiment found both the N2 wildtype and the CL2355 strain to exhibit a response to all seven frequencies, and directly compared response aspects such as reaction and response time. Thus this experiment solely concluded 4000Hz to be the only experimental frequency that decreased the difference in behavioral response between the CL2355 strain and the N2 wildtype (although, see the Limitations section).

Light Therapy Systems at 40 Hz have demonstrated potential in the non-invasive induction of neural activity in humans. Various devices using this 'invisible flicker' are available on the market, as are apps that convert display screens to ones that flicker at 40 Hz. Various clinical trials are also underway using this visual stimulus method of brain stimulation (24). Therefore, models such as the nematode model used in this study are valid and germane to study this phenomenon – using either light or auditory signal stimuli – to propose extrapolative inter species effects. This study used $A\beta_{1-42}$ expressing nematode mutants. The differences in physiology, neural complexity, and the fluid dynamics between nematodes and humans likely accounted for the variation in optimal frequencies between those in this study and those in Agger et al. (24). For instance, nematodes lack cerebrospinal fluid, and their neural networks are significantly simpler, which may account for a shift in optimal frequency for maximum stimulus.

This study built on findings from McDermott et al. (14), who conducted a clinical study observing dementia patients' external behavioral response to different types of music suggesting that music relates to an individual's sense of self and may correspond to an individual's abstract appreciation of life. The study found that although it would be unrealistic to expect considerable cognitive or memory improvements in regard to the care home residents with moderate to severe dementia, the music improved the mood of individuals at all stages of dementia (14). Exposure to music activities helped promote mental stimulation and more social interactions, creating more connectedness within the community (14).

Past studies that have also investigated the effect of music on patients with dementia from a physiological perspective have also supported the significance and relevance of music therapy. Okada et al. investigated the effect of music therapy on the autonomic nervous system and deduced that exposure to music leads to improved parasympathetic and cardiovascular status (24). Kumar et al. observed a group of patients with Alzheimer's disease during a 4-week exposure to music therapy and then at the 6-week follow-up and found the melatonin concentrations increased significantly during and after treatment (25), which improved mood, morale, and the ability to relax.

Limitations

The sound amplitude was not recorded at the pipette tips. PVC and plastic pipette tips could have absorbed the sound waves emitted from the earbuds such that the actual emitted sound at the pipette tips was < 80 dB.

The CL2335 strain shows deficits in chemotaxis (6). The experiments in this study were performed at 23 ^{o}C, not 16 ^{o}C. Consequently, this study cannot exclude the possibility that the differential response to sound at a particular frequency was not strain-specific and independent of the expression of $A\beta_{1-42}$.

In their study, Holbrook et al. (14) played the sound for 30 seconds preceded and succeeded by 30 seconds of silence. The worms were then recorded for 90 seconds total to ensure that the change in movement in response to the sound was captured and that the movement could in fact be assigned to the sound. Consequently, this study cannot exclude the possibility that

playing the sound for 2 seconds, as was done in this study, provided enough duration to form or to record an adequate sensory response. Additionally, substrate-borne vibrations – which were not investigated in this study – could be a better indicator of auditory sensory responses than than airborne frequencies.

Since the location of the sound source was fixed, this study cannot provide assurance that the nematode's reactions to various frequencies could have been direction specific. This is especially relevant considering the arrangement of mechanosensitive receptors on the worm.

This study also cannot rule out the possibility that the deposition of $A\beta_{1-42}$ does not selectively ablate the mechanosensitive receptors response to a particular frequency or change the frequency response of the mechanosensitive receptors to converge to a narrow band of frequencies (from a broad spectrum of frequencies). In order to discount this possibility, future studies could use worms deficient in ciliated neurons, similar to Holbrook et al. (14). Consequently, this study cannot provide assurance that the deposition of $A\beta_{1-42}$ does not take away from or change the mechanosensitive receptors' response to particular frequencies.

Perspectives

These results can contribute to real life, and build a platform for future research regarding frequencies as a potential treatment for organisms with dementia. The mechanisms of such frequency effects can be discerned by observing (for example changes in fluorescence (27) in tagged proteins) in different neuronal organelles.

