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A comparison of cognitive decline in aged mice and mice treated with aftin-4

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Dementia, especially Alzheimer's disease, presents a major clinical challenge, and researchers are still searching for an optimal animal model. To address this gap, we compared male and ovariectomized female C57BL/6 mice treated with 30 mg/kg aftin-4, which induces neurodegeneration, with naturally aged (15–16 months old) mice not treated with aftin-4. We performed a series of behavioral tests; measured postmortem plasma β -amyloid levels ($A\beta$ 1–40 and $A\beta$ 1–42) and the levels of the oxidative stress indicators glutathione peroxidase (GPx), superoxide dismutase (SOD) and malondialdehyde (MDA); and evaluated astrocytic reactivity in the brain using glial fibrillary acid protein (GFAP) levels. Our results revealed no behavioral changes in the aged or aftin-4-treated mice compared with the control mice. Aftin-4 mice presented lower brain MDA levels and no detectable changes in plasma $A\beta$ levels. In general, female mice had higher GPx and SOD levels and lower $A\beta$ 1–42 levels than male mice did. In contrast, aged and aftin-4-treated male mice presented elevated levels of GFAP, indicating astrocyte damage. Our results could not confirm that either aftin-4-treated or aged mice are reliable models for dementia. However, the observed molecular changes suggest that male animals may be more susceptible to oxidative stress and brain damage than females are. This study demonstrates the complexity of modeling dementia in animals and the importance of future studies in this area.

Keywords Aftin-4, Aged mice, Cognitive decline, Oxidative stress, Animal behavior

Dementia, a major global health concern, manifests as behavioral and psychological symptoms such as memory loss, depression, anxiety, apathy, agitation, aggression, and deficits in social communication that significantly affect activities of daily living¹. Neurodegenerative processes and cerebrovascular disorders contribute to the etiology of dementia, with Alzheimer's disease (AD) being the predominant form². AD is characterized by $A\beta$ aggregates and neurofibrillary tangles comprising the tau protein². Identifying the ideal animal model to study AD and other forms of dementia remains one of the most important challenges for researchers. Unfortunately, no ideal model exists because it is difficult to simulate the diversity and lifestyle differences of humans in other species. The use of transgenic animal models (genetically engineered to overexpress human genes associated with AD) raises ethical concerns and requires special care, and these animals still differ significantly from humans^{3–5}.

Numerous studies have shown that cognitive decline begins at 6 months of age, and biochemical alterations and neuronal degeneration (increased levels of endogenous $A\beta$) occur in aged wild-type mice of different strains. These studies suggest that wild-type mice may better mimic the variability observed in humans^{6–9}. However, aging affects cognition differently in each individual, leading to conflicting results, and this idea has yet to be supported by further evidence^{6–9}.

On the other hand, various pharmacological models serve as efficient tools for preclinical research. The scopolamine model, one of the most commonly used pharmacological models, is often criticized for not being versatile enough, as the action of scopolamine is limited to cholinergic neurons^{10,11}. Thus, we have been more interested in recent pharmacological models that induce more complex neurodegenerative processes. Aftin-4 is a notable inducer of neurodegeneration that increases $A\beta$ 1–42 levels in vitro and in vivo by binding to mitochondrial proteins, including VDAC1, mitofilin, and prohibitin, thereby disrupting the mitochondrial and cellular environment^{12,13}. This disruption alters γ -secretase activity and shifts its cleavage of the amyloid precursor protein (APP) to favor the production of the neurotoxic $A\beta$ 1–42. In addition, in vivo, aftin-4 induces proinflammatory cytokine production and lipid peroxidation and triggers an astrocytic response that ultimately impairs learning and memory in behavioral tasks^{12–14}.

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The aim of this study was to evaluate the cognitive performance and behavioral symptoms of two potential models carefully and to perform a comparative analysis. Specifically, we compared mice that received aftin-4, an inducer of neurodegeneration, with aged mice starting at 15 months of age. To gain comprehensive insights, we subjected the animals to a series of behavioral tests and measured postmortem A β levels along with other important indicators of oxidative stress and glial activity in the brain. We also included both sexes in the study to determine whether there was an interaction effect between sex and condition.

Results

Elevated plus maze (EPM) test

We observed no interaction effect between sex and the proportion of time spent in the closed arms ($F(2, 63) = 0.13, p = 0.872$) or in the open arms ($F(2, 63) = 0.07, p = 0.928$). Simple effects analysis revealed that the sex of the animal affected the proportion of time spent in the open arms ($F(1, 63) = 6.10, p = 0.016$). On average, males spent 7.5% more time in the open arms than females did. Sex and condition affected the proportion of time spent in the closed arms ($F(1, 63) = 4.45, p = 0.039$), $F(2, 63) = 3.16, p = 0.049$). On average, aged mice spent 8.5% more time in the closed arms than did the control ($p = 0.071$) and aftin-4 ($p = 0.086$) mice, although the effects were only borderline significant according to Tukey's post hoc test (data not shown). The control mice tended to visit the open arms 3.25 times more frequently, whereas the aftin-4-treated mice did so 2.3 times more frequently than did the aged mice; again, the difference was not statistically significant ($F(2, 63) = 3.03, p = 0.055$). Figure 1 graphically presents the data.

Spontaneous alternations and spatial recognition in the Y maze

In the spontaneous alternation test, the spontaneous alternation percentage data revealed that the mice in all groups had an equivalent ability to enter the three arms in succession ($H(5) = 10.40, p = 0.064$). In the spatial recognition memory test in the Y-maze, the mice explored both the familiar arm and the new arm to the same extent. Each arm was explored on average approximately 30% of the time by each group ($H(5) = 7.12, p = 0.211$). Figure 2 shows the data graphically.

Three-chamber sociability test

A simple main effect analysis revealed that in the first phase of the sociability test, condition had an effect on the amount of time spent sniffing the empty box ($F(2, 62) = 5.54, p = 0.006$). On average, aged mice spent 22 fewer seconds sniffing the empty box than did control mice. We expected that this was related to a greater SI in aged mice, but borderline statistical significance was observed ($F(2, 62) = 3.04, p = 0.055$). The analysis revealed no interaction effect between sex and the time spent sniffing the empty box ($F(2, 62) = 0.44, p = 0.641$) or the SI ($F(2, 63) = 0.19, p = 0.825$).

In the second phase of the test, the behavior of the mice in all the groups was similar, regardless of sex and condition. Neither group showed a preference for novelty, expressed as the social novelty preference index ($H(6) = 1.93, p = 0.858$). Figure 3 shows the data graphically.

Screening of locomotor activity in all behavioral tests

Throughout the entire battery of behavioral tests, we measured locomotor activity, namely, the distance traveled and average speed, in each test. For these parameters, we were not interested in the effects of sex; therefore, we disregarded sex for this analysis and pooled the data into three groups because we were more interested in the

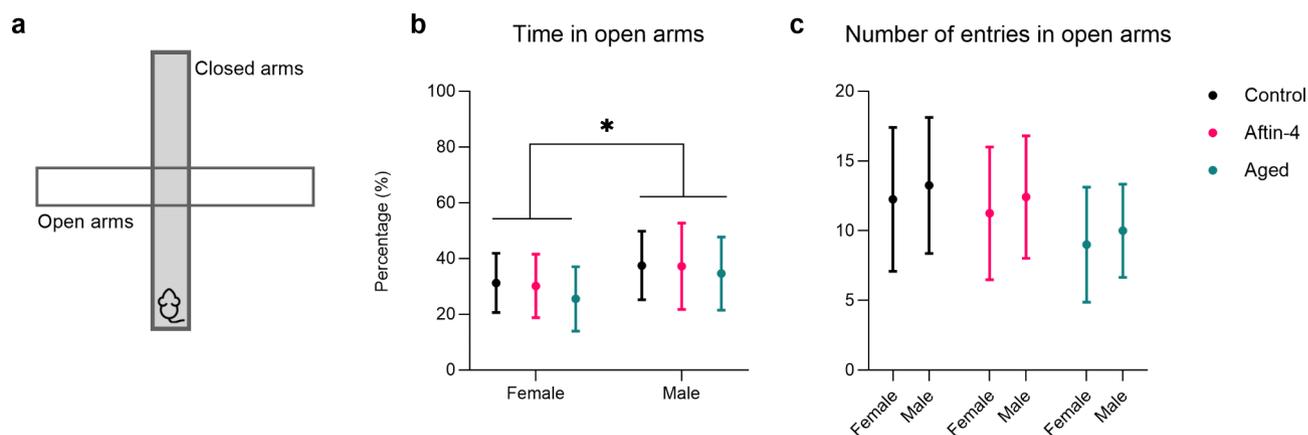


Fig. 1. Behavior of all the groups in the EPM test. **(A)** Schematic representation of the EPM apparatus with two open and two closed arms. **(B)** Time spent in the open arms. Males spent more time in the open arms than females did. * $P < 0.05$ females vs. males under all conditions as assessed using two-way ANOVA. **(C)** Total number of entries into the open arms in the EPM test. The control and aftin-4-treated mice entered the open arms more frequently than the aged mice did, but the difference was borderline significant ($P = 0.055$) according to two-way ANOVA. The data are expressed as the means \pm SDs. Aftin-4 group ($n = 12$ females, 12 males), control group ($n = 12$ females, 12 males), and aged group ($n = 11$ females, 10 males).

