International Journal of
Life Sciences
International Peer Reviewed Open Access Refereed Journal

Int. J. of Life Sciences, 2023; 11 (3): 249-256
ISSN:2320-7817(p) | 2320-964X(o)
https://doi.org/10.5281/zenodo.8397203

Research Article Open Access

The Effect of *Paeonia lactiflora* root on the Memory of *Drosophila melanogaster* with Autism Spectrum Disorder

Pandrate Prajith

Academies of Loudoun, Leesburg, USA
Email: prajith.s.pandrate@gmail.com

Manuscript details:
Received: 15.08.2023
Accepted: 26.09.2023
Published: 30.09.2023

Cite this article as:
Pandrate Prajith (2023) The Effect of *Paeonia lactiflora* root on the Memory of *Drosophila melanogaster* with Autism Spectrum Disorder, *Int. J. of Life Sciences*, 11 (3): 249-256.
https://doi.org/10.5281/zenodo.8397203

Available online on http://www.ijlsci.in
ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

ABSTRACT

Autism spectrum disorder (ASD) is a prevalent problem within the United States, with approximately 1 in 44 children diagnosed. The frontal cortex of ASD brains have 67% more neurons compared with control brains. Increased neuron accumulation negatively impacts memory, as the frontal cortex plays a critical role in tasks containing memory and cannot be overstimulated. Previous research found that *Paeonia lactiflora* root lowered neurotrophin levels (essential proteins for neuron survival) in the frontal cortex. Due to the connection between ASD and neuron levels, the purpose and novelty of this study is to determine the effect of *P. lactiflora* root on the memory retention of Drosophila melanogaster by using an Aversive Phototaxic Suppression Assay. The results of the study show a statistically significant difference (P-value = 0.0217) between ASD flies without the root in diet and ASD flies with the root in diet, indicating an increase in memory retention for the flies that were administered the extract. These findings support the original hypothesis that *P. lactiflora* root would increase memory retention in Drosophila with ASD. By better understanding the effect of *P. lactiflora* root on the memory retention of ASD, a treatment for this chronic disorder can be found.

Keywords: Autism Spectrum Disorder, behavioral neuroscience, *Paeonia lactiflora* root, *Drosophila melanogaster*, memory retention

INTRODUCTION

Autism Spectrum Disorder

Autism Spectrum Disorder (ASD) is a complex neurodevelopmental condition characterized by persistent challenges in social interaction, communication difficulties, and repetitive patterns of behavior. In recent years, there has been a significant rise in the prevalence of ASD, leading to increased concern about its impact on individuals, families, and society as a whole. The most recent data from the Centers for Disease Control and Prevention (CDC) indicate that approximately 1 in 54 children in the United States have ASD (Centers

for Disease Control and Prevention, 2022). Additionally, ASD poses substantial costs on the healthcare system and imposes considerable financial burdens on affected individuals and their families.

A study published in JAMA Pediatrics estimated the average lifetime cost of caring for an individual with ASD to be approximately $1.4 million in the United States (Lavelle et al., 2019). These costs include direct medical expenses, special education, therapies, caregiver time, and productivity losses. The study further highlights the disproportionate financial burden on families, as households with a member diagnosed with ASD experience significantly higher healthcare expenditures and reduced work productivity. Despite the increasing prevalence and substantial costs associated with ASD, the underlying causes and mechanisms of the disorder remain poorly understood. This lack of understanding poses significant challenges in developing effective treatments or cures for individuals with ASD.

