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Testing a Choline Diet in Neuroligin-Deficient *C. elegans* to Reduce Repetitive Behavior in Autism

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ABSTRACT

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by obsessive adherence to repetitive motion and behavior. In my previous study, my research tested whether neuroligin, a brain molecule and potential determinant of behavioral traits in autism, could be used in neuroligin-deficient C.elegans (strain NLG-1) to model autism. That experiment studied the sensitivity of neuroligin-deficient C. elegans to sensory stimuli, a core symptom of autism, and the results led to the conclusion that NLG-1 C. elegans could be used as a potential model for autism in worms. This project is a continuation study that tested whether choline supplementation reduced repetitive locomotion in neuroligin-deficient C. elegans. Since humans with neurological disorders are often deficient in the neurotransmitter acetylcholine, this experiment hypothesized that increasing the amount of choline, a building block of acetylcholine, would reduce repetitive behaviors. The C. elegans were age-synched to test three different age groups of worms, and over a 1-minute period, the repetitive movement of the worms exposed to choline was compared to worms without choline using microscope camera software. The results showed the addition of choline in the diet significantly reduced the amount of repetitive locomotion in NLG-1 C. elegans in all 3 age groups. This study suggests that, for people with ASD, adding choline to the diet in their daily schedule may help them reduce small repetitive behavior. On a larger scale, scientists can use NLG-1 C. elegans to target neurochemical systems to develop early treatments, and ultimately reduce significant deficits of autism.

Introduction

Autism Spectrum Disorder (ASD) is a complex neurodevelopmental disorder characterized by impairments in human social interaction, difficulties in communication, and restrictive and repetitive behaviors (Faras et al., 2010). Autism Spectrum Disorder affects 1 in 44 children in the United States, and there is no cure (CDC, 2022). Despite being one of the longest subjects of research since 1934, there is still a vast gap of knowledge and understanding for the spectrum of symptoms that are encompassed in autism. According to the Diagnostic and Statistical Manual of Mental Disorders, there are two criteria for diagnosing autism: social difficulties and Restricted Interest andRepetitive Behaviors (American Psychiatric Association 2000). However, much of the focus in autism research studies social difficulties, which leaves repetitive and restrictive behavior widely unexplored. The symptom domain of restrictive, repetitive behavior is historically highly understudied compared to any other core symptom domains in ASD (Soorya et al., 2008). There is also a high medication-related stigma of humans with Autism associated with the fear of taking several medical prescriptions on a daily basis. Many families are often hesitant to give their children with autism medications, as many children with ASD are at higher risk for adverse reactions and unknown side effects with medications (Nadeau et al., 2013). Thus, this leaves a high demand for natural and more accessible ways to help reduce symptoms.

The repetitive behavior domain in ASD consists of diverse behaviors characterized by repetition, rigid behavioral patterns, severe adherence to routine, and intense interests in unusual subjects (Soorya et al., 2008). The

Diagnostic and Statistical Manual of Mental Disorders describes repetitive behavior as a preoccupation with stereotyped patterns of interest, inflexibility in adhering to routines, and repetitive motor mannerisms (American Psychiatric Association 2000). Although they may seem like innocent symptoms, in a study done on the predictors of perceived negative impact in mothers of children with autism, "Repetitive and Restrictive Behavior are reported to be the most distressing aspect of ASD for patients and families that profoundly impact daily living" (Bishop et al., 2007). However, due to the scarce medical and behavioral intervention research in this area, there is no reliable method for treating repetitive behavior in autism (Soorya et al., 2008). Although previous studies have found significant treatment improvements in other cognitive and social-communicative skills, there is still no corresponding improvement in the repetitive behavior domain (Dawson et al.2010). Thus, this leaves a large gap for scientific research to discover potential treatments to reduce repetitive behavior in autism.

Acetylcholine (ACh) is a fast-acting neurotransmitter in the brain that influences synaptic transmission (Piccioto et al., 2013). Low levels of ACh expression and function has shown to be associated with neurodegenerative diseases such as Alzheimer's and Parkinson's (Tata et al., 2014). However, the role of acetylcholine deficits in autism has not been explored until recently (Lam et al., 2006). The cholinergic system is instrumental in brain development and is largely dependent on the presence of acetylcholine(Ahmed et al., 2019). As the cholinergic system is vital for brain development and early function, disruptions in the cholinergic system can be linked to several cognitive deficits in ASD(Lam et al., 2006). Since there have been studies of low levels of acetylcholine in humans with autism, it could cause a disruption in the cholinergic system, and therefore lead to cognitive deficits including repetitive behavior. The problem is that there is no reliable way for humans to directly get acetylcholine into the diet to increase their acetylcholine levels. Common household foods such as egg yolks, fish, and cruciferous vegetables contain high amounts of choline, an essential nutrient found in several foods. Choline is a building block of acetylcholine and can be converted to acetylcholine through the enzyme choline acetyltransferase (Bellier et al., 2007). Thus, this experiment hypothesized that choline can raise acetylcholine levels to help reduce the cognitive deficit of repetition in autism. However, in order to test whether a choline diet can reduce repetitive behavior in autism, there first needed to be an organism to model autism. Since this research study was conducted in a highschool lab setting, due to safety and ethical concerns, vertebrate models and humans with ASD were not feasible to test.

