THE DIFFERENTIAL EFFECTS OF SHORT-TERM PHYSICAL EXERCISE, ENVIRONMENTAL ENRICHMENT AND THEIR COMBINATION, AND SHORT- AND LONG-TERM ENVIRONMENTAL ENRICHMENT ON BEHAVIOR AND ASSOCIATED MOLECULAR CHANGES IN THE BRAIN OF C57BL/6 MICE

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Declaration

I certify that:

- this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.
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Gaurav Singhal

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2. **Singhal, G**, Morgan, J, Jawahar, MC, Corrigan, F, Jaehne, E, Toben, C, Manavis, J, Hannan, AJ & Baune, BT (2020), **'**Duration of environmental enrichment determines astrocyte and cervical lymph node T lymphocyte numbers but not microglial number in middle-aged C57BL/6 mice', Frontiers of Cellular Neuroscience, vol. 14, no. 57. **IF: 3.900.**
3. **Singhal, G**, Morgan, J, Jawahar, MC, Corrigan, F, Jaehne, E, Jawahar, MC, Jaehne, E, Toben, C, Breen, J, Pederson, SM, Manavis, J, Hannan, AJ & Baune, BT (2020), 'Effects of aging on the motor, cognitive and affective behaviors, neuroimmune responses and hippocampal gene expression', Behavioral Brain research, p. 112501. **IF: 2.770.**
4. **Singhal, G**, Morgan, J, Jawahar, MC, Corrigan, F, Jaehne, E, Toben, C, Breen, J, Pederson, SM, Hannan, AJ, Baune, BT (2019), 'Short-term environmental enrichment, and not physical exercise, alleviate cognitive decline and anxiety from middle age onwards without affecting hippocampal gene expression', Cognitive, Affective, & Behavioral Neuroscience, p. 1-27. **IF: 2.661**
5. **Singhal, G**, Morgan, J, Jawahar, MC, Corrigan, F, Jaehne, E, Jawahar, MC, Jaehne, E, Toben, C, Breen, J, Pederson, SM, Hannan, AJ, Baune, BT (2019), 'The effects of short-term and long-term environmental enrichment on locomotion, mood-like behavior, cognition and hippocampal gene expression', Behavioral Brain Research, p. 111917. **IF: 2.770.**
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childhood trauma but not to recent stressors', Psychoneuroendocrinology, vol. 73, pp. 24-31. **IF: 4.013.**

Scientific Reviews:

1. Fourrier, C, **Singhal, G**, Baune, BT (2019), 'Neuroinflammation and cognition across psychiatric conditions', CNS spectrums, vol. 24(1): p. 4-15. **2018 IF: 3.940.**
2. **Singhal, G,** and Baune, BT (2017), 'Microglia: an interface between the loss of neuroplasticity and depression', Frontiers in Cellular Neuroscience, vol. 11, p. 270. **2018 IF: 3.900. Google Scholar citations > 50.**
3. Stuart, MJ, **Singhal, G**, Baune, BT (2015), 'Systematic review of the neurobiological relevance of chemokines to psychiatric disorders', Frontiers in Cellular Neuroscience, vol. 9, p. 357. **2018 IF: 3.900. Google Scholar citations > 50.**
4. **Singhal, G**, Jaehne, EJ, Corrigan, F, Toben, C, Baune, BT (2014), 'Inflammasomes in neuroinflammation and changes in brain function: a focused review', Frontiers in Neuroscience, vol. 8. **2018 IF: 3.566. Google Scholar citations > 250.**
5. **Singhal, G**, Jaehne, EJ, Corrigan, F, Baune, BT (2014), 'Cellular and molecular mechanisms of immunomodulation in the brain through environmental enrichment', Frontiers of Cellular Neuroscience, vol. 8:97. **2018 IF: 3.900. Google Scholar citations > 50.**

Book chapters:

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2. **Singhal, G**. & Baune, BT (2020), 'Chapter 6 - Inflammatory Abnormalities in Major Depressive Disorder', Major Depressive Disorder, Elsevier: Philadelphia (PA). pp. 75-89.
3. **Singhal, G,** and Baune, BT (2019), 'Immune-Based Biomarkers and Therapies in Psychiatry: Future Prospects', Oxford University Press. Available online.
4. **Singhal, G.** and Baune, BT (2018), 'Do Chemokines Have a Role in the Pathophysiology of Depression?', Inflammation and Immunity in Depression, Elsevier, pp. 135-159.
5. **Singhal, G.** and Baune, BT (2018), 'Inflammasomes Action as an Important Mechanism in Experimental and Clinical Depression', Inflammation and Immunity in Depression, Elsevier, pp. 161-171.

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1. **Singhal, G**, Morgan, J, Jawahar, MC, Corrigan, F, Jaehne, E, Hannan, AJ & Baune, BT, 'TNF signaling via the TNF receptors does not mediate the effects of short-term exercise on cognition-, anxiety- and depressive-like behaviors'. Submitted to Brain Research. **2018 IF: 3.1. Status:** Revisions requested.

Note: All the above manuscripts were written and published/submitted while doing PhD, however, only the highlighted publications (all published/accepted) are part of this Ph.D. thesis.

