HHS Public Access

Author manuscript

Behav Processes. Author manuscript; available in PMC 2024 September 30.

Published in final edited form as:

Behav Processes. 2023 September ; 211: 104929. doi:10.1016/j.beproc.2023.104929.

Aging to 24 Months Increased C56BL/6J Mouse Social Sniffing and Hippocampal Neto1 Levels, and Impaired Female Spatial Learning

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Abstract

Understanding how natural aging impacts rodent performance in translational behavior tests is critical to teasing apart impairments due to age-related decline from neurodegenerative disorder modeling. Reduced neuropilin and tolloid-like 1 (NETO1), an accessory protein of ionotropic glutamate receptors involved in synaptic plasticity, was associated with Alzheimer's disease, yet aging effects on Neto1 remain unclear. For these reasons, our goal was to characterize how Neto1 expression corresponded with social, repetitive, and spatial learning behaviors and stress response across the C57BL/6J mouse lifespan. We measured social preferences in three-chamber tests, and motor stereotypies by marble burying. Cognitive flexibility is typically assessed in the Morris water maze (MWM), wherein C57BL/6J mice exhibit deficits with age. However, fatigue or locomotor impairment may confound interpretation of MWM performance. Therefore, we used a less arduous water T-maze (WTM) to compare spatial learning flexibility in 2, 9–15, and 24-month-old male and female mice to test the hypothesis that deficits would emerge with age. In both sexes, 9–15-month-olds made more chamber entries during social preference tests, while 2-month-olds did less social sniffing than aged mice. No age or sex differences emerged in marble burying or serum corticosterone measurements. In 24-month-olds hippocampal Neto1was increased relative to 2-month-olds, and male cognitive flexibility was strong, while spatial learning and reversal learning of 24-month-old females was impaired in WTM irrespective of Neto1 expression. The WTM is a useful alternative assessment for cognitive flexibility deficits in aged mice, and the role of hippocampal Neto1 in promoting social sniffing is of interest.

Graphical Abstract

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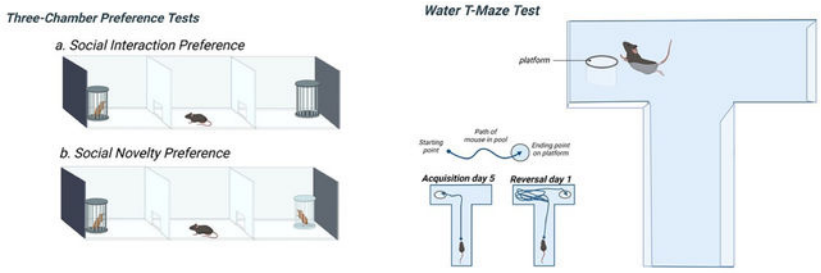

Social preferences of young (2-month-old), middle-aged (9-15-month-old) and aged (24-month-old) aged male and female C57BL/6 mice toward same sex strangers were compared in three chamber preference for: a. social interaction and b. social novelty tests. Spatial learning was subsequently assessed in the water T-maze (WTM) test. Images created with BioRender.com

Keywords

Alzheimer's disease; cognitive flexibility; NETO1; social preference

1. Introduction

As the aging human population grows, preserving quality of life and cognitive function is an emergent research priority (Brodaty et al., 2013; Ferreira et al., 2014; Eshkoor et al., 2015). Cognitive decline ranges from mild-cognitive impairment (MCI) to dementia and is hallmarked by memory impairments. The definition of what constitutes MCI is evolving, it is four times more common than dementia, and diagnosis involves assessing a patient's functional status, medication history, and neurological and psychological function (Langa & Levine, 2014, Eshkoor et al., 2015). Primary care physicians often make initial diagnoses of these disorders with differing criteria and limited screening tools (Galvin & Sadowsky, 2012).

Mice are essential research tools for mechanistic studies of aging disorders (Galvin & Sadowsky, 2012; Bellantuono et al., 2020). Yet about 90% of published research on aging was conducted in mice younger than 18 months, including in genetic models of Alzheimer's disease such as Aβ Tg2576, APP23, APP/PS1, triple transgene (3xTg) and Familial Alzheimer's Disease (5xFAD) mice (Bilkei-Gorzo, 2014; Dutta & Sengupta, 2016; Esquerda-Canals et al., 2017; Kluever & Fornasiero, 2021; Santana-Santana et al., 2021). Yet many physical biomarkers in mice paralleling human aging, such as eye lens thickening, osteoporosis, insulin resistance and cardiac dysfunction, are only evident after18 months in C57BL/6J mice (Dutta & Sengupta, 2016; Bellantuono et al., 2020). Cognitive deficits corresponding with hippocampal volume loss occurred by 18–24 months in R26R mice, but onset in C57BL/6J was not reported (Reichel et al., 2017).

A variety of factors influence risk for MCI and dementia; chief among them are sex, stress, and social environment (Levine et al., 2021). Sex differences occur in MCI onset, with males showing accelerated brain atrophy and cognitive decline, while females typically develop MCI and dementia over a longer timespan (Brodaty et al., 2013; Levine et

al., 2021). Additional environmental factors such loneliness and isolation are linked to stress, cognitive decline, and increased MCI and dementia risk (Kim, et al., 2017; Hsiao, et al., 2018; Yu et al., 2021). Yet social preferences were rarely studied in aged mice and/or mouse Alzheimer's disease models. Just one study considered social preferences in 6-month-old 3xTg AD mice and found deficient social novelty preference followed a higher stress-induced corticosterone release in females (Nguyen et al., 2020). Added corticosterone promoted Alzheimer's-relevant pathology in mice (Green et al., 2006). Likewise high cortisol, the human equivalent of corticosterone, in cerebral spinal fluid was positively associated with the rate of cognitive decline (Ouanes & Popp 2019).

Movement stereotypies are common to all forms of degenerative dementias (Prioni et al., 2012). Stereotypical involuntary movements such as hand rubbing, or behaviors like taking, burying or misplacing objects (e.g., keys) occur with MCI, Alzheimer's disease or dementia (Mendez et al., 2005; Cipriani et al., 2013). The stereotypical, perseverative behavior of marble burying in mice may parallel this trait. For example, 1 year old 3xTg AD male mice buried more marbles than controls or female AD mice (Santana-Santana et al., 2021). However, no prior reports compared marble burying by 24 month-old C57BL/6J to younger mice of either sex.

Advanced cognitive deficits such as temporal and spatial disorientation hallmark mid-stage Alzheimer's disease. Recent transcriptomic evidence from the International Genomics of Alzheimer's Project (IGAP) and other bioinformatic sources show hippocampal neuropilin and tolloid-like 1 (NETO1), an auxiliary subunit of ionotropic glutamate receptors, mainly kainite sensitive ones, is downregulated or deficient in patients with Alzheimer's (Walter et al., 2011; Feng et al., 2015; Katsumata et al., 2019). In mice NETO1 is densely expressed in hippocampus and frontal cortex, where it modulates the functions of glutamate receptor complexes involved in spatial learning, and Neto1 knockout mice show spatial learning deficits in Morris water maze (MWM) tests (Ng et al., 2009; Straub et al., 2011; Wyeth et al., 2017; Orav et al., 2017; Mennesson et al., 2019). NETO1 may hold therapeutic promise to manage symptoms of MCI, Alzheimer's disease or other dementias, which is essential since available treatments for these disorders have limited efficacy. Yet, the trajectory of NETO1 expression with age in people and mice is unknown, and studies on Neto1 level changes in mice over 18 months old were lacking.

Performance of aged C57BL/6J and Alzheimer's disease models were previously assessed in MWM, and mice >18 months exhibited reversal learning and working memory deficits (Magnusson et al., 2003; Bromley-Brits, et al, 2011; Tian et al., 2019; Hamieh et al., 2021). However, concerns were raised about use of MWM for studies in aged mice, such as the impact of stress due to high physical demand of swimming and locomotor deficits developing with age on MWM performance (Lissner et al., 2010; O'Leary and Brown, 2022), as well as lack of aged females in such studies (Zhvania et al., 2021). The water T maze (WTM) for mice is derived from the MWM (Guariglia & Chadman, 2013). WTM tests use error-based instead of path-based learning, which imposes less physical strain from extended swimming (Locchi et al., 2007; Guariglia & Chadman, 2013). Errors and latency to escape are measured, offering spatial learning and cognitive flexibility assessments less prone to confound by locomotor deficits.