The next viable option for a model organism were roundworms, or *Caenorhabditis elegans* (*C. elegans*). *C. elegans* are often used in biological research due to their inexpensive cost, simple genome, and quick life cycle. The genetic homology between the *C. elegans* and mammalian genome leads the *C. elegans* to serve as a model in a wide variety of neurological studies using single gene analysis(Rawsthorne et al., 2021). However, there is no designated autism model of *C. elegans*. This is why the results of the research I conducted last year were crucial to the goal of the research study this year. Prior to this study, my research last year tested if a strain of *C. elegans* called neuroligin-deficient *C. elegans* could be used as a potential model for autism. Neuroligin is a synaptic adhesion molecule in the brain that is involved with the formation of synapses and e a potential determinant of behavioral traits of signature behaviors of autism (Calahorro et al., 2020). Many humans with autism have a mutation or deficiency in their Neuroligin-3 gene, and thus that experiment hypothesized neuroligin-deficient *C. elegans* could serve as a model for autism. To test whether a neuroligin-deficient *C. elegan* strain (NLG-1 *C. Elegans)* could model autism, the study exposed NLG-1 *C. elegans* and N2 *C. elegans* (a wild-type mutant) to a tapping motor. A core symptom of autism is hypersensitivity to sensory stimuli, which can be defined as anything that evokes senses such as loud sound or movement. For this reason, a tapping motor that vibrated the petri dish was used to elicit a form of sensory stress to the worms. Through the exposure of the tapping motor on the petri dish, the distance each worm traveled, when exposed to the tapping motor versus without the tapping motor, was tracked by associating the higher the amount of distance traveled as a way of showing the worm's sensitivity to the tapping stimulant.

The results of the research last year, showed a significant increase in the movement of NLG-1 *C. elegans* (worms modeling autism) when exposed to the tapping motor. In comparison, the N2 (wild-type) had no reaction to the tapping motor, and there was no significant difference in distance traveled when exposed to a tapping motor versus without. As mentioned above, it was hypothesized that the amount of distance the worm traveled when exposed to the

tapping motor could be correlated with the worm's sensitivity to the tapping motor. Thus, since the NLG-1 worms moved a higher amount when exposed to a sensory stimulant, they modeled a similar reaction to humans with ASD that are hypersensitive to movement and noise. Contrastingly, the N2 worms had no reaction to the tapping motor, which can be compared to humans without ASD since they are not hypersensitive to sensory stimuli. Thus, last year's study demonstrated that NLG-1 *C. elegans* could be a potential model for autism.

This project builds off the results from my previous study and this current study uses neuroligin-deficient C. elegans as a potential model for autism to reduce repetitive behavior in autism. The role of acetylcholine deficiency in autism has not been explored until very recently and still has large gaps in how it can correlate to ASD (Lam et al., 2006). Furthermore, current research has not studied the link of choline and acetylcholine in repetitive behaviors in *C. elegans*. Therefore, this study focuses on testing the research question: Can the addition of choline in a diet reduce the repetitive behavior in a potential model of autism in *C. elegans*?

Methods

As previously stated in the Introduction, the results last year supported that neurologin-deficient C. Elegans could be used as a model for autism. Since NLG-1 C. elegans are a potential model for autism, the NLG-1 C. elegans should hypothetically display more repetitive behavior than N2 C. elegans since repetition is a core symptom in autism. However, this statement needed to be supported by evidence to show that NLG-1 C. elegans can serve as a model for repetitive behavior. To test this, first, there needed to be significant data to support that NLG-1 C. elegans display a significantly higher number of repetitive movements compared to N2 Wild-type C. elegans.

To ensure that NLG-1 C. elegans could be a model for repetitive behavior, I referred to my data collected last year, which was taken in 1-minute video recordings of the NLG-1 and N2 C. elegans using microscope and computer software. Those videos were rewatched to count the number of repetitive behaviors the worms from each strain exhibited. However, in order to compare the repetitive movement, there first needed to be a method established for what should count as a repetitive behavior in C. elegans.

