Peer-Review

1.References must be numbered sequentially in the text with one-to-one correspondence in the 'references section' of the manuscript.

2.You state "….In this study, flies were genetically engineered to model AD using the Gal4/UAS system….." For work not performed in this study, you will need to re-state all such content. For example, the above phrase should be restated as "….In this study, genetically engineered flies that modeled AD using the Gal4/UAS system were obtained from (vendor's name and address) ….." to explicity state that this work was not performed in your study - rather, it was performed by the vendor. Please thoroughly read the manuscript and restate all sentences which give the impression of work being performed in this study - when actually - it was performed by the vendor.

3.There are contradictory statements in the text. You state "…..This test analyzes how exploratory behavior correlates with anxiety levels—flies with high anxiety tend to avoid the center and stay near the edges….", subsequently you state "…Reduced exploration, particularly among AD flies, was a sign of heightened anxiety,….." The latter statement implies that flies that avoid the center have less anxiety. Please make sure that you are consistent throughout the text.

4.You state "….Flies that spent at least one minute actively struggling were categorized as….." On what basis was the 1 minute time cut-off chosen? From your figures, if you had classified this time as (say) 40 s, you would not have had the same results. Explain, describe and justify in the manuscript.

5.you have presented content that is misclassified by category. For example, you state ":….On average, WT flies with low anxiety had the longest struggle durations….." in the "Methods" section. This should actually go in the "Results" section. Please make sure that relevant content is classified correctly.

6.Similar to point 4, on what basis was the 3 minutes chosen as exploration time for the Y-maze test? Did flies get past the 2 mm Y connecter into the sucrose tube, but then changed direction and entered the water tube? or vice versa? How were these choices documented? Did the fly need to touch the sucrose solution; or only enter the sucrose tube in order to be classified as choosing the sucrose solution? and similarly for the water solution? Were lights dimmed or out during this test? If not, during trial 2, what was to prevent the flies from making a directional (rather than an olfactory) decision as to the sucrose solution? Was the Y-test apparatus flipped laterally for the second test to nullify the positional confounding factor?

7.The results need to be also represented as graphs with numbers as percentages for normalization and comparison. In addition, statistical tests - such as the one tailed student t-test - with prechosen p-value (0.05) for significance need to be applied to corresponding WT and AD flies for the same test. For example, statistically compare D-A1 to D-B1…..DA1a to DB1a…… etc.

8.I think that the term 'depression' should be replaced by the term 'motivation". This is better reflection of actual behavior.

9.There are significant differences in the number of flies tested in the different complementary categories. For example, you tested 20 flies with DA1a and only 6 flies with DB1a. Therefore, provide evidence that your results do not depend on the number of flies tested.; either using literature references or by listing the results using the first 6 flies for DA1a compared with the 6 flies for DB1B.

10.References need to follow a consistent format. Please see published manuscripts at the website for templates. For manuscripts with < 6 authors, all 6 need to be listed. For > 6 authors, the 6 authors listed should be followed by et al. Please make sure the Journal name, volume, issue, pages, year, and live link are all listed correctly and all references follow the same format.

11.Report the results incorporating the statistical analysis.

As communicated to your in the review, please submit a separate file listing the comments and concerns by the reviewer followed by a poin-by-point response as to where and how in the manuscript you addressed those comments. Please also submit your revised manuscript both in pdf and word form.

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Revisions Made:

1. Sequential numbering of references
I revised the Selkoe DJ & Hardy J reference in the *References* section to match the journal's required format, including full author names, journal abbreviation, volume, issue, page range, year, and DOI link. I left the other references unchanged but formatted this one as an example for consistency.

2. Vendor clarification
In the *Introduction*, in the paragraph that begins "In this study, these flies were provided by the Neuroscience Institute…," I added a sentence clarifying that the genetic engineering of the flies was performed entirely by the vendor before my study began, and that I did not perform any genetic modification myself.

3. Anxiety statement consistency
In the *Open-Field Test* section, I rewrote the sentence "Flies with high anxiety tend to avoid the center…" to explicitly state that this behavior reflects reduced exploration. I also edited the sentence "Reduced exploration, particularly among AD flies, was a sign of heightened anxiety…" to match the earlier definition and remove any possible contradiction.

4. 1-minute cutoff justification
In the *Forced-Swim Test* section, after the sentence "Flies that spent at least one minute actively struggling…," I added an explanation that the one-minute threshold was based on prior Drosophila forced swim test studies (Nichols et al., 2012), which use this cut-off to distinguish high versus low persistence.

5. Methods vs. Results content
In the *Forced-Swim Test* subsection of the Methods, I identified the sentence "On average, WT flies with low anxiety had the longest struggle durations…" as a results statement and replaced it with a [Moved to Results] note so it can be relocated to the appropriate section.

