Detecting Behavioral Changes in an Autism Mouse Model after Treatment with Ergothioneine

Submitted by

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Abstract

Autism Spectrum Disorder (ASD) is a neurological disorder that can impair developmental, behavioral, and social skills. Although there are currently no curative remedies authorized for the treatment of autism spectrum disorder, certain dietary supplements may act as a form of therapy for some of the atypical behaviors associated with this diagnosis.

Ergothioneine (ET) is an amino acid derivative known for its antioxidant properties and its ability to enhance neurological function. Here we utilized ET supplementation to examine the potential benefits it may have on autism phenotypes. A BTBR autistic mouse model was used to test the effects of ET treatment followed by behavioral examination. Quantitative analyses of behavioral changes observed between the treatment group along with controls were performed to determine if ET could improve autism-like behaviors. The long-term goal of this study is to establish the possible application of a safe ET supplementation to ameliorate autistic manifestations in ASD children.

Introduction

Detecting behavioral changes in an autism mouse model after treatment with ergothioneine

Autism Spectrum Disorder

An increase in the diagnosis of autism spectrum disorder (ASD) has been reported throughout the early 2000s. The Centers for Disease Control (CDC) and Prevention have quantified the prevalence of this neurodevelopmental disorder among 8-year-olds, and their assessment revealed a clear linear relationship between ASD cases and time progression. In the year 2000, 1 out of every 150 children was diagnosed with ASD, yet in the year 2020, this ratio escalated to 1 out of every 36 children reported to be affected by the disorder (Centers for Disease Control). ASD is a disorder that ranges in severity and is characterized by a wide range of abnormal behaviors relating to social interactions and developmental milestones. Autism-related behaviors are often initially noticed in children during their first two years of life. A diagnosis of ASD can be considered if any combination of the following mannerisms is noted: decreased interest in social interactions and connectedness with others, deficits in social skills and awareness, lack of eye contact, abnormal play, absence of pretend play, decreased attention, peculiar behaviors and/or self-injuries, compulsions, obsessions, lack of or delayed speech, and repetitive use of words or use of words out of context, etc (Sanchack & Thomas, 2016).

To understand why ASD causes these uncommon behaviors, researchers have identified several notable biochemical alterations present in individuals with this diagnosis. One of the major molecules studied concerning neurological disorders is reactive oxygen species (ROS). Evidence has indicated that ASD is associated with a greater sensitivity to ROS than seen in individuals without this disorder, and ASD is characterized by high levels of ROS within the

body (Bonomini et al., 2022). This sensitivity, in combination with increased levels of ROS, elicits a neurotoxic effect on proteins, lipids, and polynucleotides in the cells. Elevated levels of ROS induce an environment of high oxidative stress which negatively impacts neurodevelopment and cognitive function. These complications of ROS abundance are also demonstrated in other neurological complications such as Alzheimer's and Parkinson's disease (Brieger et al, 2012).

Additional neurological anomalies associated with ASD include anxiety, depression, and sleeping difficulties (Bonomini et al., 2022). While these adverse effects are treatable through a variety of therapies and medications, there is currently no preventive or curative treatment for ASD. Some specialists recommend managing symptoms through holistic approaches including massage therapy, applied behavioral analysis (ABA), or vitamin supplementation. On the other hand, providers may recommend medical intervention to help manage the more severe symptoms. Medications such as Aripiprazole and Risperidone are within the class of antipsychotics and are used to regulate aggressive behaviors displayed by children with ASD who may be harmful to others or themselves (Sanchack & Thomas, 2016). While these remedies are often beneficial, there is much room for improved management and treatment of ASD impairments.

Ergothioneine use in ASD

Recent studies have begun to examine the benefits of utilizing amino acid supplementation as a form of therapeutic treatment for ASD. Ergothioneine (ET) is an amino acid derivative of histidine (Figure 1), and this molecule has been utilized in neurological research because of its valuable antioxidant properties (Ishimoto & Kato, 2022). As previously

stated, ASD is associated with an increase in oxidative stress that remarkably negatively impacts cellular health and function within the body (Bonomini et al., 2022). Therefore, antioxidants may be used as therapy for ASD as they are said to reduce some of the harm caused by highly reactive free radicals (Ishimoto & Kato, 2022).