To address these gaps in knowledge regarding mice of advanced (>18 months) age, the aims of the present study were to discern during the natural aging process in male and female C57BL/6J mice the timing of onset of social behavior deficits, increases in repetitive behavior, spatial learning, and cognitive flexibility deficits, and how they correspond with brain Neto1 and serum corticosterone levels. Age groupings for C57BL/6J were based on Dutta & Sengupta (2016) and Bellantuono et al (2020), with 2 months as young adults, 9–15 months as middle age, and 24 months as old age. We hypothesized deficits in social preference, spatial learning and cognitive flexibility, and stereotypies such as marble burying would emerge with advancing age.

2. Methods

2.1 Animals

C57BL/6J breeders (8) were purchased from the Jackson labs (Jax strain # 000664) and bred in house to produce 10 each male and female 2-month-old C57BL/6J test subjects. This was done to avoid confound due to genetic drift from in house colony breeding. Four each of 8 and 14-month-old male and female C57BL/6J were also purchased from the Jackson labs (Jax strain # 000664) to be subjects in this study. Also 5 each of 14-month-old males and females, plus 15 male and 5 female 23-month-old C57BL/6J mice were obtained from the National Institute on Aging (NIA) rodent ordering system (ROS, https://ros.nia.nih.gov/) to be used as study subjects. The NIA mice were bred from Jax strain # 000664 founders that are rederived from pedigreed stock every 6–7 years at the NIA facility (NIA, 2022). All subject mice from Jackson labs or NIA ROS acclimated for a week after arrival before behavioral testing onset. One 23-month-old male was humanely euthanized soon after arrival due to low body weight and pronounced spine curvature. All subject C57BL/6J mice were 2 (n = 10 male, 10 female), 9 to 15 (n = 13 male, 13 female), or 24 (n = 14 male, 5 female) months of age at the start of the behavioral tests. Stimulus stranger mice used in behavior tests were same sex and weight range 129S1/SvImJ, (Jax strain #002448) bred in house and kept on a separate ventilated rodent housing rack. Mice were maintained at 22–25°C, with 14:10 h light/dark cycle, with lights (300 Lx) on at 0700 h. All mice were housed 2 to 5 mice per sterilized cage, with wood chip bedding floor changed bi-weekly in a ventilated housing rack with cotton nestlet squares for enrichment and ad libitum access to chow (Teklad #7912, Harlan, Madison, WI) and water (reverse-osmosis, acidified to pH 2.5–3.0) refreshed weekly. Subjects were humanely euthanized for tissue collection after behavior testing by cervical dislocation and decapitation by a trained and experienced researcher. All procedures involving live mice complied with the National Research Council (2011) Guide for Care and Use of Laboratory Animals and were approved by the University of Texas Health Science Center at San Antonio's Institutional Animal Care and Use Committee.

2.2 Social Interaction and Novelty

Three chamber tests for social interaction and novelty preference were performed in all subject mice as in prior studies (Moy et al., 2007; Greene et al., 2021). Stranger mice were preconditioned under wire mesh cups for 3 30-minute sessions prior to testing. Tests took place between 1100 and 1600 h, with 6 mice tested simultaneously in different arenas under low red lighting (16 lx, measured by Lux Light Meter Pro App for iOS). Mice were

conditioned prior to testing for 10 minutes in the middle chamber, and another 10 minutes with access to all three chambers. For social interaction testing a stranger mouse of the same sex was placed in one chamber while an empty wire cup cage (novel object) was placed in the chamber at the opposite end. Mice began in the middle and were video recorded for 10 minutes before being returned to the middle chamber. Social novelty testing, where mice were video recorded for another 10 minutes, began directly after and a new stranger was added under the empty cup cage. All apparatuses were cleaned with 70% ethanol after testing. Video recordings were then coded by observers blinded to mouse age and sex (unrelated identification numbers were assigned to the mice in videos) for time spent in each chamber and time sniffing (nose pointed toward the cup and no further than a head length away) the stranger mice and novel object. Social interaction and novelty preference were then calculated by subtracting the time spent in each chamber or sniffing the novel object from the stranger mouse or the old from the new stranger.

2.3 Marble Burying

Directly after sociability tests mice were placed individually in a 40 × 20 cm sterilized rat cage with 8 cm wood chip bedding (Teklad, Harlan, Indianapolis, IN) and 18 flattened blue glass marbles placed in a 3 × 6 grid format. Room lighting was as described for social interaction tests and mice were left for 30 minutes. Afterwards all marbles at least 2/3 buried were tallied.

2.4 Water T-Maze

Cognitive deficits were assessed in a lit room (300 lx) using the water T-maze, a modified version of the Morris Water maze for mice as described in (Guariglia & Chadman, 2013; Greene et al., 2021). This maze consists of two aquatic cross mazes, 71 cm × 51 cm (zebrafish cross maze, Noldus, Leesburg, VA, USA) where one functions as a top to keep mice from climbing out. Room temperature deionized water was approximately 10 cm deep and obscured with 10 ml white tempera paint (Sargent, Hazelton, PA, USA). The top of the cross was blocked so mice were trained to use the 50 cm runway and 20 cm arms of the maze for testing. Initially, in a pre-training swim the first maze side arm that any given mouse entered was designated as its “preferred” arm as in Guariglia & Chadman (2013), and each mouse was subsequently removed from the WTM after pre-training and dried with a clean cotton towel. The platform (inverted plastic container 6 cm deep) was added to the opposite arm for the first and all subsequent training swims to train the mouse to acquire a memory of the platform location in order to escape the WTM in the opposite arm. Cues in the room were metal piping high on the wall on the left side of the room and a doorway on the right and both mazes were oriented the same way for all trials. Mice were given 10 daily trials for 5 consecutive days to allow the group to learn to reach the platform with a criterion of at least 80% of trials being correct for 3 consecutive days. Time between trials lasted 1–2 minutes during which time mice were dried and rested in a pile of cotton towels.

Following acquisition training, for each mouse the platform was then moved to the opposite arm (the initially “preferred” arm) to measure cognitive flexibility via reversal learning on days 6–9. Time between trials lasted 1–2 minutes as mice were dried and rested in a pile of cotton towels. Once all mice were tested, using the same water, mazes were drained and

cleaned. Up to two mazes were used a day to allow separate testing mazes for males and females. Days 1–5 served as acquisition training days while 6–9 were used to test cognitive flexibility. The mazes have marks set at even intervals. If a mouse entered the side of the chamber not containing the platform and passed the interval marker (halfway point in the arm) they were considered to make an error. Additionally, if the mice swam back toward the starting point and passed an interval marker, they were considered to make an error. The number of times they entered the wrong area and passed the interval marker were counted. Acquisition criteria for mice is set at group averages of 80% error free trials for 3 consecutive days. Latency to the platform, number of errors per day, and % of error free trials were collected daily and analyzed to assess performance.

2.5 Corticosterone Measurements in Serum

Mice were euthanized 2–3 days after behavior tests via cervical dislocation and decapitation. Trunk blood was collected and clotted overnight at 4°C in a walk- in cold-room before centrifugation at 2000 × G for 10 minutes to isolate serum. Serum was stored at −80 °C until use and was only defrosted once at 4°C at the time of use in the assay. Serum was thawed and 10 μl from each tube was added to a well in an uncoated 96 well plate. Each 10 μl serum sample was treated with 10 μl 1:100 steroid displacement reagent (ADI-900–097, Enzo Life Sciences, Farmingdale, NY, USA) to release corticosterone from corticosteroid-binding globulin and shaken for 10 min before dilution in 300 μl assay buffer from the corticosterone enzyme linked immunosorbent assay (ELISA) kit (Cayman Chemical Co., Ann Arbor, Michigan, USA). These diluted samples were measured against the corticosterone standards provided, using the corticosterone ELISA kit as instructed (Cayman Chemical Company: Item No. 501320). After washes and dark incubation in Ellman's reagent, the plates were read on a microplate reader at 405 nm (SpectraMax 190, Molecular Devices LLC, San Jose, CA, USA). A standard curve was generated using a sigmoidal 4PL nonlinear regression model in GraphPad Prism 9 (La Jolla, CA, USA), and concentration values were corrected for the 1:32 dilution. All samples except for three, two 15-month-old females and one 15-month-old male, were included in the analysis. One of the two excluded females had a corticosterone level above 212 ng/ml that fell so far outside the linear range of the standards that it could not be accurately extrapolated, and the excluded male and other female serum samples had insufficient volume to accurately measure in the assay.

