**Exploring the complex interplay between anxiety, aging, and behavior in CB6F1 and C57BL/6 mice: Implications for cognitive function**

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**Abstract**

Anxiety is a pervasive emotional response that can profoundly impact well-being and cognitive function in both humans and animals. The relationship between anxiety and aging remains complex and multifaceted. In order to study this relationship in more detail, an open-field photobeam system was used to quantify anxiety-related behaviors in aging CB6F1 and C57BL/6 male mice and determine associations with aging phenotypes including short- and long-term memory, grip strength, rotarod performance, and self-motivated wheel running. The findings indicated a heightened anxiety in novel environments with increasing age as evidenced by preference for peripheral areas during the open-field test. Elevated anxiety levels were not associated with decreased cognitive performance, suggesting that anxiety and cognition operate somewhat independently of each other. A negative correlation was observed between anxiety level and distance ran in the voluntary wheel running assessment, while no associations were seen with grip strength or rotarod performance. These observations contribute to an increased understanding of anxiety and its consequences in aging mice, providing insights into potential therapeutic interventions aimed at delaying aging through anxiety management.

**Key words.** Anxiety, Aging, Behavioral assessment, Cognition, CB6F1 mice, C57BL/6 mice.

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**Introduction**

Anxiety is a pervasive emotion that can manifest as an unpleasant state of internal confusion and a sense of fear toward impending events [1-3]. While anxiety is a common physiological response to a stressful situation, the constant presence is a sign of a clinical diagnosis of an anxiety disorder [4-7]. The numerous types of anxiety disorders have common risk factors but distinct symptoms, with generalized anxiety disorder (GAD) and obsessive-compulsive disorder (OCD) being the most common [8-9]. There is published evidence of an association between anxiety and memory, with the presence of anxiety serving as a strong predictor of future cognitive deterioration [10-13]. Symptoms of memory impairment often emerge along with those of anxiety [14-17].

Anxiety in mice parallels the physiological responses observed in humans, which include increased alertness, avoidance of certain environments or stimuli, changes in motor activity, changes in social interactions, and physiological changes such as increased heart rate or blood pressure [18]. Several behavioral tests have been designed to measure anxiety in mice based on the introduction to novel environments that may elicit anxiety secondary to an intrinsic fear response. The open-field test (OFT) is a popular paradigm that employs a floor grid and video tracking system to record movements within predefined grid zones over various time intervals [19]. The photobeam-based variant of this test allows for increased tracking frequencies, enabling more precise observations of anxiety-related behaviors [20]. Anxious tendencies in mice are often characterized by an aversion to open and exposed spaces, leading to increased time spent in the peripheral areas of the testing area while avoiding exploration of the central region [21-22].

This study examines the relationship between anxiety and established aging parameters in mice to help understand the associations and mechanisms underlying the aging process and its effect on various physiological and behavioral aspects. The findings develop further insight on age-related disorders and contribute to the development of novel therapeutic approaches and interventions to promote healthy aging.

**Methods**

***Animals.*** CB6F1 (C57BL/6 X Balb/c F1 cross) and C57BL/6 male mice in age groups of 4, 12, 20, 28 months were obtained from the National Institute on Aging Aged Rodent Colony, contracted by Charles River, Inc. Mice were housed in a specific pathogen free mouse facility at the University of Washington (UW) main campus in Seattle, WA. The status of the room was monitored under the guidance of the Rodent Health Monitoring Program within the purview of the UW Department of Comparative Medicine. Mice were group housed, up to five per cage, and given nestlets (Ancare Corp, Bellmore, NY) for environmental enrichment. Mice were acclimated for two weeks before starting test procedures. All procedures were approved by the University of Washington IACUC (Animal Care and Use Committee).

***Anxiety Assessment.*** An open-field photobeam testing system (OFT) (Columbus Instruments, Inc) was employed to evaluate anxiety-related behaviors of mice in a novel environment as previously described [23]. The apparatus simulates a standard mouse cage, featuring a clear rectangular container and infrared beams arranged in a grid pattern, three horizontally and four vertically. The open-field photobeam system was configured with two sets of infrared beams to measure both lateral and vertical activity. Beam breaks, which occurred when mice crossed an infrared beam, were counted for each activity. The data collected were subsequently categorized into two distinct zones: the central and peripheral areas of the container. This categorization allowed for the assessment of anxiety levels based on preference for exploring specific regions. Increased time spent in the central area suggested reduced anxiety, whereas a preference for peripheral regions suggested heightened anxiety. Each mouse was placed inside the testing container for a period of five minutes. This standard duration ensured consistent evaluation of anxiety-related behaviors and minimized potential habituation effects or stress-related responses.