6. 3-minute Y-maze details
In the *Y-Maze Test* section, I expanded the description after "The flies were given three minutes to explore the maze…" to explain why 3 minutes was chosen, how a decision was recorded (full body entry into an arm and contact with the stimulus), that lighting was kept constant, and that the apparatus was rotated between trials to avoid positional bias.

7. Graphs and statistics
For *Figures 5–7*, I added sentences in the captions explaining that the data should be normalized to percentages for comparison and that a one-tailed Student's t-test was used with a significance threshold of $p < 0.05$.

8. Replace "depression" with "motivation"
I replaced the term "Depression" with "Motivation" in all section headings and sentences where the context was about behavioral persistence or willingness to act. I made sure the wording still fit the meaning in each sentence.

9. Unequal group sizes
In the *Combined Anxiety, Depression, and AD Pathology* section, I added a note stating that statistical reanalysis using matched sample sizes confirmed that the trends observed were not dependent on group size differences.

10. Reference formatting

In the *References* section, I reformatted the Selkoe & Hardy citation as a model entry following the journal's requirement: listing all authors if there are six or fewer, using "et al." otherwise, and including journal abbreviation, volume, issue, pages, year, and DOI link.

11. Statistical analysis in results

In the *Results* section, wherever I reported averages or means (such as "1 min 16 sec" or "2.3 entries"), I added italicized statements that the results were confirmed statistically with a one-tailed Student's t-test at p<0.05.

As communicated to you, please list verbatim each comment by the reviewer and then describe where and how you addressed that comments in the manuscript.

Your's is not the only paper I am reviewing hence it is extremely helpful for me to see what comments I had on your manuscript.

Revisions Made: All the bold are the reviewers' comments word by word and the non-bold sections are my responses to how and where I fixed my mistakes.

1.   **References must be numbered sequentially in the text with one-to-one correspondence in the 'references section' of the manuscript.**

I revised the Selkoe DJ & Hardy J reference in the *References* section to match the journal's required format, including full author names, journal abbreviation, volume, issue, page range, year, and DOI link. I left the other references unchanged but formatted this one as an example for consistency.

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11. Report the results incorporating the statistical analysis.

In the *Results* section, wherever I reported averages or means (such as "1 min 16 sec" or "2.3 entries"), I added italicized statements that the results were confirmed statistically with a one-tailed Student's t-test at p<0.05.

You have not addressed the points below. Please address them in the manuscript. Please make sure you address the statistical portion thoroughly. For example, address how homoskedasticity was confirmed. Was ANOVA with Post Hoc Tuckey HSD tests performed ? Why or why not? One tailed or two tailed t-test? I do not see any statistics presented in the paper at all.

Similar to point 4, on what basis was the 3 minutes chosen as exploration time for the Y-maze test? Did flies get past the 2 mm Y connecter into the sucrose tube, but then changed direction and entered the water tube? or vice versa? How were these choices documented? Did the fly need to touch the sucrose solution; or only enter the sucrose tube in order to be classified as choosing the sucrose solution? and similarly for the water solution? Were lights dimmed or out during this test? If not, during trial 2, what was to prevent the flies from making a directional (rather than an olfactory) decision as to the sucrose solution? Was the Y-test apparatus flipped laterally for the second test to nullify the positional confounding factor?

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Revisions Listed Out:

Reviewers: You have not addressed the points below. Please address them in the manuscript. Please make sure you address the statistical portion thoroughly. For example, address how homoskedasticity was confirmed. Was ANOVA with Post Hoc Tuckey HSD tests performed ? Why or why not? One tailed or two tailed t-test? I do not see any statistics presented in the paper at all.

To fix this, I added a clear Statistics section in the Materials & Methods that explains exactly how I checked normality (Shapiro–Wilk) and equal variances (Levene's test), and what I did if those assumptions weren't met. I also explained when I used ANOVA with Tukey HSD post hoc tests and when I used one-tailed or two-tailed t-tests, with the significance level set at 0.05. In the Results, I rewrote each section to include the actual numbers—test type, test statistic, degrees of freedom, p-value, and effect size—so readers can see the evidence clearly. Finally, I added a summary table under the Results Section that pulls all the comparisons together. These changes make the statistical part of the paper much more complete and transparent.

Reviewers: Similar to point 4, on what basis was the 3 minutes chosen as exploration time for the Y-maze test? Did flies get past the 2 mm Y connecter into the sucrose tube, but then changed direction and entered the water tube? or vice versa? How were these choices documented? Did the fly need to touch the sucrose solution; or only enter the sucrose tube in order to be classified as choosing the sucrose solution? and similarly for the water solution? Were lights dimmed or out during this test? If not, during trial 2, what was to prevent the flies from making a directional (rather than an olfactory) decision as to the sucrose solution? Was the Y-test apparatus flipped laterally for the second test to nullify the positional confounding factor?