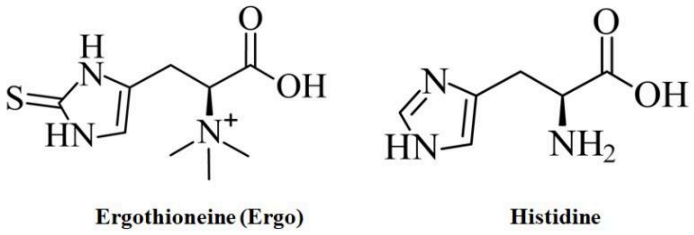


Figure 1

Molecular structure of Ergothioneine in comparison to Histidine (Apparoo et al., 2022)

ET is not naturally produced in the human body, instead, it is introduced into the digestive tract through plant-rich diets. In particular, mushrooms have been found to contain high levels of ET. Water-soluble supplements may be used in lieu of dietary consumption of ET-containing foods for easy accessibility to the benefits of this molecule. Alongside its antioxidant properties, ET offers a broad range of advantages that have a notable impact on neurological function. In a study conducted by Ishimoto and Kato (2021), the researchers performed a double-blind experiment on humans in which cognitive function was assessed following treatment with ET. Significantly higher scores on a Cognitrax examination were detected from the ET treatment groups as opposed to the control groups. Protection from oxidative stress, induction of neuronal maturation, improved quality of sleep, and antidepressant effects were also attributed to regular ET consumption in this study.

Research exploring the influence of ET supplementation on brain function has demonstrated an impressive array of favorable outcomes. Its use in dementia, epilepsy and Parkinson's disease research shows promising results for future experimentation with this amino acid derivative (Ishimoto & Kato, 2021). Given the neurologically-enhancing nature of this supplement, the following study intends to examine the use of an ET supplement in an ASD mouse model to determine possible therapeutic effects on ASD-related behaviors. Our preliminary results showed that mice under ET treatment have shown some increased social interest and locomotor activity, but changes in regard to light-sensitivity and exploratory behaviors did not prove significant throughout this study.

Materials and Methods

BTBR Mice

A BTBR mouse model was purchased from The Jackson Laboratory and used for the duration of testing. The *T*+ *Itpr3tf*/J strain is commonly used in autism research because these mice exhibit a unique brain anatomy that lacks the corpus callosum and has a decreased hippocampal commissure. These discrepancies are deemed responsible for the abnormal behaviors displayed by this mouse strain and have been shown to change the dynamic of the intraspecific relationships between the mice (BTBR). The BTBR mice were housed in an Institutional Animal Care and Use Committee (IACUC) approved animal facility where they were treated with standard protocols. Unlimited access to water and a Teklad rodent diet were available throughout the studies, and 250mg/L of ET was dissolved into the water of the treatment groups. Two to four mice of identical age and gender were housed in each cage, and treatment/control groups were determined by cage number. After treatment, the groups were placed in a variety of devices and their behaviors were analyzed.

Light/dark box

The light/dark box (Figure 2a) is a device used to examine sensitivity and preference to areas of high light exposure. This device was created by joining two 40cm x 20cm x 30cm rectangular boxes, and a small 8cm x 8cm passageway was cut to allow easy movement between the two rooms. The light side of the box was made from clear plexiglass while the dark side was crafted from black plexiglass. The dark side also contained a black plexiglass hinge-top which prevented excessive light from entering the area and allowed easy access to the mice upon completion of the experiment. Individually, mice were placed into the light area of the device, and exploration was permitted for a period of four minutes. The location of the mice within the box was noted and time stamps were recorded for time spent in both the light and dark areas of the box. The total times spent in both areas were added and results were compared between treatment and control groups.

Figure 2a

Photograph of the elevated plus maze

Elevated plus maze

The elevated plus maze (Figure 2b) contains four arms of equal length that have a central attachment, allowing the formation to appear as a plus shape. The arms are 25cm x 5cm, and two nonadjacent arms are bordered by walls 16cm high. The plus was then placed on a cylinder with a height of 45cm to elevate the platform. Individually, mice were placed in the center of the plus and allowed to explore the arrangement for five minutes. Throughout the experiment, three factors were measured: total time spent on the enclosed arm, total time spent on the open arms and time spent peeking out of the enclosed arms. Peeking behavior was noted when the body of the mouse remained inside the enclosed arm but the head of the mouse was seen to be outstretched and peering toward the exposed areas. The elevated plus maze is used to measure the frequency of exploratory behaviors while determining the degree of exploration. Once collected, the data from exploration time was compiled and results were compared between treatment and control groups.