NLG4 Knockdown Model

The NLG4 gene, also known as Neuroligin-4, is a gene that has been extensively studied in the context of ASD due to its involvement in synaptic function and neuronal connectivity. Research has shown that NLG4 plays a crucial role in mediating excitatory and inhibitory synaptic transmission, as well as in the formation and maintenance of neural circuits involved in social behaviors. The NLG4 knockdown model in *Drosophila* serves as a valid method for modeling ASD, as it successfully mirrors essential behavioral and molecular characteristics linked to the disorder. Research utilizing *Drosophila* with reduced NLG4 expression through RNA interference (RNAi) displayed distinct changes in social behavior, impaired courtship behavior, and deficits in learning and memory tasks (Banerjee et al., 2016). These observed behavioral abnormalities closely resemble the core features observed in individuals with ASD, supporting the model's capability to capture relevant aspects of ASD pathology. Another study used the NLG4 knockdown model in *Drosophila* to investigate the molecular mechanisms underlying ASD (Sulkowski et al., 2016). By reducing NLG4 expression, the researchers identified disruptions in synapse formation and function, resulting in altered neural connectivity patterns. The NLG4 knockdown model in *Drosophila* provides valuable insights into the role of NLG4 in ASD-related behaviors and synaptic functioning, thus establishing it as a useful tool for modeling Autism Spectrum Disorder.

ASD & Memory

ASD has been linked to deficiencies in working memory, which refers to the capacity to temporarily store and manipulate information for the completion of mental tasks (Habib et al., 2019). Working memory plays a vital role in multiple cognitive processes, including memory, problem-solving, decision-making, and learning. These deficits result in difficulties in retaining and manipulating information, which in turn can hinder memory retention. Furthermore, research has revealed structural differences in the frontal cortex of individuals with ASD. In one study, it was reported that ASD brains exhibit a 67% increase in the number of neurons in the frontal cortex compared to control brains (Courchesne et al., 2011). This excess of neurons in the frontal cortex can have implications for memory processes, as the region plays a critical role in tasks involving memory and attention. The increased neuronal density in the frontal cortex leads to overstimulation and disrupted neural signaling, impacting memory functions in individuals with ASD. Taken together, these findings suggest that ASD-associated deficits in working memory can be linked to alterations in the frontal cortex.

***Paeonia lactiflora* root**

Previous research has shown that *P. lactiflora* root lowers neurotrophin levels in the frontal cortex (Lan et al., 2013). Neurotrophins are proteins that promote the growth of neurons in the brain. However, the *P. lactiflora* root, by decreasing neurotrophin levels, may offer a potential treatment for memory deficiencies associated with ASD. By regulating the excessive neuronal growth in the frontal cortex, the root could potentially help restore proper memory functioning.

Aversive Phototaxic Suppression (APS) Assay

An Aversive Phototaxic suppression (APS) assay is utilized to measure memory retention in *Drosophila*. As the assay does not require special equipment or a large amount of space, it can easily be stored and used repeatedly. APS is simple yet inexpensive and can be used to assess how genetic manipulations alter short-term memory. To start, *Drosophila* are trained in a light vial with quinine solution (negative reinforcer). As the flies are instinctively phototaxic and prefer the lighted vial more frequently, the training allows them to associate the light vial with the negative reinforcer,

which they do not prefer. After being in the light vial for a minute, the flies are then shifted to a dark vial that does not contain the quinine solution for 30 seconds. This process of shifting the flies from light to dark vials is repeated numerous times until the training phase is concluded. To test memory, the light and dark vials are taped together with flies inside, and the number of times that the flies go to the dark chamber is recorded. This use of the APS assay has continued to be justified and repeated through various modes of research, including evaluation of memory (Seugnet et al., 2009).

NLG4 Knockdown *Drosophila* Model

The *Drosophila* model for ASD can be generated by reducing the expression of the NLG4 gene. The model can be achieved using the GAL4/UAS system, a technique commonly used for gene knockdown in *Drosophila*. The knockdown is accomplished by crossing a UAS line with a GAL4 driver line. The GAL4 driver line contains a GAL4 transcription factor that facilitates mRNA production specifically in the targeted tissue where the gene expression is to be altered. On the other hand, the UAS responder line carries an upstream activation sequence (UAS) integrated into the gene of interest. When these lines are crossed, the GAL4 driver activates the expression of the gene with the UAS marker in the GAL4-promoted tissue, resulting in the desired gene expression patterns in the offspring. To achieve under expression of the gene, an RNA interference (RNAi) construct is introduced into the UAS responder line. This RNAi construct hampers protein translation by binding to the mRNA of the target gene, interfering with its ability to produce specific proteins.

