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Research report

Effects of aging on the motor, cognitive and affective behaviors, neuroimmune responses and hippocampal gene expression

Gaurav Singhal[a], Julie Morgan[a], Magdalene C. Jawahar[a], Frances Corrigan[b], Emily J. Jaehne[a,c], Catherine Toben[a], James Breen[d,e], Stephen M. Pederson[e], Jim Manavis[f], Anthony J. Hannan[g], Bernhard T. Baune[g,h,i,*]

[a] Psychiatric Neuroscience Lab, Discipline of Psychiatry, The University of Adelaide, Adelaide, SA, Australia
[b] Division of Health Sciences, The University of South Australia, Adelaide, SA, Australia
[c] School of Psychology and Public Health, LIMS2, Room 204, La Trobe University, Bundoora, Melbourne, Vic, Australia
[d] Robinson Research Institute, The University of Adelaide, SA, Australia
[e] Bioinformatics Hub, School of Biological Sciences, The University of Adelaide, Adelaide, SA, Australia
[f] Centre for Neurological Diseases, School of Medicine, Faculty of Health, The University of Adelaide, Adelaide, SA, Australia
[g] Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Melbourne, VIC, Australia
[h] Department of Psychiatry, Melbourne Medical School, The University of Melbourne, Melbourne, VIC, Australia
[i] Department of Psychiatry, University of Münster, Münster, Germany

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ABSTRACT

The known effects of aging on the brain and behavior include impaired cognition, increases in anxiety and depressive-like behaviors, and reduced locomotor activity. Environmental exposures and interventions also influence brain functions during aging. We investigated the effects of normal aging under controlled environmental conditions and in the absence of external interventions on locomotor activity, cognition, anxiety and depressive-like behaviors, immune function and hippocampal gene expression in C57BL/6 mice. Healthy mice at 4, 9, and 14 months of age underwent behavioral testing using an established behavioral battery, followed by cellular and molecular analysis using flow cytometry, immunohistochemistry, and quantitative PCR. We found that 14-month-old mice showed significantly reduced baseline locomotion, increased anxiety, and impaired spatial memory compared to younger counterparts. However, no significant differences were observed for depressive-like behavior in the forced-swim test. Microglia numbers in the dentate gyrus, as well as CD8+ memory T cells increased towards late middle age. Aging processes exerted a significant effect on the expression of 43 genes of interest in the hippocampus. We conclude that aging is associated with specific changes in locomotor activity, cognition, anxiety-like behaviors, neuroimmune responses and hippocampal gene expression.

1. Introduction

Aging is a known risk factor for degenerative changes in various brain regions [1,2], which, in turn, also results in functional loss, such as progressive decline in learning, memory, and cognitive and motor functions [3–10]. Studies in the past have shown a significant increase in anxiety [11–14] and depressive-like [13,15] behaviors, and decline in spatial learning and memory [11,16–19] and locomotion [12,13] in aging C57BL/6 mice, particularly 12 month onwards. However, aging alone is not the only risk factor and other extrinsic (e.g., air pollution, psychological stress) and intrinsic (e.g., genetics, neurotransmitters) factors may also play a role in the impairment of brain functions during normal aging [20–24].

Research has shown that several underlying molecular changes are responsible for the change in behavior during normal aging. For example, during aging microglia and astrocytes become increasingly dysfunctional and lose neuroprotective properties [25–28]. The dysfunctional glial cells mediate immune reactivity and inflammation by

* Corresponding author at: University Hospital Department of Psychiatry and Psychotherapy, University of Münster, Albert-Schweitzer Campus 1, Building A 9, 48149 Münster, Germany.
E-mail addresses: Gaurav.Singhal@adelaide.edu.au (G. Singhal), Julie.Morgan@adelaide.edu.au (J. Morgan), Catharine.Jawahar@adelaide.edu.au (M.C. Jawahar), Frances.Corrigan@unisa.edu.au (F. Corrigan), E.Jaehne@latrobe.edu.au (E.J. Jaehne), Catherine.Toben@adelaide.edu.au (C. Toben), Jimmy.Breen@adelaide.edu.au (J. Breen), Stephen.Pederson@adelaide.edu.au (S.M. Pederson), Jim.Manavis@adelaide.edu.au (J. Manavis), Anthony.Hannan@florey.edu.au (A.J. Hannan), Bernhard.Baune@ukmuenster.de (B.T. Baune).

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expressing proinflammatory cytokines (e.g., TNF-α, IL-1β, IL-6, IL-10, interferons α, β, and γ) and chemokines (e.g., Ccl2, Ccl5) in the aging brain, particularly in the hippocampus, hypothalamus and cerebral cortex regions [29,30]. The chronic pro-inflammatory environment may predispose the brain to neurodegenerative disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD) [31–33].

Besides creating an inflammatory state, the dysregulated glial functions and the overexpressed proinflammatory cytokines impair serotoninergic and glutamatergic neurotransmission systems by activating the tryptophan- and serotonin-degrading enzyme indoleamine 2, 3-dioxygenase leading to increased consumption of serotonin and tryptophan [27,29,34–36]. The decline in dopamine and serotonin levels during normal aging has been shown to result in impaired cognitive and motor performances [8,37], and dysregulated synaptic plasticity and neurogenesis [38]. This has also been shown to alter hippocampal synaptic plasticity in aging rodents [39]. Indeed, there are substantial evidence suggesting that hippocampus, especially the dentate gyrus region, play a vital role in the regulation of mood behaviors, cognition and memory [40–47]. Dysregulated neurotransmission with reduced synaptic plasticity and impaired neurogenesis during neuronal aging are associated with various cognitive and affective disorders, including major depressive disorder (MDD) [8,37,38]. Aging has also been shown to impair growth hormones and glucose metabolism which may result in cognitive impairment and white matter lesions [8,38,48].

The expression of various genes in the brain, particularly in the hippocampus, also changes during aging. For example, the expression of the *BDNF* gene, which is important for neuronal survival in the adult brain and plays a role in the biology of mood disorders is reduced during old age [49,50]. Similarly, the expression of the gene that encodes the transcription factor CREB1 has been shown to decrease in the hippocampus of aging rats [51]. In contrast, the increased expression of proinflammatory factors, such as NLRP3 inflammasomes, TNF-α and its receptors Tnfrsf1a and Tnfrsf1b, and IL-6 also suggest that there is an increase in the expression of *Nlrp3*, *Tnf*, *Tnfrsf1a*, *Tnfrsf1b*, and *Il-6* genes in the brain of aging rodents [52–54]. The increase in the number of glial cells during normal aging is also due to the upregulation of hippocampal and striatal genes expressed in reactive microglia and astrocytes [25]. It is, therefore, possible that this downregulation of protective genes and upregulation of harmful genes during normal aging may be responsible for aging-related adverse effects on brain functions.

Environmental exposures and interventions significantly influence brain functions, and cellular and molecular mechanisms associated with neuroplasticity during normal aging. Hostile factors, for example psychological stress as mentioned previously, have been shown to generate metabolic oxidative stress and result in cognitive deficits, including memory loss, and behavioral impairments during old age [24,55]. On the contrary, favorable external factors such as physical exercise and enriched environment have been shown to stimulate a supportive immune response and gene expression in the brain, and thereby help to maintain brain homeostasis and in improving the brain functions [56–77].

Hence, to the author's knowledge, while there are a large number of studies that have reported the correlation between the age-associated change in brain functions, underlying molecular changes and external environmental factors, the current literature lacks to explain the effects of aging on brain functions in the absence of all external interventions. During this study, we bred and housed C57BL/6 mice in an ambient artificial environment devoid of all external interventions. We hypothesized that normal aging in mice would result in impairment in cognition, anxiety- and depressive-like behaviors, as well as a reduction in locomotor activity, especially middle age (9 month) onwards. We analyzed behavior using a standard behavioral battery designed to minimize the potential effects of various confounding factors and to ensure more accurate interpretations of behavioral phenotypes. Since the sex of the animal may regulate various behaviors during aging [11], we also performed sex analysis for various behaviors. The dentate gyrus region of the hippocampus was selected for immunohistochemistry analysis due to its known role in the regulation of behavior and memory as mentioned previously. Our results will help to understand the effects of normal aging on mouse behavior and may find practical application during the planning of future aging-related behavioral studies.

2. Methods

2.1. Animals

Wild-type (C57BL/6) mice (n = 46; 25 males and 21 females), parental sub-strain Nhsd (derived from a colony from the National Institutes of Health), were bred in-house in the laboratory animal services facility at the University of Adelaide and maintained under controlled conditions of temperature (22 ± 1 °C), humidity (55 %) and a 12 − 12 -h dark-light cycle. The C57BL/6Nhsd line has inbred between 9–13 generations. Mice were housed in same-sex groups of 4–5 in individually ventilated cages (IVCs) of dimensions 37 cm × 20.5 cm x 13.5 cm and were given adequate bedding and *ad libitum* access to standard laboratory food and water. The ethics approval for performing experiments on C57BL/6 mice was received from the University of Adelaide Animal Ethics Committee, and all guidelines as prescribed for handling the experimental animals were followed during the study.

2.2. Experimental design

Once of the desired age, i.e., 3, 8 and 13 months of age, mice that showed no signs of injury and sickness were randomly paired (males and females paired separately) and distributed equally into open-top cages for four weeks. The latter was provided to mice to allow them to acclimatize to the environmental conditions outside IVCs. Behavioral effects were determined at 4, 9, and 14 months of age. Each group had 12–19 (approximately 50 % male and 50 % female) mice per age group (Table 1). Following behavioral analysis, mice were sacrificed for molecular analysis and were divided about equally to perform fluorescence-activated cell sorting (FACS) and real-time quantitative PCR (RT-qPCR), and immunohistochemistry (IHC) (Table 1).

The cages had the following dimensions: 48.5 cm × 15.5 cm × 12 cm. Mice received no intervention and were provided with ample bedding and ad libitum standard laboratory food pellets and water. Mice were inspected daily but handled only once a week while transferring them to clean cages on every Friday morning, starting week 1. Friday was selected for change of cages to allow mice to acclimatize to the fresh bedding for two days before the Home Cage test was performed after four weeks of treatment. At the same time, mice were also weighed on a digital weighing scale. Mice were monitored for dominancy throughout the experiments, and those found to be dominant were segregated to prevent dominance effects on behavior and gene expression.

2.3. Behavioral analysis

After four weeks of acclimatization in open-top cages, mice undertook a behavioral battery following established procedures in the

Table 1
Mouse numbers for behavioral and molecular analysis at three age points.

Age	Behavioral analysis n (males: females)	FACS and RT-qPCR n	IHC n
4M	19 (10:9)	8	7
9M	15 (9:6)	7	6
14M	12 (6:6)	6	6

M: month, FACS: fluorescence-activated cell sorting, RT-qPCR: real-time quantitative PCR, IHC: immunohistochemistry.

laboratory and as per the below schedule:

Week 1: Home Cage (Monday), Open Field (Tuesday)
Week 2: Elevated-Zero Maze (Monday)
Week 3: Barnes Maze (Monday to Friday)
Week 4: Forced-Swim Test (Monday)

The behavioral testing was done at each time point in order of the least to most stressful tests. All trials were recorded by a ceiling-mounted camera and analyzed using Any-maze software version 4.70 from Stoelting, USA. To remove the olfactory traces, F10SC veterinary disinfectant was used to clean the testing area during and between behavioral tests.