Figure 2b

Photograph of the elevated plus maze

3-chamber maze

The 3-chamber maze (Figure 2c) is used to evaluate interest in social interaction and assess the curiosity of unrelated individuals. The intricate layout of this maze allows controlled interactions between the experimental individual and one to two other mice while also providing a secluded area for the mouse to remain when solitude is preferred. Three 17cm x 24cm x 23cm rectangles were adjacently connected, and openings of 8cm x 8cm were cut into the adjacent walls allowing accessibility into each of the compartments. Within each of the outermost chambers, a 12cm x 8cm x 23cm cell was placed in the center of the rooms. Both of the cells contained small slits on the lower half of the wall which faced the center chamber, and the center chamber remained empty. The 3-chamber maze experiment was conducted twice for each mouse, and different variables were introduced to best determine how treatment affects social behavior in the BTBR mice. Initially, the experimental mouse was allotted a period of two minutes to explore the maze layout. This pre-trial time allowed the mouse to become familiar with the device so during the official trial period, the only new variable presented was other mice. Sexual interest was also eliminated as all of the mice present in the maze during testing were of the same sex. To begin the official trial period, a stranger mouse from a non-BTBR strain was placed into one of the two cells, then the experimental mouse was placed into the center chamber. The mouse was given five minutes to navigate the maze while data on chamber location was recorded. On a separate day, the 3-chamber experiment was repeated, but a stranger mouse was placed into one of the cells and a familiar mouse, a mouse from the same cage as the experimental mouse, was placed into the remaining cell. For five minutes, the position of the mouse within the maze was noted, and preference for familiar versus unfamiliar social interactions was recorded.

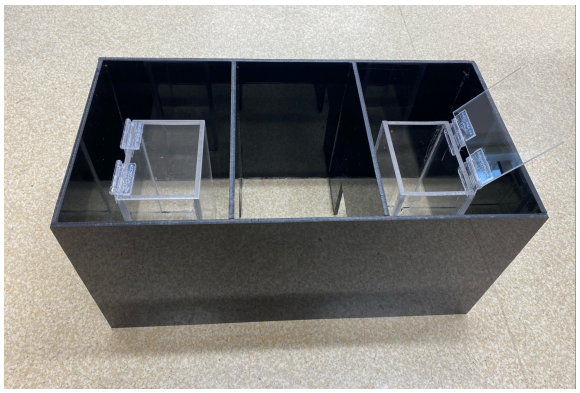


Figure 2c

Photograph of the 3-chamber maze

Open field test

To further investigate exploratory behaviors and measure movement patterns among the mice, a SuperFlex Open Field apparatus (Figure 2d) (*SuperFlex,* 2023) was used. This apparatus contains clear acrylic walls of 40cm on all sides, and the floor is made of a smooth white acrylic material. Each mouse was individually placed into the device and given a period of 30 minutes to acclimate to the new environment. During this pre-trial and trial, the room lights were turned off to encourage mobility of the mice. Proceeding the pre-trial was a 30-minute trial in which movement patterns were measured by the Fusion Locomotor Activity Plotter Analysis Module (*Fusion,* 2023) created by Omnitech Electronics. Data collected from this program was used to generate heat maps. Heat maps of treatment and control groups were visually compared to establish variation in movement patterns of the BTBR mice.

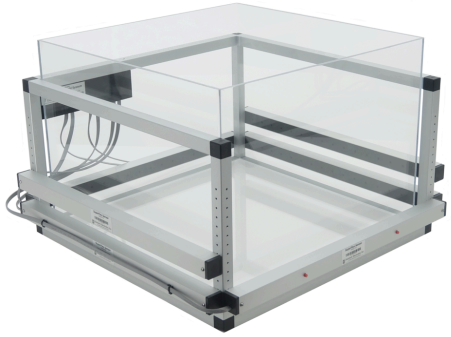


Figure 2d

Photograph of the open field test (*SuperFlex*, 2023)

Data Analysis

All of the data collected from behavioral experimentation was compiled, graphed, and analyzed through the GraphPad Prism application (*Prism*). Outliers outside of the interquartile range were excluded for the light/dark box and unmodified 3-chamber testing. The elevated plus maze data did not contain outliers, and the modified 3-chamber contained many outliers. Therefore, it was determined that removing outliers from the modified 3-chamber experiment would heavily skew the data. For the light/dark box, elevated plus maze and 3-chamber maze, a two-way ANOVA was performed. This statistical analysis provided information concerning the significant differences seen among the male and female mice, the ET treatment and control groups, and the interaction between gender and treatment type. A p-value of .05 was selected to determine the significance of each relationship.