***Memory Assessment.*** A radial water tread maze was used to assess short-term and long-term memory [24]. The maze consisted of a circular basin with nine holes, eight decoys leading to dead ends, and one escape hole leading to a dark safety box equipped with a heating pad to simulate a standard mouse cage. The basin contained approximately one inch of water and an overhead light placed above the cage as an escape incentive. Mice had three trials in the maze for four consecutive days of training, followed by testing on the fifth day and retesting on the twelfth day.

***Grip Strength Assessment.*** Forelimb paw strength was measured using a grip strength meter [25]. Each mouse was positioned horizontally with forepaws on a metal grip bar (Columbus Instruments, Inc), and the mouse was pulled back at a uniform rate until releasing the bar. The machine recorded the maximum force exerted by the mouse for a total of five trials. Mice were weighed on the test day and peak force was expressed relative to body weight to normalize grip strength measurements.

***Agility Assessment.*** Agility was assessed using a Rotamax 4/8 rotating bar machine (Columbus Instruments, Inc) [25]. The machine tested the ability of mice to maintain walking speed on a rotating bar. The initial speed was set to 0 RPM and gradually increased by 0.1 RPM/s over a 5-minute duration until all mice fell off and were detected by a sensor. The time in seconds was recorded for each mouse over three trials.

***Voluntary Wheel Running Assessment.*** Total distance ran over three days was measured with a running wheel added to a standard mouse cage [26]. Mice were individually housed in standard cages with a slanted running wheel wirelessly connected to a computer (Med Associates, Inc). There was a two-day acclimation period with the wheels locked and on the third day the wheel was unlocked, and data collection began. Running distances were continuously monitored over a 72-hour period with total distances ran every minute recorded in kilometers.

***Data Analysis.*** All data were grouped according to strain and age. A Shapiro-Wilk test was used to assess data under each group to determine whether there was normal distribution. For normally distributed data, statistical comparisons were performed using parametric tests. Data that were not normally distributed were analyzed using non-parametric tests. The Wilcoxon rank sum test was used to determine significant differences between two groups when the data were not normally distributed. The Kruskal-Wallis H test was used to determine significant differences between multiple groups when data was not normally distributed. In cases where there was no interaction between factors, but a factor was found to be statistically significant, a post-hoc analysis of means was conducted using the Bonferroni adjustment method. Spearman’s rank test was used to measure the strength of association between aging and each parameter while the point-biserial correlation coefficient was used for assessing the association between a continuous variable and a binary variable. All statistical analyses were performed using GraphPad Prism (version 10.0.3).

**Results**

***Age-related differences in total movement in the open field test were detected in CB6F1 mice.***

The results of the two-way ANOVA on total movement in the open field test (OFT) revealed significant main effects of both strain (F(1,84) = 4.313, p < 0.0001) and age (F(1, 84) = 29.19, p < 0.05) without a significant interaction between strain and age (F(1,84) = 1.678, p = 0.199). Post-hoc analysis indicated that older mice at 20 to 28 months of age exhibited prolonged periods of movement compared to younger mice at 4 to 12 months of age in CB6F1 but not C57BL/6 (B6) strains (Figure 1A & 1B, respectively). Furthermore, older CB6F1 mice displayed more extensive movement compared to their B6 cohorts (Figure 1C).

A. **CB6F1 Total Movement in OFT** B. **C57BL/6 Total Movement in OFT** C. **Inter-Strain Total Movement in OFT**

  

**Figure 1**. **Total movement in OFT. A.** CB6F1 mice at20and 28 months of age exhibited significant differences when compared to 4 and 12-month-old cohorts and showed a positive association between movement and age. **B.** B6 mice at the same age groups did not show the same significant differences and age-related increase in movement as seen in CB6F1 mice. **C.** Comparison of older B6 and CB6F1 mice showed a significant difference in total movement (\*\*\*\*p < 0.0001, ns = not significant (p > 0.05), N = 19-28/cohort, OFT = open-field photobeam testing system).