Literature review of previous scientific studies helped guide the methodology for how to track repetition in worms. However, many of the studies observed repetitive behaviors in mice. In a well-known mouse model of autism called BTBR, researchers tracked repetitive behaviors through self-grooming and marble burying, both regular habits of rodents. Then they counted the amount of "repetitive" motion a mouse showed by counting the number of marbles buried and the amount of times a mouse showed a self-grooming behavior (Amodeo et al., 2014). Mice are more complex model organisms compared to C. elegans and therefore were able to display this complex social behavior. However since working with vertebrates was infeasible in a school setting, this study showed the limitations of working with C. elegans because this study could not replicate a similar model that previous studies used with mice to track repetition. One journal research study that was beneficial to this project's methods was a study done by Menachem Katz and Francis Corzron in 2019, where researchers observed repetitive behavior in C. elegans through recording worm locomotion to analyze the movement of a worm. To define a "reversal" behavior of the C. elegans, the researchers tracked the orientation of the worm movement on a one-dimensional coordinate system and recorded a backward movement as repetitive behavior (Katz et al., 2019). Although the details given by this paper were limited, it was helpful to observe what other researchers identified as repetitive behavior in different model organisms. In this project, repetitive behavior was defined as a movement with the head, then movement with tail, then movement with the head. This 'head-tail-head 'movement causes the worms to repeatedly move back and forth in place, showing a "repetitive" behavior. Furthermore, it was noted that it should not be counted as repetitive behavior if another worm interferes with a worm's movement because that could change the way the worm responds, and thus trying to eliminate as much variability as possible.

Using the established method for repetition detailed above, the video recordings of N2 worms and NLG-1 worms from the data last year was rewatched and counted the repetitive behavior each worm displayed. The graphs below show the repetitive behavior of NLG-1 C. elegans at 1 day old (Figure 1) and 3-days old (Figure 2) compared

to the repetitive behavior of N2 C. elegans over a 1-minute period. At both ages, the NLG-1 strain had a significantly higher amount of repetitive behavior compared to the N2 strain. The error bars on the graph represent +/- 2SEM (Standard Error of the Mean) which mean that 95% of the values will fall within it and display a large representation of my sample size. The error bars do not overlap which helps suggest that the NLG-1 C. elegans had a significantly higher amount or repetition. To further confirm there is a significant difference, a one-way ANOVA test was ran to determine the significance which showed the probability value was less than 0.01 (with $p<0.01$ indicating significance), showing that this comparison between the increased difference of NLG-1 repetitive behavior compared to N2 worms was statistically significant. By creating a method for repetition and comparing the repetitive behavior of NLG-1 C. elegans to N2 C. elegans, it supported that because NLG-1 C. elegans have a statistically significant increased amount of repetitive behavior, NLG-1 C. elegans could be used as a potential model for reducing repetitive behavior in this year's study.

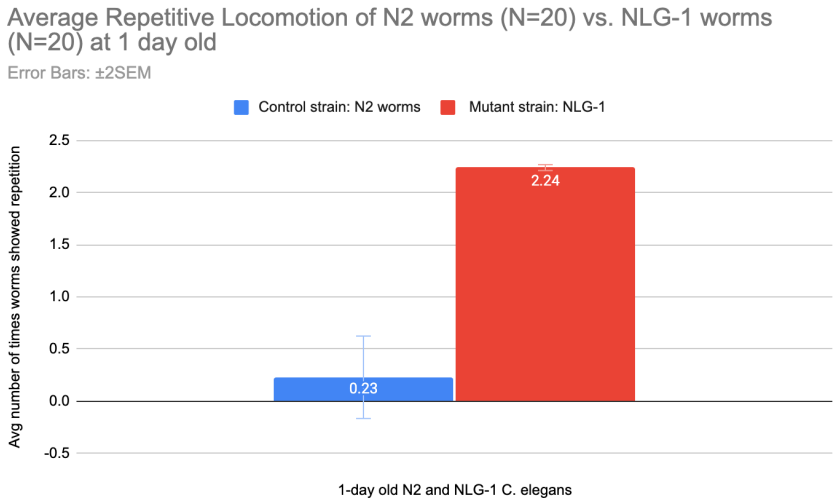

Figure 1- Graph of repetitive behavior of NLG-1 C. elegans vs N2 C. Elegant at 1 day old (+/- 2SEM Error Bars)

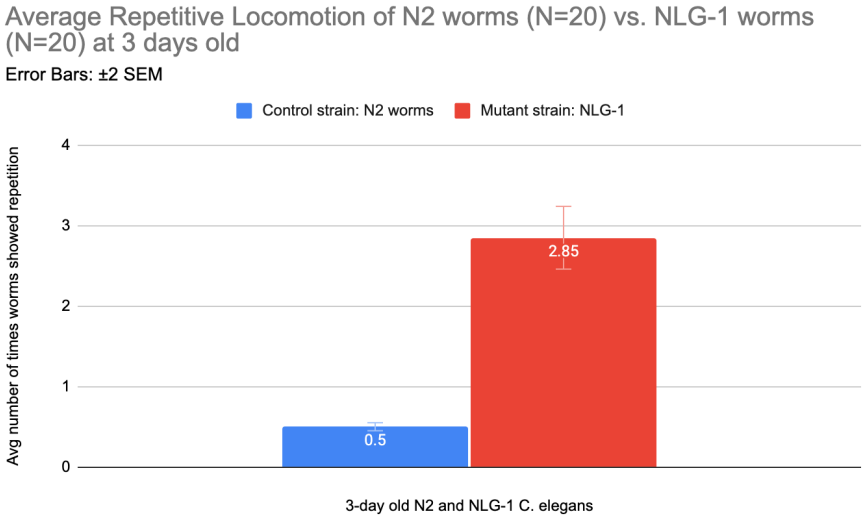

Figure 2- Graph of repetitive behavior of NLG-1 C. elegans vs N2 C. Elegant at 3 day old (+/- 2SEM Error Bars)