Purpose and Variables

The proposed research will determine the effect of *P. lactiflora* root on the memory retention of D. melanogaster with ASD by comparing with an ASD model of *D. melanogaster* with a standard diet through the use of an Aversive Phototaxic Suppression Assay to measure memory. ASD will suppress memory through its high levels of neurons in the prefrontal cortex that impacts function of memory and attention tasks. The ASD *D. melanogaster* model will be made by crossing a UAS-RNAi line and Gal4 line, which will under-express the NLG4 gene (Li et al., 2013). It is hypothesized that *P. lactiflora* root will increase memory retention in *D. melanogaster* with Autism Spectrum Disorder (ASD). Previous studies have shown that *P. lactiflora* root lowers neurotrophin levels (which promote excess growth of neurons) in the frontal cortex (Lan et al., 2013). ASD causes a high volume of neurons in the prefrontal cortex, reaching up to 67% higher levels than non-ASD brains. Due to this, memory retention is negatively impacted so a decrease in volume of neurons caused by the *P. lactiflora* root will treat memory deficiencies.

The independent variables in the experiment are the presence of *P.lactiflora* in the diet of flies, and the presence of Autism Spectrum Disorder (ASD) in the *D. melanogaster*. The flies will have a diet consisting of a control in which base fly food will be used, and an experimental diet in which *P. lactiflora* root extract will be added at a 0.021 M concentration. ASD in the *D. melanogaster* will be attained by crossing a NLG4-Gal4 line and UAS-NLG4-RNAi, resulting in offspring with an under expression in NLG4. The offspring would be the experimental group while just the NLG4-Gal4 line would be the control group. The dependent variable is the memory retention of the *D. melanogaster*, which will be tested through the use of an Aversive Phototaxic Suppression assay (APS). Through the assay, memory retention will be determined by using a pass rate percentage for the flies. A fly will constitute a pass based on it being able to remember a relationship between light and negative stimulus.

MATERIALS AND METHODS

Fly Food Preparation

The following dry ingredients were measured with the use of an electronic balance and weight boat: 3.90 grams of soy flour, 6.75 grams of yeast, 28.50 grams of yellow cornmeal, 2.25 grams of agar. Then the following wet ingredients were measured out with a graduated cylinder: 390 mL of distilled water, 30 mL light of corn syrup. All ingredients were then thoroughly mixed in a 500mL beaker using a stirring rod. Once done with mixing, the beaker of contents was placed into the microwave for 30 seconds intervals, while stirring after each completed interval. The intervals were continued until the mixture was seen boiling (indicated by bubbles on the surface). Whenever taking the beaker in and out of the microwave, hot hands and goggles were worn. After allowing the contents to cool, 1.88mL of propionic acid was added to the beaker using a 1000 µl micropipette. The contents of the beaker were then poured into

empty vials using a funnel. Each vial had 10mL of Drosophila food, and when done pouring, a cheesecloth was placed over the vials to prevent contamination. After the contents cooled to room temperature, the vials were plugged with flugs. To prepare the treatment food, 3.93 grams of *P. lactiflora* extract (Amazon #B07FLD4K6M) was added to the base food preparation at a 0.021 M concentration.