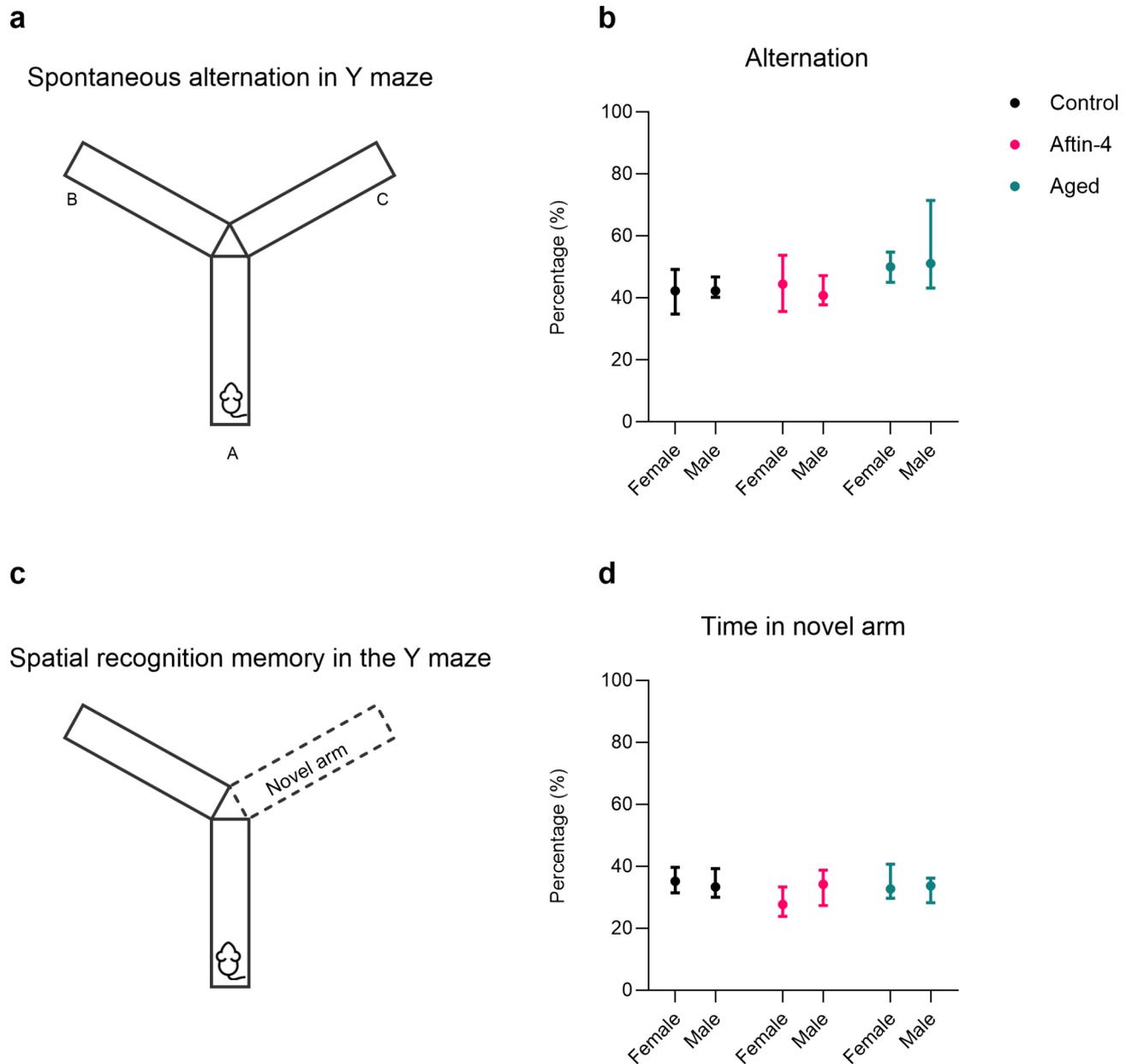


Fig. 2. The behavior of all the groups in two different Y-maze tests. **(A)** Schematic representation of the spontaneous alternation test in the Y-maze. **(B)** Percentage of spontaneous alternations. Behavior was comparable among all groups; no significant differences were found. **(C)** Schematic representation of the spatial recognition memory test in the Y-maze. **(D)** Percentage of time spent in the new arm after the 1-hour interval. All groups explored the new arm for a similar percentage of time. The data are presented as medians and interquartile ranges. Aftin-4 group ($n=12$ females, 12 males), control group ($n=12$ females, 12 males), and aged group ($n=11$ females, 10 males).

effects of condition. Normally distributed data were analyzed via one-way analysis of variance (ANOVA) with condition as the dependent variable. If the data were not normally distributed, the nonparametric Kruskal-Wallis test was performed. The data for the three-chamber sociability test and the distance traveled in the Y-maze (spatial recognition test) were transformed to logarithmic values to obtain normally distributed data. The details of the analyses are presented in Table 1.

Measurement of the levels of markers of oxidative stress

Two-way ANOVA of the MDA level data revealed a simple main effect of the condition ($F(2, 63) = 4.53, p = 0.014$) on the extent of lipid peroxidation. The average MDA concentration in the brains of aftin-4-treated mice was 0.3 units lower than that in the brains of control mice. We observed no interaction between sex and condition ($F(2, 60) = 0.30, p = 0.73$).