2.3.1. Locomotor and exploratory behaviour

a **Home cage activity**: The baseline locomotor activity of mice were analyzed in home cages with 2-day old bedding under basal non-stressful conditions according to previously published protocols [78]. The total distance covered over five minutes was recorded as a measure of baseline locomotor activity.
b **Open Field**: Another test used to analyze the locomotor activity of mice was Open Field where mice were individually tested in a brightly lit (approximately 680 lx) square arena of 40 cm x 40 cm, with clear 35 cm high walls, according to previously published protocols [78–80]. The floor was divided into inner (26.6 cm × 26.6 cm) and outer (13.4 cm × 13.4 cm) zones. Total distance traveled over 5 min in Open Field was measured as an indication of locomotor activity and exploratory behavior.

2.3.2. Anxiety-like behaviour

a **Elevated-zero maze**: The elevated zero maze (EZM) is a round maze, 105 cm diameter, with a 5 cm wide platform 40 cm above the floor and divided into four equal quadrants [81]. Two quadrants have 15 cm high walls (closed), and two are open in alternate order. The EZM was placed in a brightly lit (approximately 175 lx) area during the behavioral testing. The mouse was placed on the open quadrant and allowed to explore for 5 min according to published protocols [82]. The time spent by the mouse in closed and open quadrants, and number of head dips were recorded as a measure of anxiety in mice. Anxious mice tend to avoid exposure to open quadrant, spend more time in quadrant with high walls and show a lower number of head dips [83].
b **Open Field**: In the Open Field, time spent by mice in the inner and outer zones over five minutes was recorded as a measure of anxiety-like behavior. Anxious mice tend to spend less time in the inner zone [78–80].

2.3.3. Spatial learning and memory

Barnes Maze: The Barnes maze, which is a circular grey platform 91 cm in diameter and 90 cm above the ground, with 20 holes on the perimeter, one with a hidden escape box and the rest with false boxes, was used to measure changes in spatial learning and retention of spatial memory in mice at 4, 9 and 14 months of age. Since the false boxes are too small to enter and looked the same as the target escape box to the mouse, they removed visual cues that might be observed through an open hole. Barnes Maze was placed in a brightly lit (approximately 230 lx) area and the procedures were carried out according to published protocols [78,84–87]. The mice were placed in the center of the maze and were allowed to locate the escape box for three minutes. Three trials separated by 15 min were performed for each mouse on a day. Time to find the location of the escape box over four days of training was recorded. Latency to find the location was used to assess the spatial learning and visual memory of the mouse in the new environment [88]. On day 5, the position of escape box was changed from the original training position (in NW quadrant) to the probe trial position (in NE quadrant – at 90 degrees). Latency to find the location of the escape box in the NW quadrant was recorded to assess the retention of spatial memory of the mouse in the new environment [88]. Latency to find the location of the escape box in NE quadrant was recorded to assess cognitive flexibility [88]. Since decreased drive to explore is a key factor underlying many aspects of reduced behavioral performance, including cognitive capacity during aging [12], we also statistically analyzed the distances traveled by mice while finding the location of the escape box on training days and during probe trial.

We also calculated the entry errors, i.e., searches of any holes that did not contain the hidden escape box beneath it following the protocol published by Nithianantharajah et al. [89]. This included nose pokes and head deflections over a hole. Increased number of entry errors were considered indices of spatial learning and memory impairment.

2.3.4. Depression-like behaviour

a Forced-swim test: Duration of immobility (when mice were floating with no movement of limbs in any direction) in a 4 L circular container, 20 cm in diameter and 45 cm high, filled 2/3rd with water at 23–24 degree Celsius and placed in a brightly lit (approximately 375 lx) area was recorded using a ceiling-mounted camera as a measure of despair and depressive-like behavior [83,87,90–93]. One trial of six minutes was conducted per animal to minimize distress associated with repeated testing.

2.4. Molecular analysis

After the behavioral testing at each time point, mice were randomly divided for FACS and RT-qPCR, and IHC analysis in numbers as mentioned before. Mice were terminally anesthetized with a lethal intraperitoneal dose of pentobarbital (60 mg/kg IP), and blood was collected through cardiac puncture [94]. Animals utilized for IHC were perfused via transcardiac injection with 10 % neutral buffered formalin, with the brains rapidly removed and placed in 10 % formalin until the further procedure. The other animals had cervical lymph nodes and brains extracted for FACS and gene expression analysis, respectively. The brains for gene expression analysis were stored in RNAlater (Ambion, Life Technologies) at -80 degrees C until further processing.

2.4.1. Peripheral T cell immunophenotyping

2.4.1.1. Fluorescence-activated cell sorting. FACS was applied for the detection of T cell numbers and characterization of their phenotype in the cervical lymph nodes of mice. This included $CD4^+$ and $CD8^+$ T cell subpopulations (Naïve or T_N, Central memory or T_{CM} and Effector memory or T_{EM}), their phenotype ($CD25^+$ and $CD69^+$) and CD4:CD8 T cells ratio.

The cervical lymph nodes from each mouse were retrieved one day after behavioral testing ended and collected in Roswell Park Memorial Institute (RPMI+) medium. Lymph nodes were passed through a 0.1μ sieve (BD) using RPMI + and centrifuged to separate cells from tissue debris. Retrieved lymph node cells were counted on a hemocytometer and resuspended in PBS to a final concentration of 2×10^6 cells/ml. 250 μL of the cell solution was then washed once with FACS buffer (PBS with 1 % heat-inactivated bovine serum albumin) and blocked with 10 μL 0.5 mg/mL Fc block. Eight color staining panel was used to characterize the $CD4^+$ and $CD8^+$ T cells. Unstained cells were used to gate out autofluorescent cells while single stained and fluorescence minus one (FMO) stained cells were used to control for spectral overlap or distinguishing between negative and positive cells respectively (non-specific bindings). Cells were incubated for 30 min at room temperature with the respective mAbs (as shown in Table 2) after which they were washed twice before resuspension in 300 μL FACS buffer. Cells were analyzed using the Gallios flow cytometer, and 100,000 events were acquired. The data obtained were analyzed using FCS Express software (version 4). Forward side scatter gating on acquired data distinguished singlet from doublet cell populations from which $CD45^+$ cells were

Table 2
Monoclonal antibodies used for FACS.

mAb	Clone	Fluorochromes	BD Biosciences catalog no.	Conc. (mg/mL)	Antigen Distribution/Function
CD3	145-2C11	FITC	553061	1.0×10^3	T-cell identification marker
CD45	30-F11	V500	561487	2.0×10^3	Nucleated hematopoietic cell lineage marker; common leukocyte antigen
CD4	GK1.5	APC-H7	560181	2.0×10^4	T helper cell co-receptor for MHC II-restricted antigen induced T-cell activation
CD8	53-6.7	PerCP-Cy5.5	551162	2.0×10^4	Cytotoxic T-cell Co-receptor for MHC I restricted antigen induced T-cell activation
CD25	3C7	PE	561065	2.0×10^3	Early T-cell activation marker
CD44	IM7	PerCy7	560569	5.0×10^4	Activation marker for effector or memory T-cells; attachment and rolling
CD62L	MEL-14	V450	560507	5.0×10^5	T-cell homing receptor; transmigration
CD69	H1.2F3	APC	560689	2.0×10^3	Early T-cell activation marker

gated. Percentages of $CD3^+$ $CD4^+$ or $CD3^+$ $CD8^+$ positively gated cells were used to calculate total cell numbers in combination with cell counts. Gating on $CD25^+$, $CD44^+$, $CD62L^{+,}$ and $CD69^+$ positive cell populations enabled further phenotyping of T cells.

2.4.2. Glial cell immunophenotyping

2.4.2.1. Immunohistochemistry. Brains preserved in formalin were cut into five 3 mm coronal slices and following overnight treatment with increasing concentrations and durations of ethanol, xylene and paraffin baths, the sliced brain samples were embedded in paraffin wax. The hippocampus was then serial sectioned, with six sections 150 μm apart. Each section was 5 μm thick.

For IHC, on day 1, sections were dewaxed and dehydrated in xylene and ethanol, and endogenous peroxidase activity was blocked by incubation with 0.5 % hydrogen peroxide in methanol for 30 min. Antigen retrieval was performed by heating at close to boiling point for 10 min. in citrate buffer, and slides were then allowed to cool below 40 °C before further processing. The appropriate primary antibody IBA1 for microglia, 1: 10,000; GFAP for astrocytes 1: 40,000; Abcam was applied to the slides which were then left to incubate overnight for allowing primary antibodies to bind to the target antigen. On day 2, the IgG biotinylated antibody of rabbit same as primary antibodies was added and allowed to react with primary antibodies for 30 min. The formed immune complex was then further amplified by incubating slides with a biotin-binding protein, streptavidin-peroxidase conjugate, for 60 min. The immune complex was then visualized with precipitation of DAB in the presence of hydrogen peroxide. Slides were washed to remove excess DAB and lightly counterstained with hematoxylin, dehydrated and mounted with DePeX.

All slides were digitally scanned (Nanozoomer, Hamamatsu City, Japan) and then viewed with the associated software (NDP view version 1.2.2.5). Immunopositive cells in the dentate gyrus region of the hippocampus were counted manually for statistical analysis. Freehand boxes were drawn to cover the entire dentate gyrus regions in the right hemisphere of hippocampus of the six stained sections followed by counting of the cells within the boxes. For each section, the total number of cells was then divided with the area of the box (in mm^2) to get the number of cells/mm^2. The average of six sections represented the value for one mouse and was utilized during statistical analysis.

2.4.3. Gene expression analysis

2.4.3.1. Real-time quantitative PCR. Quantification of the levels of mRNA of 43 genes of interest (GOI) across various cytokines, monoamines, neurotrophins and other genes along with 4 endogeneous reference genes (Table 3) was performed using TaqMan assays (Life Technologies, ThermoFisher, Australia) in the high-throughput qPCR system BioMarkHD™(Fluidigm Inc., USA). Briefly, total RNA was extracted from HC tissues stored in RNA later (Ambion, Life Technologies) using PureLink RNA mini extraction kit (Ambion) following the manufacturer's instructions. Total RNA was then subjected to reverse transcription using the SuperScript III first-strand cDNA synthesis system (Invitrogen, Australia) according to the manufacturer's instructions. The samples were then prepared for High-throughput qPCR in BioMark HD™(Fluidigm Inc., USA) using a single 14-cycle Pre-amplification consisting of 20 ng of each cDNA samples mixed with pooled TaqMan assays (47 assays listed in supplementary material 1) and PreAmp Master Mix (Fluidigm Inc., USA).

RT-qPCR was performed for each TaqMan assay for each sample in a 96.96 dynamic array nanofluidic chip (Fluidigm Inc., USA). A total of 47 × 48 (Assays x Samples in duplicates) PCR reactions were performed. Cycle threshold (Ct) values were generated by Fluidigm Real-time PCR analysis software (Fluidigm Inc., USA).