Another possible future application could include staining the amyloid-β transgene through the use of a Congo-Red stain, exposing the nematodes to frequencies for an extended period and observing changes in gene expression through successive generations. These study designs can also be applied cross-species; for example in zerbrafish.

Conclusion

Caenorhabditis elegans mutant strain *smg-1* CL2355, containing a human Aβ transgene engineered into its pan-neuronal cells (mimicking the human dementia phenotype), and its counterpart N2 wildtype, were exposed to different airborne auditory frequencies, ranging from 100 Hz to 5000 Hz. All frequencies had a detectable effect on the organisms, generating a backward sinusoidal movement response followed by recovery. The response and recovery times for the mutant strain were found to be significantly lesser than its wildtype counterpart only at 4000 Hz. Assuming a shorter response in this nematode model to be a surrogate for decreased neurodegeneration, these findings may have implications for using auditory stimuli at specific frequencies for treating dementia.

Acknowledgments

The contributions of the Belldegrun Center for Innovative Leadership, Chris Chen, and Casey Wu for their lab space and support are acknowledged. The contribution of nematodes from the CGC (Caenorhabditis Genetics Center) is appreciated.

References

1. ADI - Dementia statistics. (n.d.). https://www.alzint.org/about/dementia-facts-figures/dementia-statistics/

2. Arvanitakis, Z., Shah, R. C., Bennett, D. A. (2019). Diagnosis and Management of Dementia: Review. *JAMA*, 322(16), 1589–1599. https://doi.org/10.1001/jama.2019.4782

3. Medications for Memory, Cognition and Dementia-Related Behaviors. *Alzheimer's Disease and Dementia*. https://alz.org/alzheimers-dementia/treatments/medications-for-memory

4. Deep brain stimulation—Mayo Clinic. https://www.mayoclinic.org/tests-procedures/deep-brain-stimulation/about/pac-20384562

5. Smg-1 (gene)—WormBase: Nematode Information Resource. (n.d.). https://wormbase.org/species/c_elegans/gene/WBGene00004879#0-9fc-10

6. Wu, Y., Wu, Z., Butko, P., Christen, Y., Lambert, M. P., Klein, W., et al. (2006). Amyloid-β-Induced Pathological Behaviors Are Suppressed by *Ginkgo biloba* Extract EGb 761 and Ginkgolides in Transgenic *Caenorhabditis elegans*. *The Journal of Neuroscience*, 26(50), 13102–13113. https://doi.org/10.1523/JNEUROSCI.3448-06.2006

7. Sinnige, T., Ciryam, P., Casford, S., Dobson, C. M., De Bono, M., Vendruscolo, M. (2019). Expression of the amyloid-β peptide in a single pair of C. elegans sensory neurons modulates the associated behavioural response. *PLOS ONE*, 14(5), e0217746. https://doi.org/10.1371/journal.pone.0217746

8. Jack, C. R., Bennett, D. A., Blennow, K., Carrillo, M. C., Feldman, H. H., Frisoni, G. B. et al. (2016) A/T/N: An unbiased descriptive classification scheme for Alzheimer disease biomarkers. *Neurology*, 87(5), 539–547. https://doi.org/10.1212/WNL.0000000000002923

9. Alvarez, J., Alvarez-Illera, P., Santo-Domingo, J., Fonteriz, R. I., Montero, M. (2022). Modeling Alzheimer's Disease in Caenorhabditis elegans. *Biomedicines*, 10(2), 288. https://doi.org/10.3390/biomedicines10020288

10. Li, Y., Zhao, Y., Huang, X., Lin, X., Guo, Y., Wang, D., Li, C. et al. (2013). Serotonin Control of Thermotaxis Memory Behavior in Nematode Caenorhabditis elegans. *PLoS ONE*, 8(11), e77779. https://doi.org/10.1371/journal.pone.0077779

11. Zhao, B., Khare, P., Feldman, L., Dent, J. A. (2003). Reversal Frequency in *Caenorhabditis elegans* Represents an Integrated Response to the State of the Animal and Its Environment. *The*

Journal of Neuroscience, 23(12), 5319–5328. https://doi.org/10.1523/JNEUROSCI.23-12-05319.2003