Results

Light/dark box

Figure 3a provides a visual representation of the data collected from the light/dark box experiment. For this experiment, data was collected on 11 adult male control and treatment mice and 10 female control and treatment mice. The amount of time each mouse spent in the light area of the device was measured and analyzed through the GraphPad Prism application. The results were calculated to reveal the mean±standard deviation (SD) for the differing variable groups. The male control group spent an average of 58.229±35.016 seconds in the light side while the female control group spent an average of 45.122±23.088 seconds in the light. The male treatment group averaged 37.899±23.870 seconds in the light area, and the female treatment group

averaged 44.008±26.102 seconds in the light area. Next, a two-way ANOVA was performed to reveal the p-values for each of the variable relationships. The p-value for the differences observed between the male and female groups is p=0.6839. For the differences seen in treatment versus control groups, a p-value of p=0.2160 was computed. To establish the role of ET treatment on gender, a p-value was calculated for the variation in both of these groups. This p-value was reported to be p=0.2666.

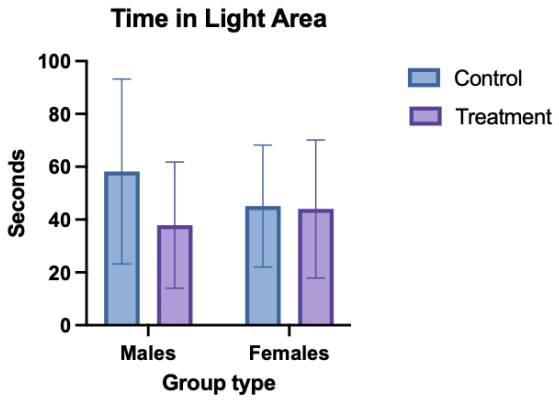


Figure 3a

Graphical representation of the light/dark box analysis. The mean±SD of the time spent in the light area for males and females was recorded, and the gender groups were further separated based on exposure to treatment or no treatment.

Elevated plus maze

Data gathered from the elevated plus maze (Figure 3b) was collected from 13 control males, 10 control females, 11 treatment males and 11 treatment females. The amount of time spent exhibiting exploratory behavior was documented during the trial period. Exploratory behavior was recorded when the mouse was observed to be walking or standing on one of the open arms, or if the mouse was seen to be *peaking* as described earlier in the research. Total

exploratory behavior was calculated for each mouse, and the mean±SD was calculated using the GraphPad Prism application. The male control group expressed such behaviors for an average of 55.846±41.380 seconds, and the female control group exhibited exploratory behavior for an average of 60.800±44.803 seconds. For the treatment groups, the males had a mean exploration time of 50.182±27.989 seconds, and the females had a mean of 64.546±30.559 seconds. Next, a two-way ANOVA was performed to determine p-values for the differing variable groups. The p-value for the relationships between male and female mice was determined to be p=0.3870. The data from the control versus treatment groups revealed a p-value of 0.9312. Regarding the relationship between gender and ET exposure, a p-value of p=0.6724 was calculated.

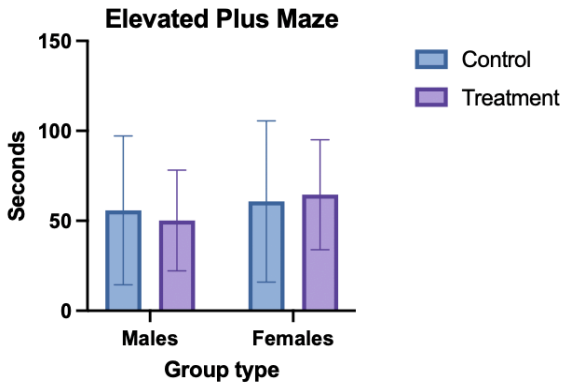


Figure 3b

Graphical representation of the elevated plus maze. The data displayed represents the mean±SD of the explorative data of the treatment/control groups, and they are separated based on gender.