2.5. Statistical analysis

Statistical analyses of mouse body weights and behavioral data were conducted using GraphPad Prism version 7.02 (GraphPad Software Inc.). All data outliers were removed using the ROUT method, and normality of data distribution was determined by visual inspection of histograms. The two-way interaction between age and sex was determined using two-way ANOVA. If the two-way interaction was non-significant, a one-way ANOVA was conducted to determine the effects of age on the dependent variable. If the two-way interaction was significant, the data were entered in SPSS statistics version 25.0 (IBM Corporation) and a one-way ANCOVA was conducted to control for sex differences while determining the effects of age on the dependent variable. Similarly, the behavioral data obtained during the four days of training in the Barnes maze were statistically analyzed using three-way ANOVA in SPSS statistics, followed by a two-way repeated-measures ANOVA or two-way ANCOVA. The analysis of sex differences for molecular data was not possible due to the relatively smaller sample size (n) compared to behavioral data. This also limits us from performing the correlation analysis between behavioral and molecular data. Hence, the analyses of data from immunohistochemistry and flow cytometry were carried out using one-way ANOVA in GraphPad Prism to determine the effects of age on the dependent variable. Post hoc analysis was conducted using Tukey's multiple comparison tests. Results are presented as F-test, p values and mean ± SEM for ANOVAs, and F-test, p values and covariate-adjusted means (estimated marginal means, EMM) ± 2 SE for ANCOVAs. Differences were considered statistically significant when $p < .05$.

Differentially expressed genes were identified by analysis of Ct data measurements taken from the BioMarkHD arrays in R. Briefly, Input expression values (Ct) of house-keeping genes (*B2m*, *Gapdh*, *Gusb*, and *Hprt*) were compared across all samples to identify outlier samples. Delta Ct values were normalized against the geometric mean of all sample expression values. Linear Mixed-Effects Models were then used to compare normalized expression between the groups and adjusted for multiple comparisons using the R package multcomp [95]. Genes were identified as differentially expressed when the adjusted p values were < 0.05 with Z scores indicating the direction of expression change. David (functional annotation clustering tool) and GeneMANIA (Multiple Association Network Integration Algorithm) prediction servers were used to identify the molecular pathways modulated by the differentially expressed genes at false discovery rate (FDR) < 0.05

Table 3
Genes of Interest quantified using high-throughput qPCR.

Gene Symbol	Gene Name	TaqMan Assay ID
Monoamine		
Slc6a3	solute carrier family 6 (neurotransmitter transporter; dopamine); member 3	Mm00438388_m1
Slc6a4	solute carrier family 6 (neurotransmitter transporter; serotonin); member 4	Mm00439391_m1
Htr1a	5-hydroxytryptamine (serotonin) receptor 1A	Mm00434106_s1
Htr1b	5-hydroxytryptamine (serotonin) receptor 1B	Mm00439377_s1
Htr2a	5-hydroxytryptamine (serotonin) receptor 2A	Mm00555764_m1
Grin2a	glutamate receptor; ionotropic; NMDA2A (epsilon 1)	Mm00433802_m1
Gria1	glutamate receptor; ionotropic; AMPA1 (alpha 1)	Mm00433753_m1
Gria2	glutamate receptor; ionotropic; AMPA2 (alpha 2)	Mm00442822_m1
Tph2	tryptophan hydroxylase 2	Mm00557715_m1
Growth factors		
Bdnf	brain-derived neurotrophic factor	Mm04230607_s1
Ntrk1	neurotrophic tyrosine kinase; receptor; type 1	Mm01219406_m1
Creb1	cAMP responsive element binding protein 1	Mm00501607_m1
Ngf	nerve growth factor	Mm00443039_m1
Igf1	insulin-like growth factor 1	Mm00439560_m1
Immune factors and receptors		
Il1r1	interleukin 1 receptor; type I	Mm00434237_m1
Il1b	interleukin 1 beta	Mm00434228_m1
Il6	interleukin 6	Mm00446190_m1
Il10	interleukin 10	Mm01288386_m1
Il12a	interleukin 12a	Mm00434169_m1
Tnf	tumor necrosis factor	Mm00443258_m1
Tnfrsf1a	tumor necrosis factor receptor superfamily; member 1a	Mm00441883_g1
Tnfrsf1b	tumor necrosis factor receptor superfamily; member 1b	Mm00441889_m1
Ifng	interferon-gamma	Mm01168134_m1
Foxp3	forkhead box P3	Mm00475162_m1
Smad2	SMAD family member 2	Mm00487530_m1
Smad3	SMAD family member 3	Mm01170760_m1
Foxo3	forkhead box O3	Mm01185722_m1
Nlrp3	NLR family; pyrin domain containing 3	Mm00840904_m1
Aif1	allograft inflammatory factor 1	Mm00479862_g1
Gfap	glial fibrillary acidic protein	Mm01253033_m1
HPA axis/Stress response		
Nr3c1	nuclear receptor subfamily 3; group C; member 1	Mm00433832_m1
Crhr1	corticotropin-releasing hormone receptor 1	Mm00432670_m1
Crh	corticotropin-releasing hormone	Mm01293920_s1
Mitochondrial function/ROS and Oxidation		
Sirt1	silent mating type information regulation 2 homolog 1	Mm01168521_m1
Cs	cytrate synthase	Mm00466043_m1
Uqcrc1	ubiquinol-cytochrome c reductase core protein 1	Mm00445911_m1
Actb	beta-actin	Mm02619580_g1
Sod1	superoxide dismutase 1	Mm01344233_g1
Sod2	superoxide dismutase 2	Mm01313000_m1
Gpx1	glutathione peroxidase	Mm00656767_g1
Cat	catalase	Mm00437992_m1
Prkaa1	AMPK-activated protein kinase 1	Mm01296700_m1
Prkaa2	AMPK-activated protein kinase 2	Mm01264789_m1
Endogenous references		
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1
Hprt	hypoxanthine-guanine phosphoribosyl transferase	Mm03024075_m1
Gusb	glucuronidase; beta	Mm01197698_m1
B2m	Beta-2-Microglobulin	Mm00437762_m1

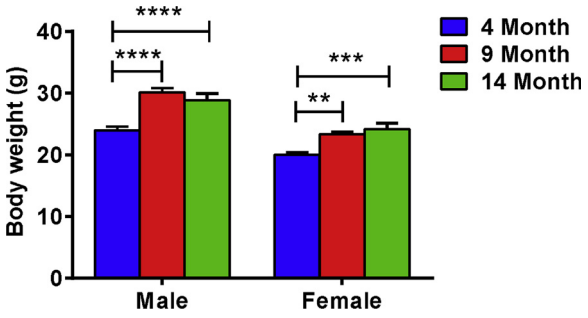

Fig. 1. Body weights of male and female C57BL/6 mice at 4, 9, and 14 months of age. Two-way ANOVA, n = 6–10 per group. All data represented as mean ± SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

[96,97].

3. Results

3.1. Body weights

Body weights at 4, 9 and 14 months of age were analyzed using a two-way ANOVA. The interaction between age and sex was found to be non-significant ($F_{(2, 40)} = 2.346$, $p = .109$), but the simple main effects of both age and sex were significant ($F_{(2, 40)} = 33.73$, $p < .0001$ and $F_{(1, 40)} = 82.95$, $p < .0001$). Simple comparisons revealed that the mean body weights of 4-month-old male and female mice were significantly lower than 9 and 14-month-old male (24.0 ± .55 vs. 30.1 ± .72 and 28.8 ± 1.08, $p < .0001$) and female (20.0 ± .39 vs. 23.3 ± .42, $p < .01$ and 24.2 ± .95, $p < .001$) cohorts (Fig. 1).

Further analysis was carried out in SPSS statistics to control for sex differences. A one-way ANCOVA with sex as a covariate revealed the main effect of age on body weights as still significant ($F_{(2, 42)} = 32.87$, $p < .001$; Fig. 2). The body weights of 4-month-old mice were significantly less than those of 9- and 14-month-old mice (22.9 ± 0.5 vs. 27.4 ± 0.8 and 27.3 ± 1.1, $p < .001$). No significant differences were observed between the body weights of 9- and 14-month-old mice.

3.2. Behaviour

3.2.1. Locomotor activity

Baseline locomotor activity was measured in the home cage (Fig. 3). Two-way ANOVA revealed a non-significant two-way interaction between age and sex for baseline locomotion ($F_{(2, 40)} = .11$, $p = .89$). Similarly, the main effect of sex was also non-significant ($F_{(1, 40)} = .98$, $p = .327$), but the main effect of age was significant ($F_{(2, 40)} = 3.76$, $p = .032$). Hence, further analysis was carried out using a one-way

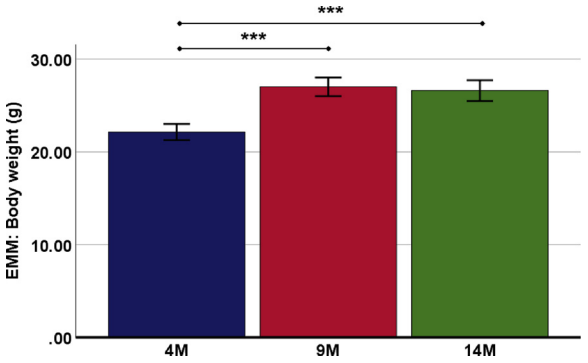

Fig. 2. Body weights of C57BL/6 mice at 4, 9, and 14 months of age. One-way ANCOVA with sex used as a covariate at the following value: 1.48, n = 12–19 per group. All data represented as estimated marginal means (EMM) ± 2 SE. *** $p < .001$.

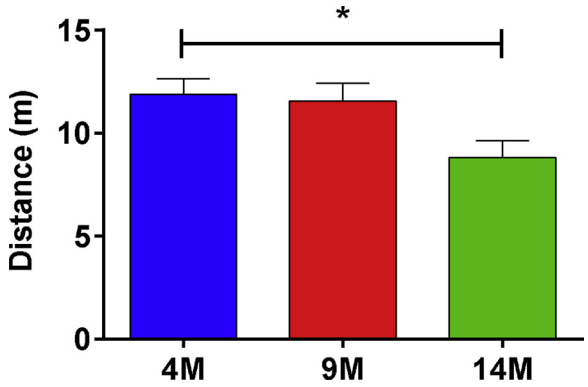

Fig. 3. Baseline locomotor activity of C57BL/6 mice in the Home Cage at 4, 9, and 14 months of age. All data represented as mean ± SEM, n = 12–19 per group. * p < .05.

ANOVA which showed that aging significantly affects baseline locomotion in the Home Cage ($F_{(2, 43)} = 3.84$, $p = 0.029$). On post-hoc analysis, significant differences were noted between the 4 month and 14-month age groups (11.9 ± 0.7 vs. 8.8 ± 0.9).

We also measured locomotion under the more stressful environment of Open Field. Two-way ANOVA found a non-significant interaction between age and sex ($F_{(2, 40)} = .13$, $p = .876$), as well as non-significant main effects of both age ($F_{(2, 40)} = .12$, $p = .871$) and sex ($F_{(1, 40)} = 1.18$, $p = .283$).