Abstract

Background: Animal studies have shown that with increasing age, there is impaired cognition, enhanced anxiety- and depressive-like behaviors, and reduced locomotor activity, accompanied by neurobiological changes, such as enhanced inflammation, decreased neurotrophins, and altered neurotransmitter levels. Evidence suggests that external environmental conditions can modify the effects of aging. Environmental enrichment (EE), where the immediate environment of experimental animals is modified, has been reported to reverse the adverse effects of brain aging. Past studies on EE have, however, also utilized physical exercise (PE) as a part of EE. Additionally, the duration of EE and the age of rodents varies greatly across studies. Hence, the current literature fails to explain the effects that PE has in EE studies across age, as well as the differential effects of the duration of EE, if any, on behavior and underlying molecular biology.

Methods: We analyzed the differential effects of short-term PE, EE, and PE+EE, and the effects of short- vs. long-term EE on cognition-, anxiety- and depressive-like behaviors in 4-, 9- and 14-month-old C57BL/6 mice using an established behavioral battery. Changes in the expression of hippocampal genes, microglial and astrocyte numbers in the dentate gyrus, and T cell subsets in the cervical lymph nodes were also analyzed.

Results: We found that short-term EE reversed the adverse effects of age on cognitive- and anxiety-like behaviors at 14 months. Conversely, short-term PE was found to impair cognition and enhance depressive-like behavior at 4 months. Furthermore, PE and PE+EE, but not EE alone, were observed to modify the expression of several hippocampal genes at 9 months compared to control mice. EE also enhanced the number of microglia in the dentate gyrus at 4 and 9 months while PE and PE+EE increased it only at 9 months. Significant differences were also noted between the treatment- and age-matched cohorts for the proportion of $CD4^+$ and $CD8^+$ T cell subsets in the cervical lymph nodes. At 9 months, both short-term and long-term EE significantly enhanced locomotion in the home cage and reduced depressive-like behavior in the forced swim test. Conversely, long-term EE reduced locomotion in the open-field test. Additionally, short-term EE showed anxiolytic effects in the elevated zero maze, while these effects were lost after long-term EE. EE, irrespective of duration, increased microglia number within the dentate gyrus and had no significant effects on the expression of 43 hippocampal genes of interest. Long-term EE, however, increased astrocytic numbers in the dentate gyrus, as well as total $CD8^+$ and proportions of $CD8^+$ T_{Naive} cells in the cervical lymph nodes.

Conclusion: The above results suggest that short-term EE is effective in alleviating the age-related impaired cognition and enhanced anxiety-like behavior in the absence of altered hippocampal gene expression. Conversely, short-term PE adversely affects cognition and affective behaviors at an early age, with modification of hippocampal genes only occurring at middle age. Finally, the longer duration of EE affects locomotion adversely in a threatening environment, reverses the beneficial effects of short-term EE on anxiety-like behavior, and may alter the neuroinflammatory response during middle age.

Chapter 1: Thesis Introduction

Preamble to chapter 1

The objective of this chapter is to provide an appropriate background for the study. Our review, titled 'Cellular and molecular mechanisms of immunomodulation in the brain through environmental enrichment', that was published in the journal Frontiers of Cellular Neuroscience discussed in detail the effects of physical exercise and environmental enrichment (with or without physical exercise) on brain functions and neuroimmune mechanisms. In this introductory chapter, the review has been cited as and where required. For this reason, we first provide the details of the review. We then move on to define aging and discuss the pathophysiological changes that occur during neuronal aging. Subsequently, a short explanation is provided on the role of aging in neuroinflammatory disorders. We then proceed to discuss the definitions, prevalence, and pathogenesis of anxiety, depression, and cognitive impairment. Following this and based on the available literature, we outline the effects of aging on various behaviors, glial cell expression, T cell-mediated immunity, and hippocampal gene expression, and discuss how PE and EE modulate these parameters during normal aging. We then provide the relevance of our results for translation to clinical trials. Finally, we discuss research questions, develop hypotheses, and set aims for the first sets of experiments that we conducted during this study.

In the subsequent chapters of this theses, we provide methodology, develop hypotheses and set aims for the second and third set of experiments, summarize and discuss results, and conclude. Finally, we discuss the limitations of our study and discuss future directions based on our research.

Review: Cellular and molecular mechanisms of immunomodulation in the brain through environmental enrichment

Singhal, G. [1], Jaehne, E. J. [1], Corrigan, F. [2], & Baune, B. T. [1] (2014). *Frontiers in cellular neuroscience, 8*.

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CELLULAR NEUROSCIENCE

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Cellular and molecular mechanisms of immunomodulation in the brain through environmental enrichment

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Recent studies on environmental enrichment (EE) have shown cytokines, cellular immune components [e.g., T lymphocytes, natural killer (NK) cells], and glial cells in causal relationship to EE in bringing out changes to neurobiology and behavior. The purpose of this review is to evaluate these neuroimmune mechanisms associated with neurobiological and behavioral changes in response to different EE methods. We systematically reviewed common research databases. After applying all inclusion and exclusion criteria, 328 articles remained for this review. Physical exercise (PE), a form of EE, elicits anti-inflammatory and neuromodulatory effects through interaction with several immune pathways including interleukin (IL)-6 secretion from muscle fibers, reduced expression of Toll-like receptors on monocytes and macrophages, reduced secretion of adipokines, modulation of hippocampal T cells, priming of microglia, and upregulation of mitogen-activated protein kinase phosphatase-1 in central nervous system. In contrast, immunomodulatory roles of other enrichment methods are not studied extensively. Nonetheless, studies showing reduction in the expression of IL-1β and tumor necrosis factor-α in response to enrichment with novel objects and accessories suggest anti-inflammatory effects of novel environment. Likewise, social enrichment, though considered a necessity for healthy behavior, results in immunosuppression in socially defeated animals. This has been attributed to reduction in T lymphocytes, NK cells and IL-10 in subordinate animals. EE through sensory stimuli has been investigated to a lesser extent and the effect on immune factors has not been evaluated yet. Discovery of this multidimensional relationship between immune system, brain functioning, and EE has paved a way toward formulating environ-immuno therapies for treating psychiatric illnesses with minimal use of pharmacotherapy. While the immunomodulatory role of PE has been evaluated extensively, more research is required to investigate neuroimmune changes associated with other enrichment methods.