2.6 Neto1 Measurements in Frontal Cortex and Hippocampus

At the time of euthanasia, mouse brains were dissected to collect hippocampus and frontal cortex into microcentrifuge tubes submerged in powdered dry ice. The samples remained frozen until use in an enzyme linked immunoassay (ELISA) for Neto1 using a kit (Catalog #E5240m, Wuhan EIAab Science Co., LTD, Wuhan, China). Samples in tubes were thawed at 4°C and homogenized in 1 ml phosphate buffered saline using a pellet pestle motor with disposable tips (Kimble Chase, Vineland, NJ, USA) on ice, and then stored overnight at −20°C, defrosted at 4°C for 1 h, refrozen overnight at −20°C (2 freeze-thaw cycles) as directed to break cell membranes. The samples were finally thawed at 4°C and centrifuged at 1000 × G for 10 min to isolate the Neto1 protein from the tissue, and samples were kept on a bed of ice water for use in ELISA the same day. Protein content of the supernatant was determined using Bradford reagent and standards of bovine serum albumen dissolved

in sodium hydroxide (all from Sigma Aldrich, St. Louis, MO, USA), plates were read on a plate reader at 595 nm (SpectraMax 190). The frontal cortex and hippocampal samples were diluted 1: 5 in sample diluent and 100 µl was used in each sample well of the Neto1 ELISA plate, and the assay incubation was performed at 37°C, and plates were washed, detection and stop reagents were added and the plates were read at 450 nm as directed. A standard curve was generated using sigmoidal 4PL nonlinear regression in GraphPad Prism to determine Neto1 levels which were then normalized to the protein content of each sample.

2.7 Statistical Analysis

Since group sample sizes were uneven, for all analyses of variance (ANOVA) a mixed effects model, that uses a restricted maximum likelihood method, was selected in Prism 9 (GraphPad, La Jolla, CA). Social interaction and novelty data for analyses included time spent in each chamber and time sniffing, along with social interaction (time in chamber or sniffing stranger mouse – novel object) and novelty (time in chamber or sniffing new stranger– old stranger) preference. Since male and female responses were nearly identical, and both three-way (sex × age × chamber side) and two-way (sex × age) ANOVAs for social preferences revealed no significant sex differences in either test phase at any age for any chamber time or sniff time measurements, sex was pooled to reduce the number of groups shown in figures to emphasize age-specific social choices in the analyses and graphs and symbols are triangles in graphs. Time spent by subject mice in each chamber and time sniffing in social interaction and novelty preference tests were analyzed using 2-way ANOVA (chamber side × age), and then the interaction preference and novelty preference time differences were analyzed by one-way ANOVA (age). Analyses of all other variables include sex and age, using standard genealogy symbols for sex, since there were half as many dependent variables as in social preference tests. Marbles buried after 30 minutes were compared for 2, 9–15, and 24-month-old males and females with a 2-way ANOVA (sex × age). For Water T-Maze, data analyzed include average trial time, number of errors made, and percentage of error-free trials. For acquisition all five days were compared using a repeated measures ANOVA (sex × age × day). For analysis of reversal day 1 (R1) results, a 2-way ANOVA (sex × age) was performed. Furthermore, days A5 and R1 were compared using repeated measures ANOVA (age × day) to assess cognitive flexibility and impairment. Bonferroni and Šidák's multiple comparison tests were used to further compare groups when a significant main effect or interaction was present. Finally, corticosterone levels in serum and Neto1 in brain were compared between males and females at all age groups with a 2-Way ANOVA (sex × age). Assumptions of normality (skewness between −2 to +2 and Kurtosis between −7 to +7) were tested using the Anderson-Darling test in Prism 9, and homogeneity of variance (Levene's Test), and multicollinearity (VIF<10) were tested using SPSS. If the assumption for normality was violated for the variables a non-parametric test such as Mann-Whitney U or Kruskal-Wallis test were run and reported in addition to parametric tests. For the Water T-Maze, since repeated measures ANOVA was used, if Mauchly's Test of Sphericity was violated a Greenhouse-Geisser correction was used. Prism 9 (Graph Pad, La Jolla, CA) and SPSS (IBM Inc., USA) were used for analyses, and figures were generated in Prism 9.

3. Results

3.1 Social Interaction and Novelty

Mice of all ages spent more time in the chamber with the stranger mouse than the novel object during the social interaction test ($F(1, 124) = 88.03$, $P<0.0001$, $\eta^2=0.415$, Fig 1a). For social interaction sniffing time (Fig 1b) mice aged 9–15 and 24 months spent more time sniffing the stranger mouse than the novel object while 2-month-old mice did not ($F(1, 124) = 43.81$, $P<0.0001$, $\eta^2=0.261$). Additionally, mice aged 2 months sniffed the stranger mouse less than mice aged 9–15 and 24 months ($F(2, 124) = 16.41$, $P<0.0001$, $\eta^2=0.209$). No differences were present for social interaction time in chamber preference ($F(2, 62) = 1.204$, $P=0.3070$, $\eta^2=0.037$) or social interaction sniffing preference ($F(2, 62) = 1.327$, $P=0.2727$, $\eta^2=0.041$), or social novelty for time in chamber preference ($F<1$). However, social novelty time sniffing mice aged 2 months spent less time sniffing both the old and new stranger ($F(2, 124) = 22.41$, $P<0.0001$, $\eta^2=0.265$, Fig 1d), and had a lower novelty sniffing preference ($F(2, 62) = 5.101$, $P<0.01$, $\eta^2=0.141$) than other age groups, resulting in an interaction between age and chamber ($F(2, 124) = 3.954$, $P<0.05$, $\eta^2=0.06$). Mice aged 9–15 months made more chamber entries than mice aged 2 and 24 months during both the social interaction ($F(2, 62) = 16.39$, $P<0.0001$, $\eta^2=0.346$, Fig 2a) and novelty ($F(2, 62) = 24.77$, $P<0.0001$, $\eta^2=0.444$, Fig 2b) tests.

3.2 Marble Burying

No differences among age ($F<1$) or sex ($F(1, 59) = 1.506$, $P<0.2247$, $\eta^2=0.025$, Fig 3) were evident in marbles buried after 30 minutes.

3.3 Water T-Maze

All age groups were able to reach 80 percent trials correct by day 5 of acquisition and day 3 of reversal learning (Fig 3). Latency to the platform, number of errors made, and percentage of error-free trials were compared for age and sex differences for acquisition using repeated measures ANOVA. During acquisition 24-month-old mice had longer latencies to the platform than younger mice (day × age) ($F(2.277, 58) = 19.421$, $P<0.001$, $\eta^2=0.495$, Fig 4a) with 24-month-old female mice having longer latencies than male mice (day × sex) ($F(1.138, 58) = 13.644$, $P<0.001$, $\eta^2=0.19$, Fig 4a) resulting in an interaction (day, age, and sex) ($F(2.277, 58) = 7.49$, $P<0.001$, $\eta^2=0.205$). Additionally, 24-month-old females made more errors (sex) ($F(1,58) = 9.309$, $P<0.01$, $\eta^2=0.138$) and had fewer error-free trials ($F(1,58) = 9.599$, $P<0.01$, $\eta^2=0.142$) than males and had more errors (age) ($F(2,58) = 11.831$, $P<0.001$, $\eta^2=0.29$) and fewer error-free trials ($F(2,58) = 13.843$, $P<0.001$, $\eta^2=0.323$) than younger females (Fig 4b,c). All mice but 24-month-old females made fewer errors after A1 resulting in a day effect for errors ($F(1.771, 58) = 37.42$, $P<0.001$, $\eta^2=0.392$) and error-free trials ($F(2.026, 58) = 50.678$, $P<0.001$, $\eta^2=0.466$).