3.2.2. Anxiety-like behavior

Time spent in the open quadrant of the EZM is a measure of anxiety-like behavior, with less anxious mice tending to spend more time in the open quadrant. The interaction between age and sex was non-significant in two-way ANOVA ($F_{(2, 33)} = 2.00$, $p = .152$). The main effect of sex was also non-significant ($F_{(1, 33)} = .04$, $p = .844$), but the main effect of age was significant ($F_{(2, 33)} = 4.07$, $p = .026$). Hence, further analysis was carried out using a one-way ANOVA which showed a significant effect of age on time spent in the open arm of EZM between the three age groups ($F_{(2, 36)} = 4.13$, $p = 0.024$; Fig. 4A). Post-hoc analysis using Tukey's multiple comparison test showed that 4-month-old mice spent significantly more time in the open quadrant of EZM than 14-month-old mice (45.8 ± 3.7 vs. 27.7 ± 5.9, $p = 0.021$).

The number of head dips along the open quadrant in EZM is a measure of anxiety-like behavior, with less anxious mice show more number of head dips. Two-way ANOVA found a non-significant interaction between age and sex ($F_{(2, 39)} = 2.44$, $p = .1$). The main effect of sex was also non-significant ($F_{(1, 39)} = 2.0$, $p = .167$) but the main effect of age was significant ($F_{(2, 39)} = 5.24$, $p = .001$). Further analysis was carried out using a one-way ANOVA which showed a significant effect of age on head dips in the EZM between the three age groups ($F_{(2, 42)} = 4.97$, $p = 0.012$; Fig. 4B). Post hoc analysis revealed that 4-month-old mice showed a significantly higher number of head dips than 14-month-old mice (26.2 ± 1.5 vs. 15.9 ± 3.4, $p = 0.009$).

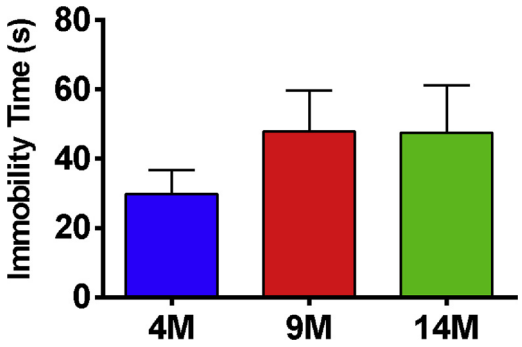

Fig. 5. Depressive-like behavior. Immobility time in FST. All data represented as mean ± SEM, n = 12–19 per group.

Time spent in the inner zone of the Open Field is a measure of anxiety-like behavior, with less anxious mice tend to spend more time in the inner zone. Similar to EZM, two-way ANOVA found a non-significant interaction between age and sex ($F_{(2, 36)} = 1.05$, $p = .36$). The main effect of sex was also non-significant ($F_{(1, 36)} = 1.08$, $p = .306$), however, the main effect of age was again found to be significant ($F_{(2, 36)} = 9.70$, $p < .001$). Hence, further analysis was carried out using a one-way ANOVA which showed a significant effect of age on time spent in the inner zone of the Open Field between the three age groups ($F_{(2, 39)} = 6.88$, $p = 0.003$; Fig. 4C). Post-hoc analysis using Tukey's multiple comparison test showed that 9-month-old mice spent significantly more time in the inner zone than 4-month-old mice (118.3 ± 9.3 vs. 70.8 ± 8.8, $p = 0.002$).

3.2.3. Depressive-like behaviour

Mice were tested for depressive-like behaviour in the forced-swim test. Two-way ANOVA found non-significant interaction between age and sex for immobility time ($F_{(2, 39)} = .24$, $p = .791$), as well as non-significant main effects of age ($F_{(2, 39)} = 1.48$, $p = .24$) and sex ($F_{(1, 39)} = 1.01$, $p = .322$). One-way ANOVA confirmed that age has no significant effect on the immobility time in FST ($F_{(2, 41)} = 1.06$, $p = .355$; Fig. 5).

3.2.4. Spatial learning, retention of spatial memory and cognitive flexibility

Spatial learning in separate cohorts of mice of different ages was tested by measuring the latency to find the escape box over four days of training in Barnes maze. Three-way ANOVA in SPSS statistics found a non-significant interaction between age and sex for latency to escape over four days of training ($F_{(2, 160)} = 2.83$, $p = .062$). The main effect of sex was also non-significant ($F_{(1, 160)} = 2.47$, $p = .118$) but the main effect of age was significant ($F_{(2, 160)} = 16.54$, $p < .001$). Hence, further analysis was carried out using a two-way repeated-measures ANOVA which confirmed the significant effect of age on spatial learning duirng four days of training in Barnes maze ($F_{(2, 43)} = 6.30$, $p = .004$; Fig. 6A). Post hoc analysis revealed that 14-month-old mice spent a significantly longer time to find the escape box than 4-month-

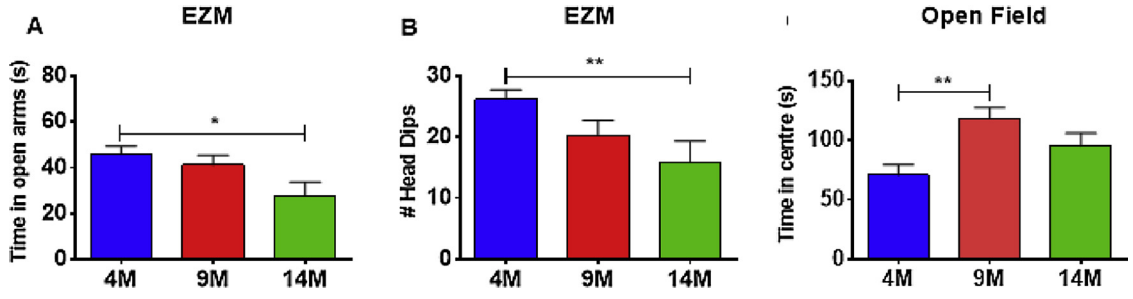

Fig. 4. Anxiety-like behavior. (A) EZM: time in open arms, (B) EZM: number of head dips and (C) Open Field: Time in the inner zone. All data represented as mean ± SEM, n = 12–19 per group. * p < 0.05, ** p < 0.01.

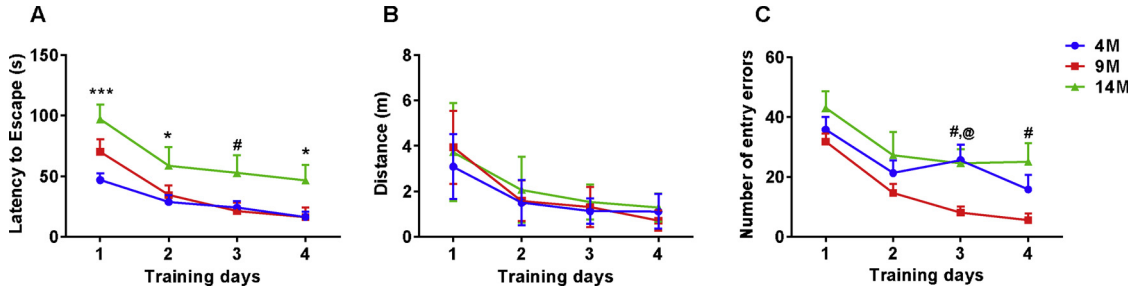

Fig. 6. Spatial learning: (A) Latency to find the escape box, (B) Distance traveled while locating the escape box, and (C) Entry errors over 4 days of training in Barnes maze. All data represented mean ± SEM, n = 12–19 per group. * 4 M vs. 14 M, $^{@}$ 4 M vs. 9 M, $^{\#}$ 9 M vs. 14 M. $^{*, \#, @}$ $p < .05$, *** $p < .001$.

old mice on days 1 (97.0 ± 12.2 vs. 46.8 ± 5.7, $p < .001$), 2 (58.6 ± 15.4 vs. 28.6 ± 5.9, $p = .046$) and 4 (46.6 ± 12.7 vs. 16.4 ± 4.2, $p = .044$) of traning (Fig. 6A). Likewise, 14-month-old mice showed higher latency to escape than 9-month-old mice on day 3 (52.6 ± 14.7 vs. 21.3 ± 7.1, $p = .048$) of traning. Also, although not significant at $p < .05$, a trend at $p < .1$ of 14-month-old mice showing significantly higher latency to escape than 4-month-old mice on days 3 ($p = .062$), and 9-month-old mice on days 4 ($p = .056$) of training was also observed.

We also analyzed the distance of the path to the escape box over four days of training in the Barnes maze. The three-way ANOVA showed a non-significant interaction between age and sex ($F_{(2, 160)} = .57$, $p = .565$), as well as non-significant main effects of both age ($F_{(2, 160)} = 2.26$, $p = .108$) and sex ($F_{(1, 160)} = 1.17$, $p = .282$) for distance traveled over four days of training. A two-way repeated-measures ANOVA confirmed the non-significant effect of age on distance traveled during four days of training in Barnes maze ($F_{(2, 43)} = 1.23$, $p = .303$; Fig. 6B).

Spatial learning in separate cohorts of mice of different ages was also tested by measuring the number of entry errors in finding the escape box over four days of training in Barnes maze. Three-way ANOVA found a significant two-way interaction between age and sex ($F_{(2,160)} = 5.32$, $p = .006$) and main effect of age on entry errors ($F_{(2,160)} = 11.14$, $p < 0.001$), but the main effect of sex was non-significant ($F_{(1,160)} = 3.52$, $p = .062$) over four days of training. Further analysis using a two-way repeated-measures ANOVA showed that age significantly affected the entry errors during four days of training in Barnes maze ($F_{(2, 43)} = 4.62$, $p = .015$; Fig. 6C). During post hoc analysis, we observed that 9-month-old mice showed significantly less entry errors than 4-month-old mice on day 3 (8.1 ± 2.0 vs. 25.7 ± 5.1, $p = .014$) and 14-month-old mice on days 3 (8.1 ± 2.0 vs. 24.7 ± 4.6, $p = .046$) and 4 (5.6 ± 2.1 vs. 25.1 ± 6.2, $p = .015$) of training in Barnes maze (Fig. 5B).

After 4 days of training on Barnes maze, mice were tested for retention of spatial memory and cognitive flexibility on day 5. Two-way ANOVA found non-significant interaction between age and sex for latency to escape in the original location (NW quadrant) of the escape box ($F_{(2,40)} = .61$, $p = .551$). The main effect of sex was also non-significant ($F_{(1,40)} = 2.84$, $p = 0.099$), but the main effect of age was significant ($F_{(2,40)} = 7.59$, $p = 0.002$). One-way ANOVA found significant differences in latency to escape due to age effects ($F_{(2,36)} = 27.3$, $p < .0001$; Fig. 7A). Post-hoc analysis using a Tukey's multiple comparison test showed that 14-month-old mice spent significantly higher time than both 4- and 9-month-old mice to find the location of the escape box in the NW quadrant of Barnes maze (110.5 ± 21.6 vs. 3.7 ± 0.7 and 4.8 ± 1.1, $p < .0001$).