After confirming the validity of using NLG-1 *C. Elegans* as a model for this study, the neuroligin-deficient C. elegans or C. elegans strain, OK2599 and were purchased from the Caenorhabditis Genetics Center (University of Minnesota). The worms were cultured and then grown on 60mm petri dishes with Nematode Growth Medium Agar premix from Teknova and 0.2 mL of Escherichia coli (E. coli) K-12 broth from Carolina Biological Supply for food source for the C. elegans. The purpose of age-synchronizing is to standardize the ages of the worms so all the worms remain the same age when recorded. This experiment also wanted to test whether the age of worms impacts the amount of repetitive behavior it displayed. In Autistic humans, some symptoms worsen or lessen as children progress to adults (Volkmar 2013). Thus, repetitive behavior was recorded of 3 different ages of the worms to see if repetition is affected as age increases. To age-synchronize, the worms were first washed with a M9 Buffer to collect the eggs off of the previous worm plates. Then, the plates were bleached using a 20% Alkaline Hypochlorite solution (5mL of NaOH with 10mL of 5% Bleach) and centrifuged three times to kill the adult worms and leave only the eggs remaining. After replating onto new plates, only NLG-1 C. elegans eggs remained and all were the same age. This process is visualized in Figure 3 below.

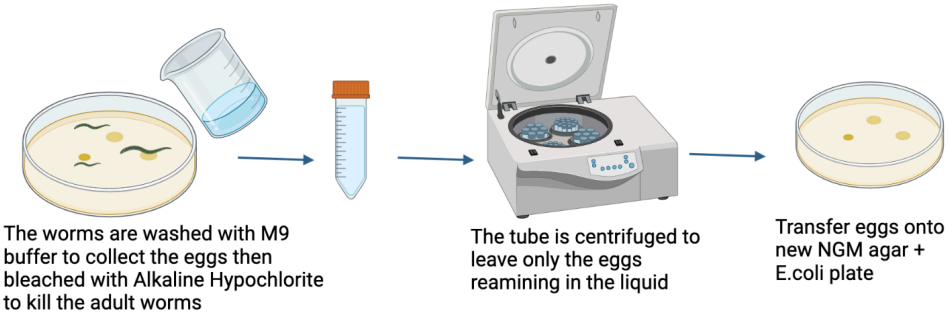

Figure 3: Visual representation of standardizing the ages of the C. Elegans to get all the worms of the same age-group (image created with Biorender)

Once all the NLG-1 C. elegans were age-synchronized, the first trials of recording videos of NLG-1 C.elegans began. As shown in the visual diagram in Figure 4, first a dissection microscope connected to a microscope camera, connected to a computer was used. Then, the petri dish of age-synched worms under the microscope was placed to record 1-minute videos of the worm movement through the microscope camera. Using the microscope camera software, ToupView, 1-minute videos of each petri dish on the computer were recorded. Then the amount of repetitive behavior (per methods described above) of each worm was counted and recorded the data on a spreadsheet. This procedure was repeated for the worms 1-day after age-synching, 3-days after age-synch, and 5-days after age-synch to get 3 different stages of the life cycle.

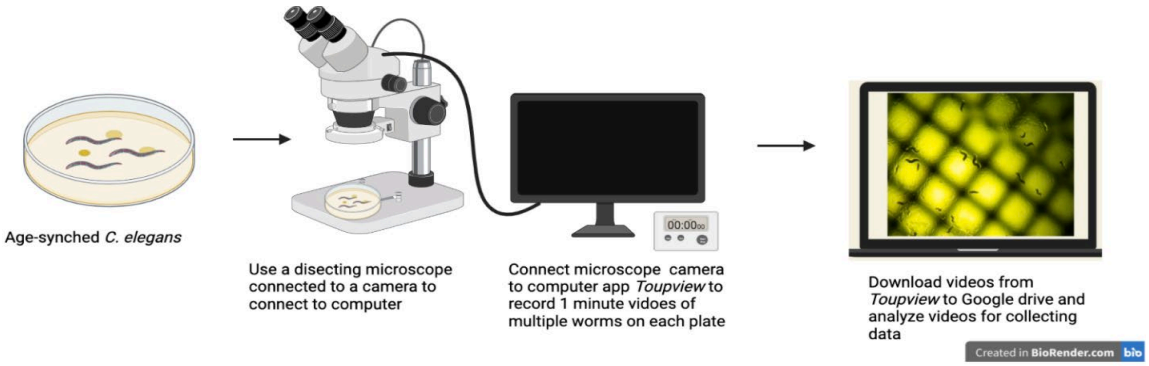

Figure 4: Visual representation of recording the C. Elegans movement on microscope camera and transferring to the computer for analysis (image created with Biorender)