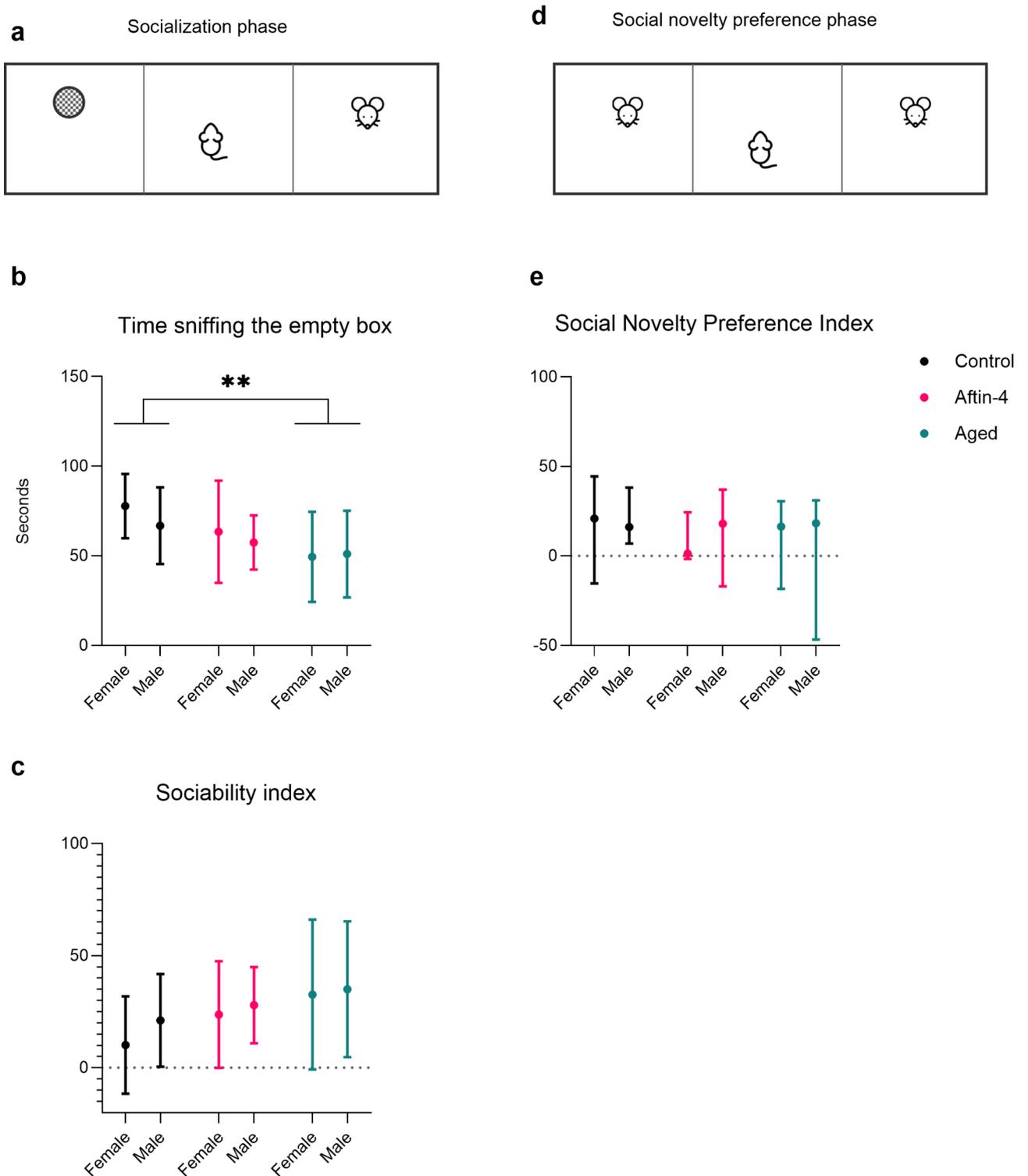


Fig. 3. Behavior of all the groups in the three-chamber sociability test. **(A)** Schematic representation of the first socialization phase. **(B)** Time spent sniffing the empty box by the animals during the first phase. The animals in the aged group spent less time exploring the empty box than did the animals in the control group. $**P < 0.01$ aged mice vs. the control group by two-way ANOVA followed by Tukey's post hoc analysis. **(C)** SI. No real difference was found between the mean values, although the mean SI tended to be greater in aged animals ($p = 0.05$). **(D)** Schematic representation of the second phase, the social novelty preference phase. **(E)** No difference was found between the groups in terms of the SNI. The data are presented as the means \pm SDs, except for the SNI data (shown as medians and interquartile ranges). Aftin-4 group ($n = 11$ females, 12 males), control group ($n = 12$ females, 12 males), and aged group ($n = 11$ females, 10 males).

Test		Experimental groups			Statistics	Post hoc test
		Control (n = 24)	Aftin-4 (n = 24) *EPM test (n = 23) *SOC test (n = 23)	Aged (n = 21)		
EPM test	Distance traveled (m)	0.82 ± 0.24	0.84 ± 0.27	0.62 ± 0.15	F (2, 65) = 6.00 p = 0.004	Control vs. aged p = 0.0114 Aftin-4 vs. aged p = 0.0083
	Speed (cm/s)	0.3 ± 0.1	0.3 ± 0.2	0.2 ± 0.0	H (2) = 11.67 p = 0.003	Control vs. aged p = 0.0077 Aftin-4 vs. aged p = 0.0093
Y-maze test	Distance traveled (m)	1.92 ± 0.54	1.84 ± 0.39	1.29 ± 0.26	F (2, 66) = 14.57 p < 0.0001	Control vs. aged p < 0.0001 Aftin-4 vs. aged p = 0.0001
	Speed (cm/s)	0.4 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	H (2) = 19.48 p < 0.0001	Control vs. aged p = 0.0001 Aftin-4 vs. aged p = 0.001
Three-chamber sociability test	Distance (m)	20.90 ± 5.69	19.95 ± 3.35	17.79 ± 4.41	F (2, 65) = 3.11 p = 0.055	Control vs. aged p = 0.06
	Speed (cm/s)	3.48 ± 0.94	3.33 ± 0.55	2.967 ± 0.74	F (2, 65) = 3.14 p = 0.049	Control vs. aged p = 0.06
Y-maze spatial recognition test	Distance traveled (m)	1.20 ± 0.37	1.23 ± 0.32	0.84 ± 0.29	F (2, 66) = 11.63 p < 0.0001	Control vs. aged p = 0.0004 Aftin-4 vs. aged p = 0.0001
	Speed (cm/s)	0.4 ± 0.1	0.4 ± 0.2	0.3 ± 0.1	H (3) = 15.18 p = 0.0005	Control vs. aged p = 0.0042 Aftin-4 vs. aged p = 0.0010

Table 1. Distance traveled (m) and average speed (cm/s) in each behavioral test. The data are presented as the means ± SDs or medians ± interquartile ranges for nonnormally distributed data.

The Kruskal-Wallis test revealed that, on average, SOD levels were 26.3 units higher in control females than in control and aged males ($H(5) = 21.96, p = 0.0005$). The difference in GPx activity was significant ($H(5) = 40.17, p < 0.0001$), and multiple comparison tests revealed that females had greater GPx activity in their blood than did males. Figure 4 shows the data graphically.

Plasma A β 1–40 and A β 1–42 concentrations

To determine whether plasma A β concentrations differ based on condition or sex, we measured plasma A β 1–42 concentrations using ELISA kits. The analysis revealed similar plasma A β 1–40 concentrations ($H(5) = 7.38, p = 0.1939$). However, because the results were not statistically significant, the data are not shown. Two-way ANOVA of the A β 1–42 level data revealed main effects of sex ($F(1, 60) = 23.87, p < 0.001$) and condition ($F(2, 60) = 3.5, p = 0.036$). Tukey's post hoc test revealed that A β 1–42 levels were lower in all females in each group. Figure 5 shows the data graphically.

Preliminary qualitative analysis of glial fibrillary acidic protein (GFAP) expression

Western blot analysis of protein samples from mice in the aftin-4-treated, control, and aged groups using an anti-GFAP antibody revealed the presence of a protein with a size of approximately 50–55 kDa, which is consistent with the relative molecular weight of GFAP reported in the literature (50 kDa). The Western blots also revealed four strong bands and seven weaker bands, with similar patterns of differences in expression between the groups. However, the intensity of the band for the sample from one male aftin-4-treated mouse stood out as being stronger, and the intensities of the protein bands in the samples from the aged mice were also slightly stronger. Figure 6 shows the final blot. All the preliminary blots are available in the Supplementary Material.

Discussion

A reliable conventional (nontransgenic) animal model of cognitive decline would be a useful tool for preclinical drug testing. Therefore, we performed a comparative analysis of two potential models, namely, the aftin-4-treated mouse model and the aged mouse model, to evaluate their research value. We included both sexes in the study; assessed cognitive performance and behavioral symptoms; and analyzed SOD and GPx levels in the blood, MDA and GFAP expression in the brain, and A β levels in the plasma. We administered aftin-4 i.p. 14 days before the start of the behavioral tests, and 15- to 16-month-old mice that received only vehicle served as aged mice. Cognitive deficits typically include impairments in short-term memory and spatial perception and psychological symptoms such as anxiety and a lack of social desire. To assess all aspects of these behaviors, the animals were subjected to four different behavioral tests.

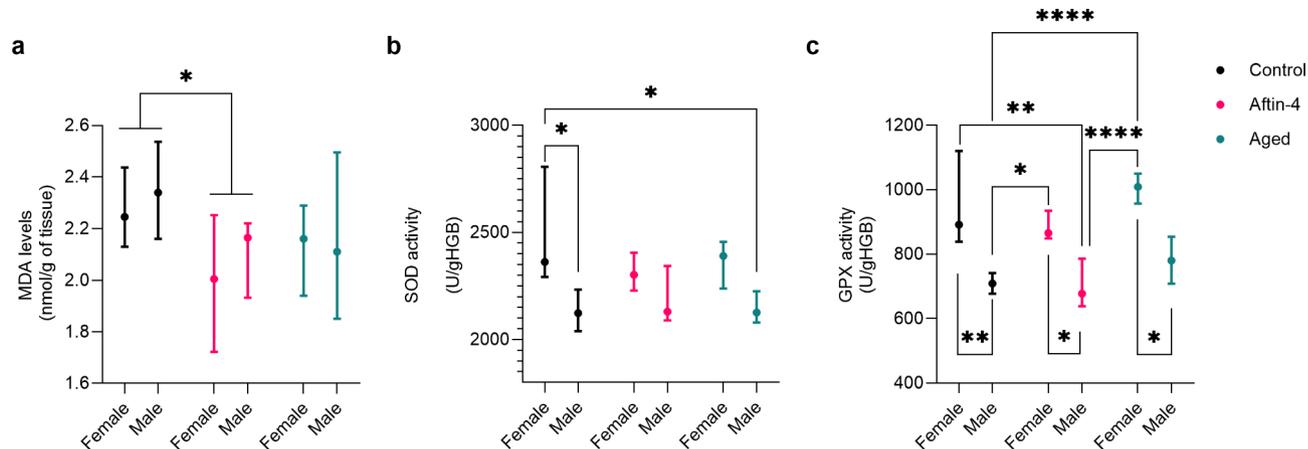


Fig. 4. Changes in the levels of oxidative stress markers in the cerebral cortex and blood of mice. **(A)** MDA levels. $*P < 0.05$ for aftin-4-treated mice vs. control mice by two-way ANOVA followed by Tukey's post hoc test. Aftin-4 group ($n = 12$ females, 12 males), control group ($n = 12$ females, 12 males), and aged group ($n = 11$ females, 10 males). **(B)** SOD activity. $*P < 0.05$ female control mice vs. male control mice and male aged mice according to the Kruskal-Wallis test followed by Dunn's post hoc test. Aftin-4 group ($n = 12$ females, 11 males), control group ($n = 11$ females, 12 males), and aged group ($n = 11$ females, 10 males). **(C)** GPX activity. $**P < 0.01$ control females vs. control males; $**P < 0.01$ control females vs. aftin-4-treated males; $*P < 0.05$ aftin-4-treated females vs. control males; $*P < 0.005$ aftin-4-treated females vs. aftin-4-treated males; $****P < 0.0001$ aged females vs. control males; $****P < 0.0001$ aged females vs. aftin-4-treated males; $*P < 0.05$ aged females vs. aged males using the Kruskal-Wallis test followed by Dunn's post hoc test. The data are presented as medians and interquartile ranges.