Latency to the platform, number of errors made, and the percentage of error-free trials were compared for age and sex differences on R1. Age differences for latency to the platform were present, with 24-month-old mice showing the longest latencies to the platform of all age groups (($F(2, 58) = 14.80$, $P<0.0001$, $\eta^2=0.338$) ($H(2) = 22.027$, $P < 0.001$, $\varepsilon^2_R = 0.35$)). Additionally, 24-month-old female mice had increased latency compared to other

groups resulting in sex differences ((F (1, 58) = 7.540, P<0.01, η^2=0.115) (Mann-Whitney U = 320.5, P > 0.05, η^2=0.094)).

To determine the strength of the association mice made with the platform's location during acquisition days A5 and R1 were compared for latency to the platform, the number of errors made, and the percentage of error-free trials. Males and females were compared with separate repeated measures analyses to account for sex differences present during comparisons during days acquisition and R1. Predictably, latency increased from A5 to R1 for both male (day) (F (1, 34) = 20.96, P<0.0001, η^2=0.381) and female (F (1, 24) = 17.94, P<0.001, η^2=0.428) mice, with the longest latencies for 24-month-old male (age) (F (2, 34) = 11.91, P=0.0001, η^2=0.412, Fig 5d) and female (F (2, 24) = 21.63, P<0.0001, η^2=0.643, Fig 5a) mice compared to younger mice. While an interaction for latency between day and age was present for male (F (2, 34) = 3.888, P<0.05, η^2=0.186) mice, it was not for females (F<1). Additionally for error-based learning, males of all age groups had an increase in errors (day) (F (1, 34) = 114.55, P<0.0001, η^2=0.771, Fig 5e) and fewer error-free trials (F (1, 34) = 162.7, P<0.0001, η^2=0.827, Fig 5f) on R1 than A5. There were no main effects of age for number of errors made (F (2, 34) = 1.797, P=0.1812, η^2=0.096) and error-free trials (F (2, 34) = 2.028, P=0.1473, η^2=0.107) or interactions between day and age for number of errors (F (2, 34) = 1.334, P=0.2768, η^2=0.073) or error-free trials (F (2, 34) = 2.363, P=0.1094, η^2=0.122) by males between days. However, for females all age groups except 24-month-old females had an increase in errors (day) (F (1, 24) = 34.26, P<0.0001, η^2=0.588, Fig 5b) and fewer error-free trials (F (1, 24) = 61.29, P<0.0001, η^2=0.719, Fig 5c) on R1 than A5, with interactions between day and age approaching significance for errors made (F (2, 24) = 3.001, P=0.069, η^2=0.2) but not number of error-free trials (F (2, 24) = 2.362, P=0.1158, η^2=0.164). There were no main effects of age for number of errors made (F<1) and error-free trials (F<1) by females between days. This indicates weak learning for the platform location during acquisition for 24-month-old females.

3.4 Corticosterone in Serum

A 2-way ANOVA found no differences in serum corticosterone for sex (F (1, 57) = 2.151, P = 0.148, η^2=0.036) or age (F (2, 57) = 1.665, P = 0.198, η^2=0.055) (Fig 6).

3.5 Neto1 Expression in Frontal Cortex and Hippocampus

In the frontal cortex there were no significant effects of sex (F (1,59) = 0.43, P = 0.52, η2 = 0.01) or age ((F (2,59) = 1.1, P = 0.34, η^2 = 0.036) and no sex × age interaction (F (2,59) = 1.44, P = 0.25, η^2 = 0.047), as shown in Fig 7a. In the hippocampus, there was no significant effect of sex (F (1,59) = 0.49, P = 0.49, η^2 = 0.01), or sex × age interaction (F (2, 59) = 1.14, P = 0.33, η^2=0.04). The effect of age was significant (F (2, 59) = 5.43, P < 0.01, η^2=0.16), as 2-month-old mice had lower Neto1 levels than 24-month-old mice (Fig 7b).

4. Discussion

Overall, preference for social interaction was enhanced in C57BL/6J mice with age, as evidenced by increased social sniffing time in three-chamber social interaction and social novelty preference tests by all mice older than 9 months relative to 2-month-olds. We

observed no significant differences in the social behaviors of male and female mice, and uniform behaviors at all ages with respect to responses to a same-sexed stranger mice, so male and female mice were pooled in each age group to simplify the interpretation of these findings. We did not observe any social deficits in interaction or novelty preference tests in the C57BL/6J mice at 24 months, as mice of all ages tested spent more time in the chamber and sniffing the stranger mouse than the novel object. However, 24-month-old mice spent much more time than 2-month-old mice sniffing stranger mice in both the interaction and novelty preference tests.

Corresponding with this, Neto1 levels in the hippocampus were higher in 24-month-old-mice versus 2-month-olds. Neto1 is densely expressed in hippocampal cornu Ammonis 3 (CA 3) pyramidal cells, especially early in development (Tomita & Castillo, 2012, Wyeth et al., 2017; Mennesson et al., 2019). Therein Neto1 is expressed on glutamatergic neurons and GABAergic interneurons, modulating long-term potentiation (LTP), long-term depression (LTD), synaptic plasticity, and cognitive flexibility (Ng et al., 2009; Wyeth et al., 2014; 2017; Orav et al., 2017). Neto1 knock-out mice exhibit depressed hippocampal LTP and deficient cognitive flexibility (Ng et al., 2009; Wyeth et al., 2017). Neto1 interacts primarily with kainate (KA)-sensitive glutamate receptors and may also modulate aminomethylisoxazole propionic acid (AMPA) and/or N-methyl-D-aspartate (NMDA) sensitive glutamate receptors (Tomita, 2010; Straub et al., 2011; Tang et al., 2011; Li et al., 2019). NETO1 may modulate the circuit between hippocampus nucleus reuniens and frontal cortex to shape working memory (Dollerman-vander Weel et al., 2019). While the role of NETO1 in development is currently unfolding, far less is known about the fate or importance of NETO1 with age and in Alzheimer's disease. Recent bioinformatic studies revealed that reduced NETO1 expression is associated with Alzheimer's disease (Walter et al., 2011; Feng et al., 2015; Katsumata et al., 2019), but whether NETO1 deficit is a cause or consequence remains unclear. Taken together these findings indicate a positive correspondence between advanced age, social sniffing and Neto1 levels in the hippocampus of C57BL/6J mice that is of interest for aging research and social behavior studies. A potential limitation to this study is that the two freeze-thaw cycles necessary to break cell membranes and isolate the Neto1 protein from brain tissue samples may have damaged the Neto1 protein, so while relative level comparisons are valid, since all samples were treated the same, the actual Neto1 protein content may not be accurate.

During these same three-chamber social preference tests, a reduction in ambulatory exploration, as evidenced by reduced number of chamber entries, was evident in both 2 and 24-month-old C57BL/6J mice relative to 9–15-month-olds. This could potentially relate to a combination of decreased chamber entries due to impaired locomotion in the aged C57BL/6J mice as reported in this and other strains (Traschütz et al., 2018; O'Leary and Brown, 2022) and/or increased anxiety in the 2-month-olds (Gould et al., 2014) relative to 9–15-month-olds. Prior studies comparing male C57BL/6J mice to the socially impaired black and tan brachury tufted strain (BTBR T + Itpr3tf/J or BTBR) revealed no change in their strain specific social preference phenotypes at 15 months old (Jasien et al., 2014). At 19–21 months of age, male BTBR mice still displayed more anxious behaviors and impaired social novelty recognition relative to C57BL/6J mice (O'Connor et al., 2021). However, neither of these prior studies of social preference in older mice examined sex

differences, as only male mice were studied, most likely due to their emphasis on autism which is more common in males. Yet much of ageing related social processes may be sex dependent in rodents (Zhvania et al., 2021). The present study is the first to take sex and age into consideration in the context of social preferences, and to demonstrate that social sniffing behavior in both interaction and novelty preference test is enhanced in 24-month-old C57BL/6J mice, independent of their sex.