Keywords: environmental enrichment, immune, cytokines, glial cells, T cells, neurobiology, cognition, behavior

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Name of Principal Author (Candidate)	Gaurav Singhal		
Contribution to the Paper	Reviewing of the articles, organizing them in Endnote, analysis of reported findings, interpretation, writing and redrafting manuscripts.		
Overall percentage (%)	70 %		
Certification:	This paper reviewed articles reporting the effects of external interventions physical exercise and environmental enrichment on brain functions and neuroimmune mechanisms. I wrote this review during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

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2. permission is granted for the candidate in include the publication in the thesis; and
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Preamble to the Review

In the published review, the studies on the role of EE in behavioral and neuroimmune modulation are reviewed and analyzed for validity. Evidence suggests that PE and EE (with or without PE) have a significant influence on brain functions and associated neurobiology. Recent studies have shown that both PE and EE modulate the expression of neuroimmune factors, such as cytokines, cellular immune components (e.g. T lymphocytes, Natural Killer cells) and glial cells, which, in turn, influences neurobiology and behavior. The objective of the review, therefore, was to evaluate neuroimmune mechanisms associated with neurobiological and behavioral changes in response to PE, EE and the combination of PE and EE.

To achieve our objective, we systematically retrieved articles from PubMed, Google Scholar and Science Direct using a combination of relevant search terms. After applying all inclusion and exclusion criteria, 278 articles were utilized for the construction of the review using the PRISMA guidelines. The review provides the foundation for Ph.D. work and thesis. A short summary of the review is provided next, with relevant sections presented.

Review Summary

PE alone or in combination with EE elicits anti-neuroinflammatory and neuromodulatory effects through influencing several neuroimmune pathways, including priming of glial cells, reduction in proinflammatory cytokines levels and modulation of hippocampal T cells. Although the immunomodulatory roles of environmental enrichment with novel objects and other accessories (in the absence of a running wheel) in rodents have been studied less extensively, it has been shown to reduce the expression of pro-inflammatory cytokines including IL-1β and TNF-α, suggesting anti-neuroinflammatory effects associated with EE. More research is required to elucidate the differential effects of PE and EE on brain functions and associated neuroimmune mechanisms. The multidimensional relationship of PE, EE, and PE+EE with brain functions and underlying neuroimmune mechanisms can help to formulate environ-immune therapies for treating psychiatric illnesses either as standalone or adjunctive with pharmacotherapy.

The excerpts and figures 1.1, 1.2 and 1.3 from the review below built the foundation for this thesis.

“PE can modulate a number of brain regions which may, in turn, result in varied functional outcomes, such as improvement in memory [1], learning [2], behavior [3], cognition [4] and motor activity [5], and this has been highly regarded by researchers. It mostly affects the humoral immune system; however, its role in the modulation of the cellular immune system cannot be ignored. Post-exercise, production and expression of anti-inflammatory factors, particularly anti-inflammatory cytokines (e.g., IL-6, IL-10) are enhanced in both the systemic circulation as well as within the brain. This subsequently reduces the level of pro-inflammatory factors, such as the cytokines TNF-α and IL-1β [6], chemokines [7], TLR’s [8] and CRP [9], helping in alleviating both systemic and neuroinflammation, the latter being the causal factor for most psychiatric disorders. An increase in the number of T lymphocytes and NK cells after PE [10] strengthens adaptive immunity. Modulation of glial cells, T cells and macrophages in the brain by PE also helps in reducing the neurotoxic effects and enhances neurogenesis in the brain, particularly in the hippocampus [6].”

“‘Novelty seeking’ behavior is the inherent tendency to explore novel objects and accessories and has been investigated in many enrichment studies. Novel objects and accessories used in conjunction with PE have been reported to reduce the expression of IL-1β and TNF-α in the hippocampus [11] suggestive of anti-inflammatory effects. This, however, makes it difficult to conclude whether these anti-inflammatory effects were seen in response to PE and/or to the novel objects and accessories. Nevertheless, the role of novel objects in cell-mediated immunity cannot be disregarded as improvement in macrophage chemotaxis and phagocytosis, lymphocyte chemotaxis, and NK cell activity were seen in the peritoneal suspension of mice enriched with two novel objects at a time [12].”