12. Gjorgjieva, J., Biron, D., Haspel, G. (2014). Neurobiology of Caenorhabditis elegans Locomotion: Where Do We Stand? *BioScience*, 64(6), 476–486. https://doi.org/10.1093/biosci/biu058

13. Alexander, A. G., Marfil, V., Li, C. (2014). Use of Caenorhabditis elegans as a model to study Alzheimer's disease and other neurodegenerative diseases. *Frontiers in Genetics*, 5. https://doi.org/10.3389/fgene.2014.00279

14. McDermott, O., Orrell, M., Ridder, H. M. (2014). The importance of music for people with dementia: The perspectives of people with dementia, family carers, staff and music therapists. *Aging & Mental Health*, 18(6), 706–716. https://doi.org/10.1080/13607863.2013.875124

15. Iliff, A. J., Wang, C., Ronan, E. A., Hake, A. E., Guo, Y., Li, X. et al. (2021). The nematode C. elegans senses airborne sound. *Neuron*, 109(22), 3633-3646.e7. https://doi.org/10.1016/j.neuron.2021.08.035

16. Van Pelt, K. M., Truttmann, M. C. (2020). Caenorhabditis elegans as a model system for studying aging-associated neurodegenerative diseases. *Translational Medicine of Aging*, 4, 60–72. https://doi.org/10.1016/j.tma.2020.05.001

17. Kiontke, K., Fitch, D. H. A. (2013). Nematodes. *Current Biology*, 23(19), R862–R864. https://doi.org/10.1016/j.cub.2013.08.009

18. Iliff, A. J., Xu, X. Z. S. (2020). *C. elegans:* a sensible model for sensory biology. *J Neurogenet*, 34(3–4), 347–50. https://doi.org/10.1080/01677063.2020.1823386

19. Holbrook, R. I., Mortimer, B. (2018). Vibration sensitivity found in *Caenorhabditis elegans*. *Journal of Experimental Biology*, 221(15), jeb178947. https://doi.org/10.1242/jeb.178947

20. Gray, J., Lissmann, H. W. (1964). The Locomotion of Nematodes. *Journal of Experimental Biology*, 41(1), 135–154. https://doi.org/10.1242/jeb.41.1.135

21. Hart, A. (2006). Behavior. *WormBook*. https://doi.org/10.1895/wormbook.1.87.1

22. Anderton, E., Chamoli, M., Bhaumik, D., King, C. D., Xie, X., Foulger, A., et al. (2024). Amyloid β accelerates age-related proteome-wide protein insolubility. *GeroScience*, 46(5), 4585–4602. https://doi.org/10.1007/s11357-024-01169-1

23. Brown, D., Christian, W., Hanson, R. (2020). Tracker Video Analysis and Modeling JS (Version 5.9) https://tracker.physlets.org/trackerJS/

24. Agger, M. P., Horning, M., Carstensen, M. S., Danielsen, E. R., Baandrup, A. O., Nguyen, M., et al. (2023). Study on the effect of 40 Hz non-invasive light therapy system. A protocol for a randomized, double-blinded, placebo-controlled clinical trial. *Frontiers in Aging Neuroscience*, 15. https://doi.org/10.3389/fnagi.2023.1250626

25. Okada, K., Kurita, A., Takase, B., Otsuka, T., Kodani, E., Kusama, Y., et al. (2009). Effects of Music Therapy on Autonomic Nervous System Activity, Incidence of Heart Failure Events, and Plasma Cytokine and Catecholamine Levels in Elderly Patients With Cerebrovascular Disease and Dementia. *International Heart Journal*, 50(1), 95–110. https://doi.org/10.1536/ihj.50.95

26. Kumar, A. M., Tims, F., Cruess, D. G., Mintzer, M. J., Ironson, G., Loewenstein, D., et al. (1999). Music therapy increases serum melatonin levels in patients with Alzheimer's disease. *Alternative Therapies in Health and Medicine*, 5(6), 49–57.

27. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., Prasher, D. C. (1994). Green Fluorescent Protein as a Marker for Gene Expression. *Science*, 263(5148), 802–805. https://doi.org/10.1126/science.8303295