***Mice showed area preferences in the open field test in an age and strain-dependent manner.***

Older CB6F1 mice exhibited significantly more time in both central and peripheral areas than their younger counterparts as revealed by the results of a one-way ANOVA with significant main effects of both central (F (3,82) = 8.144, p < 0.0001) and peripheral (F (3,82) = 10.30, p < 0.0001) area region preferences with respect to age after post-hoc analyses (Figures 2A & 2B, respectively). Older B6 mice displayed such distinctions for central areas, as demonstrated by the results of a one-way ANOVA (F (3,77) = 7.587, p < 0.0005), but not for peripheral areas (F (3,77) = 1.526, p = 0.214) after post-hoc analyses (Figures 2C & 2D, respectively).

CB6F1 mice at 28 months of age allocated significantly more time to peripheral areas than 28-month-old B6 mice (Figure 2E). A two-way ANOVA of time spent in the peripheral area revealed a significant interaction between strain and age (F (3,159) = 5.292, p < 0.005) with post-hoc analysis. These observations collectively suggest that with increasing age CB6F1 mice have a higher likelihood than B6 mice for prolonged occupancy in peripheral areas.

A. **CB6F1 Central Movement in OFT** B. **CB6F1 Peripheral Movement in OFT**

 

C. **C57BL/6 Central Movement in OFT** D. **C57BL/6 Peripheral Movement in OFT** E. **Inter-Strain Peripheral Movement in OFT**

 

**Figure 2.** **Total time spent in central and peripheral areas.** **A/B.** Older CB6F1 mice spent significantly more time in central and peripheral areas than their younger counterparts. **C/D.** Older B6 mice spent significantly more time in central but not in peripheral areas compared to their younger counterparts. **E.** CB6F1 mice at 20- and 28-months of age spent significantly more time in peripheral areas compared to B6 mice (\*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001, ns = not significant (p > 0.05), N = 19-28/cohort, OFT = open-field photobeam testing system).

***Time spent rearing was age dependent in CB6F1 mice.***

An interaction between time spent rearing and age was demonstrated by a Kruskal-Wallis test for both CB6F1 (H = 19.69, df = 3, p < 0.0005) and B6 mice (H = 9.355, df = 3, p < 0.05), although post-hoc analysis indicated that older CB6F1 mice spent significantly more time rearing than their younger counterparts (Figure 3A) while time rearing was not age dependent in B6 mice (Figure 3B). Time spent rearing was area dependent as both CB6F1 and C57BL/6 mice spent significantly more time rearing in the periphery compared to the central regions as demonstrated by Kruskal-Wallis tests ([H = 83.83, df = 7, p < 0.0001], [H = 136.3, df = 7, p < 0.0001], respectively) with post-hoc analysis (Figures 3D & 3E, respectively). A two-way ANOVA revealed significant main effects of age (F (1,159) = 20.64, p<0.0001) but not strain (F(3,159) = 1.368, p=0.255) or interaction between strain and age (F(3,159) = 1.452, p=0.230)) on preference for rearing over activity. Overall, both CB6F1 and B6 mice spent significantly less time rearing than moving laterally (Figure 3E & 3F, respectively).

A. **CB6F1 Overall Rearing Time in OFT** B. **C57BL/6 Overall Rearing Time in OFT**

 

C. **CB6F1 Rearing Area Preference in OFT** D. **C57BL/6 Rearing Area Preference in OFT  **

E. **CB6F1 Movement Preference in OFT** F. **C57BL/6 Movement Preference in OFT**

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**Figures 3.** **Time spent in each area analyzed according to age and strain.** **A/B.** Older CB6F1 mice tended to spend more time rearing compared to younger counterparts while there was no age-dependent preference observed in C57BL/6 mice. **C/D.** Rearing was area-dependent, as both CB6F1 and C57BL/6 mice tended to rear in peripheral areas for longer compared to central areas. **E/F.** CB6F1 and C57BL/6 across all age groups preferred activity compared to rearing (\*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001, ns = not significant (p > 0.05), N = 19-28/cohort, OFT = open field photobeam testing system).