Groups

The experiment will contain 3 control groups. The first control group is the negative control, and will consist of Gal4-Nlg4 line (Non-ASD) flies along with a standard diet. This group will establish a baseline that can be utilized in analyzing the effect of the *P. lactiflora* root when comparing between the control groups. The expected outcome for the first group is that the flies will have better memory retention compared to the flies with ASD. The second control group, the toxicity control, will contain Gal4-Nlg4 line (Non-ASD) flies with an addition of a *P. lactiflora* root extract in the diet. This group will signify if there is an impact on memory retention for flies without ASD. The expected outcome for the second group is that there will be better or no change in memory retention compared to group 1, as a lower retention would indicate a likely error in the experimental design. The third control group will contain ASD line (Nlg4 knockdown) flies with a standard diet. The purpose of this group is to compare the experimental group against. It is expected that this group will have lower memory retention compared to groups 1 and 2 since ASD causes a high volume of neurons in the prefrontal cortex which negatively impacts memory. The experimental group will contain ASD line (Nlg4 knockdown) flies with the *P. lactiflora* root diet.

Crossing

To begin crossing, the knob on the CO_2 tank was loosened until a light stream of air was felt on the back of a hand from the CO_2 gun. The vial was then turned upside down and the flug on the vial was loosened until barely on. The needle was slid flush with the vial and then the CO_2 gun was activated until all the flies stopped moving. The needle was then removed from the vial and the flies were poured onto the CO_2 pad under a microscope (Labomed Luxeo 6z). Using proper technique, male and female flies were separated while identifying the virgins. Male flies are identified by a round and dark tip, are generally smaller than female flies, have sex combs and claspers, have blunt flat genitalia, and have black rounded abdomen. Female flies have a light and pointed tip, are generally larger than male flies, have no sex combs or claspers, have pointy genitalia, and have striped rear-abdomen. Any flies with folded wings signals immediately that they are virgins, and female virgins have a white or black spot on the stomach or back. Once done with identification, the CO_2 sorter was turned off and 5-6 virgin female UAS-nlg4-RNAi Drosophila (Bloomington Stock #58119) flies and 4 male Gal4-Nlg4 Drosophila (Bloomington Stock #23608) flies were added to a vial. This method was performed twice, with one vial containing a standard diet and one containing an experimental diet. After 3-4 days, the adults were tapped out and the newly laid eggs were left. This process was repeated every 3-4 days or whenever eggs were laid. Flies were tapped into a new vial twice, and then the original vial was discarded by freezing at -20° C for around 1 hour, adjusting as needed based on if all flies were dead. Then the flies were placed 70% ethanol and ensured they were dead before disposing of them in the trash.

APS Assay

To set up an APS Assay in order to measure memory of the flies, two vials were obtained and one was covered completely with foil so that no light would pass through. The second vial was then taped with LED strips with equal spacing.

Cold Sorting

After the experimental flies were tapped into a new vial, the cold sorter was plugged in and turned on. Then, the cold sorter was set to 2^{o} C by changing the set digit using the down arrow and selecting the digit by pressing the left arrow key once and confirming by pressing the "SET" button. The fly vials were then submerged into an ice bath for 3-4 minutes, while being checked on throughout the process to ensure that they were asleep before taking out. After taking the vials out, a weigh paper was placed onto the cold sorter and then the flies were slowly tapped onto the paper. Using a magnifying glass and feather, the flies were sorted by moving them around gently while looking for indicators of their sex. The male flies were disposed of while placing female flies into a vial.

Training

To train the flies in the APS assay, a 0.1M stock solution of Quinine Hydrochloride was first created using 0.74g Quinine Hydrochloride and 20 mL water.

Then, 1 mL of the 0.1M quinine solution was placed on a flug by using a pipette, and the light vial's flug was replaced with the new flug (solution side going in first). The previously sorted female flies were tapped into the light vial and the led strips were turned on while simultaneously setting a timer for 60 seconds. After the 60 second timer, the LED light was turned off and the flies were tapped into the dark vial while simultaneously setting a timer for 30 seconds. The light and dark vials were rotated between with their respective timings until 5 training sessions were complete.

Testing

Flies were tested around 16 days after being laid, and cold sorting was done 2 days prior to testing. For data collection, the flug was replaced with a newly applied quinine solution flug between each trial. The flies were tapped so they were close to the flug, and the vial was laid flat horizontally. Before beginning, the number of flies in the vial were recorded. Then both flugs were carefully removed and the openings of each vial were attached together with tape and the assay was placed horizontally. Once ready, the light was turned on and simultaneously a timer for 60 seconds was set. After 60 seconds the number of flies in the light vial and dark vial (count one and subtract) were recorded. Percentages were created for the failed flies in proportion to the total flies tested in each trial. This process was repeated for 10 trials for each of the four groups.