For the trials of worms exposed to choline, it followed a very similar procedure as shown above in Figure 4. However, the first step was to expose the choline chemical by creating a stock solution of Choline Bitartrate (C9H19NO7) purchased from Fisher Scientific. Each plate was exposed to 0.2 mL of a 2.4 mg/ml of choline solution. To make this solution, 240 mg of Choline Bitartrate powder was dissolved in 100 mL of distilled water. The dosage and concentrations were scaled from the human daily choline supplement intake recommended by the CDC, which is 550 milligrams a day, to a C. elegan mass intake of 2.2 micrograms (ug). Then 0.2 mL of the choline solution was added to each plate of worms on the surface of the NGM Agar + E. coli plate. (See Figure 5). After leaving the plates exposed to the choline solution for 24 hours, the same procedure as shown in Figure 4 was repeated to record 1 minute videos of the worms and count the repetitive behavior of each worm. The videos were recorded 1-day, 3-day, and 5-day after exposure to the choline solution in order to match the ages of the previous trials to compare age groups.

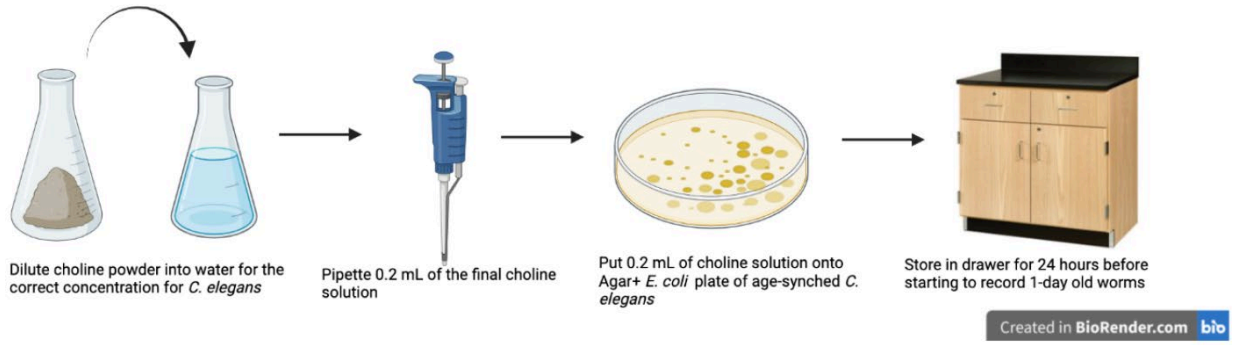

Figure 5: Visual representation of creating the solution of choline and exposing the C. Elegans to the choline solution for certain trials (image created with Biorender)

This process was repeated each week where the worms were age-synchronized and then videos were recorded periodically throughout the week at 1-day, 3-day, and 5-day old stages. In total, 2 trials of NLG-1 C. elegans without choline (150 worms total), 2 trials of NLG-1 C. elegans with choline (150 worms total), and 4 trials of video recordings from last year were analyzed for repetitive movement. Ultimately, there was 6 trials with 400 worms analyzed for repetitive behavior.

Results

To analyze the data from the recordings of the NLG-1 *C. elegans* and test whether choline reduces repetitive behavior, the number of repetitive behavior of NLG-1 *C. elegans* when exposed to choline was compared to without choline at 1-day old, 3-day old, and 5-day old.

The graph below (Figure 6) shows the Average Repetitive Locomotion the 1-day old NLG-1 *C. elegans* displayed when exposed to choline compared to without choline over a 1 minute period. The blue experimental group represents the worms without choline and the red experimental group represents the worms with choline. As visually shown, the red experimental group is significantly lower than the blue experimental group. The error bars on the graph represent +/- 2SEM that show that 95% of my values will fall within. These error bars do not overlap and the data was significantly different which leads to support the claim that choline reduced the amount of repetitive behavior in 1-day old NLG-1 *C. elegans*.