***Memory was associated with age but not peripheral area preference in CB6F1 mice.***

The multiple linear regression analysis revealed that age was a significant predictor of long-term memory (ß= 3.02, p < 0.0005), with post-hoc analysis indicating that older CB6F1 mice exhibited a significant increase in escape times on day 12 of the radial water tread maze (Figure 4A). However, the Open Field Ratio (OFR) was not a significant predictor of long-term memory for any age-group (ß= -53.58, p = 0.466) (Figures 4C-F) and the overall model did not account for a significant portion of the variance in long-term memory scores (R2 = 0.162, F (2,75) = 7.234, p = 0.093). OFR was calculated as the time spent in peripheral areas divided by the total time recorded. Long-term memory was quantified by time spent to find the escape on day 12.

Regarding short-term memory, the multiple linear regression analysis revealed that age was a significant predictor (ß=2.98, p < 0.0001), with post-hoc analysis indicating that older CB6F1 mice exhibited a significant increase in escape times on day 5 of the radial water tread maze (Figure 4B). However, OFR was not a significant predictor of short-term memory for any age-group (ß= -99.18, p = 0.110) (Figures 4C-F). The overall model accounted for a significant portion of the variance in short-term memory scores (R2 = 0.208, F (2,82) = 10.78, p < 0.0001). Short-term memory was measured by time spent to find the escape on day 5.

A. **CB6F1 LTM-Age Correlation** B. **CB6F1 STM-Age Correlation**

 

C. **CB6F1 4-Month Memory Correlation** D. **CB6F1 12-Month Memory Correlation**

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E. **CB6F1 20-Month Memory Correlation** F. **CB6F1 28-Month Memory Correlation**

 

**Figure 4.** **Relationship between memory and Open Field Ratio (OFR) in CB6F1 mice.** **A/B.** There were statistically significant differences across CB6F1 age groups for both long- and short-term memory. **C/D/E/F.** There were no significant correlations between either long- or short-term memory with anxiety scores (\*p < 0.05, \*\*p < 0.01, ns = not significant (p > 0.05), N = 19-28/cohort, LTM = long-term memory, STM = short-term memory).

For C57BL/6 mice, the multiple linear regression and post-hoc analyses revealed that neither age (ß = 0.895, p = 0.281) (Figure 5A) or OFR (ß = 180.9, p = 0.067) (Figures 5C-F) were significant predictors of long-term memory with the overall model accounting for a significant portion of the variance in long-term memory scores (R2 = 0.081, F (2,75) = 3.291, p < 0.05). However, for short-term memory, age was a significant predictor (ß = 2.029, p < 0.05) (Figure 5B), but not OFR (ß = 127.0, p = 0.144) (Figures 5C-F), with the overall model accounting for a significant portion of the variance in short-term memory scores (R2 = 0.151, F (2,78) = 6.911, p < 0.005). Post-hoc analysis indicated that older C57BL/6 mice exhibited a significant increase in escape times on day 5 of the radial water tread maze (Figure 5B). Overall, there were no statistically significant correlations between memory performance and peripheral area preference across ages groups in C57BL/6 mice.

A. **C57BL/6 LTM-Age Correlation** C. **C57BL/6 4-Month Memory Correlation** D. **C57BL/6 12-Month Memory Correlation**

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B. **C57BL/6 STM-Age Correlation** E. **C57BL/6 20-Month Memory Correlation** F. **C57BL/6 28-Month Memory Correlation**

 

**Figure 5.** **Relationship between memory and Open Field Ratio (OFR) in C57BL/6 mice.** **A/B.** There were statistically significant differences across C57BL/6 age groups for short- but not long-term memory. **C/D/E/F.** There were no significant correlations between either long- or short-term memory with anxiety scores (\*p < 0.05, \*\*p < 0.01, ns = not significant (p > 0.05), N = 19-28/cohort, LTM = long-term memory, STM = short-term memory).

***Peripheral area preference was associated with reduced running distance in 28-month-old C57BL/6 mice, but no associations in either strain were found with grip strength or rotarod.***

For CB6F1 mice in voluntary wheel running, the multiple linear regression analysis revealed that age was a significant predictor of distance traveled (ß= -0.501, p < 0.0001), with post-hoc analyses indicating that older CB6F1 mice exhibited a significant reduction in self-motivated running distances (Figure 6A). However, OFR was not a significant predictor of distance traveled (ß= -3.661, p = 0.659) (Figures 6B-E). The overall model accounted for a significant portion of the variance in distance traveled (R2 = 0.332, F (2,65) = 16.12, p < 0.0001).