The genetic background of aged mice can make dramatic contributions to the rate of cognitive decline and/or manifestation of pathological biomarkers in translational mouse models of Alzheimer's disease. For example, the C57BL/6 genome appears to harbor epistatic modifiers that attenuate the rate and severity of cognitive decline produced by introduction of the 5×FAD transgene, despite substantial Aβ accumulation relative to other strains (Neuner et al., 2019). Natural aging in C57BL/6NJ mice was previously reported to impair female performance by 15-months, an earlier age than in males, in novel object recognition tests (Fahlström et al., 2011). However, a recent study found locomotor and exploratory activity were reduced to such an extent that novel object preference could not be reliably detected even in male 15–24-month-old C57BL/6N mice (Traschütz et al., 2018). This reduction in exploratory locomotion may be due in part to higher incidence of retinal degeneration for which a mutation, rd8 in the crumbs homolog 1 (CRB1) gene, renders C57BL/6N mice more prone to vision impairment as they age (Mattapallil et al., 2012; Aredo et al., 2015). Based on the NIA aged rodent colony handbook, the mice supplied by NIA ROS for this study were most likely C57BL/6J mice restocked from the Jackson strain # 000664 every 6–7 years and would therefore have been less likely to harbor the rd8 mutation and resulting age related vision loss than C57BL/6N mice. However, reliance on NIA ROS for 24-month-old mice lead to a major weakness in this study, specifically only 5 aged females were sent, a sample size sufficiently low to increase the odds of sampling errors.

The MWM was extensively used at in aged male C57BL/6N and C57BL/6J mice, with mice of both strains >18 months showing deficits in reversal learning and working memory (Magnusson et al., 2003; Hamieh et al., 2021). However, detection of cognitive deficits can be confounded not only by vision impairments, but also by fatigue or physical stress from extended swimming time in MWM tests, especially in aged mice (Locchi et al., 2007; Guariglia & Chadman, 2013), and/or from locomotor deficits in age-related disease models (O'Leary and Brown, 2022). For these reasons, we characterized the effects of natural aging of both male and female C57BL/6J mice in a battery of less commonly used behavioral tests that are relevant both to aging and neurodegenerative disorders: Specifically social preferences, marble burying, and spatial learning and cognitive flexibility in WTM test.

Marble burying behavior can provide insight into stereotypical movements as well as anxiety states, but in the present study it did not reveal any age or sex-related differences and was typical of C57BL/6J mice (Gould et al., 2014; Greene et al., 2021). This contrasts with prior studies in the 3xTg AD mice wherein sex-specific phenotypes were evident (Santana-Santana et al., 2021). This finding may be indicative of marble burying behavior being a better index of Alzheimer's disease or neurodegenerative status than ageing per se.

In the WTM, 24-month-old females made more errors and had longer latency to find the platform in acquisition training days 1 and had greater latency to find the platform and made more errors on day 5 of acquisition training. By contrast in the 24-month-old males there were fewer errors made than by same aged females. Additionally, 24-month-old female mice appear to have the longest times, even significantly longer than same aged males on the first day of testing. These deficits in WTM performance together with reduced exploration of the 3-chamber arena during sociability preference tests may be indicative of vision impairments in 24-month-old female mice: Alternatively, if age impaired their swimming ability so that they were not able to move as easily, it may have contributed to their slower swim times. A major limitation of this study was that locomotor behavior was not tested directly in an open field or in an apparatus measuring beam breaks in an open arena. Yet based on other studies, locomotor activity is expected to decline with age, so testing cognitive ability independent of locomotor ability can be a challenge in MWM (O'Leary and Brown, 2022).

To determine if stress response was a possible factor impacting performance of older female mice in the WTM corticosterone was measured in the frozen mouse serum samples. Corticosterone levels were not found to differ in 24-month-old females from 9–15-month-olds, consistent with the MWM subjects of Lissner et al. (2021). Baseline corticosterone levels are more likely to vary in Alzheimer's disease models (Green et al., 2006), than in aged mice.

By using the WTM instead to assess spatial learning, and cognitive flexibility, the physical exertion and locomotor ability dependence was reduced as compared to MWM tests. Furthermore, the focus on error-based learning over path-based learning gives a unique insight into assessing the ability and strength of the mice to encode and re-learn location information (Lissner et al., 2010; Guariglia & Chadman, 2013). The ability to measure errors made and error-free trials in the WTM also provides for a measure that better reflects cognitive function, when compared to latency time, which may be impacted by both cognitive function and locomotor function (Locchi et al., 2007). WTM may prove beneficial in studying therapeutic response to treatments for cognitive impairment in aged mice, as it lessens the effects of locomotion and physical strain as confounding variables when comparing cognitive performance among mice.

Cognitive deficits in spatial learning were evident in the 24-month-old females, but not the 24-month-old males, as compared to younger mice. Older female mice had longer latencies, more errors, and fewer error free trials throughout WTM testing than younger mice and same aged males. Additionally, when comparing days A5, the last day of acquisition training, to R1, the first day of reversal learning, to measure the strength of animal's association with platform location during acquisition, it appears female animals had weak association with platform location, as the number of errors did not increase and the percentage of error free trials did not decrease, unlike other age groups and same age males. Females barely reached the 80% error free trial criteria after five training days. This shows older females were not able to form a strong association or effectively learn the WTM task as well as younger mice or same age males, highlighting spatial learning deficits consistent with prior MWM findings (Magnusson et al., 2003; Tian et al., 2019; Hamieh et al., 2021; Zhvania et al., 2021). Cognitive deficits were previously found in younger female

mice, and while other studies found deficits in aged males in MWM (Magnusson et al., 2003), none occurred in 24-month-old males in our WTM study.

Involvement of the hippocampus is assumed but has yet to be demonstrated in the WTM test through ablation studies. However it was shown that an in-tact hippocampus is critical to good performance in a win-shift version of the 8-arm radial maze but not to win-stay or conditioned cue preference versions of the task that are ascribed to the amygdala, and dorsal striatum, respectively (McDonald and White, 1993). In the MWM adult neurogenesis in the hippocampus derived from the dentate gyrus and allocentric map formation are not always quantifiable in many of the behaviors typically assessed such as path length and latency to find the platform (Garthe and Kempermann, 2013). In the 5xFAD mouse model, mild learning, but not memory deficits were evident in MWM tests, and this was described as indicative of ventral hippocampus-based deficiencies (O'Leary and Brown, 2022). Alternatively, if escape from the water is rewarding, then is possible that performance of mice in the WTM is due to cued learning that is more dependent on striatal function than hippocampal neurogenesis. The brain region specificity of WTM tests, along with measurements of Neto1 expression measured by qPCR will be the focus of future studies in aged C57BL/6J mice, toward future studies of their involvement in Alzheimer's disease and/or age-related neurodegeneration.

5. Conclusion

Social preferences for interactions or social novelty with same-sex stranger mice remain robust as C57BL/6J mice reach advanced ages, independent of sex. Corresponding with this NETO1, an auxiliary protein to ionotropic glutamate receptors in hippocampus increased in 24-month-old mice relative to 2-month-olds. The WTM appears promising to assess spatial learning, cognitive flexibility and/or memory even in models of neurodegenerative disorders or with locomotor deficiencies in aged mice.

Acknowledgements

This work was supported by a pilot grant award to GGG from the San Antonio Nathan Shock Center of Excellence in the Biology of Aging (P30 AG013319, PI Peter Hornsby), a postdoctoral fellowship award to SMG from Cardiovascular Training in South Texas (T32 HL007446, PI James D Stockand), and training awards to GAA, IB and AJ from the Summer Physiology Undergraduate Researcher Program (R25 NS115552, PI Manzoor Bhat). We thank Dr. Jannine Cody, Dr. Kevin Bieniek, Dr. Peter Hornsby, and Dr. James Nelson for their helpful suggestions.