Our results revealed no interaction effect between sex and condition, indicating consistent effects of both variables at all levels. Notably, the conditions had similar effects on males and females, supporting the generalizability of the observed sex-specific effects.

The goal of our study was to evaluate the cognitive ability of the observed model mice. Thus, the EPM test was used to assess anxiety-like behavior. Our results indicate that anxiety levels did not change in males or females treated with aftin-4, suggesting that aftin-4 has no effect on brain processes related to anxiety. Aged mice presented slightly increased anxiety-like behavior; however, the difference between the groups did not reach statistical significance (Fig. 1). Increased anxiety could be one of the concomitant factors of aging and affect performance in other behavioral tests, but the results of studies examining this topic are inconsistent. For example, research by Shoji and Miyakawa¹⁵ revealed no changes in the behavior of aged C57BL/6J mice in the EPM test, whereas aged mice spent less time in the open arms than younger mice did, suggesting increased anxiety in aged mice. In contrast, Yanai and Endo¹⁶ reported increased anxiety in aged mice only under light conditions in the open field test, whereas no differences were observed in the dark. In addition, they reported increased anxiety only in 22-month-old mice compared with other cohorts of mice that were similar in age (12 and 18 months) to our mice¹⁶. Similarly, inconsistent and contradictory results regarding anxiety have been reported in studies with rodent models of AD, as summarized by Pentkowsky et al.¹⁷. Regardless, for a complete analysis of anxiety, additional tests in addition to the EPM test should be used, and mice should be tested under different conditions. This could be the focus of a future study.

In the Y-maze test, all the mice exhibited similar behaviors (Fig. 2). This result was unexpected, especially for the aftin-4 group, because Meunier et al.¹⁴ reported a significant decrease in the percentage of spontaneous alternations 14 days after treatment with 30 mg/kg i.p. aftin-4, a protocol identical to ours. However, several factors could contribute to the observed discrepancy. First, Meunier et al.¹⁴ conducted their experiments using male Swiss OF-1 mice that were singly housed at the time of testing. In contrast, our study used C57BL/6J mice that were housed in pairs throughout the experimental period. Different strains of mice can exhibit different behavioral patterns¹⁸. Second, the Y-maze test data were not normally distributed. This could have been due to the variability between the animals, which was greatest in the aged males. This finding suggests that the effect of aging depends on the individual, as previously reported^{19,20}.

In the three-chamber sociability test, aged mice sniffed the empty cup less often than did the aftin-4-treated and control mice, but the SIs were similar among the groups (Fig. 3). Very few studies have used the three-chamber test to analyze the sociability of aged mice. Shoji and Miyakawa reported similar conclusions as we did, and in the same study, the social interaction test revealed no differences between 2- and 17-month-old mice¹⁵. They reported that 22-month-old mice had a greater duration of sniffing per contact than younger mice did, suggesting that social behavior was affected only at an older age¹⁵. In contrast, in a previous study, Shoji et al.²¹ reported that social interaction decreased with age when different groups of mice aged 2 to 12 months were compared.

In the second phase of the three-chamber sociability test, social novelty preference was comparable among all groups of mice, with no group showing a greater preference for a stranger. This lack of differences suggests that

Levels of A β 42 in plasma

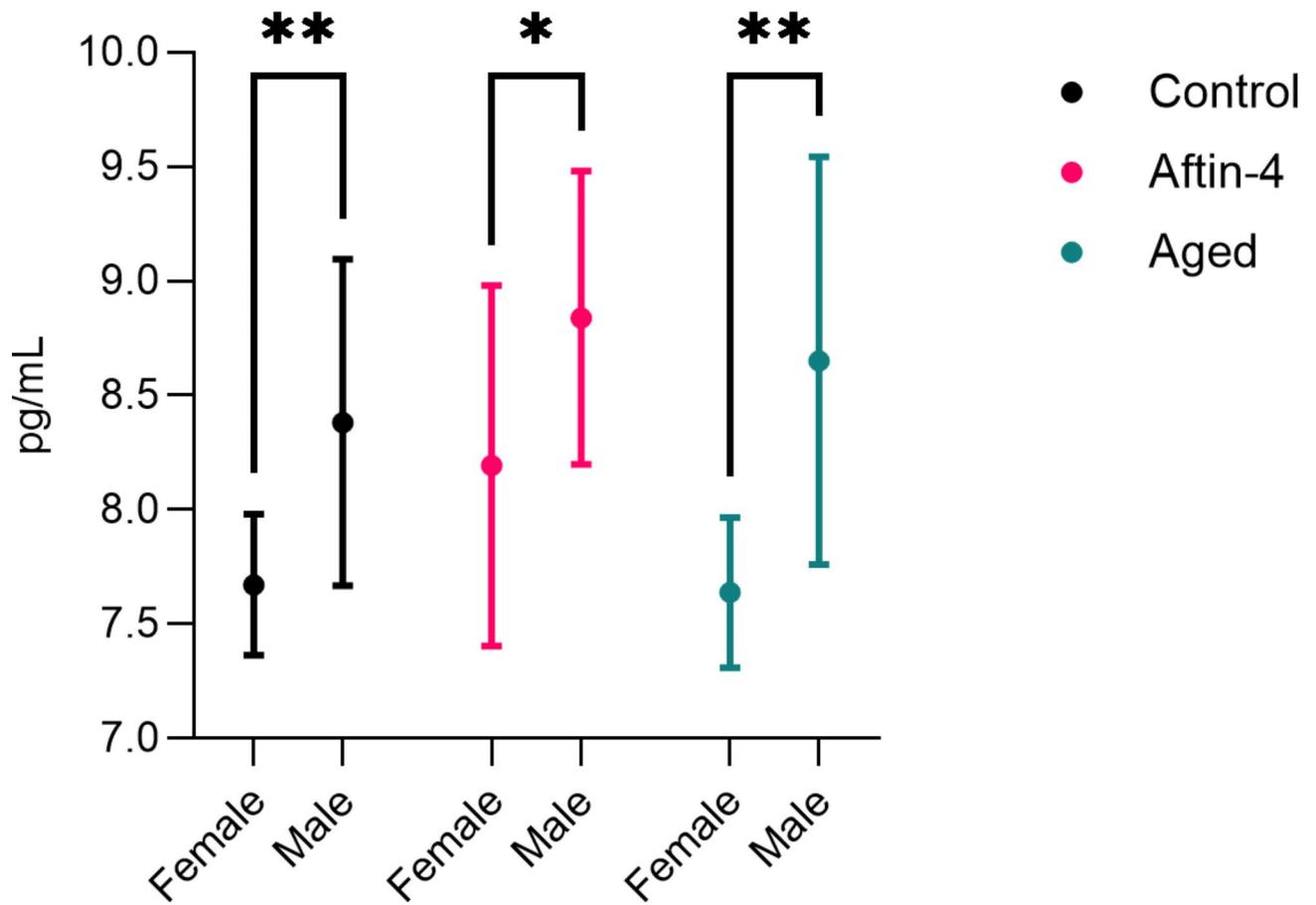


Fig. 5. Plasma A β 1-42 levels in mice. *** $P < 0.01$ control females vs. males; * $P < 0.05$ aftin-4-treated females vs. males; ** $P < 0.01$ aged females vs. males by two-way ANOVA followed by Tukey's post hoc test. Aftin-4 group ($n = 12$ females, 11 males), control group ($n = 12$ females, 12 males), and aged group ($n = 9$ females, 10 males).

the 15- to 16-month-old mice presented social skills similar to those of middle-aged mice. Previous studies have reported an age-dependent decline in social interaction in both wild-type mice and models of AD²¹⁻²⁴. However, our results challenge this assumption, as the mice in our study did not show significant differences in the sociability test. An alternative hypothesis is that the simultaneous requirement to navigate a novel environment and interact with a stranger during the three-chamber sociability test may complicate the interpretation of the results²⁴. Furthermore, our study suggested that aftin-4 has no effect on social behavior, as the behavior of aftin-4-treated mice was similar to that of control mice.