Furthermore, a two-way ANOVA found non-significant interaction between age and sex for latency to escape in the new location (NE quadrant) of escape box ($F_{(2,40)} = 2.99$, $p = .062$). The main effects of sex and age were also non-significant ($F_{(1,40)} = 0.96$, $p = 0.333$ and $F_{(2,40)} = 1.56$, $p = 0.223$). Since the interaction was significant at $p < .1$, we conducted one-way ANOVA to analyze if age has any effect on cognitive flexibility and observed significant differences ($F_{(2,40)} = 3.83$, $p = .030$; Fig. 7B). Post hoc analysis revealed that 14-month-old mice took significantly longer to find the NE location of escape box than 9-month-old mice (77.7 ± 20.1 vs. 30.8 ± 6.1, $p = .028$).

For the analysis of distance traveled during probe trial, we conducted a two-way ANOVA that showed non-significant interecation between age and sex for distance traveled to find both the original (NW quadrant) and new (NE quadrant) locations of the escape box ($F_{(2,40)} = 1.89$, $p = .164$ and $F_{(2,40)} = .82$, $p = .447$). Similarly, the main effect of age and sex were also non-significant for distance traveled during the probe trial of Barnes maze ($p > .05$), thereby suggesting non-significant differences in the mobility of mice of the three age groups during Barnes maze.

Mice were also tested for entry errors in the probe trial on Barnes maze. Two-way ANOVA found a non-significant two-way interaction between age and sex ($F_{(2,40)} = 2.33$, $p = .111$). The main effects of age was also non-significant ($F_{(2,40)} = 2.15$, $p = .130$), but the main effect of sex was significant ($F_{(1,40)} = 4.64$, $p = .037$). However, simple comparisons revealed no significant differences between males and between females of the three age groups ($p > .1$; Fig. 8A). A one-way ANCOVA after controlling for sex differences also showed non-significant effects of age on entry errors in the probe trial of Barnes maze ($F_{(2,41)} = 1.56$, $p = .223$; Fig. 8B).

3.3. Molecular analyses

3.3.1. Alteration in the number of microglia and astrocytes within the dentate gyrus in C57BL/6 mice with aging

The microglial response with age was measured by counting the IBA1^{+} cells in the dentate gyrus and statistically analyzing the data using a one-way ANOVA. We observed a significant main effect of age ($F_{(2,16)} = 6.98$, $p = .007$, Fig. 9), with decrease in the number of immunopositive microglia from 4 month to 9 month (71.6 ± 4.9 vs 51.7 ± 5.3, $p = 0.036$) followed by an increase from 9 month to 14 month of age (51.7 ± 5.3 vs 78.7 ± 5.3, $p = 0.007$).

We also measured astrocytic response by counting the GFAP^{+} cells in the dentate gyrus and statistically analyzing using a one-way ANOVA and observed non-significant effects of age ($F_{(2,15)} = 2.27$, $p = .138$, Fig. 10).

3.3.2. Immunophenotyping of T cells in the brain of C57BL/6 mice at 4, 9 and 14 months of age

There was no statistically significant effect of age on the proportion of CD4^{+} and CD8^{+} T cells ($F_{(2,32)} = .641$, $p = .533$, Fig. 11A). The proportion of the CD4^{+} T cell subsets, i.e., T_N, T_{CM} and T_{EM} cells were also not significantly different between the three age groups ($F_{(4,49)} = .171$, $p = .952$; Fig. 11B). However, the proportion of CD8^{+} T cell subsets were found to be significant among the three age groups ($F_{(4,51)} = 4.989$, $p = .002$; Fig. 11C). Post hoc analysis revealed that the proportion of the CD8^{+} T_N cells was significantly higher in the 9-month-old mice in comparison to 14-month-old mice (15.2 ± 1.2 vs 10.2 ± 1.3, $p = 0.018$). Conversely, 14-month-old mice showed

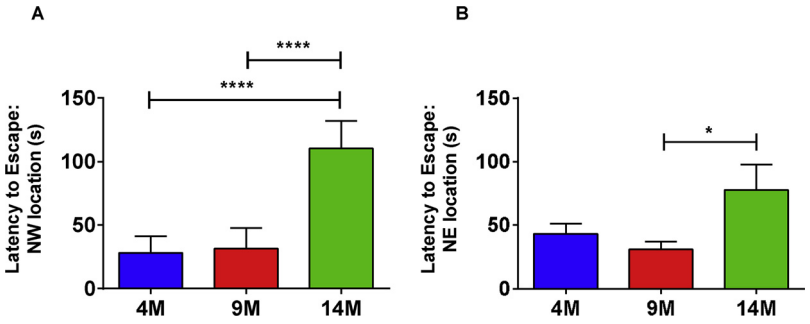

Fig. 7. Spatial memory. Latency to escape in probe trial in the (A) NW location and (B) NE location of Barnes maze on day 5. All data represented as mean ± SEM, n = 12–19 per group. * $p < 0.05$, **** $p < 0.0001$.

significantly higher proportion of $CD8^+$ T_{CM} cells than both 4- (14.3 ± 1.4 vs 9.6 ± 1.2, p = 0.040) and 9-month-old mice (14.3 ± 1.4 vs 8.2 ± 1.2, p = 0.006). There was no significant difference in the proportion of $CD8^+$ T_{EM} cells between the three age groups. Similarly, the proportion of CD4 and CD8 early activation CD25 marker showed non-significant difference between the three age groups ($F_{(2,14)} = .297$, p = .748 and $F_{(2,14)} = .426$, p = .661). The mean ± SEM for T cell subset counts in absolute numbers for the three age groups are shown in Table 4.

3.3.3. Changes in the expression of 43 GOI in the hippocampus of C57BL/6 mice with aging

Real-time high-throughput qPCR was used to analyze changes in the hippocampal expression of 43 target genes (Table 2) in response to aging.

Differential expression analysis of the 43 genes between the three age groups (i.e., 4, 9 and 14 months) identified 13, 21 and 21 GOI differentially regulated in the hippocampus of C57BL/6 mice between 4 and 9, 4 and 14, and 9 and 14-month groups respectively in response to only aging effects (see Table 5). We observed 2 GOI upregulated and 11 GOI downregulated in the 9-month-old mice when compared to 4-month-old mice. On comparing 4-month-old mice with 14-month-old mice, we observed 4 upregulated GOI, and 17 downregulated GOI in the 14-month group. Similarly, the 14-month group showed 6 upregulated GOI, and 15 downregulated GOI when compared to the 9-month group.

4. Discussion

The study was conducted to understand the effects of normal aging on behavior and the underlying molecular biology in the absence of all external interventions. The effects of normal aging on behavior and underlying molecular biology were determined at 4 (early), 9 (middle), and 14 (late-middle) months of age in C57BL/6 mice. We observed that there was a significant reduction in the baseline locomotion at 14 months when compared to 4 months in the home cage, but not within the more stressful environment of an open field. Similarly, a significant increase in anxiety-like behavior and a decrease in spatial memory was observed in 14-month-old mice compared to 4-month-old mice, which suggests that age is a controlling factor for the onset of anxiety and memory impairment. Interestingly, we observed that depressive-like behavior is independent of age. Normal aging also seems to affect neuroimmune mechanisms as microglia numbers in the dentate gyrus, as well as $CD8^+$ memory T cells increased in 14-month-old mice. However, no significant change in astroglia numbers was observed between the three age groups. Also, we observed that age significantly affects the expression of hippocampal GOIs. To make the discussion more structured, we will discuss these effects pointwise below.

4.1. Bodyweight increases significantly until middle age

The mean body weights of 9- and 14-month-old mice were significantly more than 4-month-old mice, both in males and females. No significant differences were observed between the mean body weights of 9-month-old and 14-month-old mice. This is suggestive of the rapid growth of mice until middle age which then slows down. We provided a dietary intake without malnutrition. Mice were not provided with any special diet, such as a high-fat diet as reported in other studies [98,99]. A highly significant increase in body weight has been reported in C57BL/6 mice from 2 to 18 months of age [18]. Evidence suggests that after an initial fast increase, growth continues but at a slower pace until weight peaks at 15–20 months in C57BL/6 mice [12]. During our experiments the bodyweight of mice peaked at nine months and then remained steady until the end of our experiments, i.e., at fourteen months. These variations in reported results for change in body weight across studies likely reflect the fact that external environmental and genetic factors also modulate growth during normal aging.

4.2. Locomotor activity reduces significantly at late-middle age under basal conditions but not in a stressful environment

Our results also showed that during normal aging, there is a decrease in locomotor activity under basal conditions in home cage, and the difference becomes significant by late-middle age. Conversely, under challenging conditions of the Open Field, no difference in locomotion was observed between the three age groups. Another study has

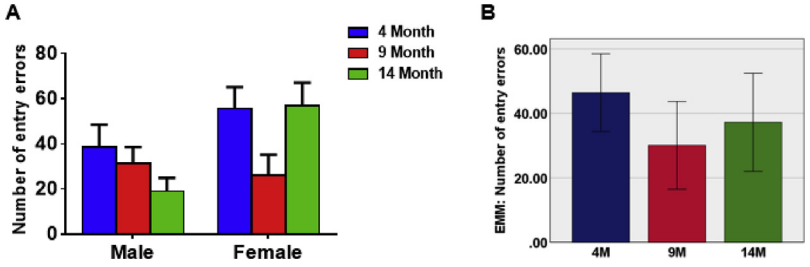

Fig. 8. Entry errors during probe trial in the Barnes maze. (A) Two-way ANOVA, n = 6–10 per group, all data represented as mean ± SEM, and (B) one-way ANCOVA with sex used as covariate at the following value: 1.4565, all data represented as estimated marginal means (EMM) ± 2 SE. n = 12–19 per group.

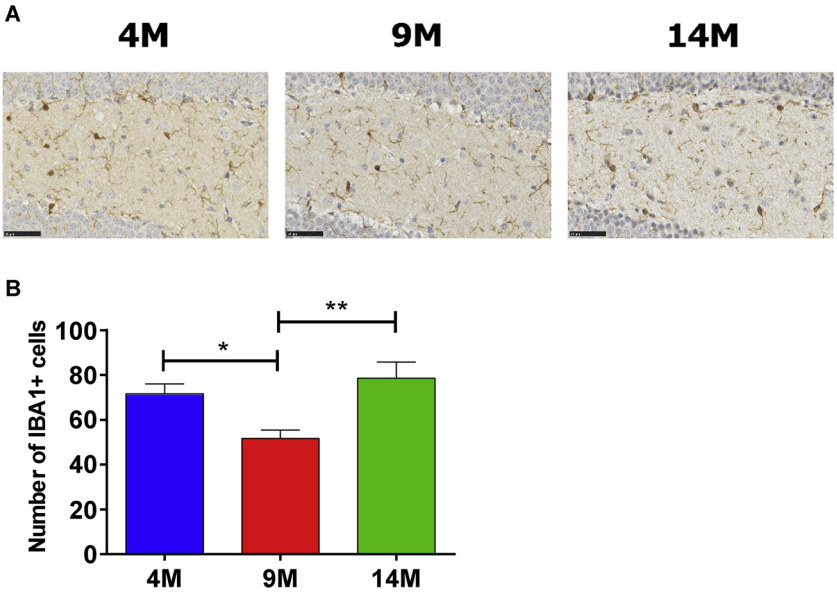

Fig. 9. Effects of aging on IBA1$^+$ cells in the dentate gyrus: (A) Representative immunohistochemical images of the number of IBA1$^+$ cells in the dentate gyrus (black scale represents 50 μm length), and (B) Number of IBA1$^+$ cells/mm^2. One-way ANOVA in GraphPad Prism. All data represented as Mean ± SEM, n = 6–7 per group. * $p < 0.05$, **$p < 0.01$.

shown no loss of locomotion in the Open field until eight months of age, but locomotion significantly reduced at 15 months, and this became highly significant at 28 months of age in C57BL/6 mice [12]. Similarly, no significant loss in locomotion was reported in 2- to 7-month-old C57BL/6 mice when tested in the open field for 5 min [13]. However, 8- to 12-month-old mice traveled significantly less distance. These results are contrary to what we report here. It is important to note that the Open Field test was conducted under an artificial light of approximately 680 lx, hence, the test was more challenging for mice than when conducted under basal conditions of home cage. It is not clear whether the authors in the above-mentioned studies used artificial light or not during the Open Field. It is possible that artificial light stimulates locomotion in middle-aged mice. Indeed, a study showed no significant difference in locomotion in 5, 13, and 15-month-old C57BL/6 mice in the Open Field when the arena was illuminated by 300 lx of light [17]. It is also important to note that we analyzed the effects of aging only until late-middle age and hence cannot validate findings of a significant decrease in locomotion at old age in Open Field as reported in another study [100].