RESULTS

5 trials of APS Assay testing were conducted for the four groups in the study. After creating a pass rate for each trial, the data was recorded as shown below.

Table 1: APS Assay Results for Negative Control (No ASD + Standard Diet)

Trial	# Flies Tested	# Flies Passed	Pass Rate %
1	10	7	70
2	10	8	80
3	9	6	67
4	9	8	89
5	9	7	78
Average	9.4	7.2	76.8

Table 2: APS Assay Results for Toxicity Control (No ASD + P. lactiflora Diet)

Trial	# Flies Tested	# Flies Passed	Pass Rate %
1	10	7	70
2	10	9	90
3	10	8	80
4	10	7	70
5	10	8	80
Average	10	7.8	78

Table 3: APS Assay Results for Positive Control (ASD + Standard Diet)

Trial	# Flies Tested	# Flies Passed	Pass Rate %
1	10	5	50
2	10	5	50
3	10	6	60
4	10	4	40
5	10	6	60
Average	10	5.2	52

Table 4: APS Assay Results for Experimental Group (ASD + P. lactiflora Diet)

Trial	# Flies Tested	# Flies Passed	Pass Rate %
1	10	6	60
2	10	6	60
3	10	7	70
4	10	7	70
5	10	6	60
Average	10	6.4	64

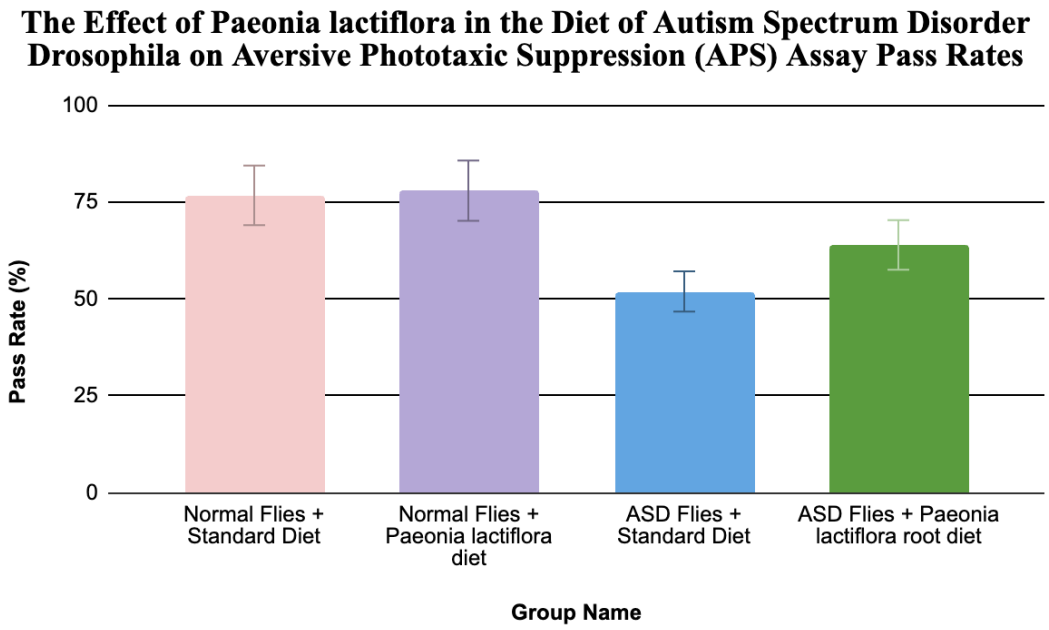

Figure 1: Average APS Pass Rates for All Groups

Table 5: Mann-Whitney U-Test p-values

Group Comparisons	p-value[1]
Control Flies vs Control Flies + *P.lactiflora* diet	0.3342
Control Flies with Standard Diet vs ASD Flies with Standard Diet	**0.0058***
ASD Flies vs ASD Flies with *P. lactiflora* diet	**0.0217***