3-chamber maze

The 3-chamber maze results are presented graphically in Figure 3c. This schematic includes data from 11 control males, 10 control females, 11 treatment males and 11 treatment females. The GraphPad Prism application was used to generate quantitative values for mean time

spent in the same chamber as the stranger mouse for both the gender and treatment groups. The male control mice were observed to spend an average of 137.600±17.711 seconds near the stranger mice while the female mice appeared to be more inclined to spend time near the stranger mouse. Their average mean time was 153.031±35.272 seconds. The male treatment mice spent 138.701±27.250 seconds and the female treatment mice spent 141.128±39.666 seconds near the stranger mouse. Using a two-way ANOVA, the p-values for the relationships between the variable groups were calculated. The p-value between the male and female groups was determined to be p=0.3513. ET treatment versus non-treatment revealed a relationship with a p-value of p=0.5715. Lastly, the male/female versus treatment/control interaction provided a p-value of p=0.4962.

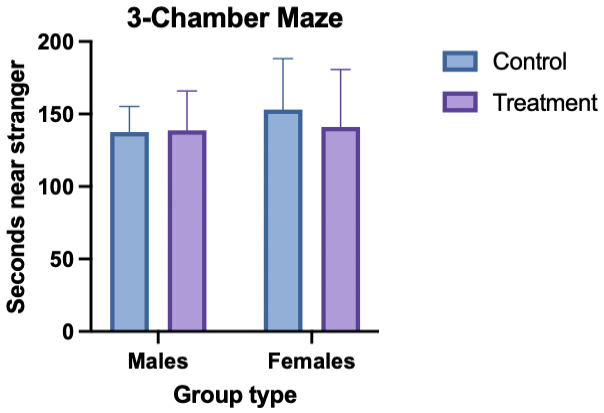


Figure 3c

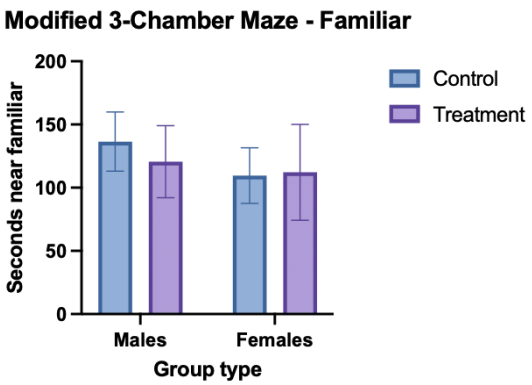
Graphical diagram of the 3-chamber maze. The mean±SD is presented for the amount of time spent near the unfamiliar mouse. Data is grouped based on gender and treatment type.

Modified 3-chamber maze

The modified 3-chamber maze, which included exposure to a familiar and stranger mouse simultaneously, yielded information that was used to evaluate the relationship between the ASD

mouse model and social preferences. The data collected from the trials is represented by Figure 3d and Figure 3e. Individually, 12 control males, 10 control females, 11 treatment males and 11 treatment females were placed into the maze for a predetermined trial period. The GraphPad Prism application was utilized for data analysis and a two-way ANOVA test. The male control group spent an average of 136.483±23.376 seconds in the same chamber as the familiar mouse, while the female control group averaged 109.572±21.954 seconds around the familiar mouse. The males treated with ET showed a decreased mean time spent near the familiar mouse. Their mean was 120.555±28.536 seconds, and the mean for the ET-treated females increased to 112.182±37.956 seconds. The p-values of the relationships between gender and treatment were computed for the time spent in the same chamber as the familiar mouse. The 2-way ANOVA indicated a p-value of $p=0.0484$ for the differences observed between the males and females. In regards to treatment type, a p-value of $p=0.4467$ was determined. The interaction of gender and treatment type for the modified 3-chamber-familiar side gave a p-value of $p=0.2912$.

The average time spent in the chamber alongside the stranger mouse was also calculated for all four groups. The control males spent an average of 114.963±29.928 seconds near the stranger mouse, and the females exhibited a higher interest in the stranger mouse with a mean time of 140.645±18.040 seconds. The males treated with ET were observed to be in the same chamber as the stranger mouse for an average of 141.344±29.129 seconds. The treatment females averaged 128.306±45.821 seconds near the stranger mice. The relationship between gender and mean was quantified by a p-value of $p=0.5234$. The differences seen in the treatment versus the control group revealed a p-value of $p=0.4788$. The relationship between the male/female group and the treatment/control group provided a p-value of $p=.0556$.