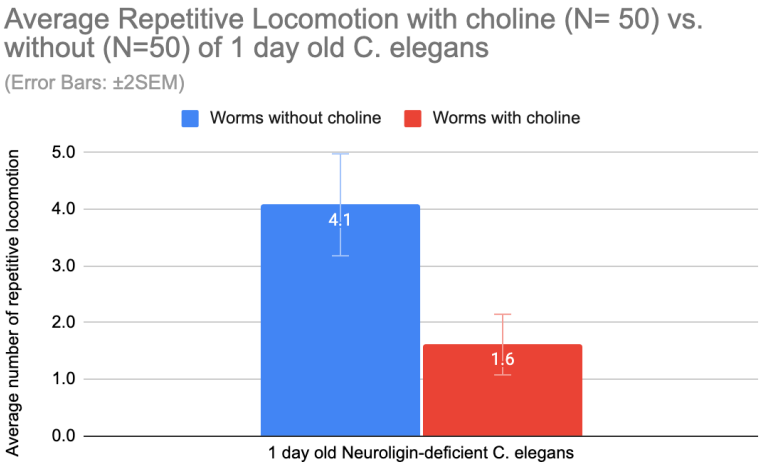

Figure 6- Graph of Average Repetitive Locomotion that 1-day old NLG-1 *C. elegans* displayed when exposed to choline vs. without choline over 1 minute period (+/- 2SEM Error bars)

The following graph below (Figure 7) follows the same pattern as 1-day old *C.elegans*, but displays the Average Repetitive Locomotion of a 3-day old NLG-1 *C. elegan* with choline versus without choline. Similar to the 1-day old *C. elegans* from Figure 6, the worms exposed to choline had significantly less amount of repetitive behavior in 1 minute compared to the worms without choline.

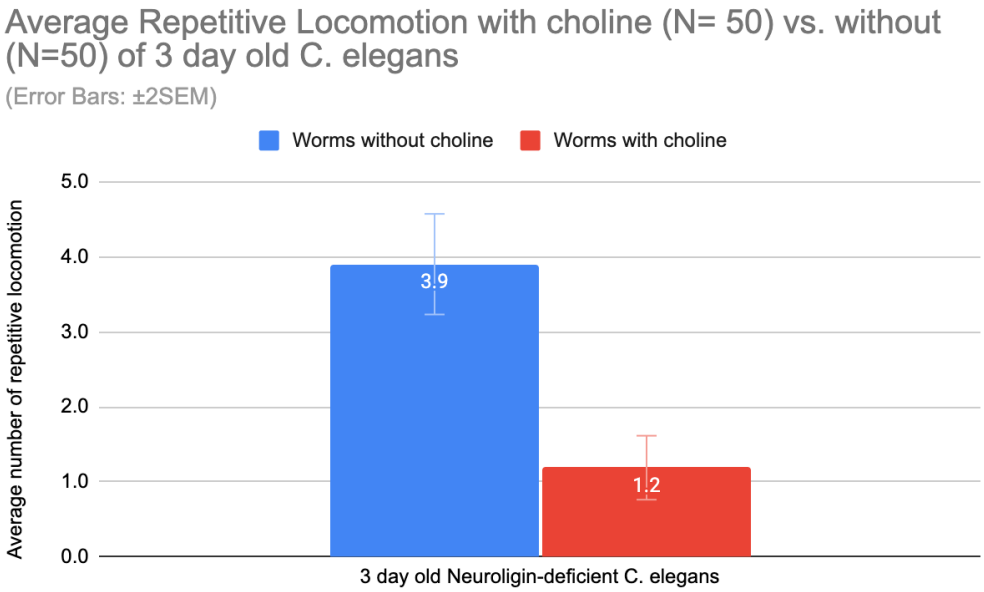

Figure 7- Graph of Average Repetitive Locomotion that 3-day old NLG-1 *C. elegans* displayed when exposed to choline vs. without choline over 1 minute period (+/- 2SEM Error bars)

The final graph (Figure 8) follows in the same pattern and this graph compares the Average Repetitive Locomotion of 5-day old NLG-1 *C. elegans* with choline versus without choline. Similar to the 1-day old and 3-day old *C. elegans*, the worms with choline significantly had less amount of repetitive behavior in 1 minute compared to the worms without choline as shown by the non-overlapping error bars and significant difference in data values.

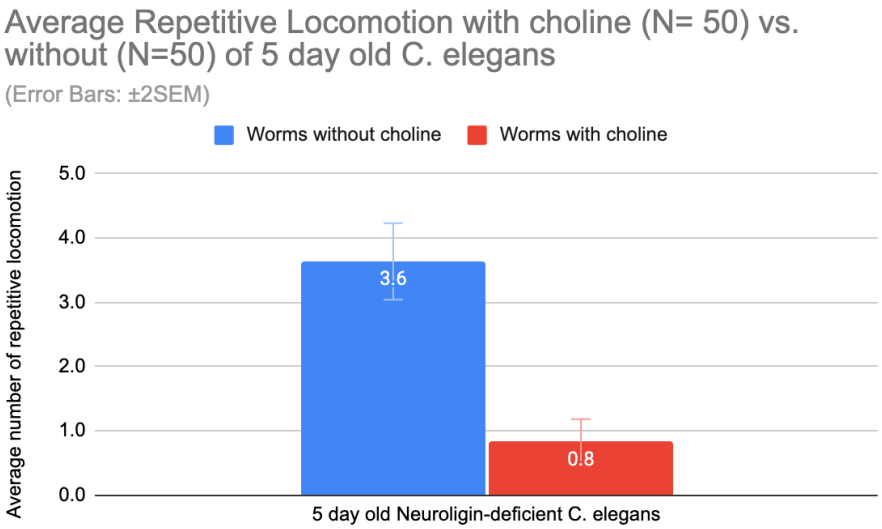

Figure 8- Graph of Average Repetitive Locomotion that 5-day old NLG-1 *C. elegans* displayed when exposed to choline vs. without choline over 1 minute period (+/- 2SEM Error bars)