**Figure 6.** **Relationship between OFR and voluntary wheel running distance in CB6F1 mice**. **A.** A statistically significant association was observed between age and wheel running. **B/C/D/E.** There were no significant correlations among CB6F1 mice across age-groups between time spent in peripheral areas and total running distance (\*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001, not significant if p > 0.05, N = 19-28/cohort).

1. **CB6F1 age-running distance correlation**



B. **CB6F1 4-month running distance correlation** C. **CB6F1 12-month running distance correlation**

 

D. **CB6F1 20-month running distance correlation** E. **CB6F1 28-month running distance correlation**

 

For C57BL/6 mice in voluntary wheel running, the multiple linear regression analysis revealed that age was a significant predictor of distance traveled (ß= -0.375, p < 0.0001), with post-hoc analyses indicating that older CB6F1 mice exhibited a significant reduction in self-motivated running distances (Figure 7A). OFR was a significant predictor of distance traveled in the 28-month-old cohort (Figure 7E) but was not a significant predictor of distance traveled (ß= 3.52, p = 0.669) for 4-, 12-, and 20-month cohorts (Figures 7B-D). The overall model accounted for a significant portion of the variance in distance traveled (R2 = 0.332, F (2,65) = 16.12, p < 0.0001).

A. **C57BL/6 age-running distance correlation**



B. **C57BL/6 4-month running distance correlation** C. **C57BL/6 12-month running distance correlation**

 

D. **C57BL/6 20-month running distance correlation** E. **C57BL/6 28-month running distance correlation**

 

**Figure 7.** **Relationship between OFR and voluntary wheel running distance in C57BL/6** **mice**. **A.** A statistically significant association was observed between age and running distance. **B/C/D.** There were no significant correlations among C57BL/6 mice 4-,12-, and 20-months old between time spent in peripheral areas and total running distance (\*p < 0.05, \*\*p < 0.01, \*\*\*\*p < 0.0001, not significant if p > 0.05, N = 19-28/cohort). **E.** There was a significant correlation between time spent in peripheral areas and total running distance in 28-month-old C57BL/6 mice (p < 0.001, N = 12).

For grip strength, *t*he multiple linear regression analysis revealed that age was a significant predictor of grip strength in CB6F1 (ß= -1.240, p < 0.0001) and C57BL/6 mice (ß= -1.434, p < 0.0001), indicating that older mice in both strains exhibited a significant reduction in grip strength (Figures 8A-B). However, OFR was not a significant predictor of grip strength in either strain (CB6F1: ß= 4.230, p = 0.7830, C57BL/6: ß= -24.19, p = 0.3078). The overall model accounted for a significant portion of the variance in grip strength for both strains (CB6F1: R2 = 0.3890, F (2,81) = 25.79, p < 0.0001, C57BL/6: R2 = 0.4525, F (2,78) = 32.24, p < 0.0001).

A. **CB6F1 age-grip strength correlation** B. **C57BL/6 age-grip strength correlation**

 

**Figure 8.** **Relationship between age and grip strengt**. **A/B.** There were significant correlations between age and grip strength in CB6F1 and C57BL/6 mice (\*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001, ns = not significant (p > 0.05), N = 19-27/cohort).

For rotarod performance, the multiple linear regression analysis revealed that age was a significant predictor of rotarod performance in both CB6F1 (ß= -2.376, p < 0.0001) and C57BL/6 mice (ß= -1.997, p < 0.0001), indicating that older mice exhibited a significant reduction in rotarod performance (Figures 9A-B). However, OFR was not a significant predictor of rotarod performance in either strain (CB6F1: ß= 50.82, p = 0.1244, C57BL/6: ß= 47.94, p = 0.1244). The overall model accounted for a significant portion of the variance in rotarod performance for both strains (CB6F1: R2 = 0.352, F (2,83) = 22.54, p < 0.0001, C57BL/6: R2 = 0.294, F (2,78) = 16.24, p < 0.0001).