“No research to date has used standardized enrichment techniques and different methods have been used randomly. This standardization of enrichment techniques is necessary, as it is clear from our previous discussion that different methods of EE can elicit diverse effects on neurobiology, behavior and neuroimmune mechanisms. The above standardization can be translated to human studies using appropriate

analogous models of enrichment for the treatment of brain disorders. This may, however, require extensive investigation as a standard human environment is similar to an enriched environment for rodents. The results from enrichment through physical exercise in rodents can successfully be translated to human intervention for psychiatric illnesses, however, findings from other enrichment methods in rodents could find less value in human studies. An intense examination of human nature and its application while formulating an environment enriched with cognitively stimulating activities is essential for similar interventions in humans."

"Besides different methods of EE, the stage of life when enrichment has been given can potentially affect results. During the neonatal and early prenatal periods, the brain develops rapidly, large numbers of new synapses are formed and growth and differentiation of the cerebrocortical region take place. Any changes in the brain induced during this period can persist throughout life. Environmental conditions during prenatal and early stages of the life cycle can have distinct effects on neurobiology and behavior [13, 14]."

"Neonatal handling with an enriched environment can reduce the signs of emotionality and anxiety, augment novelty-seeking behavior, and can have preventative actions on age-related learning impairments and hippocampal neuronal atrophy in rodents (See review by Fernandez *et al.* [15]. Likewise, enrichment with frequently changed toys after weaning provided a beneficial intervention for reversing the harmful effects of maternal separation in rats [16]."

"Controlled trials on Sprague-Dawley rats have established that enrichment in early life increases T cell numbers, enhances production of anti-inflammatory cytokines (e.g., IL-2, IL-10) and lowers production of the pro-inflammatory cytokine IL-1β in various brain regions such as the hypothalamus and frontal cortex [17, 18], suggesting attenuating effects of early life enrichment on neuroinflammation. This suggests that requisite neurological and behavioral enhancements can be more readily achieved by enriching the environment of an animal at the prenatal stage and preserving it later in life with regular novel and enriching inputs."

"It is evident that over-expression of pro-inflammatory cytokines and chemokines in the brain, in addition to decreases in cytotoxic T cell proliferation and activity may result in diminished cognitive performance and development of neuropathology [19-21]. These cytokines are primarily expressed by glial cells in the brain whose levels increase with aging. However, the neuronal aging slows down in mice raised in an enriched environment and is characterized by sustained neurogenesis and reduced neuronal damage in the cellular microenvironment of the dentate gyrus [22-24]. Indeed, EE has been shown to improve the plasticity of cognitive functions and learning performance, and reduce the impairment of spatial memory in aged rodents [25-27]. In addition, the role of cellular immunity is important when studying the immune effects of external environmental stimuli [28-30]."

"Taken together, the finding in this review, suggests that improvement and development of the environment are beneficial to preserve and enhance species-typical behavioral aspects by altering immune parameters in association with genes and neuro-chemicals. However, the immunomodulatory roles of enrichment methods other than PE have received less attention and a better understanding is required."

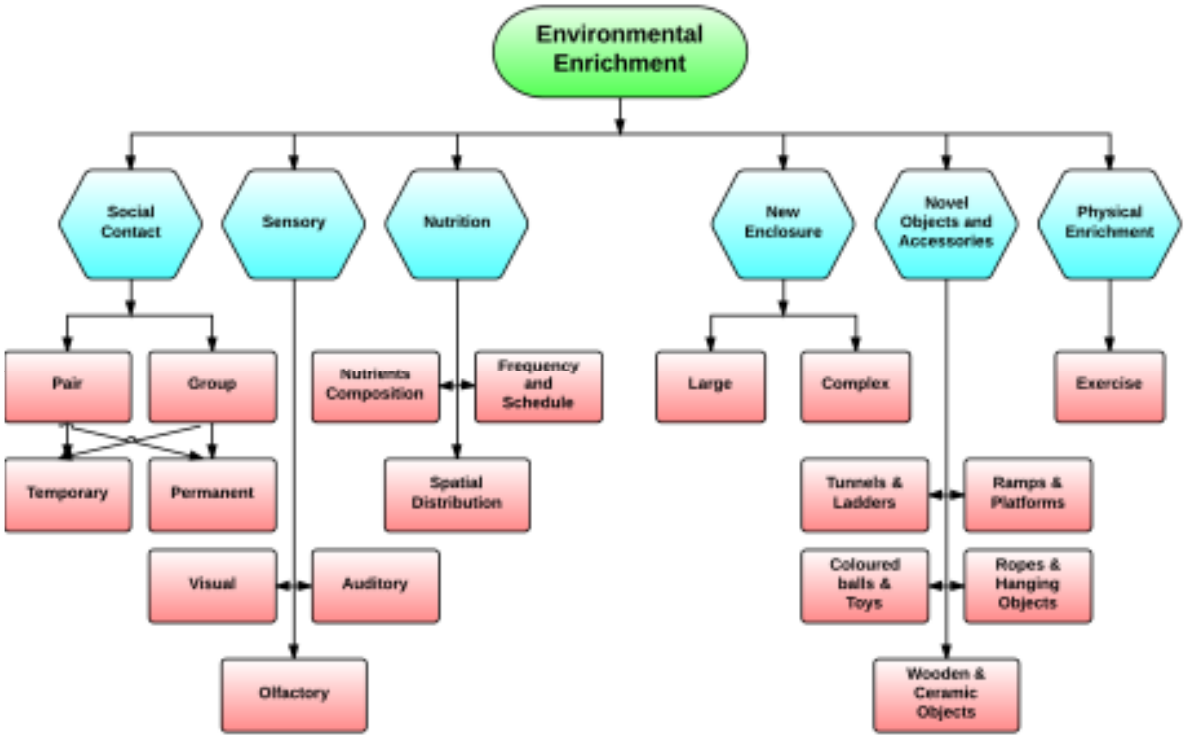

Fig. 1.1 Methods of enriching the environment for rodents in captivity [31]. **Legend:** EE for rodents in captivity can be achieved by providing them with social contact (pair, group, permanent, temporary), physical activity using running wheel, new larger and complex enclosures, novel objects and accessories, sensory stimulations (visual, auditory and olfactory) and better nutrition.