4.3. Normal aging causes a significant increase in anxiety-like behavior by late-middle age

We observed an increase in the anxiety-like behavior from 4 to 14 months in C57BL/6 mice, as evident from reduced time in open quadrant and the number of head dips by 14 months mice in the EZM. However, in the Open Field, 4-month-old mice spent significantly less time in the inner zone compared to 9 months old mice, suggesting that 4-month-old mice were more anxious than 9-month-old mice. These results comply closely with the published findings. Studies have shown a significant increase in anxiety-like behavior in 12-17-month-old and 28-month-old [11–14], but not in 5 and 11-month-old [11,101] C57BL/6 mice in the elevated-plus maze test. However, unlike a study that reported an increase in anxiety-like behavior in 17-month female and

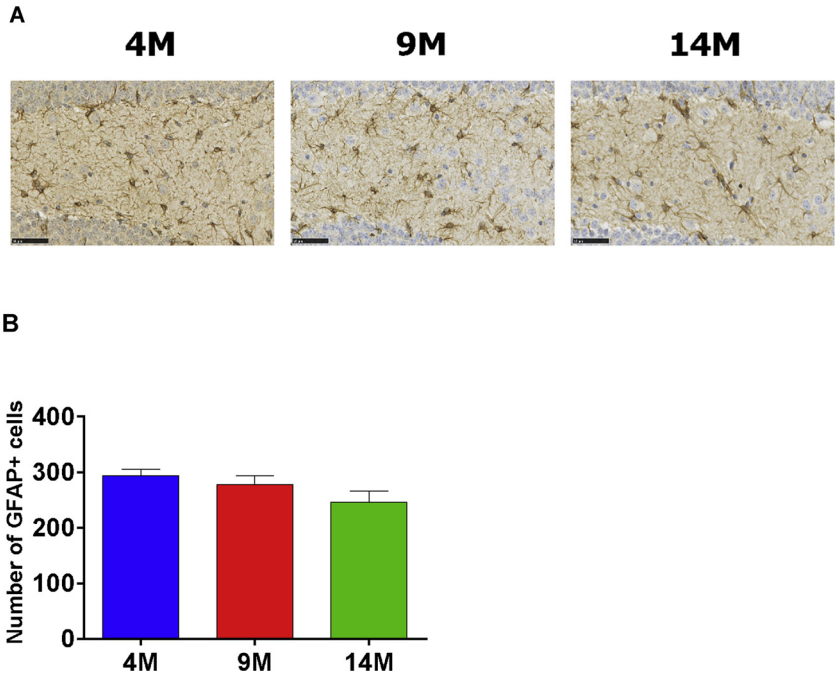

Fig. 10. Effects of aging on GFAP$^+$ cells in the dentate gyrus: (A) Representative immunohistochemical images of the number of GFAP$^+$ cells in the dentate gyrus (black scale represents 50 μm length), and (B) Number of GFAP$^+$ cells/mm^2. One-way ANOVA in GraphPad Prism. All data represented mean ± SEM, n = 6 per group.

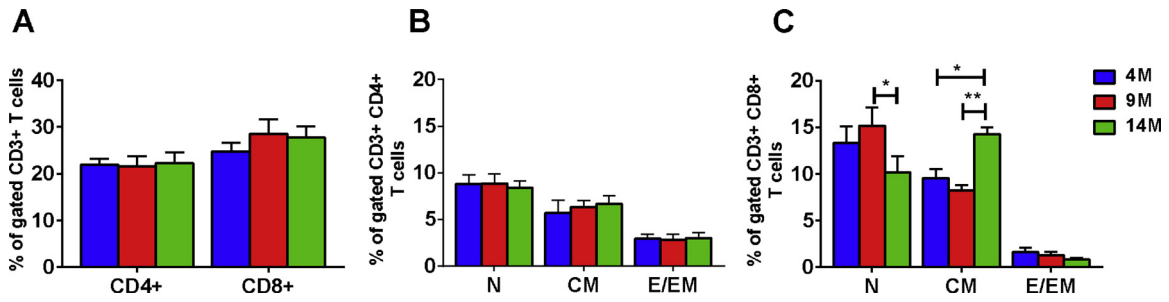

Fig. 11. T-cell subset composition. Proportions of (A) $CD4^+$ and $CD8^+$ T cells, (B) CD4 naive, central memory and effector/effector memory cell subsets and (C) CD8 naive, central memory and effector/effector memory cell subsets in the cervical lymph nodes of 4, 9 and 14-month old C57BL/6 mice. All data represented as estimated marginal means of the proportion of cells, n = 5–8 per group. * $p < 0.05$, ** $p < 0.01$. N: naïve, CM: central memory, EM: effector memory.

Table 4
T-cell subset composition. Mean count ± SEM of (A) $CD4^+$ and $CD8^+$ T cells, (B) CD4 naive, central memory and effector/effector memory cell subsets and (C) CD8 naive, central memory and effector/effector memory cell subsets in the cervical lymph nodes of 4, 9 and 14-month old C57BL/6 mice. n = 5–8 per group.

T cell type	4 months	9 months	14 months
$CD4^+$ T cells	9.7 * 10e5 ± 1.0 * 10e5	8.9 * 10e5 ± 1.4 * 10e5	9.1 * 10e5 ± 1.1 * 10e5
$CD8^+$ T cells	14.2 * 10e5 ± 1.6 * 10e5	17.2 ± 10e5 ± 1.7 * 10e5	15.3 * 10e5 ± 3.1 * 10e5
$CD4^+$ T_N cells	8.5 * 10e4 ± 9.1 * 10e3	7.2 * 10e4 ± 9.3 * 10e3	7.6 * 10e4 ± 6.8 * 10e3
$CD4^+$ T_{CM} cells	5.5 * 10e4 ± 1.3 * 10e4	5.6 * 10e4 ± 6.5 * 10e3	6.0 * 10e4 ± 8.2 * 10e3
$CD4^+$ T_{EM} cells	2.8 * 10e4 ± 4.7 * 10e3	2.3 * 10e4 ± 5.0 * 10e3	2.7 * 10e4 ± 5.2 * 10e3
$CD8^+$ T_N cells	1.9 * 10e5 ± 2.5 * 10e4	2.6 * 10e5 ± 3.4 * 10e4	1.6 * 10e5 ± 2.6 * 10e4
$CD8^+$ T_{CM} cells	1.4 * 10e5 ± 1.4 * 10e4	1.4 * 10e5 ± 9.7 * 10e3	2.2 * 10e5 ± 1.2 * 10e4
$CD8^+$ T_{EM} cells	2.3 * 10e4 ± 6.9 * 10e3	2.2 * 10e4 ± 5.9 * 10e3	1.3 * 10e4 ± 2.8 * 10e3

N: naïve, CM: central memory, EM: effector memory.

not male mice [11], we observed no significant effect of sex on anxiety-like behavior. Interestingly, there is also evidence for no significant change in anxiety-like behavior of C57BL/6 mice from 3 to 18 months of age [102]. The authors purchased mice two weeks before the experiment from the supplier and tested mice on EZM during the dark cycle. Our experiments differed in that we bred mice ourselves (suggesting that we were in better control of the environmental conditions of the mice), and we tested them on EZM during the light cycle. Our findings from EZM, therefore, suggest that normal aging is directly associated with the onset of anxiety-like behavior after middle age. However, less time spent by 4-month-old mice in the inner zone of the Open Field during our experiments contradicts findings from the EZM and suggest that 4-month-old mice showed more anxiety-like behavior than older mice. We believe that this could be attributed to the increased exploration of outer zones by early age mice, and it may not necessarily indicate anxiety-like behavior in 4-month-old mice. More research is required to understand the reason for the observed difference in anxiety-like behavior between the EZM and Open Field tests.

4.4. Normal aging alone is not a controlling factor for depressive-like behavior

No significant differences between any of the age groups for depressive-like behavior suggest that aging is not a limiting factor for the onset of depression in the absence of external stimulation. Studies in the past have been varying on reporting depression-related behavior in C57BL/6 mice, with some reporting no effects of aging on depressive-like behavior [101,102] while others are suggesting that aging has a significant effect on depressive-like behavior [13,15]. C57BL/6 mice, 11 and 18 months old, showed no differences in the parameters of behavioral despair when evaluated against young mice (2 to 3-month-old) in the FST [101,102]. On the contrary, significantly lower immobility of 12-month-old C57BL/6 mice than their younger (2 to 3-month-old) counterparts when tested in FST over a 10-min period has been reported [13]. Similarly, a significant decrease in immobility time has been reported for the 22-month-old C57BL/6 mice versus the 17-month and 11-month-old mice [15]. It is possible that subtle environmental factors may be responsible for the onset of depressive-like behavior in C57BL/6 mice until late-middle age and aging may play a significant role only during old age. The differences in genetic strains, behavioral test procedures and prior experience with stress may be responsible for the discrepancy between studies [103]. Also, we conducted FST towards the end of the behavioral testing schedule which may have influenced results, since prior test experience and frequent handling of mice has been reported to alter subsequent behavioral responses [104,105].

4.5. Normal aging causes a significant reduction in spatial learning and memory by late-middle age when compared to early and middle age

During our experiments, 14-month-old mice showed significant impairment of spatial learning on training days, and impaired retention of spatial memory and cognitive flexibility during the probe trial when compared to both 4 and 9-month-old mice. Over the four days of training, the latency to escape was significantly high for 14-month-old mice on days 1, 2 and 4 of training when compared to 4-month-old mice, and on day 3 of training when compared to 9-month-old mice. No significant differences were observed between the 4- and 9-month-old mice on any training days. During the probe trial, 14-month-old mice showed significantly higher latency to escape in the NW location when compared to both 4- and 9-month-old mice, suggesting significantly reduced retention of spatial memory at 14 months. The 14-month-old mice also showed significantly higher latency to escape in the NE quadrant during the probe trial when compared to 9-month-old mice, indicating significantly less cognitive flexibility at the late-middle age. Since the decreased drive to explore during aging could result in increased latency to escape, we also statistically analyzed the distance traveled by mice while locating the escape box. We observed no differences in distance traveled between the three age groups, which confirmed that the abovementioned higher latency at 14 months

Table 5
Differentially expressed genes between aging 4-, 9- and 14-month-old C57BL/6 mice (listed in order of increasing adjusted p values).