All three graphs (Figures 6-8) of the three age-groups displayed that NLG-1 *C. elegans* exposed to choline had significantly reduced repetitive behavior compared to NLG-1 *C. elegans* without choline. To further confirm the significant difference, a one-way ANOVA and Tukey HSD test was used to determine significance between the repetitive behavior of NLG-1 *C. elegans* when exposed to choline versus without. An ANOVA test is a statistical significance test that compares the amount of variance within the groups and a Tukey HSD test indicates specifically where the significance occurs. Using the online statistical analysis calculator by Navendu Vasavada (astatsa.com), a p-value, representing the probability that the difference between the data sets is due to chance, is given. In all three age groups of this study, the p-value was less than 0.05 ($p<0.05$) which correlates to making a conclusion with a 95% confidence rate that the difference of repetitive behavior with choline versus without was not due to chance. As stated previously in the methods, the worms were also age-synchronized to identify whether the age of the *C. elegans* affected the amount of repetitive behavior it showed. However, after running ANOVA test to determine if there was a difference between repetitive behavior of 1-day old vs. 3-day old vs. 5-day old *C. elegans*, the results showed insignificant. As supported by the results of the ANOVA test and the Tukey HSD values, it can be concluded that the addition of choline significantly reduced the repetitive behavior in NLG-1 *C. elegans* but the age of *C. elegans* had no significant effect on the amount of repetition they show.

Discussion

The data from this study supports the claim that the addition of choline can significantly reduce the amount of repetitive behavior in NLG-1 *C. elegans* modeling autism in the three age groups tested. As mentioned in the Results, the statistical analysis tests showed an insignificant correlation between age and the amount of repetition behavior. In 2013, Fred Volkmore detailed a study describing the progression of children with ASD when they become adults. The researchers discussed that while many symptoms of autism worsen or lessen as age increases, repetitive behaviors tend to persist and remain constant into adulthood (Volkmar 2013). Thus, the results of this research study are supportive of professional literature showing that repetitive behaviors of NLG-1 *C. elegans* and humans with Autism are both not impacted by age. An interesting observation during data collection was observed with higher amount of repetition in crowded worm environments compared to isolated environments (See Figure 9). Because the increase of proximity of worms may have an effect on the worm's repetitive behavior, a future study to consider is there might be

some correlation to hypersensitivity to large populations which may increase the amount of repetitive behavior exhibited by the worms. This should be further studied with future research.

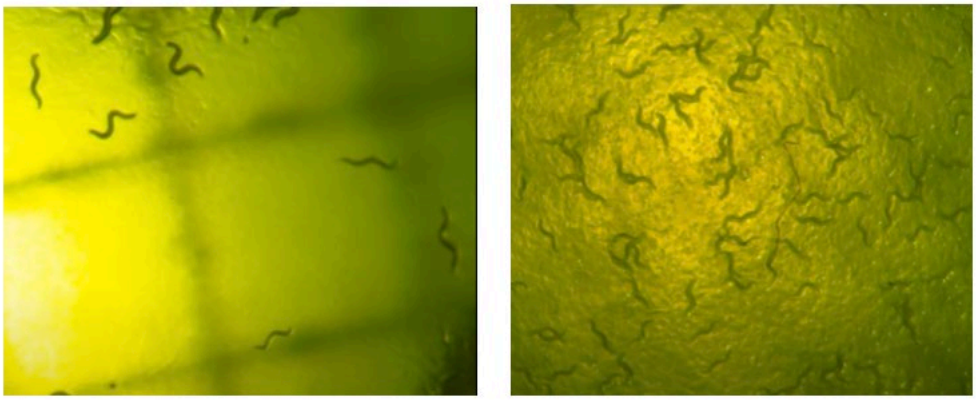

Figure 9- Comparison of Isolated worm environment (left image) vs. crowded worm environment(right image)- image taken on Toupview microscope software

Conclusion and Applications

The hypothesis that choline should improve the repetitive symptoms in a potential model for autism in NLG-1 C. elegans was supported by the data collected. In previous existing research, choline supplementation was correlated to some counterraction of social anxiety and repetitive behavior in mice models (Agam et al., 2020). As observed through the results in this study, repetitive symptoms were decreased for all of the age groups through choline supplementation. This study's results supports those of previous studies in other model organisms of Autism and shows this study can be used with a new type of model organism not previously been used in ASD research. Through this research, it can be concluded that scientists can use neuroligin-deficient C. elegans as a cost-effective model to test treatments against repetition that humans with ASD have. By increasing the amount of choline in a diet through eggs, fish, and broccoli, choline can be a strong natural reductant for all age groups. Furthermore, this research also serves as more evidence that my research last year can be supported that neuroligin-deficient *C. elegans* can be used as a model for autism as it was able to serve as a model for repetitive behaviors for this study as well as a model for sensory stimuli from last year.