A. **CB6F1 age-rotarod performance correlation** B. **C57BL/6 age-rotarod performance correlation**

 

**Figure 9.** **Relationship between age and rotarod performance**. **A/B.** There were significant correlations between age and rotarod performance in CB6F1 and C57BL/6 mice (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001, ns = not significant (p > 0.05), N =19-29/cohort).

**Discussion**

Analysis of anxiety-related behaviors in the open-field photobeam testing system (OFT) in relation to cognitive and performance functions revealed insights into the relationships between anxiety, aging, and behavior in mice. Total movement, area region preference, rearing time, and memory were all age-dependent in CB6F1 mice while no correlation between the open-field ratio (OFR) and memory as well as general motor function (as measured by the voluntary wheel running task, grip strength, and rotarod performance) were observed in either strain. The OFT is useful to measure anxiety in mice based on thigmotaxis, the natural instinct of rodents to avoid open spaces and especially with high intensity light due to their vulnerability to predation [27]. This well-documented behavioral manifestation of anxiety is rooted in evolutionary survival mechanisms and is associated with difficulty in emotional and spatial learning [28]. The amount of time spent in peripheral regions emerged as a critical parameter of anxiety. Older mice across strains exhibited heightened anxiety when exposed to a novel environment, as shown by increased time spent in peripheral areas and time spent rearing. However, mice that displayed elevated anxiety levels did not demonstrate worse performance in cognitive tasks, suggesting that anxiety and cognition operate independently of each other. A negative correlation was also revealed between anxiety level and distance traveled on the voluntary wheel running assessment, but only in 28-month-old C57BL/6 mice.

The impact of anxiety on learning and memory was not apparent in this study as we did not observe an association of peripheral region preference and decreased long-term memory. However, previous research has emphasized the role of neuronal circuits and neurotransmitter systems in anxiety-related cognitive impairment [29-30]. Stress hormones such as cortisol and corticosterone are released during anxiety states and are known to impact the functioning of brain regions involved in memory processing, such as the hippocampus and prefrontal cortex [31-36]. Furthermore, the rate of adult neurogenesis (AN), the concept of neuronal production in selective regions of the brain well after the typical brain morphogenesis associated with neonatal development has also been shown to independently affect cognition and anxiety-like behaviors in rodents [37-39]. As the ventral dentate gyrus (DG) of the hippocampus is a well-documented area of AN in rodents [40] and has been associated with anxiety-related behaviors and cognitive function [27, 41-43], further investigation into these variables would be highly relevant for future studies to elucidate the relationship between anxiety and cognition.

Interestingly, while neither strain had statistically significant associations between memory performance and peripheral area preference, CB6F1 mice demonstrated an age-related decrease in long-term memory while C57BL/6 mice did not, and associations between anxiety-related behaviors and age were overall stronger. As C57BL/6 mice are often considered to have a lower baseline level of anxiety without clearly understood reasons [44-45], this observation suggests that it would be beneficial to conduct research in elucidating differences and similarities in behavior and aging between C57BL/6 and CB6F1 mice.

A reduction in running distance in the voluntary wheel-running assessment among mice with increased anxiety can be attributed to reduced exploratory behavior and a diminished willingness to engage in physical activity [46]. Such cautiousness, reluctance to take risks, and decreased engagement in high-energy activities align with anxiety-induced behaviors, although a statistically significant association was only observed in 28-month-old C57BL/6 mice. Furthermore, no significant correlations with anxiety scores were found in either strain for the rotarod or grip strength tests. The absence of a significant correlation between anxiety and motor function across ages and strains, as assessed by voluntary wheel running, rotarod, and grip strength tests, may be attributed to several factors. Performance on these tests primarily reflects motor coordination and physical strength, while anxiety primarily affects emotional and cognitive domains, with evidence from previous studies showing that cognitive changes may operate independently of gross motor function [47]. While these tests may not directly elicit anxiety-specific responses in mice, previous studies have found positive correlations between AN and exercise without affecting anxiety-related behavior in rodents [48]. As the open-field test was performed within a month after the voluntary wheel running and rotarod performance tasks and a few days prior to the grip strength assessment, the chronological order of behavioral-assays may also have impacted our findings between behavior and cognition. Individual variability in mouse responses to anxiety and motor function capabilities could also contribute to the observed absence of a significant correlation. Further investigations employing more specific anxiety-related assays, or a broader array of behavioral tests may provide a more comprehensive understanding of the relationship between anxiety and motor function in aging mice.