Groups compared	Gene	Estimates	Std. Error	Z	p values	Adj p	Gene expression change
Gene expression in 9m compared to 4m							
1	*Uqcrc1*	−3.1544	0.1977	−15.9565	2.24E-10	8.74E-09	↓
2	*Gpx1*	−2.3555	0.214	−11.0054	2.82E-08	5.49E-07	↓
3	*Tnfrsf1b*	−2.0717	0.3306	−6.2663	2.90E-05	0.0004	↓
4	*Htr1a*	1.1003	0.1889	5.8249	4.41E-05	0.0004	↑
5	*Tnfrsf1a*	−1.4400	0.2736	−5.2635	0.0001	0.0009	↓
6	*Sod1*	−0.6684	0.1379	−4.8455	0.0003	0.0017	↓
7	*Crh*	−1.5453	0.3603	−4.2885	0.0009	0.0049	↓
8	*Il1r1*	1.3243	0.3864	3.4274	0.0045	0.0219	↑
9	*Nr3c1*	−0.7425	0.2295	−3.2355	0.006	0.0259	↓
10	*Ntrk1*	−4.1410	1.2475	−3.3196	0.0068	0.0267	↓
11	*Gfap*	−0.9865	0.3254	−3.032	0.009	0.0318	↓
12	*Crhr1*	−1.3608	0.4955	−2.7464	0.0158	0.0473	↓
13	*Nlrp3*	−1.0430	0.3747	−2.7837	0.0146	0.0473	↓
Gene expression in 14m compared to 4m							
1	*Creb1*	−1.66754937	0.2035	−8.1924	1.04E-06	4.05E-05	↓
2	*Cs*	−1.07619729	0.166	−6.4829	1.44E-05	0.0002	↓
3	*Prkaa1*	−2.03602677	0.3079	−6.6125	1.17E-05	0.0002	↓
4	*Nr3c1*	−1.34313442	0.2407	−5.5804	0.0001	0.0007	↓
5	*Prkaa2*	−1.79367949	0.3356	−5.3451	0.0001	0.0008	↓
6	*Htr1b*	−2.81806552	0.5681	−4.9603	0.0002	0.0014	↓
7	*Grin2a*	−1.74970485	0.3759	−4.6549	0.0004	0.0021	↓
8	*Nlrp3*	−1.70599891	0.393	−4.3412	0.0007	0.0033	↓
9	*Cat*	−0.47033557	0.1106	−4.2544	0.0008	0.0035	↓
10	*Ntrk1*	−6.76684625	1.5045	−4.4978	0.0009	0.0035	↓
11	*Htr2a*	−1.88479928	0.4607	−4.0909	0.0011	0.0039	↓
12	*Crh*	−1.35166094	0.3763	−3.5915	0.0033	0.0107	↓
13	*Gria1*	−0.93344729	0.2722	−3.4298	0.0041	0.0122	↓
14	*Bdnf*	1.68989016	0.514	3.2876	0.0054	0.014	↑
15	*Sirt1*	−0.91077276	0.2766	−3.2924	0.0053	0.014	↓
16	*Tnfrsf1b*	−1.17768109	0.3696	−3.1862	0.0072	0.0174	↓
17	*Uqcrc1*	−0.63917176	0.2073	−3.0828	0.0081	0.0186	↓
18	*Gfap*	0.98439656	0.3413	2.8846	0.012	0.026	↑
19	*Aif1*	0.52312261	0.1853	2.8226	0.0136	0.0264	↑
20	*Htr1a*	0.55928402	0.1981	2.8229	0.0136	0.0264	↑
21	*Il1r1*	−1.08338084	0.432	−2.5079	0.0262	0.0487	↓
Gene expression in 14m compared to 9m							
1	*Uqcrc1*	2.51524616	0.2073	12.1312	8.13E-09	3.17E-07	↑
2	*Creb1*	−2.04990498	0.2035	−10.0708	8.56E-08	1.67E-06	↓
3	*Gpx1*	2.09978438	0.2245	9.354	2.12E-07	2.76E-06	↑
4	*Cs*	−1.29170572	0.166	−7.7811	1.89E-06	1.84E-05	↓
5	*Prkaa1*	−2.05264222	0.3079	−6.6665	1.07E-05	6.94E-05	↓
6	*Sod1*	0.97723091	0.1447	6.7549	9.25E-06	6.94E-05	↑
7	*Prkaa2*	−2.03002306	0.3356	−6.0493	2.99E-05	0.0002	↓
8	*Gfap*	1.97093737	0.3413	5.7754	4.81E-05	0.0002	↑
9	*Grin2a*	−2.04138052	0.3759	−5.4309	8.86E-05	0.0004	↓
10	*Il1r1*	−2.40766579	0.432	−5.5734	9.02E-05	0.0004	↓
11	*Tnfrsf1a*	1.3490632	0.2869	4.7017	0.0003	0.0012	↑
12	*Aif1*	0.83458187	0.1853	4.5032	0.0005	0.0016	↑
13	*Sirt1*	−1.12800946	0.2766	−4.0777	0.0011	0.0034	↓
14	*Htr2a*	−1.76034264	0.4607	−3.8208	0.0019	0.0052	↓
15	*Gria1*	−0.9200402	0.2722	−3.3806	0.0045	0.0117	↓
16	*Igf1*	−1.21997698	0.3734	−3.2669	0.0056	0.0137	↓
17	*Gria2*	−0.89922917	0.306	−2.9384	0.0108	0.0248	↓
18	*Smad2*	−0.44499452	0.1609	−2.7648	0.0152	0.0329	↓
19	*Htr1a*	−0.5410603	0.1981	−2.7309	0.0162	0.0333	↓
20	*Htr1b*	−1.45439309	0.5681	−2.56	0.0227	0.0442	↓
21	*Nr3c1*	−0.60063241	0.2407	−2.4955	0.0257	0.0477	↓

Estimate: Regression coefficients for the effect of the independent variable (EE) on the dependent variable (Gene expression). Std. Error: Standard Error of the difference between two means of the gene expression, i.e., means of the groups being compared. Z: Z-score: the number of standard deviations from the mean of all the values within the same gene. Positive Z-score indicates upregulation of gene expression. Negative Z-score indicates downregulation of gene expression. Adj. p: adjusted p-value.

indicates impaired spatial learning, memory, and cognitive flexibility.

As described previously [89], we also measured the number of entry errors by 4-, 9- and 14-month-old mice during the Barnes maze test to determine spatial learning and memory impairment with aging. The 14-month-old mice again showed a significantly higher number of entry errors than 9-month-old mice on days 3 and 4 of training, but not during the probe trial. This again confirmed impaired spatial learning at late-middle age. Furthermore, no significant differences in entry errors between the three age groups during the probe trial suggested no change in drive to explore with age until the late-middle age. Interestingly, we also observed a significantly higher number of entry errors by 4-month-old mice compared to 9-month-old mice on day 3 of training. However, in the absence of similar results on other training days and during the probe trials, as well as after considering results for latency to escape and distance traveled, we believe it would be incorrect to conclude that the 4-month-old mice showed significantly reduced spatial learning compared to 9-month-old mice.

We measure the effects of aging on cognition until late-middle age,

and not old age, in C57BL/6 mice. Nonetheless, our results for age-related effects on cognitive behavior are consistent with previously reported findings. For example, significant impairment of spatial memory in 17 and 25-month-old C57BL/6 mice in comparison to younger (5-month-old) mice has been reported when tested on a Morris water maze (MWM) [11]. The authors of the study also found that 17-month-old female mice were significantly more impaired than 17-month-old male mice, unlike our results which suggest no significant effect of sex on spatial learning and memory in the Barnes maze until late-middle age. In another study, 15 and 23-month-old C57BL/6 mice showed significant spatial learning impairment relative to young controls (5-month-old) in MWM [16]. Similar to MWM, a test on Barnes maze has shown significant impairment of spatial memory and learning in aged (23-month-old) mice when compared to young (3-month-old) and middle age (11-month-old) C57BL/6 mice [17]. Like our findings, the authors reported no significant differences between young and middle age mice for spatial learning and memory. Significant impairment in spatial learning and memory was also recorded in 18-month-old C57BL/6 mice when compared with 2-month-old mice on Barnes maze [18]. Evidence suggests that while the spatial learning and memory functions significantly decline from 12 months of age [19], this process starts as early as 8 months of age in C57BL/6 mice [13]. During our study, however, while impaired cognition was observed at 14 months, no significant impairment in cognition was observed at 9 months of age.

4.6. Normal aging significantly affects microglial and not astrocyte numbers in the dentate gyrus

The significant reduction in the expression of microglia in the dentate gyrus from 4 to 9 months was followed by a significant increase from 9 to 14 months. This suggests that aging is a controlling factor for microglial number and may have significant implications in the microglia-mediated neuroimmune pathways. The quiescent forms of microglia in the CNS in early age assist in neuronal migration and repair, recycling of neurotransmitters, regulating ion balance and buffering pH, and maintaining neuronal homeostasis, however, they lack phenotypical markers required for antigen presentation [29]. During aging, there is an increase in the expression of cellular senescence proteins. This results in an increased expression of reactive microglia with age [29], which quickly proliferate and express major histocompatibility complex (MHC) class I and II proteins, receptors for various cytokines, toll-like receptors, Nod-like receptors and antigens for T-cells subsets essential to mounting an innate immune response. The increased presence of reactive microglia has been reported to be associated with aging-related cognitive and memory impairment [26,106], depression [107], and neurodegenerative diseases such as AD [32]. During our work, we investigated mice aged from early to late-middle age, and not old age, in a controlled and non-stressful environment. We labeled microglia with IBA1 antibody that doesn't differentiate between quiescent or reactive forms. The observed significant decrease in microglia number from 4 to 9 months of age could be due to faster growth rates, less presence of cellular products of senescence and the slow transition of quiescent forms of microglia to reactive forms under controlled environment conditions until middle age. Perhaps the increased expression of senescence-associated proteins after middle age resulted in increased conversion of quiescent to reactive forms and proliferation of reactive microglia, which could also explain the increase in anxiety-like behavior and impaired spatial memory at 14 months during our experiments. It is, however, interesting to note that microglia-associated aging processes until late-middle age may not be responsible for the onset of depressive-like behavior in the absence of any external (e.g. stress) interventions.

Our results also suggest that aging is not a controlling factor for the expression of astrocytes in the dentate gyrus until late-middle age, hence may play little role in the astrocyte-mediated neuroimmune pathways. Astrocytes regulate the innate and adaptive immune response in the developing and adult brain. They provide mechanical and trophic support to neurons [108], mediate neural development [109,110], function [111,112] and protection [113,114], as well as neurotransmission and synaptic plasticity [8,34,37,38]. It has been reported that during aging, in the presence of cellular products of senescence, quiescent astrocytes reduce in number and turn into reactive phenotype characteristic of neuroinflammatory reactive astrocytes [25]. The reactive astrocytes, in turn, express proinflammatory cytokines (e.g., TNF-α, IL-1β, IL-6, IL-10, interferons α, β, and γ) and chemokines (e.g., Ccl2, Ccl5), and mediate inflammation and immune reactivity in the brain in association with microglia. We observed that although there was a constant increase in astrocyte numbers, the difference was non-significant from early to late-middle age. We immunolabeled astrocytes with a GFAP antibody that doesn't differentiate between quiescent or reactive forms. Perhaps the increase in reactive astrocytes, and hence astrocyte numbers, may become significant during old age in the absence of any interventions. The results also suggest that unlike microglia, astrocytes play no or minimal role in the increase in anxiety-like behavior and cognitive impairment during late-middle age in C57BL/6 mice.