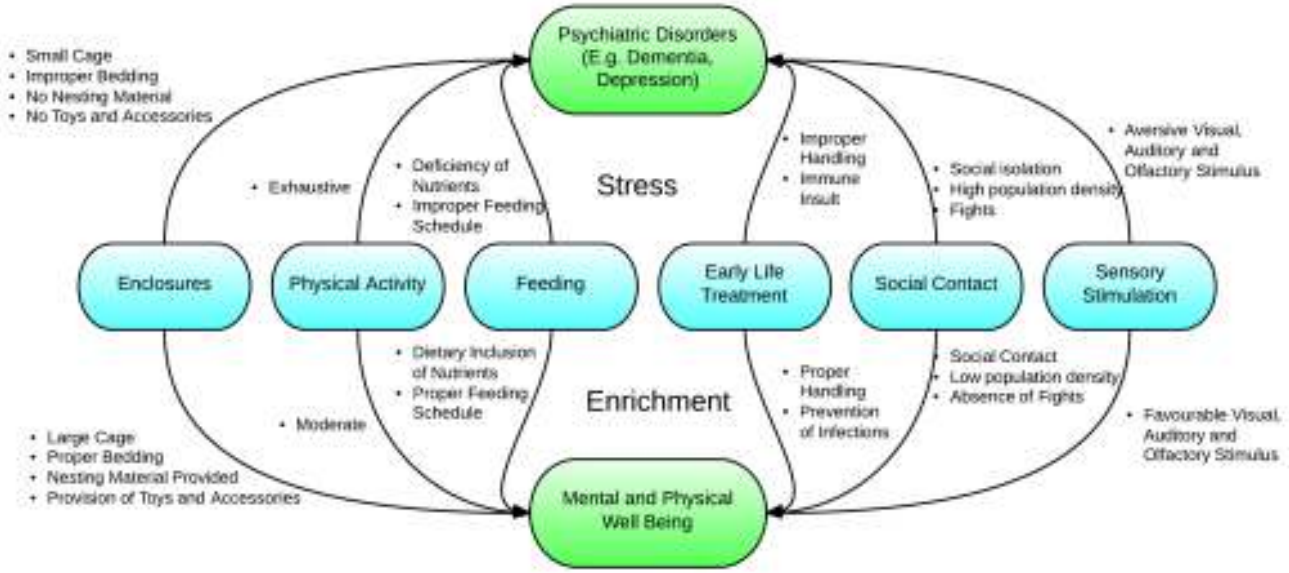

Fig. 1.2 Differential effects of enrichment methods in rodents [31]. **Legend:** The same stressful factor when subtly modified can become enrichment for the animals in captivity. This suggests that environment for rodents can be enriched by just changing the existing arrangements of different factors around them and no special efforts are required.