In addition, we monitored basic locomotor activity, distance traveled, and average speed in each behavioral test. The locomotor activity of the mice treated with aftin-4 was comparable to that of the control mice, suggesting that aftin-4 treatment had no effect on motor function. However, the aged mice were slower and moved a shorter distance in the EPM and Y-maze tests than the control and aftin-4 mice (Table 1). The activity of the aftin-4-treated mice was comparable to that of the control mice in all four tests. Our results are consistent with many studies investigating the effect of age on locomotor activity^{9,16,21}. Although it is theoretically possible that the observed decrease in locomotion in aged mice could affect their performance in behavioral tests, our results, together with those of previous studies, suggest that this decrease is not the main factor²⁵⁻²⁷. Research shows that when cognitive impairments are not strongly correlated with locomotor function, the observed behavioral differences are due to specific cognitive deficits rather than reduced locomotion^{25,26}. Our brief analysis (data not shown) revealed weak to moderate correlations between cognitive performance and distance traveled, suggesting that reduced locomotion alone is unlikely to explain the observed cognitive deficits. These impairments are likely to be influenced by multiple factors, including neurological, biochemical and environmental changes, as previous studies have shown^{21,26,27}. Furthermore, many of the observed performance deficits appear to be attributed to a reduced urge to explore rather than reduced muscle strength or motor coordination. This finding is consistent with findings that aged C57BL/6 mice show lower activity in several behavioral tests designed to measure anxiety-like behavior, such as the open field test, the elevated plus maze, and social interaction

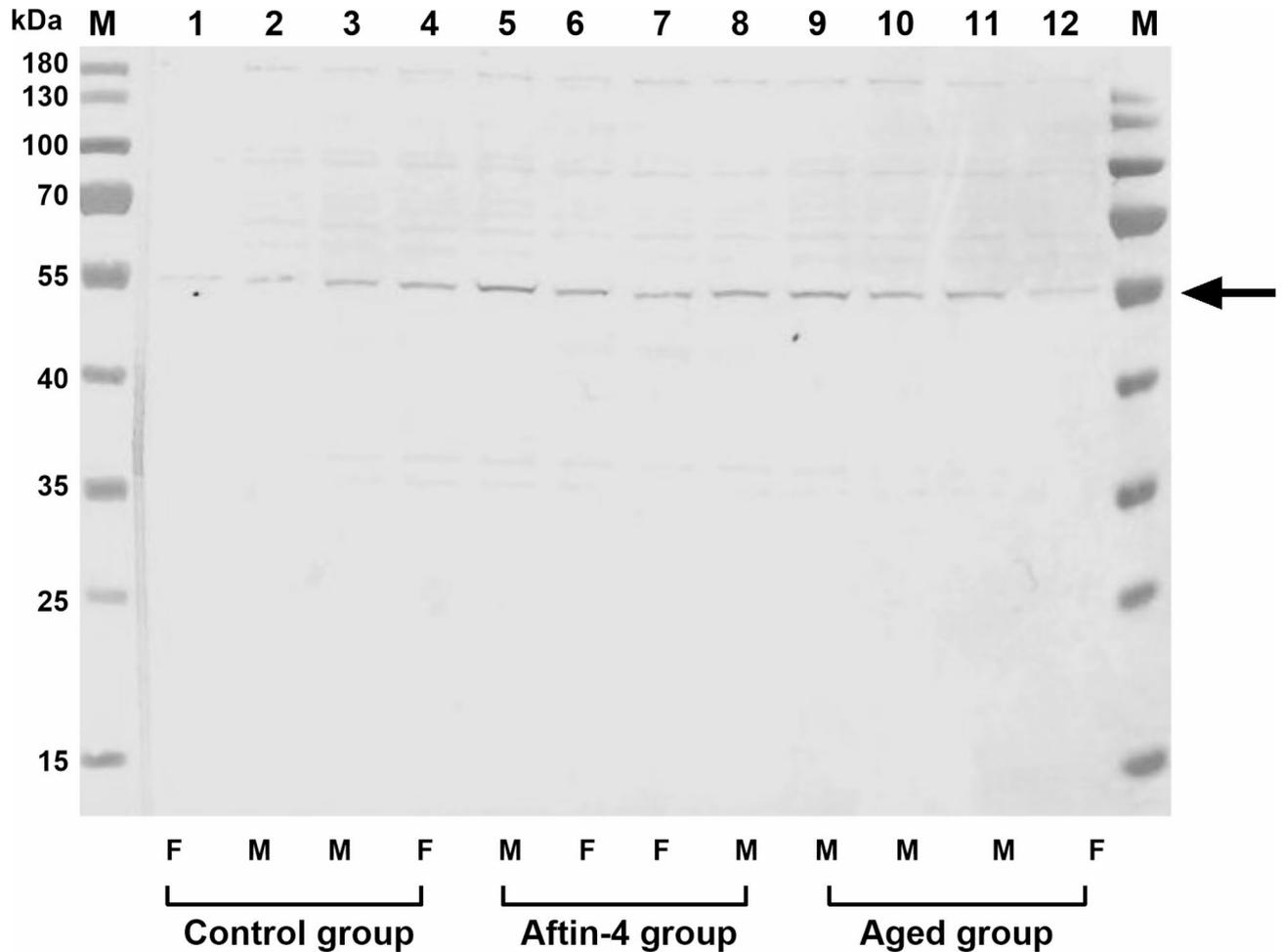


Fig. 6. Western blot analysis of supernatants from cerebral cortex homogenates. Lane 1, supernatant from females in the control group; lane 2, supernatant from males in the control group; lane 3, supernatant from males in the control group; lane 4, supernatant from females in the control group; lane 5, supernatant from males in the aftin-4 group; lane 6, supernatant from females in the aftin-4 group; lane 7, supernatant from females in the aftin-4 group; lane 8, supernatant from males in the aftin-4 group; lanes 9, 10, 11, supernatants from males in the aged group; lane 12, supernatants from females in the aged group; lane M, PageRuler™ prestained protein ladder (26616, Thermo Scientific™). The arrowhead on the right indicates an approximately 55 kDa band representing GFAP.

assessments, as evidenced by their shorter distances traveled and reduced time spent in central areas, indicating increased anxiety. In contrast, in the less anxiety-provoking dark chamber of the light-dark test, there was no significant difference in distance traveled compared to the younger mice, suggesting that anxiety levels may not influence behavior in this less challenging environment^{21,24}.

After behavioral testing, the animals were euthanized under isoflurane inhalation anesthesia, and tissue and blood samples were collected for molecular analyses. Next, we determined the concentration or activity of markers of oxidative stress. As shown in Fig. 4, MDA levels in brain tissue were lower in aftin-4-treated mice of both sexes than in control mice, and no difference was observed between aftin-4-treated mice and aged mice. MDA is a marker for lipid peroxidation in the brain. This result was not expected because MDA is formed by the peroxidation of omega-3 and omega-6 fatty acids; contrary to expectations, aftin-4 does not lead to an increase in lipid peroxidation in brain tissue²⁸. Given that aftin-4 is known to increase A β 1–42 levels by binding to mitochondrial proteins and disrupting the mitochondrial and cellular environment, further investigations of mitochondrial damage are warranted. Methods such as electron microscopy to visualize the mitochondrial ultrastructure, measurement of the mitochondrial membrane potential (JC-1), and assessment of reactive oxygen species production with fluorescent probes can be used^{14,29}. Furthermore, we found no significant differences in MDA levels between control mice and aged mice, which was also reported by Aliahmat et al.; however, some studies reported higher MDA levels in aged mice^{30,31}. We used the same strain of mice as Aliahmat et al., and the ages of the animals used in both studies were comparable. Thus, the MDA levels observed in our study are consistent with those reported in previously published studies. The results show that lipid peroxidation in 18-month-old mice of both sexes is not severe enough to affect MDA levels³¹.