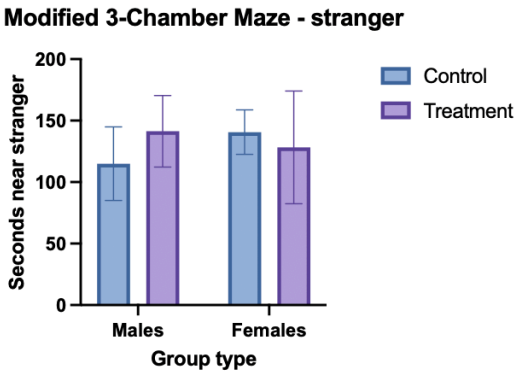


Figure 3d **Figure 3e**

Graphical representations of the modified 3-chamber maze. The mean±SD was recorded for both the time spent near the familiar and stranger mouse, respectively. The data was separated into two graphs and further grouped into categories of gender and treatment type. The time for all testing was recorded in seconds.

Open field test

Figure 4 provides a visual display of the locomotor activity and movement patterns of the mice while inside the open field device. Heat maps were generated using photo sensors and infrared beams incorporated in the SuperFlex Open Field Device (*Superflex,* 2023). The surveillance equipment pairs with specialized Fusion Software (*Fusion*, 2023) to generate data from the animal's displacement. The heat maps selected adequately represent the experimental population of 12 control males, 10 control females, 11 treatment males, and 11 treatment females. It is clearly seen that the control males exhibited a limited amount of exploratory behavior. Three out of the four selected control males remained mostly in the upper left corner of the box for the duration of testing while the other mouse spent some time in each corner with a preference for the left side corners. While most of the treatment male mice spent a significant

amount of time in the upper left corner, all of the treatment males moved a measurable distance along the walls. Upon initial observation, it is clear that both the control and treatment female mice spent more time being active than the male mice. The female control mice showed a preference for the corners and walls of the box, and almost no central movement was detected. On the other hand, the female mice treated with ET spent a considerable amount of time exploring a majority of the open field. Yet again, centralized activity was still observed less often than movement along the perimeter. The Fusion program also constructed a line graph that demonstrates total locomotion versus time in the open field. According to the graph, all of the mice were most active during the first portion of the trial period, and the female treatment mice were the most active of all the mice.

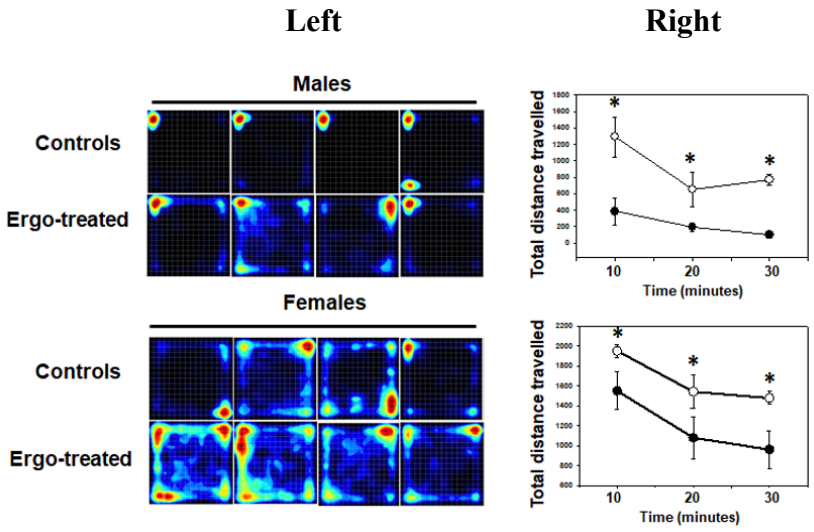


Figure 4

Left, Heat maps of four control male and female mice and four treatment male and female mice. Right, graphical representation of the data displayed in the heat maps. For both graphs, time was measured in minutes and distance was measured in centimeters.

Discussion

Light/dark box

The mean times and standard deviations generated from the light/dark box experiment are relatively close in magnitude. Both control groups spent the greatest amount of time in the light area, and the control males appeared to be the most light-insensitive. The difference seen in the mean averages of the control males versus the treatment males offers the largest variation with a second differential of 20.330 seconds. The female control and treatment groups have a much smaller differential in mean times spent in the light area, with a decrease in the treatment group by 1.114 seconds. The p-values posed insignificant for all variable relationships, yet the most significant value was calculated from the comparison of treatment versus non-treatment groups. Although the use of ET in the analysis of light-sensitivity did not reveal a significant relationship, this initial data can be used to improve future studies involving ASD diagnosis and luminar preference.