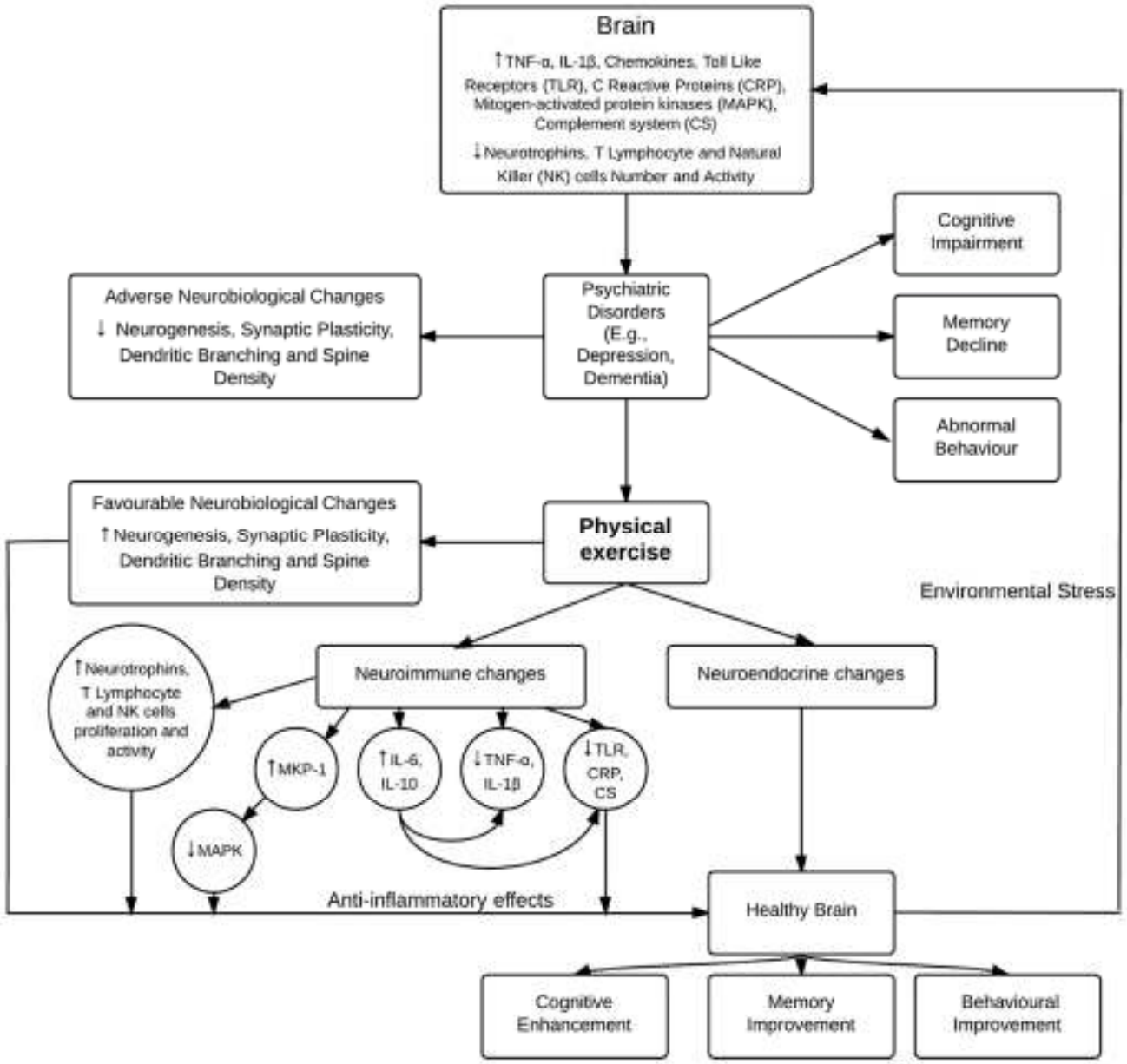

Fig. 1.3 PE modulates neuroimmune and neuroendocrine mechanisms [31]. **Legend:** This figure shows that psychiatric disorders are the result of aversive neurobiological and neuroimmune changes in the brain. However, these are reversed by physical exercise which also facilitates improvement in cognition, memory and behavior.

Introduction

With the advancement in medicine over the last two centuries, the life expectancy of humans worldwide from birth has increased from <30 years in the year 1800 to nearly 67 years in the year 2000 [32] and 71 years in the year 2015 [33] with a continuing rise. This poses an ever-increasing challenge to treat aging health disorders, including those that affect the functioning of the brain. The brain is one of the most complex organs of the body consisting of several anatomical and sub-anatomical structures communicating through neural circuitry while performing a multitude of tasks. It has been hypothesized that the modulation of the external environment can alter neuronal plasticity during aging and can, therefore, be implemented for treating age-related brain disorders. This hypothesis has been tested numerous times during the last two decades with physical exercise (PE) and environmental enrichment (EE) being used widely as two of the primary sources of environmental modulation. Improvement in neural morphology and function, as well as in neuroimmune mechanisms have been reported extensively after both PE and EE in rodents [31]. During PE and EE protocols, the external environment of rodents is modified by providing them with running wheels and/or novel objects, puzzles (mazes, plastic tubes in different configurations) and accessories (toys, ropes, ladders, tunnels, hanging objects, house, ramps, and platforms). Recent evidence suggests that voluntary physical activity through a running wheel is a strong modulator of behavior and memory, and underlying neuroimmune mechanisms, which, therefore, creates uncertainty about the effectiveness of using other toys and objects in EE paradigms. The primary purpose of this study, therefore, is to elucidate the differential effects of PE and EE during normal aging.

Definition of normal aging and neuronal aging

Rose, in 1991, defined aging as 'the persistent decline in the age-specific fitness components of an organism due to internal physiological deterioration' [34]. With advances in molecular biology in the 21st century, it slowly became clear that the above definition of aging is obsolete since it fails to explain the underlying pathophysiology of aging. Increasingly becoming evident is that the process of aging and associated physiological changes are the results of a continuous rise in the rate of cellular senescence with age accompanied by a plethora of underlying molecular changes [35]. The rate of cellular senescence with age is, also dependent on the external environmental conditions, including the presence of pathogenic stimuli which aggravate the process of cellular senescence. Normal aging in laboratory rodents, therefore, is the process of natural aging in a controlled environment, which includes ambient temperature and humidity, ad libitum food and water and the absence of all external pathogenic and stressful stimuli. The latter could also include individual housing of mice or housing together with dominant males. It is expected that animals undergoing normal aging will show the unique effects of aging in the absence of external confounders.

Aging of the brain or neuronal aging is the process of physiological deterioration resulting in functional impairment. The senescence of brain cells with age, including loss of neurons in the grey area and myelin sheath in the white matter, leads to a loss in brain volume after late-middle age [36-39]. This loss of cellular integrity during advancing age also adversely affects dendritic arbor, spines, and synapses. [40, 41]. Furthermore, aging has a significant effect on the integrity of glial cells, i.e., microglia,

and astrocytes as well as on adaptive immunity and hippocampal gene expression [42-44]. However, aging alone is not a contributing factor to physiological neuronal impairment but the external environmental factors exert a marked influence on neurobiology and underlying molecular changes during neuronal aging [45-47]. The senescence-accelerated morphological and anatomical changes in the brain can result in exaggerated behavioral changes, including anorexia, hypersomnia, lethargy, decreased social interaction, and deficits in cognitive and motor function [48, 49]. These will be discussed in the following sections.