[1]Starred p-values are considered statistically significant (p-value < α = 0.05)

The analysis was completed utilizing the InStat software. The data from each of the four groups in the experiment were taken and inserted into four corresponding columns, while being labeled at the top. The groups were as follows: Group 1 - Control, Group 2 - Wild-Type + *P. lactiflora* diet, Group 3 - ASD + Standard diet, and Group 4 - ASD + *P. lactiflora* diet. When prompted, the non-parametric Mann-Whitney U Test was chosen to compare between two groups at a time. The Mann-Whitney U test was performed with an alpha value of 0.05 repeatedly on separate groups of two in order to compare the medians between the groups. Specifically, the comparisons were made between Groups 1 & 2, Groups 1 & 3, and Groups 3 & 4. Based on the results of the test, conclusions were drawn about the data and the presence of any statistical significance was determined.

DISCUSSION

The purpose of the experiment was to determine the effect of *P. lactiflora* root on the memory retention of *Drosophila* with Autism Spectrum Disorder. It was hypothesized that *P. lactiflora* root would increase memory retention in *Drosophila* with Autism Spectrum Disorder (ASD) because the root lowers neurotrophin levels (which promote excess growth of neurons) in the frontal cortex (Lan et al., 2013), which would decrease the excess volume of neurons in ASD. In order to test the hypothesis, an NLG4 knockdown ASD model of *Drosophila* was utilized with the effects of the root on memory being tested through the use of an Aversive Phototaxic Suppression assay.

The one tailed Mann-Whitney U test that compared the positive control (ASD flies without the root in diet) and the experimental group (ASD flies with the root in diet), yielded a p-value of 0.0217, demonstrating a statistically significant difference between the groups and indicating that there was an increase in memory retention for the flies that were administered the extract. These findings support the original hypothesis that the *P. lactiflora* root would increase memory retention in *Drosophila* with ASD.

Furthermore, *P. lactiflora* root showed no impact on the memory retention of control flies. The Mann-Whitney U test that compared the negative control (control flies without the root in diet) and the toxicity control (control flies with the root in diet) yielded a p-value of 0.3342, demonstrating that there was no statistically significant difference in the memory retention between both groups. The results of the comparison show that the concentration of *P. lactiflora* root of 0.021 M is not toxic to *Drosophila*.

Lastly, there was a difference in memory retention between the negative control (control flies with a standard diet) and the positive control (ASD flies with a standard diet). The Mann-Whitney U test between these two groups resulted in a p-value of 0.0058. These results verify that there was a successful ASD cross, and that the flies demonstrated impaired memory retention. These results are further supported by previous research conducted by Banerjee et al. in 2016, who found that a neurologin knockdown model of ASD in *Drosophila* resulted in impaired learning and memory retention.

The research conducted in this study holds importance in the field of ASD and memory retention. Memory deficits are a common feature observed in individuals with ASD, impacting their daily functioning and quality of life. Addressing these cognitive impairments is crucial for developing effective treatments or cures for

individuals with ASD. By exploring the effects of *P. lactiflora* root on memory retention in a *Drosophila* model of ASD, this research offers a novel and valuable approach to potentially ameliorate memory deficits associated with the disorder. The positive results and promising findings highlight the potential therapeutic benefits of *P. lactiflora* root and shed light on a previously unexplored natural intervention for memory enhancement in individuals with ASD.

Future research could explore the specific compounds or active ingredients present in *P. lactiflora* root that are responsible for its memory-enhancing effects. Isolating and studying these components could provide valuable insights into their molecular interactions and their potential as therapeutic agents. Furthermore, investigating the long-term effects of *P. lactiflora* root administration on memory retention in flies with ASD could provide crucial information regarding the sustainability of the observed improvements. This would be particularly relevant in translating the findings to potential human applications.