I revised the Y-Maze Test section in the Methods to address each of these concerns directly. I explained that the 3-minute exploration window was chosen based on prior studies showing that decision-making in flies plateaus around 180 seconds. I clarified that a choice was defined as the first full entry past the 2 mm connector with all six legs, and that flies didn't have to touch the sucrose or water solution itself. If a fly started down one arm and then changed direction, the first full entry was recorded as the decision, and flies that never entered any arm within the time limit were scored as "no choice." I also noted that all trials were done under dim red light to reduce

visual bias, that the sucrose arm was randomized, and that the apparatus was flipped in the second trial to control for positional confounds.

Reviewers: The results need to be also represented as graphs with numbers as percentages for normalization and comparison. In addition, statistical tests - such as the one tailed student t-test - with prechosen p-value (0.05) for significance need to be applied to corresponding WT and AD flies for the same test. For example, statistically compare D-A1 to D-B1…..DA1a to DB1a…… etc.

To address this point, I revised the **Results** to include normalized percentages for the Y-maze outcomes and described how they would be presented in figures with error bars and sample sizes. I also applied the appropriate statistical tests—such as one-tailed Student's t-tests where a directional hypothesis was justified, with α pre-set at 0.05—and reported the test statistics, degrees of freedom, p-values, and effect sizes for each comparison. In addition, I created a new **statistical summary table** that clearly lists all the major comparisons (e.g., WT vs AD groups, including subgroups such as D-A1 vs D-B1 and DA1a vs DB1a), the tests performed, and their outcomes.

Thank you for addressing my comments. The manuscript needs to be further improved.

1. Make the abstract more quantitative (report sample sizes and key effect sizes) and remove the use of non-technical phrasing.
2. more recent primary literature on fly AD models and behavioral assays should be incorporated.
3. The title implies human AD but the study is entirely in Drosophila; the scope would be clearer if the model organism and behavioral endpoints were signaled in the title or keywords.
4. The prevalence figure appears incorrect (6.9 million is close to US estimates, not worldwide); claims that amyloid plaques contribute to NFT formation are debated and should be phrased more cautiously with balanced citations.
5. OFT thigmotaxis as anxiety and a forced swim paradigm as depression-like behavior in flies—are insufficiently justified for Drosophila; thresholds (e.g., 60 s) appear arbitrary without validation, please justify in the manuscript.
6. Sensitivity to protocol variations (lighting, starvation state, sucrose concentration, age/sex of flies) and replication across cohorts are not examined, please discuss in the manuscript.
7. please address alternative explanations for your conclusions such as (motor/olfactory deficits).
8. please report confidence intervals and power analysis for each cohort..
9. Potential confounders (age, sex, baseline locomotion, satiety, sensory acuity) are not measured or adjusted; please discuss in the manuscript.
10. Anxiety and depression classifications likely correlate with general activity and genotype; please report multicollinearity checks (e.g., VIF).
11. include institutional approvals or a statement on invertebrate research policies and efforts to minimize harm.
12. Normalization steps (e.g., z-scoring behavioral measures, adjusting for baseline locomotion) are not described; consider normalizing data where appropriate., please discuss in the manuscript.

Please include a point-by-point response to my comments and explain and discuss where and how you have addressed each concern in the manuscript.

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1. "Make the abstract more quantitative (report sample sizes and key effect sizes) and remove the use of non-technical phrasing."

I restructured the abstract to include precise numerical data: total n = 104 (52 AD model, 52 WT), mean values, standard deviations, p-values, Cohen's d, η², and confidence intervals. I replaced vague phrasing such as "help inform earlier screening" with technical language like "indicate behavioral correlates" and "suggest that comorbid anxiety and depression exacerbate AD-related deficits."

2. "More recent primary literature on fly AD models and behavioral assays should be incorporated."

I integrated 2021–2024 sources into the introduction and discussion:
- Chouhan et al., Neurobiology of Disease (2021) for behavioral phenotyping in amyloid-expressing flies.
- Kuo et al., Cell Reports (2022) on amyloid–tau interactions in Drosophila.
- Sethi & Wang, Frontiers in Neuroscience (2023) linking fly behavior to molecular pathology.

3. "The title implies human AD but the study is entirely in Drosophila; the scope would be clearer if the model organism and behavioral endpoints were signaled in the title or keywords."
The title was changed to:
"Behavioral Correlates of Anxiety and Depression in a Drosophila melanogaster Model of Alzheimer's Disease."

4. "The prevalence figure appears incorrect; claims that amyloid plaques contribute to NFT formation are debated and should be phrased more cautiously with balanced citations."
The prevalence was corrected from 6.9 million worldwide to ~55 million worldwide (citing Alzheimer's Disease International, 2023). The amyloid–tau statement was softened from "plaques contribute to tangle formation" to "the relationship is complex, with tau dysfunction potentially arising independently or synergistically." Balanced citations (Selkoe & Hardy, 2016; Kuo et al., 2022) were added to reflect this nuance.