The results of this study highlight the influence of age and strain on anxiety responses in male mice, emphasizing the need for careful consideration of these factors when interpreting open-field test results. The observed preference for peripheral areas in novel environments suggests that physiological anxiety plays a role in mouse behavior and its implications for cognitive function. This study contributes to future research aimed at a better understanding of how anxiety and aging interact in male mice. It is inherent to conduct similar studies in female mice to determine and identify any sex-dependent differences, and thus help provide the rationale to develop new strategies to delay aging through anxiety management.

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March 4, 2024

RE: Response to review of 707 “Exploring the complex interplay between anxiety, aging, and behavior in CB6F1 and C57BL/6 mice: Implications for cognitive function”

We thank the reviewers for their helpful comments and suggestions and address the issues in the following points from each reviewer.

**Reviewer #1.** In summary, the data require additional statistical analyses to support the conclusions.

*1. An important missing comparison is the effect of age on memory scores.*

**Response:** These were added (refer to Figures 4A-B for long- and short-term memory correlation with age in CB6F1 mice, refer to Figures 5A-B for analysis of C57BL6 mice.

*2. For the ANOVA of age and interspecies differences, was this a multifactor ANOVA (age and species)? No statistics were provided. What was the F-value? Degrees of freedom? It would help to have symbols to illustrate or localize significant comparisons.*

**Response:** A two-way ANOVA was conducted on total movement time for both strain and age. There was significant main effects of both strain (F (1,84) = 4.313, p < 0.0001) and age (F (1,84) = 29.19, p < 0.05) without a significant interaction between strain and age (F(1, 84) = 1.678, p = 0.199). Symbols to illustrate significant comparisons were added to the figures with “\*\*\*\*” denoting p < 0.0001 and “ns” denoting no significance (p > 0.05).

*3. A comparative analysis of time recorded within each area . . . What was the test? F-value? Degrees of freedom? If you are going to compare across species, please prove the same scale for the figures (i.e. Fig 2A and B, 2C and D, 3A and B).*

**Response:** For both mice strains, one-way ANOVA assessing central and peripheral region preferences with respect to age were conducted. In CB6F1 mice, statistics revealed significant main effects of both central (F(3,82) = 8.144, p < 0.0001) and peripheral (F (3,82) = 10.30, p < 0.0001) area region preferences with respect to age in CB6F1 mice. In C57BL/6 mice, statistics revealed significant main effects for central (F (3,77) = 7.587, p < 0.0005) but not for peripheral areas (F (3,77) = 1.526, p = 0.214). Figures were modified to have consistent scaling.

*4. Overall, both CB6F1 and B6 mice spent significantly less time rearing than moving laterally. What was the test? F-value? Degrees of freedom?*  
**Response**: A two-way ANOVA revealed significant main effects of age (F(1,159) = 20.64, p<0.0001) but not strain (F(3,159) = 1.368, p=0.255) or interaction between strain and age (F(3,159) = 1.452, p=0.230)) on preference for rearing over activity.

*5. Significantly worse long-term memory was observed in older CB6F1 mice that preferred the periphery compared to older CB6F1 mice that spent more time in the central area of the cage (Figure 4). Please provide bar graphs of the times used to estimate memory performance and the calculated long-term memory scores. These should be tested for age and species differences. Test? Statistics? Symbol to localize significance?*

**Response:** The aim was not to compare the two species in this case and so a test involving species differences would not be appropriate. In regard to differences in long-term memory between older CB6F1 mice that preferred the periphery compared to those that preferred central areas of the cage, regression analysis showed no significant relationship between memory (short and long) and open field ratio (Figures 4A-D). Furthermore, a multiple linear regression was performed between age, memory, and anxiety, as detailed in the response to #7.

*6. Any relationship could be due to differences in short-term memory or long-term memory times. For example, it is possible that a group exhibits poor short-term memory (long time). In this case, it is likely that the performance during long-term memory is not different from short-term and the calculation would suggest good memory (no differences between short and long term). In contrast, a group that exhibits good short-term memory (short times) could exhibit significant forgetting (long times).*

**Response:** Methods was edited to eradicate the mentioned possibility of confounding between short-term and long-term memory. Measurement of long-term memory was modified to be the time spent to find the escape on day 12 of the radial water tread maze. Measurement of short-term memory was modified to be the time spent to find the escape on day 5 of the radial water tread maze (after 4 days of training as discussed in the original Methods section).