Association between aging and neuroinflammatory brain disorders

Aging is often associated with an increase in the prevalence of neuroinflammatory and neurodegenerative disorders, such as Alzheimer's disease (AD) [50, 51], Parkinson's disease (PD) [52], Huntington's disease (HD) [53], multiple sclerosis [54] and amyotrophic lateral sclerosis [55]. Gene environmental interactions play a role in the onset and maintenance of aging-related neuroinflammatory brain disorders [56-58]. Aging acts as a priming stimulus which upon secondary stimulation (e.g. peripheral infection), can cause a rise in lymphoproliferative disorders, as well as the subsequent increase in the expression of proinflammatory immune proteins (e.g., cytokines) and cells (reactive glial cells, $CD8^+$ T cells) within the brain [42]. This can result in an altered state of neuroimmune homeostasis during old age. There is an increase in the incidence of neuroinflammation-induced brain disorders and associated impaired brain functions, such as dementia, anxiety, depression and cognitive and memory deficits during late-middle and old age [48, 59-61].

Clinical symptoms, pathophysiology and current treatments for anxiety disorders

According to Diagnostic and Statistical Manual-5 (DSM-V) classification, anxiety disorders refer to a group of mental disorders characterized by feelings of anxiety and fear, including generalized anxiety disorder, panic disorder, phobias, social anxiety disorder, obsessive-compulsive disorder and post-traumatic stress disorder [62]. The symptoms can range from mild to severe and it is more chronic than episodic disorder [62]. The anxiety disorder is characterized by a state of excessive restlessness, irritability, fatigue, difficulty concentrating, fear, muscle tension, sleep pattern change and related behavioral disturbances [62-64]. Anxiety was the 6^{th} major contributor to global disability, affecting around 3.6 % of the global population (264 million) in 2015 [62, 65]. Moreover, anxiety disorders are more common among females than males (4.6% compared to 2.6% at the global level) [62, 65]. A person shows excessive anxiety and worries (apprehensive expectation), occurring on more days than not for at least 6 months, about a number of events or activities (such as work or school performance) and finds it difficult to control it [62, 64]. Increased anxiety can cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.

Early life stressful experiences, such as childhood physical and sexual abuse and parental loss has been shown to be predictive factors for the development of anxiety disorders in adulthood [66]. Genetic predisposition and external environmental factors can increase the vulnerability to the effects of later life stress and, lead to anxiety disorders [67, 68]. Clinical studies have also shown that anxiety disorders can co-morbid with depressive disorder, and hence the pathophysiology for the two disorders overlap [69-73]. Monoamine dysregulation, impaired corticotrophin-releasing hormone

(CRH) stress response, abnormalities of neurotrophic factors, and immune insult are some of the factors implicated in the pathophysiology of anxiety disorders.

The monoamine hypothesis suggests abnormalities of the norepinephrine and serotonin (5-HT) neurotransmitter systems in depression and anxiety disorders [72, 74, 75]. A preclinical study in 5-HT1A receptor knock out mice showed decreased exploratory activity and increased fear of open or elevated spaces [76]. Likewise, a clinical study showed reduced binding of the 5-HT1A receptors in the amygdala and mesiofrontal areas of social anxiety disorder patients.[74]. Animals with a prior history of exposure to chronic stress show an increase in norepinephrine release upon re-exposure to an acute stimulus. This increased sensitization to stress results in the onset of anxiety disorders [75]. However, chronic stress may result in a decrease in brain norepinephrine levels with associated behavioral changes, such as learned helplessness [75]. Similarly, clinical evidence has shown that the level of CRH is elevated in cerebrospinal fluid of both depression and anxiety patients [77, 78], which is due to the reduced binding of CRH, indicating desensitization of CRH receptors in the brains of those suffering from anxiety. [79]. This dysregulation in stress HPA axis functioning can result in panic disorder, leading to a hyper anxious state [80]. Neurotrophins, particularly BDNF, have a role to play in the brain stress system, including the hippocampus and HPA axis [81]. It has been reported that precursor BDNF and mature BDNF facilitate long-term depression and long-term potentiation respectively, suggesting opposing cellular functions [82]. Hence, BDNF signaling (precursor or mature BDNF) may determine the type of mood behavior. BDNF also promotes the survival and differentiation of 5-HT neurons, thereby regulating the development and plasticity of neural circuits involved in mood disorders such as depression and anxiety [83]. Recent evidence has started pointing towards the role of immune insult in the onset of anxiety-like behavior. Neuroinflammation was induced after chronic stress, triggering biochemical, physiological and psychological changes in the brain and the development of anxiety-like behavior in rats [84]. Several preclinical studies have reported an increase in the level of proinflammatory cytokines and chemokines in the brain and the subsequent development of neuroinflammation and oxidative stress prior to the onset of anxiety-like behavior in rodents [85, 86]. Furthermore, neuroinflammation has been shown to reduce the expression of neurotrophins in the brain which is also correlated with reduced hippocampal volume [87]. Together, this predisposes the brain to the onset of affective behavior-like disorders.