CONCLUSION

Based on the results from this experiment, it can be concluded that *P. lactiflora* root has a positive effect on the memory retention of flies with ASD. The conclusion is supported by the Mann-Whitney U comparison tests shown in Table 5, as the comparison between the positive control (ASD flies without the root in diet) and the experimental group (ASD flies with the root in diet) indicates that there was an increase in memory retention for the flies that were administered the extract. The results support the original hypothesis and scope of the project, as the *P. lactiflora* root is observed to provide an effective treatment in increasing memory retention in ASD flies. This research is essential for developing future therapeutics, as *P. lactiflora* root is a natural resource that can be obtained at a relatively low cost compared to synthetic pharmaceuticals. Therefore, accessible and affordable treatment options for individuals with ASD could possibly be created.

Acknowledgements

I would like to thank my research mentor, Dr. Jessica Eliason, for her supervision, guidance, and valuable feedback towards my project. I would also like to thank the Academies of Loudoun for providing the laboratory space and equipment needed to conduct my project.

Conflict of Interest: None of the authors have any conflicts of interest to disclose.

REFERENCES

Banerjee S, Riordan M & Bhat MA (2016) Genetic aspects of autism spectrum disorders: insights from animal models. *Frontiers in Cellular Neuroscience, 8.* https://doi.org/10.3389/fncel.2016.00058

Centers for Disease Control and Prevention. (2022). Data and Statistics on Autism Spectrum Disorder. https://www.cdc.gov/ncbddd/autism/data.html.

Courchesne E, Mouton PR, Calhoun ME, Semendeferi K, Ahrens-Barbeau C, Hallet MJ, Barnes CC & Pierce K (2011) Neuron number and size in prefrontal cortex of children with autism. *JAMA*, 306(18), 2001–2010. https://doi.org/10.1001/jama.2011.1638.

Habib A, Harris L, Pollick F & Melville C (2019) A meta-analysis of working memory in individuals with autism spectrum disorders. *PloS one*, 14(4), e0216198. https://doi.org/10.1371/journal.pone.0216198 .

Lan Z, Chen L, Fu Q, Ji W, Wang S, Liang Z, Qu R, Kong L, & Ma S (2013) Paeoniflorin attenuates amyloid-beta peptide-induced neurotoxicity by ameliorating oxidative stress and regulating the NGF-mediated signaling in rats. *Brain research*, 1498, 9–19. https://doi.org/10.1016/j.brainres.2012.12.040.

Lavelle TA, Weinstein MC, Newhouse JP, Munir K, Kuhlthau K & Prosser LA (2019) Economic Burden of Childhood Autism Spectrum Disorders. *Pediatrics*, *133(3),* e520–e529. https://doi.org/10.1542/peds.2019-0763.

Li Y, Zhou Z, Zhang X, Tong H, Li P, Zhang ZC, Jia Z, Xie W & Han J (2013) Drosophila neuroligin 4 regulates sleep through modulating GABA transmission. *The Journal of neuroscience : The Official Journal of the Society for Neuroscience*, 33(39), 15545–15554. https://doi.org/10.1523/JNEUROSCI.0819-13.2013.

Seugnet L, Suzuki Y, Stidd R & Shaw PJ (2009) Aversive phototaxic suppression: evaluation of a short-term memory assay in *Drosophila melanogaster*. *Genes, brain, and behavior*, 8(4), 377–389. ttps://doi.org/10.1111/j.1601-183X.2009.00483

Sulkowski MJ, Han TH, Ott C, Wang Q, Verheyen EM, Lippincott-Schwartz J & Serpe M (2016) A Novel, Noncanonical BMP Pathway Modulates Synapse Maturation at the *Drosophila* Neuromuscular Junction. *PLOS Genetics*, 12(1), e1005810. https://doi.org/10.1371/journal.pgen.1005810.