*7. For correlations between memory and peripheral area preference, please provide statistics. What was the r-square value?*

**Response:** Statistics for linear regression models are provided in the revised figures (refer to Figures 4A-D).

*8. The authors state that the implication is that there was an associated of memory and anxiety during aging. Please perform a multiple regression using all the animals in the species (predict memory using anxiety score and age).*

**Response:** The multiple linear regression analysis revealed that age was a significant predictor of day 12 times on the radial water tread maze (ß= 3.02, p < 0.0005), indicating that older CB6F1 mice exhibited a significant reduction in long-term memory (Figure 4A). However, the Open Field Ratio (OFR) was not a significant predictor of long-term memory for any age-group (ß= -53.58, p = 0.466) (Figures 4B-E). The overall model did not account for a significant portion of the variance in long-term memory scores (R2 = 0.162, F (2,75) = 7.234, p = 0.093). (Refer to manuscript for additional analysis on B6 cohorts).

*9. It would be good to see a graph for anxiety and wheel running using all animals. Again, a multiple regression could be employed to determine that relationship.*

**Response**: For CB6F1 mice, the multiple linear regression analysis revealed that age was a significant predictor of distance traveled (ß= -0.501, p < 0.0001), with post-hoc analyses indicating that older CB6F1 mice exhibited a significant reduction in self-motivated running distances (Figure 6A). However, OFR was not a significant predictor of distance traveled (ß= -3.661, p = 0.659) (Figures 6B-E). The overall model accounted for a significant portion of the variance in distance traveled (R2 = 0.332, F (2,65) = 16.12, p < 0.0001). For C57BL/6 mice, the multiple linear regression analysis revealed that age was a significant predictor of distance traveled (ß= -0.375, p < 0.0001), with post-hoc analyses indicating that older CB6F1 mice exhibited a significant reduction in self-motivated running distances (Figure 7A). OFR was a significant predictor of distance traveled in the 28-month-old cohort (Figure 7E) but was not a significant predictor of distance traveled (ß= 3.52, p = 0.669) for 4-, 12-, and 20-month cohorts (Figures 7B-D). The overall model accounted for a significant portion of the variance in distance traveled (R2 = 0.332, F (2,65) = 16.12, p < 0.0001).

*10. A number of studies have examined anxiety measured in the open field and cognition, wheel running, and grip strength, cross sectional and longitudinal, in rodents. A discussion of this work and the relationship of  
the current study in this field is missing.*

**Response**: Refer to the updated Discussion section for the requested discussion and relationship of the current study.

*11. For the first heading of the results section, the authors need to specify that the “movement” is in the OFT. Age-related differences in movement were detected in both CB6F1 and C57BL/6 mice. This is required since  
movement is also measured on the wheel.*

**Response**: Heading was modified to reflect this comment.   
  
**Reviewer #2**

*1. What is the significance of using these types of mice? CB6F1 (C57BL/6 X Balb/c F1 cross) and C57BL/6?*

**Response**: These are commonly used mouse strains for studies in the field of aging and age-related diseases.

*2. What is the point behind grip strength?  Is this a sign of anxiety? Based on what?  What is this a proxy for?*

**Response**: The grip strength test is used to determine skeletal muscle strength, which usually decreases with increasing age in mice (as well as in people). It therefore is used as one factor for degree of frailty to determine independence or not in aging individuals. It is not a cognitive test, but a performance test. The objective of the study was to see if there was a correlation between anxiety and grip strength, with the hypothesis that those with increased anxiety would also have weaker grip strength.

*3. Is voluntary wheel running a sign of anxiety?  Based on what?  What is this a proxy for? Same for the agility assessment….I was struggling to see how these behaviors are proxies for certain human behaviors.  More clarity on this would be very helpful.*

**Response:** Voluntary wheel running is a validated method of studying aging in mice (refer to Goh et al., 2015 as referenced in the manuscript) and tests for the total distance ran by a mouse over a certain period (refer to Methods section of the manuscript). Valid for voluntary wheel running and agility assessments, as these test for basic parameters of health and function, they are therefore fundamental measures of health-related behaviors.