Elevated plus maze

The total variation seen among the mean values for all of the tested groups does not exceed 15 seconds in the elevated plus maze experiment. Interestingly, the male control and female treatment groups spent the highest amount of time exhibiting exploratory behaviors. The difference between control versus treatment for the female and male ranges from 3.746 seconds for the female group to 5.664 seconds for the male group. This slight fluctuation does not produce a p-value of significance below p=.05 for any of the mouse groups. The relationship between the genders and exploratory behavior produced the lowest p-value, and the treatment of ET as opposed to no treatment produced a p-value signifying almost no correlation. Further investigation of ET supplementation in ASD behaviors relating to exploration and movement

should be done to determine any correlation between possible therapeutic treatment and interest in adventurous behaviors.

3-chamber maze

Of the total 300 seconds given to the mice to explore the maze and interact with the stranger mouse, all of the groups spent about half of the time in the same chamber as the stranger mouse. In the 3-chamber maze experiment, the control female mice spent the greatest time near the stranger mouse while the control males spent the least. The second differential for the female group means is 11.903 seconds, and the male group means difference is 1.101 seconds. Insignificant p-values were calculated for the relationship between gender, treatment type, and interaction between the two as they relate to time spent in the same chamber as the stranger mouse. Gender differences revealed the smallest p-value indicating a need for continued study of BTBR behavioral variation between male and female mice.

Modified 3-chamber maze

In the modified 3-chamber experiment, time spent near a familiar and stranger mouse was recorded to evaluate social preferences of the BTBR mice. The male control mice spent an average of 21.520 seconds more in the same chamber as the familiar mouse rather than the stranger mouse. The control males also spent the longest period of time in the familiar mouse's chamber and the least amount of time in the stranger mouse's chamber than any other group. The female control mice exhibited the opposite behavior. They spent the most time in the same chamber as the stranger mouse and the least amount of time in the chamber that housed the familiar mouse. The differences in the means of the female mice's preferences in social company revealed a 31.073 second differential between interest in the familiar versus stranger mouse. Meanwhile, the male and female treatment groups spent a similar amount of time in the familiar

and stranger mouse chambers. The only significant p-value calculated from the modified 3-chamber data was derived from the relationship between gender and interest in the familiar mouse. The p-value of p=0.0484 signifies a positive correlation between these variables, and a notably low p-value of p=0.0556 was computed from the interaction between gender, treatment and time spent in the same chamber as the familiar mouse. More research should be conducted regarding ET's influence on gender preferences to strengthen these findings.

Open field test

The open field test provided visual and numerical results from the collected data. The heat maps display evidence that the female mice were more active than the male mice, and the treatment females exhibited the highest level of exploration out of all of the mouse groups. This finding also supports the results from the elevated plus maze. Although the male mice spent much less time in motion, the treatment male mice spent a similar amount of time as the control female mice in regard to activity and movement patterns. All of the mice showed a preference for movement along the perimeter of the open field. The total distance traveled was recorded and a line graph was generated using the Fusion software. The prepared graph demonstrates the significant difference in activity seen between the treatment and control groups (*Fusion*, 2023). A p-value of less than p=0.05 was used to determine the significance between all of these relationships.

Conclusion

ET has been shown to improve brain function in patients with a variety of neurological disorders (Ishimoto & Kato, 2021). Its antioxidant properties combat the toxic effects of ROS abundance, and because ASD is characterized by elevated levels of oxidative stress, this study evaluated potential benefits of this amino acid derivative on autism phenotypes (Bonomini et al.,

2022). Through a variety of behavioral analyses, the research uncovered some interesting findings. Most notably, variation among gender preferences in social settings and an increase in locomotor activity were shown to have a positive correlation. Therefore, further research regarding gender differences in BTBR mice should be done to determine the extent of this relationship. The use of ET as a supplement therapy for ASD did not show significant improvements in the tested behaviors. Future studies should consider altering the concentration of ET supplements provided to mice to determine if ET consumption levels play a role in acquired neurological benefits.

Acknowledgments

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