We also determined the activity of the intracellular antioxidant enzymes SOD and GPx, which are indicators of oxidative stress. We found no effect of aftin-4 treatment or age on the activity of either enzyme, suggesting that the degree of antioxidative activity was comparable among the animal groups. However, the activity of both enzymes increased in female animals independent of treatment (Fig. 4). This finding is consistent with established research showing that antioxidant enzyme activity is generally greater in females than in males of various species, including humans³². The higher antioxidant activity observed in females, characterized by increased levels of enzymes such as SOD and GPx, enhances their ability to fight reactive oxygen species. This increased antioxidant capacity likely contributes to better neuroprotection and may be associated with longer life expectancy³³. This effect is thought to be at least partly related to the protective role of estrogen^{32,34}. Interestingly, our study examined ovariectomized females, which makes the issue even more complex. Although some studies suggest that ovariectomy does not significantly affect SOD or GPx activity, the relationship between estrogen and these enzymes is not completely understood, and other mechanisms may also play a role^{34–36}. In addition, males tend to have greater levels of inflammation, possibly due to increased accumulation of oxidative damage and a weaker inflammatory response to acute challenges. Importantly, the differences between the sexes, although significant, are often subtle and may vary by tissue or context^{32,35,36}. This highlights the need for further research to clarify these hormonal and sex differences in terms of oxidative stress and antioxidant activity. In addition, we determined the concentrations of A β 1–40 and A β 1–42, which are markers of neurodegeneration, in blood plasma. It was reported previously that aftin-4 has no effect on A β 1–40 formation; therefore, in the aftin-4 group, an increase in A β 1–40 levels was not expected^{13,14}. Furthermore, Meunier et al.¹⁴ reported the formation of A β 1–42 after intracerebroventricular administration of aftin-4. Although our study revealed increased plasma A β 1–42 concentrations in animals treated with aftin-4 (Fig. 5), plasma A β 1–42 levels were not significantly greater in the treated animals compared with the controls. These findings suggest that the intraperitoneal administration of aftin-4 does not increase A β formation. We believe that further studies should focus on kinetic and dose-response experiments to reveal the distribution of aftin-4 after administration via different routes and the dose-dependent effects of the drug. Similarly, A β 1–40 levels in aged mice were comparable to those in control mice; however, Ahlemeyer et al.⁸ reported higher total A β levels in mice of the same strain and of similar ages. Given that A β 1–42 concentrations in brain tissue and plasma are correlated³⁷, increased plasma A β 1–42 plaque formation was expected. However, in our study, this was not confirmed. Additionally, we were interested in GFAP expression in brain tissue because increased GFAP expression indicates the response of astrocytes to CNS damage^{38,39}. We measured GFAP expression in brain tissue via preliminary Western blotting, which provided qualitative results. As shown in Fig. 6, more intense bands were observed in aftin-4-treated and aged mice than in control mice, indicating a potentially stronger glial response, especially in males. This finding is consistent with the results of Meunier et al.¹⁴ The authors reported a strong increase in GFAP immunolabeling in the cortex and limbic structures, and this finding was confirmed by ELISA. Our analysis of GFAP expression was preliminary and qualitative and aimed to provide initial insights. Although a semiquantitative approach with normalization against a housekeeping protein such as β -actin would provide more detailed information, this method has limitations, including variability in loading and transfer rates and nonlinear signal detection⁴⁰. We chose to use a qualitative method to confirm the presence and integrity of the target protein, which serves as an essential preparatory step for more precise techniques, such as mass spectrometry. In addition, recent studies suggest that astrocyte proliferation is not a uniform response to CNS damage. In inflammation and neurodegeneration, it is often restricted despite increased GFAP expression⁴¹. Astrocyte reactivity to CNS damage is heterogeneous, suggesting that a broader spectrum of markers and functional indicators is required to obtain a more differentiated picture of glial activity^{42,43}. In astrocytes, pro-inflammatory responses are often characterized by the rapid release of cytokines (e.g. IL-1 β , IL-6, TNF- α) and chemokines (e.g. CCL2, CXCL)^{44,45}. Complement component 3 (C3) is also upregulated in neurodegenerative diseases and is expressed by both astrocytes and microglia⁴⁵. When studying various pathophysiological CNS conditions, it was found that GFAP/C3-positive astrocytes were present in the prefrontal cortex and hippocampus in AD patients⁴⁶. Pathological changes in astrocyte function may include increased GABA synthesis, which is associated with upregulation of MAO-B^{45,47–49}. Finally, microglial activation could be assessed using markers such as ionized calcium-binding adaptor molecule 1 (Iba1), phagocytosis-associated marker (CD68) and as well as major histocompatibility complex (MHCII) expression, which reveal structural and lysosomal changes in reactive microglia^{42,50}. In the end, methods such as in vivo imaging and transcriptome analysis would be beneficial to gain a comprehensive understanding of the changes.

Aftin-4, a purine derivative, has the potential to trigger neurodegeneration^{13,14}. Our study revealed that, compared with the control and aged animals, the animals treated with aftin-4 did not exhibit any detectable behavioral changes. Despite previous reports indicating that systemic injection of 30 mg/kg aftin-4 leads to memory and learning deficits as well as neurodegeneration, our results suggest that aftin-4 did not cause neurodegeneration that was severe enough to be visibly observed or that it did not cause neurodegeneration when it was administered via the chosen route or to the selected mouse strain¹⁴. Meunier et al.¹⁴ demonstrated more pronounced neurodegeneration with intracerebroventricular administration of aftin-4. However, intracerebroventricular administration is more invasive and involves a surgical procedure with greater risks and stress, especially in aged animals. In contrast, intraperitoneal injection was chosen for our study because it has been shown to induce relevant effects, such as oxidative stress and learning deficits, and improve animal comfort. Furthermore, C57BL/6J mice, the strain employed in this study, are commonly used in aging and Alzheimer's research, and there is no evidence that they are insensitive to Aftin-4, as this has not been specifically tested. We chose C57BL/6J mice for our experiments because we wanted to ensure the general applicability of our results. As only one in vivo experiment involving aftin-4 has been reported in the literature, concerns about its efficacy are warranted. Consequently, it may be of limited use in the construction of a robust pharmacological animal model. Additionally, oxidative stress markers and amyloid-beta levels do not indicate the occurrence

of pathological processes. The only parameter in aβ4-treated mice that indicated neurodegeneration was increased GFAP levels. However, in the absence of compelling evidence, we assume that the aβ4-induced model lacks potential value. Investigating the effects of chronic treatment with lower doses and different delivery methods, including oral administration, is critical. In addition, comprehensive studies should be performed to investigate the effects of aβ4 in different strains and include detailed behavioral and biochemical analyses to completely understand its efficacy and mechanisms. In contrast, aged animals, particularly males, showed some deviations from normal behavioral patterns, suggesting the onset of cognitive decline. This finding was supported by elevated GFAP levels. However, the lack of robust findings prevents definitive confirmation. Analyzing animal behavior is one of the most complex and difficult tasks in research, as numerous variables influence the results, some of which may go unnoticed. To obtain a comprehensive understanding of the cognitive performance and behavioral symptoms of mice and to clarify whether aged mice present signs of dementia, the use of a more comprehensive set of complex tests is essential. This would allow a more comprehensive investigation and comparison of these models.

We are aware that one limitation of our study was the use of ovariectomized females which led to a decrease in estrogen levels^{34,51–53}. We used them in an attempt to control for fluctuations noted during the estrous cycle. However, another option would have been to castrate the males and administer hormone implants to both sexes to fully control for hormonal influences. However, we chose to focus on the conditions studied without introducing the additional complexity of hormone replacement. In addition, the organizational effects of sex steroid hormones during perinatal life have long-lasting, irreversible effects on adult physiology and behavior, particularly on the expression of sex-specific traits. During fetal and early postnatal development, estrogen levels are high, but the ovaries are not the primary source of these hormones, as estrogens likely originate from extraovarian sources such as the adrenal glands or the mother^{54–56}. It is possible that different results would have been observed if ovariectomy had been performed at a younger age, which would have allowed the study of long-term hormone deprivation in aged mice⁵⁷. Importantly, behavior is influenced not only by hormones but also by a complex interplay of internal states (such as immune system function and genetic predispositions) and individual behavioral preferences. Recent research has shown that sex-specific differences in the variability of traits are not the same for all traits. For example, male mice show greater variability in their morphological traits, whereas female mice show greater variability in their immunological traits⁵⁸. In addition, the measurement of luteinizing hormone and estradiol levels would help clarify the effects of ovariectomy on the hypothalamic–pituitary–gonadal axis and its role in the development of Alzheimer’s disease. Although this decision may complicate the discussion of traditional sex differences, we believe that the overall results would remain consistent, as no obvious interaction effect was found. In future studies, it would be beneficial to include both sexes without controlling for hormones to capture the full spectrum of biological and behavioral variability^{20,58}.

A second limitation of our study is that some research suggests that significant cognitive impairment in rodents typically does not occur before approximately 22 months of age. However, keeping animals until this age is challenging owing to the increased prevalence of health problems unrelated to cognition, which can lead to animal losses and smaller sample sizes, as noted in the study by Yanai and Endo¹⁶.

In conclusion, we emphasize that the search for conventional animal models of cognitive decline should continue. The results of our study revealed that the intraperitoneal administration of aβ4 to wild-type C57BL/6J*RccHsd* mice of both sexes did not result in cognitive decline, whereas increased GFAP levels were indicative of neurodegenerative processes. Aged mice of the same strain presented a nonsignificant decrease in cognitive function and increased GFAP levels. The levels of markers for oxidative stress and Ab did not significantly differ between the two animal models and the control group.