Choline is a cheap, convenient, and accessible option for potential reduction in repetitive behavior in Autistic humans. Humans with Autism Spectrum Disorder can start adding foods high in choline to their everyday diet to decrease repetitive behavior. Adding vegetables like broccoli to a human's diet is simpler and more cost-effective compared to prescription medications, and thus humans with ASD can add foods with choline in their diet to hopefully help reduce repetitive behavior without fear of any strong side effects that medications provide. In the chance it doesn't reduce repetitive behavior, there is not considerable harm in consuming more vegetables as they are all healthy foods for humans regardless.

Future Research

Future research should be on testing whether the addition of choline can reduce repetition in mice models of autism and eventually humans with ASD, to test whether the effect is comparable and as beneficial as it was in *C. elegans.* Another crucial study is to distinguish which kinds of repetitive behavior does choline reduce in Autistic humans. As discussed in the Introduction, the symptoms of repetition in ASD have a wide range that are categorized in lower-order behaviors such as hand flapping and vocal repetition, as well as higher-order repetitive behaviors,

which include severe compulsive adherence to routines (Soorya et al., 2008). Although this study supported that *C. elegans* can reduce repetitive behavior in an autism model, *C. elegans* are simply not complex enough to showcase a difference between the types of repetitive spectrum that humans with ASD display. Future research on testing which symptoms choline helps reduce would be pivotal in understanding the amount of impact choline can provide to Autistic humans. A possible way to test this study would be with humans with ASD ranging from children to adults and placing them on a choline diet to watch which of their typical repetitive symptoms reduce. Then, as established from previous studies with analyzing repetitive behaviors in ASD, researchers can use the RBS-R (Repetitive Behavior Scale-Revised), a questionnaire rating a range of behaviors that scores the number of RRB present and their intensity for restricted and repetitive behaviors (McDermott et al., 2020) before and after the choline diet. Then, researchers can experiment supplementing choline with other chemicals to form stronger treatment for reducing more prevalent and complex forms of repetitive behavior.

Another important direction that future research can take is to test more direct ways to increase acetylcholine. As established, the role of acetylcholine is highly unknown in autism and through investigating its effects and association with repetition in humans with ASD in this study, acetylcholine should be explored to investigate the roles it plays in other core symptoms of autism. It would be highly beneficial to find a more direct way to increase the amount of acetylcholine by using choline to pair with other treatments to reduce some main core deficits of neurodevelopmental disorders.

A much larger extension to this research is for ASD research to target specific neurochemical systems such as how this study explored the cholinergic system, and focus on particular neurotransmitters that affect each social and behavioral deficit of Autistic humans. Potentially, researchers can develop preliminary medication and treatment to test on NLG-1 *C.elegans* before mice and humans with ASD as a cost-effective alternative. Once a treatment is showing promising results, it can hopefully be given to reduce Autistic deficits in younger children before they grow older. Targeting the neurochemical systems to develop treatments for early pharmacological treatments that could reduce significant autism symptoms on a daily basis can hopefully be one step closer to a larger treatment for autism.

Limitations

Although this research supports the claim that choline may reduce repetitive behavior, it can only be partially conclusive for a few reasons.The largest limitation in my study was using a simple model organism of *C. elegans.* It is important to note that if given the decision to choose mice or humans as a model organism, this study would have tested on more complex organisms as they provide a stronger conclusion. Due to their simplicity, the results of *C. elegans* can not be directly linked to the outcomes of humans. Even though adding choline in NLG-1 *C. elegans* reduced *C. elegans* repetitive behavior, choline may affect repetitive behavior in humans differently. Humans with ASD are severely more complex organisms and their pathway of how food and chemicals digest is not fully represented by a *C. elegan* model. Thus, choline might not be a definitive solution of repetition due to the complexity of the human brain and further research is needed for a definite conclusion to be reached.

Another limitation of this study is the methodology of tracking the worm′s repetition. After observing all of the worm videos, it is apparent that the randomness of the worm movement often caused variability and difficulty in counting precise repetitive motion each time. Since data was collected from manually hand-counting each of the several turns and twists the worm makes, some data points might be slightly off due to mistaking or not accurately counting repetitive behavior. Similarly, a study by Katz et al., 2019 also found it difficult in their study to precisely differentiate each worm movement due to the multiple reversals of the worm every single time. In future research, creating computer software for methods to count the number of repetitive behaviors of a worm would have a higher accuracy for data collection. However, because the difference was strongly significant, this study can conclude that choline has a strong suggestion of reducing repetitive behavior in autism despite a possible small number of data miscounts.

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