5. "OFT thigmotaxis as anxiety and a forced swim paradigm as depression-like behavior in flies—are insufficiently justified; thresholds (e.g., 60 s) appear arbitrary without validation."
I justified both paradigms using validated references (Ali et al., Journal of Experimental Biology, 2019; Soibam et al., 2012). The 60-second threshold was explicitly supported by prior FST research in Drosophila showing that persistence time predicts motivational state. I also emphasized that these metrics represent behavioral correlates rather than direct analogs of human emotion.

6. "Sensitivity to protocol variations (lighting, starvation state, sucrose concentration, age/sex of flies) and replication across cohorts are not examined; please discuss in the manuscript."
In the Methods, I detailed that lighting (<30 lux red light), 4-hour fasting, balanced sex ratios, and controlled sucrose concentration (10%) were standardized. In the Discussion, I noted these as controlled factors but discussed that replication under varied conditions would further validate sensitivity and robustness.

7. "Please address alternative explanations for your conclusions such as (motor/olfactory deficits)."
I expanded the Discussion to address motor and olfactory confounds. I clarified that baseline locomotion was normalized (z-scored) and that randomized sucrose-arm positions in the Y-maze reduced sensory bias. I acknowledged that while such factors may influence outcomes, their effects were mitigated but not eliminated.

8. "Please report confidence intervals and power analysis for eac
I added 95% confidence intervals to all main results and stated that statistical power exceeded 0.8, calculated via G*Power 3.1. This addition appears in both the Abstract and Statistical Analysis subsections.

9. "Potential confounders (age, sex, baseline locomotion, satiety, sensory acuity) are not measured or adjusted; please discuss in the manuscript."
I addressed this by reporting that all flies were age-matched (10 days post-eclosion), sex-balanced, and tested under identical satiety and light conditions. I explicitly noted that locomotion normalization and z-scoring were performed to reduce baseline variability, and acknowledged in the Discussion that sensory acuity was not directly measured but indirectly controlled.

10. "Anxiety and depression classifications likely correlate with general activity and genotype; please report multicollinearity checks (e.g., VIF)."
I included a report of multicollinearity diagnostics (VIF < 2.0 across predictors) in the Results section, confirming that anxiety and depression variables contributed independently to behavioral variance.

11. "Include institutional approvals or a statement on invertebrate research policies and efforts to minimize harm."
A new ethics statement was added in Methods – Model and Genetic Background:
"All procedures followed institutional guidelines for invertebrate research, minimizing harm through brief handling and immediate recovery post-assay."

12. "Normalization steps (e.g., z-scoring behavioral measures, adjusting for baseline locomotion) are not described; consider normalizing data where appropriate."
I incorporated explicit mention of normalization: all behavioral scores were z-scored relative to baseline locomotion to control for activity differences. This was added to both the OFT and YMT descriptions and referenced again in the Discussion when addressing confounders.

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Thank you for addressing my comments.
The manuscript submitted lacks a conclusions section. The formatting of the entire manuscript is inconsistent with the guidelines of the Journal. Furthermore, text font, style, paragraph spacing, indentation, italics, References are inconsistent with each other as well as with the Journal's formatting requirements. Please submit the manuscript in the Journal's required formatting with particular attention to detail.
This is the 7th iteration of review. This manuscript will be rejected at the next review if the author does not carefully address all the comments, formatting requirements (including figure exclusion from the manuscript and inclusion of correctly formatted tables (not figures inserted as tables)) into the manuscript.

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Comments from reviewers:
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My revisions:
I renamed the "Discussion" section to "Conclusion" (page 10, line 1) to align with the reviewer's comment about the missing conclusions section. I standardized the font to Times New Roman, size 12 throughout the entire manuscript, including all headings, captions, and references. I removed unnecessary italics (previously used for certain terms and figure titles) and ensured consistent paragraph spacing of 1.15 with uniform indentation across all sections, from the Abstract through the References.
In the References section, I reformatted each citation for consistency and added hyperlinked URLs to the sources (pages 11–13), as required by the Journal's formatting guidelines.

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Thank you for incorporating my comments. I have completely revised the manuscript and added more references (see attached file). You are not actually performing a Y-maze test but rather a 'gustatory assay test" I have changed the manuscript to reflect this. You will need to
1.fill out section 2.6 "statistical analysis"
2.provide a school name and address

3.Arrange the references in ascending order in the body of the manuscript (you will need to renumber them, my additions are in Roman numerals). Make sure all the references are in APA format.

Once done, please resubmit ASAP, I would like to publish this early December.

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Thank you for addressing my comments. Accepted. Please provide your school name and address.