Methods

Animals

The experimental animals used in this study were wild-type C57BL/6J*RccHsd* mice bred at the Laboratory of Animal Genomics, Faculty of Veterinary Medicine, University of Ljubljana, or purchased from Envigo RMS Holding Corp. The mice were housed under standard conditions: a 12:12 dark–light cycle (LED lights, CCT 3000k, no more than 25 lx above the cage), temperature of 21–24 °C and humidity of 50–60%. The mice were fed an irradiated rodent diet (Teklad Global 16% Protein Rodent Diet[®], BN 2916, Envigo, Udine, Italy) and drinkable tap water *ad libitum*. The mice were conventionally bred in open polycarbonate cages (Tecniplast, Buguggiate, Italy) on wood chip bedding (Lignocel[®], Rettenmaier & Söhne, Rosenberg, Germany). To create a conducive environment and prevent aggressive behavior in adult males, the animals were housed in pairs consisting of one ovariectomized female and one male. This arrangement was intended to avoid isolation.

Ethics approval

All animal experiments were conducted in accordance with the European Community Council Directive on the use of animals in research (EU Directive 2010/63/EU). The experimental design was evaluated by the National Ethics Committee for Animal Experiments and approved by the Administration of the Republic of Slovenia for Food Safety, Veterinary and Plant Protection (document no. U34401-10/2021/8, with amendments U34401-10/2021/13 and U34401-10/2021/1). Every effort was made to minimize animal suffering and to use the minimum number of animals necessary to obtain reliable results. The work was performed in accordance with the ARRIVE guidelines.

Experimental design

A total of 69 mice were included in the study. The number of animals used in the study is based on calculations assuming an expected difference of 30–35% in the parameters of the behavioral tests between the animals in the control and experimental groups with $\alpha=0.05$ and a power of 0.8⁶⁰. The online sample calculation program

ClinCalc was used to determine that 11–12 animals should be included in each group^{60,61}. The mice were divided into groups by two factors, one with two levels (sex) and one with three levels (condition). The cages were randomly assigned to different conditions, with each animal serving as an experimental unit (total $n=69$). To minimize bias, the researcher responsible for conducting the study was kept blinded to the group assignments. Fourteen days after the administration of aifin-4 or vehicle, the mice were subjected to a series of behavioral tests. One week prior to the behavioral tests, the mice were habituated to handling. The behavioral test began with the elevated plus-maze (EPM) test, followed by the Y-maze (spontaneous alternation) test, the three-chamber sociability test, and finally the spatial recognition memory test in the Y-maze. The behavioral experiments were conducted in daylight, under light conditions of 10–20 lx (depending on the maze setting), and at the same time each day in an experimental room within the animal facility. The experimental design is graphically presented in Fig. 7.

Ovariectomy in females

All female animals were ovariectomized at least one month before the behavioral tests. Ovariectomy was performed as previously described⁶². All animals were examined postmortem to verify successful ovariectomy.

Treatment and drug administration

Aifin-4 was a gift from the Faculty of Pharmacy, University of Ljubljana. It was dissolved in 5% DMSO in saline to a concentration of 6 mg/mL and administered intraperitoneally (ip) at a dose of 30 mg/kg in a final volume of 100 μ l/20 g body weight 14 days before the start of the behavioral tests. The control and aged groups received only vehicle solution.

Behavioral testing

Elevated plus maze (EPM) test

The elevated plus maze (EPM) test was performed to assess anxiety-like behavior as previously described^{63,64}. The maze was made of black plexiglass; had two open and two closed arms, each 30 cm long and 5 cm wide; and was surrounded by 20 cm high walls. During the test, the maze was elevated 50 cm above the floor. The mice were placed in the center of the maze and allowed to move freely for 5 min. The number of entries into and the time spent in the open and closed arms, as well as the total distance traveled and average speed, were recorded via ANY-maze software (Stoelting Europe, Dublin, Ireland). The maze was cleaned with 70% ethanol after each trial.

Y-maze (spontaneous alternation) test

The spontaneous alternation percentage, which is a measure of spatial working memory, can be assessed by allowing mice to freely explore all three arms of the Y-maze⁶⁵. The gray Plexiglas Y-maze consisted of three

TIMELINE OF THE EXPERIMENT

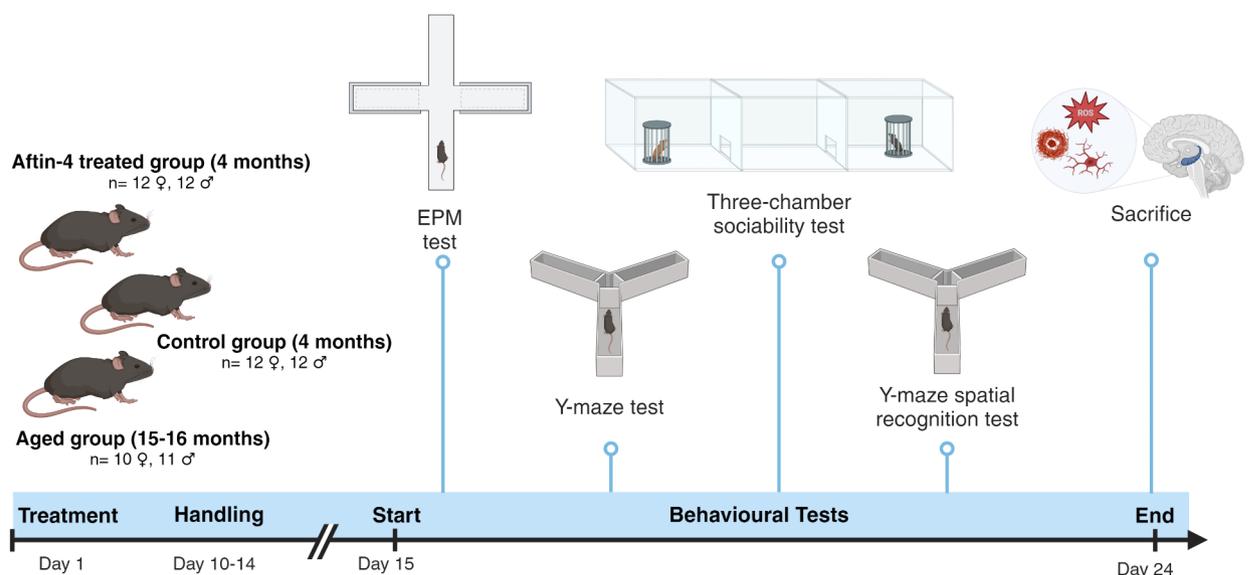


Fig. 7. Timeline of the experiment. Adapted from “Behavioral Test for Mice - timeline” using BioRender.com (2024). Retrieved from <https://app.biorender.com/biorender-templates>.

identical arms (35 × 7 × 30 cm) at 120-degree angles to one another. The arms were labeled A, B and C. A mouse was placed in the distal part of the arm labeled C and allowed to freely explore the maze for 8 min. The sequence of arm entries was recorded using the ANY-maze system (Stoelting Europe, Dublin, Ireland). Movement was considered an entry when all four limbs were within the arm. The maze was cleaned with 70% ethanol after each trial. The spontaneous alternation percentage was calculated via the following formula:

$$\text{Spontaneous alternation percentage (\%)} = \left(\frac{\text{number of alternations}}{\text{total number of arm entries} - 2} \right) \times 100$$

Spatial recognition memory test in the Y-maze

Short-term spatial recognition memory was tested with a spatial novelty preference task in the Y-maze, as described previously⁶⁶. This test is based on the natural tendency of rodents to prefer exploring novel environments over familiar environments⁶⁷. The gray Plexiglas Y-maze consisted of three identical arms (35 × 10 × 30 cm) at 120-degree angles to each other. The test consisted of two phases separated by a one-hour interval. A start arm and a familiar arm were assigned for each animal, and the animal was exposed to these two arms in the first phase. The third arm represented the new arm in the second phase (test phase). The assignment of the arms (start arm, familiar arm and new arm) was randomized. Access to the new arm was blocked during the test phase. At the beginning of each trial, an animal was released at the end of the start arm facing the center of the maze. The animal was allowed to freely explore both the start arm and the familiar arm for 5 min. After one hour, during the test phase, the animal was returned to the maze for another 5 min of exploration, during which all arms were accessible. During the intertrial interval, the animals were kept in their home cages in the test room. The maze was cleaned with 70% ethanol after each trial. The time spent in the new, unexplored arm and the distance traveled in the maze were recorded via the ANY-maze system (Stoelting Europe, Dublin, Ireland).