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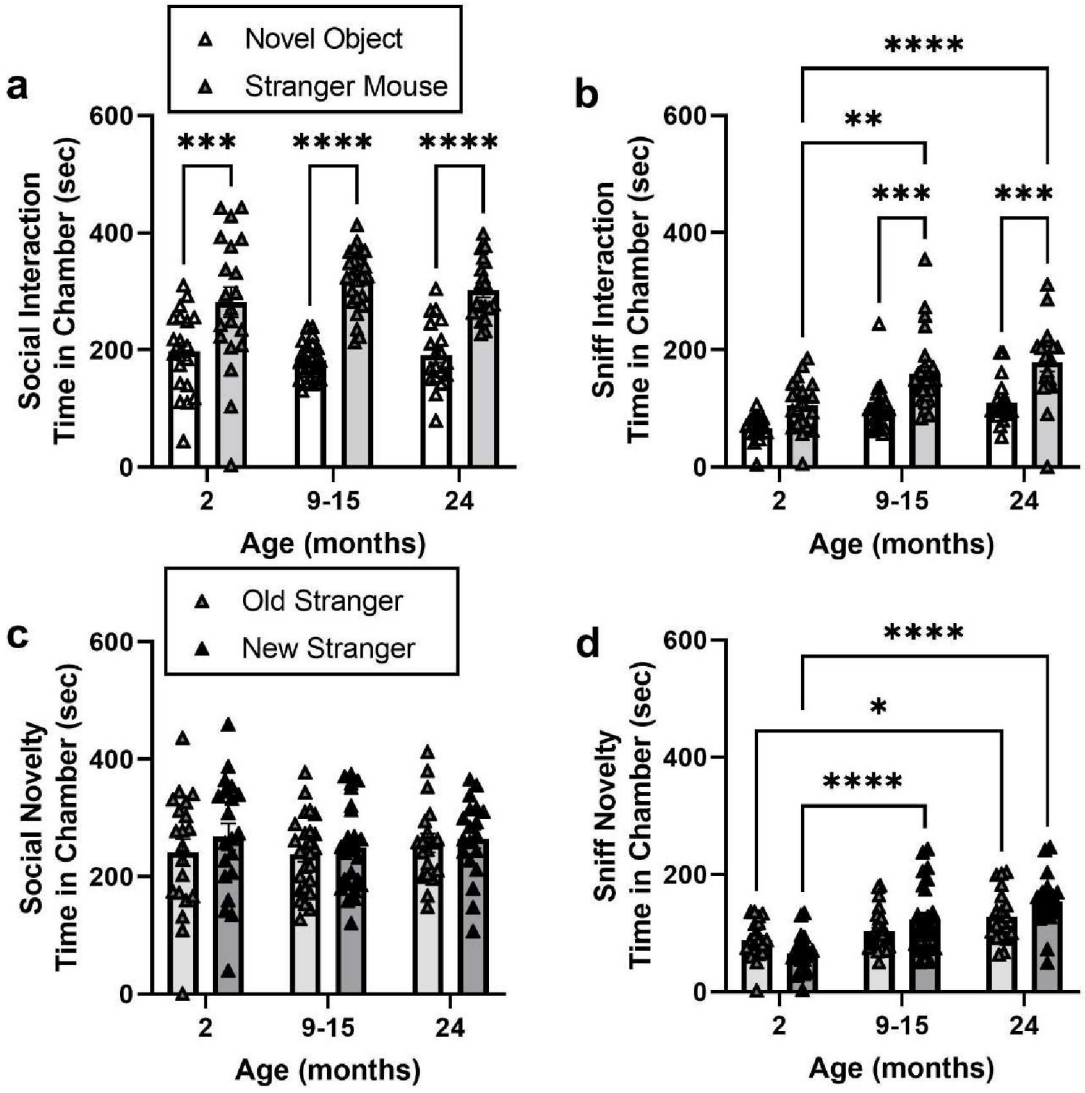

Figure 1.

Social behavior of mice aged 2, 9–15, and 24 months for 3-chamber social interaction (SI) (a,b) and novelty (SN) (c,d) by time in chamber (a,c) and time sniffing (b,d). All data shown are group averages pooled for sex with SEM. Mice tested were male and female C57BL/6 aged 2 (n= 10 male, 10 female), 9–15 (n=13 male, 13 female), and 24 (n=14 male, 5 female) months at the time of testing. All mice had spent more time in the chamber with the stranger mouse than the novel object (2 (*** $p<0.001$), 9–15 and 24 (**** $p<0.0001$) months of age) during the SI test (a). However, only 9–15 and 24 (*** $p<0.001$) month-old mice sniffed the stranger mouse more than the novel object (b). Additionally, 2-month-old mice did less sniffing of the stranger mouse than 9–15 (** $p<0.01$) and 24 (**** $p<0.0001$) month-old mice. No differences were observed for SN for time spent in the chamber (c). However, 2-month-old mice sniffed the old stranger less than 24 (* $p<0.05$) month-old mice, and the new stranger less than 9–15 and 24 (**** $p<0.0001$) month old mice.

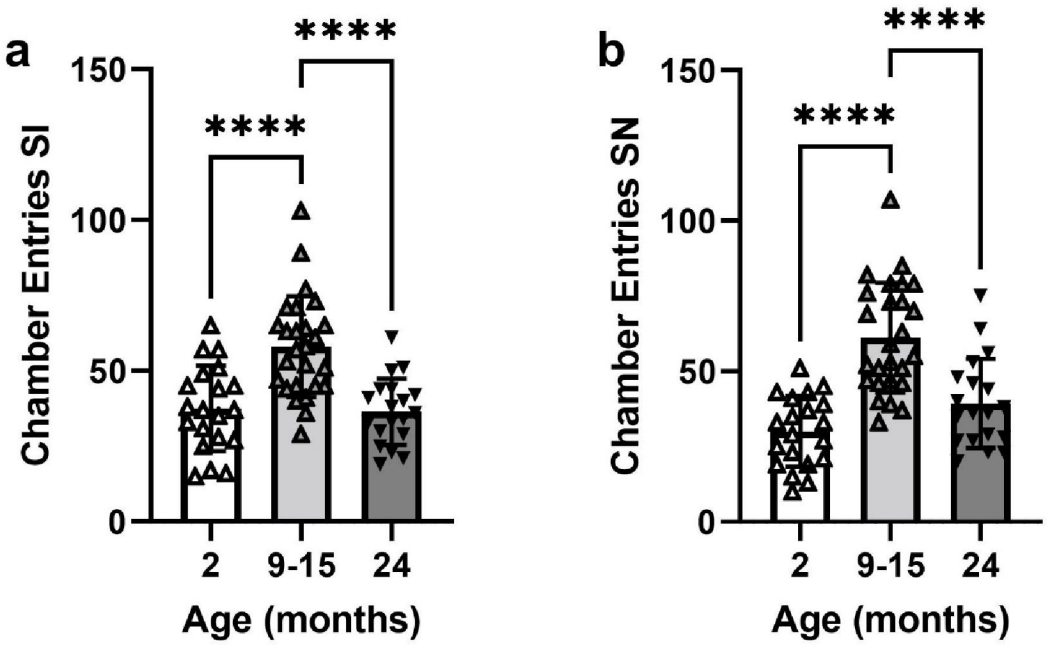

Figure 2.

Chamber entries during the social behavior tests of interaction (SI) (a) and novelty (SN) (b). All data shown are group averages pooled for sex with SEM. Mice tested were male and female C57BL/6 aged 2 (n= 10 male, 10 female), 9–15 (n= 13 male, 13 female), and 24 (n= 14 male, 5 female) months at the time of testing. During both SI and SN tests 2 and 24-month-old mice made fewer chamber entries than 9–15-month-old mice (**** $p<0.0001$).