4.7. Peripheral CD8+ naïve T cells decreases and CD8+ central memory T cells increase during normal aging

Total T cell counts, including $CD4^+$ (helper T cells) and $CD8^+$ (cytotoxic T cell), and the counts of $CD4^+$ and $CD8^+$ T cell subsets was determined to understand the effects of aging on the cellular immune response. It has been reported that early T cell activation markers decline, and memory T cells gradually increase, during aging [115]. No significant differences in the proportion of total CD4 and CD8 T cells, and early activation marker CD25 during our study indicate that aging alone does not induce proliferation of T cells or change in T cells phenotype. It is possible that during old age and/or in the presence of pathogenic stimuli, the results may differ. Furthermore, during normal aging, there is a reduction in the number of naïve T cells [116,117], which, in turn, reduces the ability to establish immunological memory in response to new antigens. T_N cells differentiate into T_{CM} or T_{EM} cells, the two cells differing in the absence or presence of immediate effector function [118,119]. We observed no change in the subsets of $CD4^+$ T cells. This could be explained by the fact that mice were raised in a pathogen and stress-free controlled environment during our study. Hence activation of $CD4^+$ T cells was minimal. However, this also suggests that aging alone has no significant effect on $CD4^+$ T cell subsets until the late-middle age. A significant number of $CD8^+$ T_N cells changed their phenotype to $CD8^+$ T_{CM} cells but not to T_{EM} cells at 14 months. It is possible that the cognate antigens encountered by $CD8^+$ T_N cells are the byproducts of cellular senescence. Indeed, a human study has shown that aging is associated with a decrease in $CD8^+$ T_N cells but not $CD4^+$ T_N cells in the cervical lymph nodes [120]. Similarly, the adverse effects of aging on the $CD8^+$ T cells receptor repertoire diversity has also been reported in another study [121]. Our results, therefore, suggest that normal aging has limited effects on the T cell number and diversity until the late-middle age.

4.8. Normal aging significantly affects the expression of hippocampal genes

Aging has been shown to influence gene expression and gene-gene interactions in the hippocampus regulating neuronal growth, structure, and functions [101]. Altered expression of hippocampal genes involved in the inflammatory response, neuronal structure and signaling, neuropeptide metabolism, amyloid precursor protein processing, and neuronal apoptosis, have been associated with aging-related memory deficits and neurodegenerative diseases in old C57BL/6 and BALB/c mice [122,123]. In humans, the changes in gene expression in the brain have been linked to psychiatric disorders associated with early

developmental stages and aging, such as autism and aging-related neurodegenerative disorders [124–129]. In this context, we analyzed the effects of aging on changes in the expression of hippocampal genes associated with immune, monoamine, transcription, growth metabolism, and HPA-axis activity. We found significant differences in the expression of 29 genes out of 43 GOI between the three age groups at adjusted $p < 0.05$.

We observed a non-significant increase in the expression of *Bdnf* gene from 4 to 9 months. However, it became significant at 14 months when compared to 4 months, which may be indicative of the constant growth of the brain, and enhanced neural plasticity, until late-middle age. BDNF protein is found in high concentrations in the hippocampus and is critical in memory formation. It has been reported that Bdnf gene plays a role in the biology of mood disorders, and its expression is reduced during aging-related neurodegenerative disorders [130–132]. Also, previous studies have shown that the level of Bdnf mRNA in the hippocampus across the life span may also depend on the external environmental conditions. For example, a decrease in the expression of hippocampal Bdnf mRNA has been reported during late-middle age in the presence of chronic stress [49] and brain pathology [50]. Conversely, the expression of the *Bdnf* gene within hippocampus has been shown to increase after external interventions, such as physical activity [72] and environmental enrichment [73]. We believe our study provides a more accurate representation of the effects of aging on the expression of *Bdnf* gene in the hippocampus since mice were housed in a controlled environment in the absence of all external interventions.

Increase in mitochondrial cytopathies with age results in the production of reactive oxygen species (ROS) leading to oxidative stress, which plays a role in the development of aging-related chronic inflammatory diseases [133,134]. The diminished expression of *Cat* and *Nr3c1* genes, which provide defense against oxidative stress, and mediates glucose metabolism, inflammatory responses, cellular proliferation, and differentiation in target tissues respectively [135–138], at 14 months suggests that oxidative and inflammatory stress increased with age. GeneMANIA confirmed that the cellular response to ROS and oxidative stress is affected at FDR < 0.05 through Cat-Nr3c1 interaction. This also explains the increase in anxiety-like behavior and spatial learning and memory impairment, as well as little growth and differentiation of $CD8^+$ T cells at 14 months. It is possible that the aging-induced neuroinflammatory diseases are the result of a disturbed balance between the expressions of protective genes, such as *Bdnf*, *Cat,* and *Nr3c1*, in the brain. Furthermore, the significantly upregulated expression of *Aif1* gene, which promotes macrophage activation and phagocytosis, growth of T-lymphocytes, and peripheral inflammation [139–141], at 14 months compared to its expression at both 4 and 9 months further points at a significant rise in inflammatory stress by late-middle age. However, unlike *Aif1* gene, the expression of *Nlrp3* gene decreased significantly between 4 and 9 months and non-significantly between 9 and 14 months. *Nlrp3* gene encodes NLRP3 inflammasomes that activate IL1 proinflammatory cytokines, thereby playing a role in innate immunity and inflammation in response to pathogens and products of cellular senescence [54]. Inflammasomes activity increases with age. The decreased expression of *Nlrp3* gene indicates lowered expression of the proteins of cellular senescence at 9 months, although the levels of senescence proteins have been reported to increase during normal aging [54]. We believe the controlled external environment for aging mice is responsible for the lowered expression of *Nlrp3* gene during our experiments.

There were nine genes (*Creb1, Cs, Prkaa1, Prkaa2, Grin2a, Gria1, Htr1b, Htr2a* and *Sirt1*) whose expression remained unchanged between 4 and 9 months but decreased significantly afterwards. The reduction in the expression of these genes at 14 months suggests impaired energy metabolism, neurotransmission, and cognition, learning and memory during late-middle age [51,142–150]. When mapped in GeneMANIA, there were 380 total links with the 20 most related genes to the nine genes that were mapped, and the mapped genes were found to be statistically related to glutamate receptor activity, glucose homeostasis, and learning and memory at FDR < 0.05. Furthermore, the expression of five genes (*Htr1a, Il1r1, Igf1, Gria2, Smad2*) that encode proteins which mediate inhibitory and excitatory neurotransmission, inflammatory responses via cytokines IL-1α and IL-1β, cell differentiation, cell proliferation, and apoptosis, hence, are important for normal growth and development during aging [147,148,151–156], increased or stayed same at 9 months when compared to 4 months but decreased from 9 to 14 months. This explains the normal increase in body weight with age from 4 to 9 months and no adverse behavioral changes until middle age that we have reported. The decrease in the expression of these genes after middle age suggests that cellular senescence overtakes growth rate after middle age and the detrimental effects of aging, such as reduced cellular proliferation and growth rate, start appearing. This also explains the increase in anxiety-like behavior and cognitive impairment at 14 months of age during our experiments. The GeneMANIA map showed 249 total links with the 20 most related genes to the five genes that were mapped. The mapped genes were found to be associated with T cell proliferation and activation, insulin-like growth factor signaling pathway, cellular response to growth factor stimulus, and response to interleukin-1 at FDR < 0.05.

In contrast to the above reported findings, the expression of *Uqcrc1* was downregulated significantly by 9 months when compared to 4 months and goes back up significantly at 14 months compared to 9 months but not the young mice levels. Likewise, the expression of *Gpx1, Sod1* and *Tnfrsf1a* reduced significantly from 4 to 9 months but increased afterwards to the young mice levels at 14 months. The expression of *Ntrk1, Crh, Crhr1* and *Tnfrsf1b* also reduced from 4 to 9 months but showed no further decrease afterwards until 14 months of age (see Table 4). These results are suggestive of improved mitochondrial metabolism and response to psychological stress, and enhanced pro-inflammatory and protective or apoptotic functions in the brain of 14-month-old mice [52,53,157–164]. However, when the genes were mapped in GeneMANIA, we found that the association between these genes was not high. The GeneMANIA map showed 72 total links with the 20 most related genes to the nine genes that were mapped. The mapped genes were found to be related to antioxidant activity at FDR < 0.05. However, at FDR > 0.05 and < 0.1, apoptotic signaling pathways and response to ROS were also affected. Together, the results from our gene expression analysis suggest that the expression of genes associated with inflammatory and oxidative changes is not high in a controlled environment until the late-middle age and external environment may have a role to play in the adverse genetic changes associated with aging.

5. Limitations of our study

It is important to note that many studies have investigated C57BL/6 mice at different ages, after providing them with some form of external interventions, for example, isolation, maternal separation, auditory or olfactory exposure, physical exercise, environmental enrichment, dietary supplements, and pharmacological drugs. Results from these studies may not comply with our reported results for normal aging in the absence of all external interventions. Similarly, the strain of animal may also affect behavioral, molecular, and gene expression results. These factors must be considered while utilizing our results as reference points during research planning in future. Also, due to low sample size, we could not perform sex differences analysis for molecular data. Hence, a future research with greater sample size is recommended to elucidate the sex differences for molecular changes in the brain of aging mice. Furthermore, it must be noted that the stains that we used to identify immunopositive microglia and astrocytes stained different phenotypes of glial cells equally and hence it is not possible to make a differential analysis of microglia and astrocytes phenotypes now. This is the reason we have only analyzed the overall change in the number of immunopositive glial cells at three age points and not mentioned about

their activation states. However, since analyzing the activation state of glial cells is very important to develop a more comprehensive understanding about the molecular changes in the brain that happen during normal aging, we recommend it to be investigated in future research.

6. Conclusion

Most of the results in the present study were consistent with, and extended, previously reported findings, for example, increase in anxiety-like behavior and cognitive impairment, and enhanced microglial numbers at late-middle age, as well as a change in hippocampal gene expression during aging. However, we report that depressive-like behavior is not affected significantly by aging in a controlled environment until the late-middle age. This is an important finding but needs further validation, perhaps through saccharin-preference test and novelty-suppressed feeding. While there are many studies on the effects of aging in the presence of external interventions, there are only a few that specifically explore the effects of only aging on behavior and underlying molecular biology.

Open practices statement

None of the data or materials for the experiments reported here is available online, and none of the experiments was preregistered.

Author's statement

The authors confirm that the ethics approval for performing experiments on C57BL/6 mice was received from the University of Adelaide Animal Ethics Committee, and all guidelines as prescribed for handling the experimental animals were followed during the study.

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