Three-chamber sociability test

The three-chamber sociability test was conducted as previously described⁶⁸ via a three-chambered Plexiglas maze for social or nonsocial stimulation. Social behavior was stimulated with a sexually naïve adult male or female BALB/c mouse, with the sex of the stimulus mouse being the same as that of the tested animal. All the stimulus-exposed mice were habituated to wire cups for several days prior to testing, and none of the test-exposed mice had previously been in contact with the stimulus-exposed mice. The position of the cup (left or right chamber) was randomly alternated between tests to avoid a possible side preference. Each test consisted of three different phases: the habituation phase, a phase in which the mice chose between an empty or stimulus-containing chamber, and a phase in which the mice chose between a known stimulus animal and a new stimulus animal. Behavioral parameters, including the time spent in the chambers, time spent sniffing the cups, and immobility, were assessed via the ANY-maze program. The maze was cleaned with 70% ethanol between tests. The sociability index (SI) and social novelty preference index (SNI) were calculated via Baronio's Eq. 7⁰ as follows:

$$SI = \left(\frac{\text{time spent social} - \text{time spent empty}}{\text{time spent social} + \text{time spent empty}} \right) \times 100,$$

$$SNI = \frac{\text{time spent novel} - \text{time spent known}}{\text{time spent novel} + \text{time spent known}} \times 100$$

Sacrifice and blood and brain collection

Blood was collected under deep isoflurane anesthesia from the sinus venosus in the periorbital cavity via a capillary tube. Blood was collected in a heparinized tube (microsample tube with lithium heparin, Sarstedt AG & Co. KG, Nümbrecht, Germany). A portion of the collected blood was separated for biochemical analyses, and the remaining blood was centrifuged at 3,000 rpm for 15 min at 4 °C to obtain plasma. Blood was also collected in an EDTA tube (Microvette® 200 K3 EDTA, Sarstedt AG & Co. KG, Nümbrecht, Germany) for measurement of the hemoglobin concentration needed for standardization of GPx and SOD activities. After all behavioral tests and blood collection, the mice were euthanized under 5% isoflurane inhalation anesthesia (Isoflurin®, VetPharma, Barcelona, Spain) and cervical dislocation. All animals from the same cage were sacrificed on the same day. The brain was removed, and the cortex was isolated and then frozen in liquid nitrogen. The brain and plasma samples were stored at -80 °C for further analysis.

Measurement of the activity of glutathione peroxidase (GPx)

GPx activity in whole-blood hemolysates was determined spectrophotometrically with an RX-Daytona Plus (Randox, Crumlin, UK) automated biochemical analyzer via a commercial Ransel kit (Randox Laboratories, Crumlin, UK) according to the methods of Paglia and Valentine⁷⁰. Whole-blood hemolysates were diluted 161-fold with Ransel diluent (Randox Laboratories, Crumlin, UK) prior to analysis. GPx activity is expressed as units per gram of hemoglobin (U/g Hgb). The hemoglobin concentration was determined via dielectric spectroscopy via a scil Vet abc Plus™ hematology analyzer (Scil Animal Care Company GmbH, Viernheim, Germany).

Measurement of superoxide dismutase (SOD) activity

SOD activity in whole-blood hemolysates was determined spectrophotometrically with an RX Daytona Plus (Randox, Crumlin, UK) automated biochemical analyzer via a commercially available Ransod kit (Randox Laboratories, Crumlin, UK) according to the methods of McCord and Fridovich⁷¹. Prior to analysis, whole-blood hemolysate samples were diluted 1:200 with Ransod sample diluent (0.01 mmol/L phosphate buffer, pH

7.0; Randox Laboratories, Crumlin, UK). SOD activity is expressed as U/g Hgb. The hemoglobin concentration was determined via dielectric spectroscopy via a scil Vet abc Plus™ hematology analyzer (scil animal care company GmbH, Germany).

Determination of the extent of lipid peroxidation

Lipid peroxidation was assessed in the cerebral cortex by measuring the malondialdehyde (MDA) concentration using MDA detection kit (Northwest Life Science, Vancouver, WA, USA) according to the manufacturer's instructions. In brief, the samples or calibrators (250 μ L) were mixed with acidic reagent and thiobarbituric acid (250 μ L) and shaken vigorously. The mixture was then incubated at 60 °C for 60 min, followed by centrifugation at 10,000 \times g for 2–3 min. The resulting reaction mixture was transferred to a cuvette, and the absorbance was measured at 532 nm. The extent of lipid peroxidation was quantified, and the results are expressed as μ m MDA/mg protein. Protein concentration was determined via a Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The MDA concentration is expressed in nmol/g brain tissue.

Measurement of the plasma levels of A β 1–40 and A β 1–42

The A β 1–40 and A β 1–42 concentrations in mouse plasma were measured via commercial mouse A β 40 (Cat. JP27720, Immuno-Biological Laboratories Co., Ltd.) and mouse A β 42 (Cat. abx254885, Abbexa LTD, Cambridge, UK) ELISA kits according to the manufacturers' protocols. The absorbance was determined at 450 nm via a Multiskan FC microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). For A β 40, the intra-assay coefficients of variation were 8.4% for low (3.1 pg/mL) and 11.0% for high (12.5 pg/mL) values. For A β 42, the intra-assay coefficients of variation were 16.2% for low (11 pg/mL) and 10.1% for high (100 pg/mL) values.

Western blotting

Glial fibrillary acid protein (GFAP) expression was measured in frozen cortical samples from the mouse brain. The protein samples were homogenized (1:9 ratio) in RIPA buffer (89900, Thermo Fischer Scientific, Waltham, MA, USA) supplemented with protease inhibitor cocktail (11836145001, Roche, Basel Swiss) and then centrifuged at 1600 \times g for 20 min at 4 °C. Then, the supernatant was collected. The protein concentration was determined via a Bio-Rad protein assay according to the manufacturer's protocol (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and a Lambda 25 spectrophotometer (US/VIS spectrophotometer, 4 PerkinElmer, Inc., Waltham, MA, USA). The samples were resuspended in loading buffer (4X Bolt™ LDS Sample Buffer, B0007, Invitrogen™, Waltham, MA, USA) and boiled for 10 min. Equal amounts of proteins (0.5 μ g) were loaded onto a pre-made Bolt™ Bis-Tris Plus Mini Protein Gel (10%, 1.0 mm) (NW00105BOX, Invitrogen™, Waltham, MA, USA), separated in Bolt™ MOPS SDS Running Buffer (B000102, Invitrogen™, Waltham, MA, USA), and then transferred to 0.2 μ m PVDF membranes (22860, Thermo Scientific™, Waltham, MA, USA) (100 V, 45 min). The membranes were blocked in 0.5% (w/v) PVP10 (WXBC6133V, Sigma-Aldrich, St. Louis, MI, USA) in phosphate-buffered saline containing 0.1% Tween 20 (PBST) overnight at 4 °C. The membranes were then washed with PBST and incubated with primary antibody (rabbit GFAP mAb, A19058, 1:2000 dilution, ABclonal, Inc., Woburn, MA, USA) for 2 h, followed by incubation with an HRP-conjugated secondary antibody (goat anti-rabbit IgG (H+L), 1:5000 dilution, 31460, Invitrogen™, Waltham, MA, USA) for 1 h at room temperature. After washing, acetate buffer (pH 5.5) supplemented with 3-amino-9-ethylcarbazole (SLCF4448, Sigma-Aldrich Chemie GmbH, St. Louis, MI, USA) and hydrogen peroxide (K52021409003, Merck KGaA, Darmstadt, Germany) was used to visualize the protein bands.

Statistical analysis

All the results were analyzed and visualized with GraphPad Prism Version 10.0.3 for Windows (GraphPad Software, Boston, MA, USA; www.graphpad.com). The distribution of the data was checked via the Shapiro-Wilk test and residual plots. Normally distributed data were analyzed with two-way ANOVA (with condition and sex as dependent variables) or one-way ANOVA (for locomotor activity data). Kruskal-Wallis ANOVA was used for nonnormally distributed data. Differences between groups (two sexes \times three conditions) were confirmed using Tukey's or Dunn's post hoc test. $p < 0.05$ was considered to indicate statistical significance. The results analyzed with ANOVA are presented as the means \pm SDs, whereas those analyzed using the Kruskal-Wallis test are presented as medians and interquartile ranges. Behavior recorded with the ANY-maze system was briefly reviewed to ensure that the program correctly tracked the experimental animals during the tests. One EPM trial was manually analyzed because the ANY-maze software was unable to correctly track the test animal during the test. Distance and mean speed data could not be manually evaluated; therefore, these data are missing for one male mouse from the aftin-4 group. One female from the aftin-4 group was excluded from the sociability test because it did not meet the inclusion criteria (excessive climbing). A few animals were not included in the analyses of SOD and GPx activity and plasma A β 1–40 and 1–42 levels because of insufficient blood volume or damaged samples.

Data availability

The datasets used and analyzed during current study are available from the corresponding author on reasonable request.

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Author contributions

N.Ž. - study design, lab work, research, analysis, writing and editing; N.G. - study design, research, analysis, writing and editing; A.N.S. - lab work, writing and editing; A.M. contributed new reagents, editing; T.S. - study design, research, writing and editing.

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Declarations

Competing interests

The authors declare no competing interests.

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