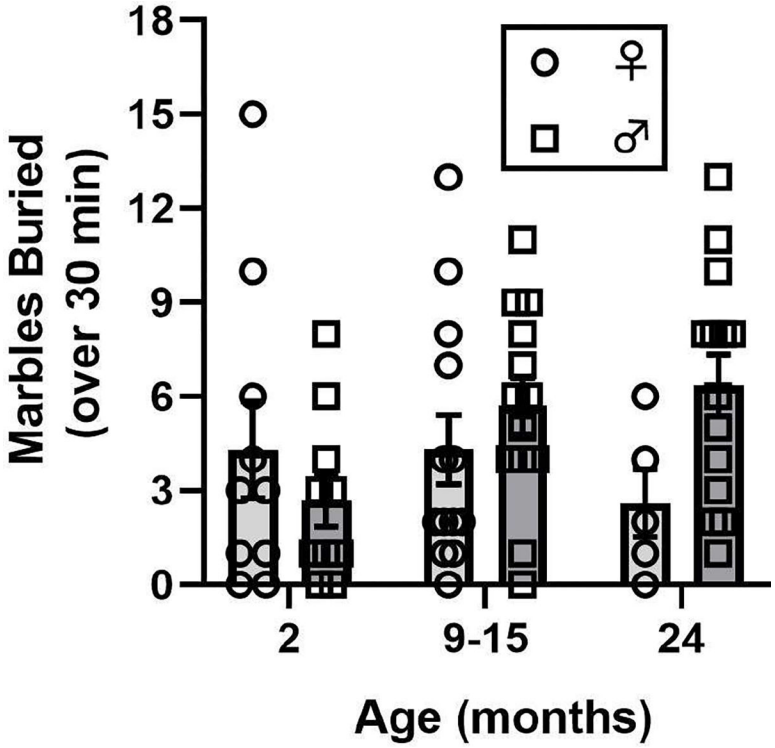

Figure 3.

Marbles buried in 30 minutes. All data shown are group averages with SEM, and Šidák's test for multiple comparisons. Mice tested were male and female C57BL/6 aged 2 (n= 10 male, 10 female), 9–15 (n=13 male, 13 female), and 24 (n=14 male, 5 female) months at the time of testing. No differences were present in the number of marbles buried for males or females of any age group.

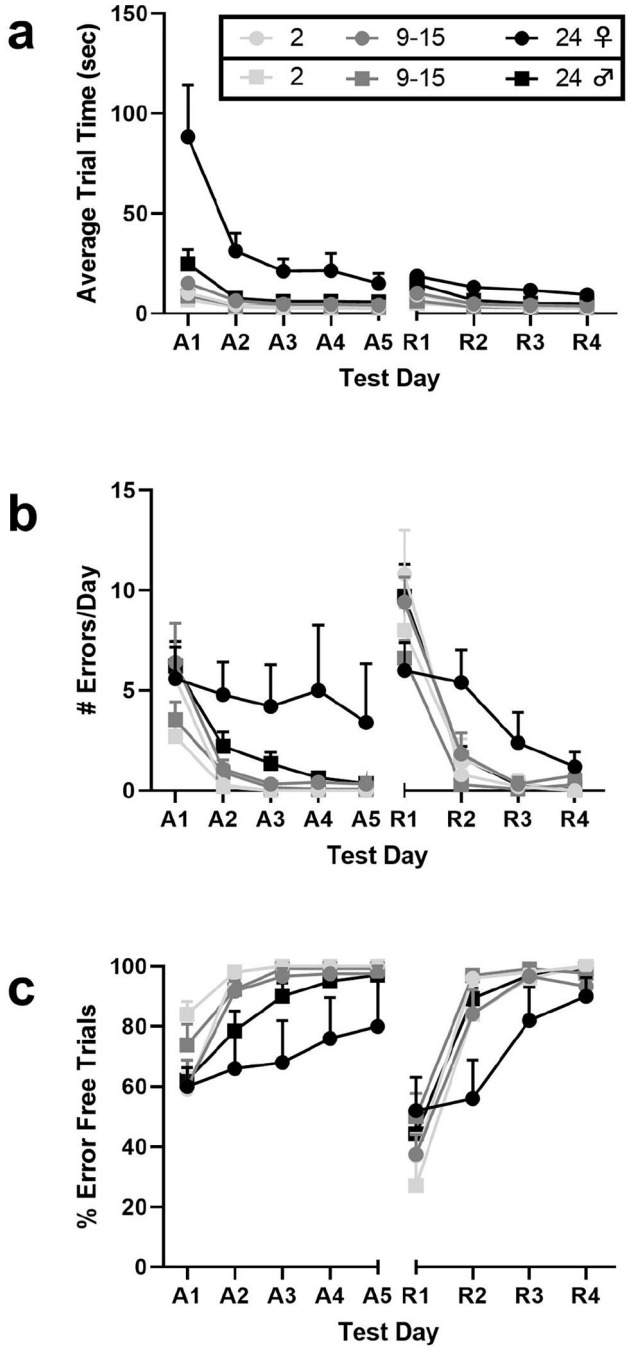

Figure 4.

Water T-Maze data for all nine days of testing. All data shown are group averages with SEM. Mice tested were male and female C57BL/6 aged 2 (n= 10 male, 10 female), 9–15 (n=13 male, 12 female), and 24 (n=14 male, 5 female) months at the time of testing. Mice were tested repeatedly for nine days, 5 acquisition days (A1-A5) and four reversal days (R1-R4) where the platform was moved to the opposite arm of the T-maze. Data shown include average trial time (a), number of errors made per day (b), and the percentage of error-free trials per day based on the 10 trials (c). Repeated measured ANOVA for the 5

acquisition days indicates 24-month-old females had longer latencies than all other groups (*** $p<0.001$) and consistently made more errors (females 2 and 9–15 *** $p<0.001$; males ** $p<0.01$) and had fewer error-free trials (females 2 and 9–15; males ** $p<0.01$) during acquisition. All groups aside from 24-month-old females made fewer errors after A1 during acquisition (*** $p<0.001$). Additionally, during R1 Šidák's test for multiple comparisons found 24-month-old females had increased trial latency when compared to younger females (2 ** $p<0.01$ and 9–15 * $p<0.05$) and 24-month-old males mice had increased trial latencies when compared to younger males (*** $p<0.001$)

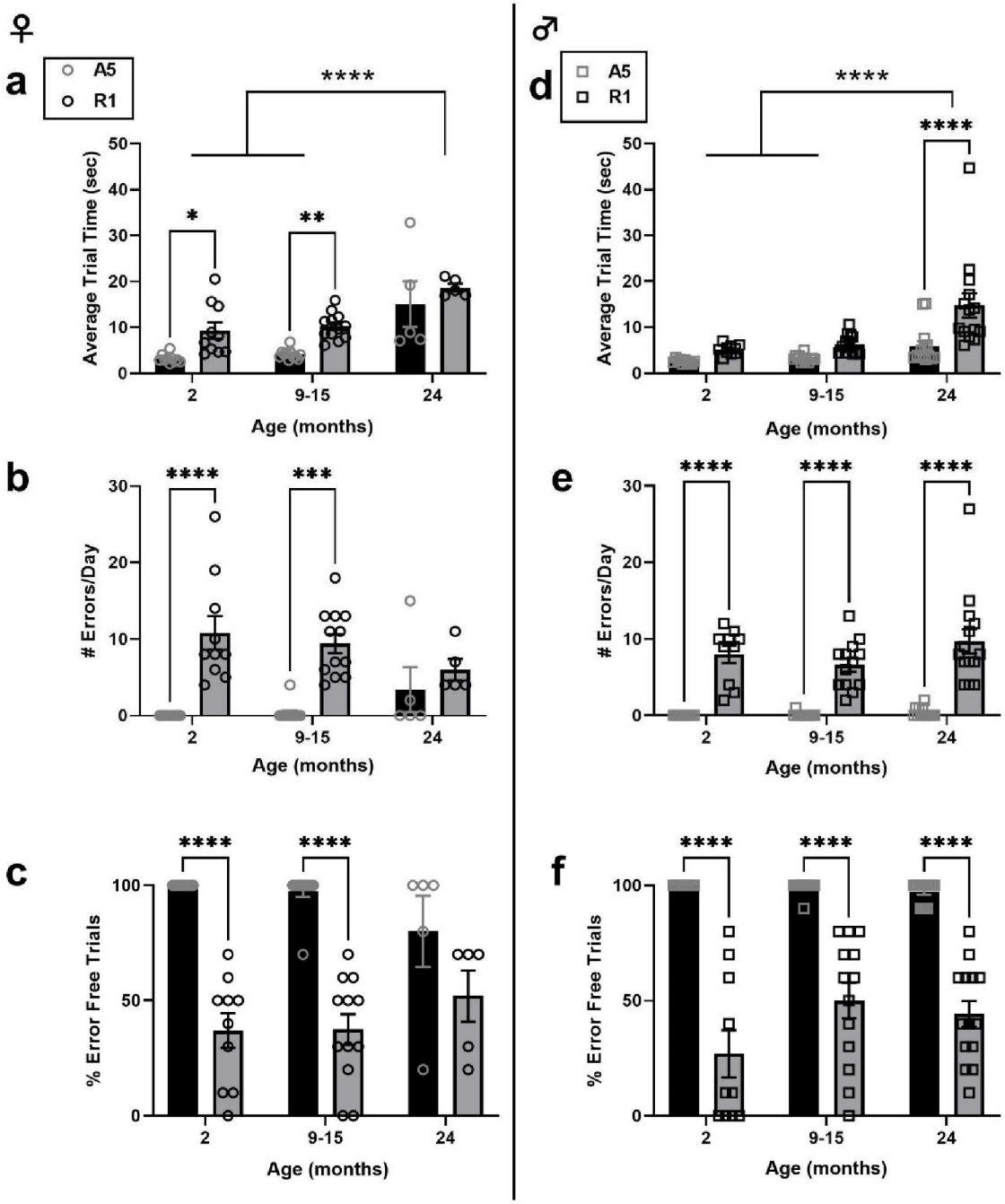

Figure 5.

Water T-Maze data comparing Acquisition Day 5 (A5) and Reversal Day 1 (R1) to determine how well animals learned platform location during acquisition. All data shown are group averages with SEM, and Bonferroni's test for multiple comparisons. Mice tested were male and female C57BL/6 aged 2 (n= 10 male, 10 female), 9–15 (n=13 male, 12 female), and 24 (n=14 male, 5 female) months at the time of testing. Due to sex differences males and females were analyzed and graphed separately. Data shown include average trial time (a,d), number of errors made per day (b,e), and the percentage of error-free trials per

day based on the 10 trials (c,f). To measure the strength of conditioning from acquisition day A5 and R1 were compared. For trial time (a,d) 24-month-old mice had higher times (**** $p<0.0001$) on R1 than A5. Additionally, 2 (* $p<0.05$) and 9–15 (** $p<0.01$) month-old females and 24-month-old males (**** $p<0.0001$) had higher trial times on R1 than A5. For error-based learning, compared to A5, on R1 all groups except the 24-month-old females made more errors (b,e) (2-month-old females and males of all ages **** $p<0.0001$; 9–15-month-old females *** $p<0.001$), and had fewer error-free trials (c,f) (**** $p<0.0001$). This shows 24-month-old females had poor platform location acquisition learning.

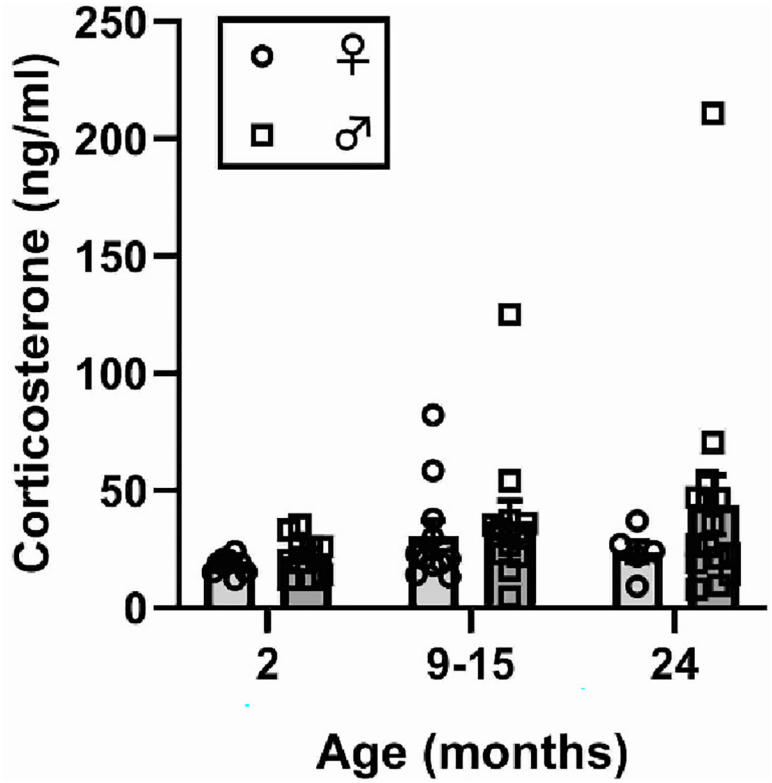

Figure 6.

Serum corticosterone was measured after behavior tests to compare stress responses of aged mice. Data shown are group averages with SEM. Mice were male and female C57BL/6 aged 2 (n= 10 male, 10 female), 9–15 (n= 13 male, 11 female), and 24 (n= 13 male, 5 female) months. ANOVA comparisons revealed no significant mean differences in serum corticosterone for sex or age.

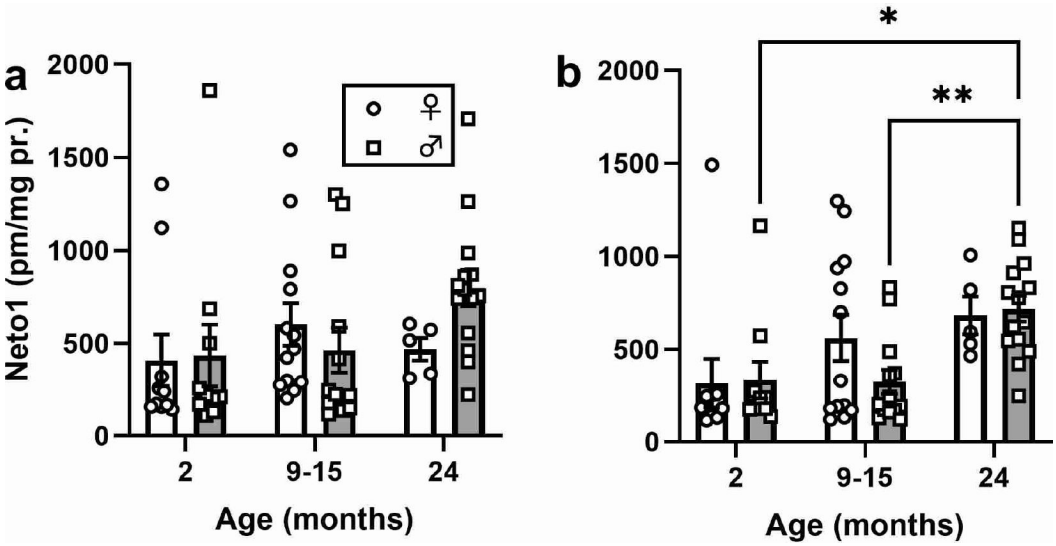

Figure 7.

Neuropilin and Tolloid-Like 1 (NETO1) levels were elevated in 24-month-old hippocampi. In (a) the frontal cortex Neto1 levels did not differ with sex or age, however in (b) the hippocampus Neto1 was higher in 24-versus 2-month-old males and females (* $p<0.05$) and in 24- versus 9–15-month-old males (** $p<0.01$). All data shown are group averages with SEM. Mice tested were male and female C57BL/6 aged 2 (n = 9–10 male, 10 female), 9–15 (n= 13 male, 13 female), and 24 (n= 14 male, 5 female) months at the time of testing.