Both pharmacological and psychotherapeutic treatments are used for the management of anxiety disorders. Since anxiety disorders are found to frequently comorbid with depressive disorder, selective serotonin reuptake inhibitors (SSRIs) and selective norepinephrine reuptake inhibitors (SNRIs) have been suggested as useful first-line agents for anxiety disorders [88-90]. Other antidepressants that are proposed for the treatment of anxiety disorders are tricyclic antidepressants [91], monoamine oxidase inhibitors [92], anticonvulsants and atypical antipsychotics [93], benzodiazepines [94] and antiepileptics [95]. However, most of the pharmacological drugs have tolerability issues and many times patients fail to adequately respond [96-98]. Hence, psychotherapeutic treatments such as cognitive behavioral therapy, as well as modulation of the external environment through physical exercise [99] and

environmental enrichment [28], are being promoted for the treatment of anxiety disorders in recent times.

Clinical symptoms, pathophysiology and current treatments for major depressive disorder

Depression is defined as experiencing a state of low mood, sense of lost-self, sadness, irritability, loss of interest in all activities and psychophysiological changes every day for more than 2 weeks [100, 101]. The DSM-V has outlined the symptoms for the clinical manifestation of depressive state in humans, also known as major depressive disorder (MDD) [63, 101]. The symptoms include loss of interest or pleasure in day to day activities, impaired social, occupational and educational functions, feeling sad, empty, guilty, worthless and tearful, loss of body weight, insomnia or hypersomnia, psychomotor agitation or retardation, indecisiveness, and thoughts of committing suicide.

Evidently, depression is foremost amongst the mental disorders that affect the human population around the globe. The most recent estimate from the World Health Organization (WHO) suggests that there are more than 300 million people (4.4 % of the global population) worldwide suffering from depression [102]. Importantly, depression is the foremost cause of suicidality (800 000 per year) [103], as well as decreased life span and impaired quality of life [104, 105]. During depression, the risk of developing life-threatening metabolic disorders such as type II diabetes and cardiovascular disease [106, 107], central obesity [108, 109], Crohn's disease [110], rheumatoid arthritis [111], osteoarthritis [112] and cancer [113] increases multifold. An estimate that reported the economic burden in excess of 80 billion dollars in the United States from depression in 2000 showed the heavy burden that it poses on the public health sector [114]. However, the biggest concern is that the number of depressed people is expected to rise and become the second leading cause of disability worldwide in the next two decades [115].

Several external factors, primarily substance abuse, lack of peer support, marital problems, low socioeconomic status, and stressful life events predisposes the brain to adverse neurobiological and molecular changes, such as chronic neuroinflammation, neurodegeneration, loss of neuroplasticity, neuroprotection and cellular senescence [116-121], noradrenergic and serotonergic neurotransmission deficiencies [122, 123], immunological insult [124-126], altered levels of growth factors [127-130] and impaired HPA axis metabolism [131, 132], subsequently leading to the onset of depression. Infection, tissue damage, allergies and antigens found in food are some of the other possible causes of immune activation triggering depression [101]. Gene and environmental interactions alone however, fail to consistently explain the pathogenesis of MDD [133], suggesting that depression is caused by other interactions as well. Clinical trials and meta-analyses reporting elevated levels of proinflammatory cytokines in the brain and serum of depressed patients seem to provide the missing link and support the inflammatory hypothesis for depression [134-136]. Proinflammatory cytokines, when overexpressed, can cause hippocampal degeneration and microglial apoptosis, both being the important etiologies of depression [137, 138]. Neuroinflammation also disrupts serotonergic and dopaminergic neurotransmission and dysregulates the HPA axis [108], all of which have been shown to regulate mood-like behavior [139-143]. Dysregulation in the monoamine pathway results in oxidative and nitrosative stress leading to

neurodegeneration and subsequently depression [144-146]. Likewise, glucocorticoids, when overexpressed, can elicit proinflammatory effects in the CNS [147] leading to atrophy of certain brain areas [148]. Moreover, excess glucocorticoids cause damage to the hippocampus [149], inhibit glucose transport in neurons and glia, and induce oxidative and nitrosative damage in the brain through interference with glutamate reuptake and increasing intracellular calcium mobilization [150, 151]. Enhanced levels of proinflammatory cytokines, IL-1β, TNF-α, and IL-6, in both peripheral blood and brain have also been reported in patients suffering from osteoarthritis [152], cancer [153], diabetes [154], central obesity [155] and CVD [156], as well as aging-associated depression [157]. Bidirectional circulation of proinflammatory cytokines between the blood-brain-barrier (BBB) and periphery is a key reason for the comorbidity of depression with metabolic disorders during old age [158, 159]. As a result, the current research focuses majorly on the ways to control the elevated levels of proinflammatory cytokines during depression and its comorbidity with metabolic disorders [116, 118, 119].

While the etiology and pathophysiology of depression is multi factorial as mentioned above, changes in gene expression in brain have also been linked to age-related neurodegenerative disorders [160-165]. With recent advances in mRNA profiling techniques, it has become possible to perform genome-wide association analysis studies (GWAS). There GWAS have confirmed the role of the expression of several genes, particularly those involved in the immune response, to be associated with the pathophysiology of depression [166-171]. Please see Figure 1.4 from one of our recently published manuscript [101] for the molecular pathways that are associated with the onset of depression.

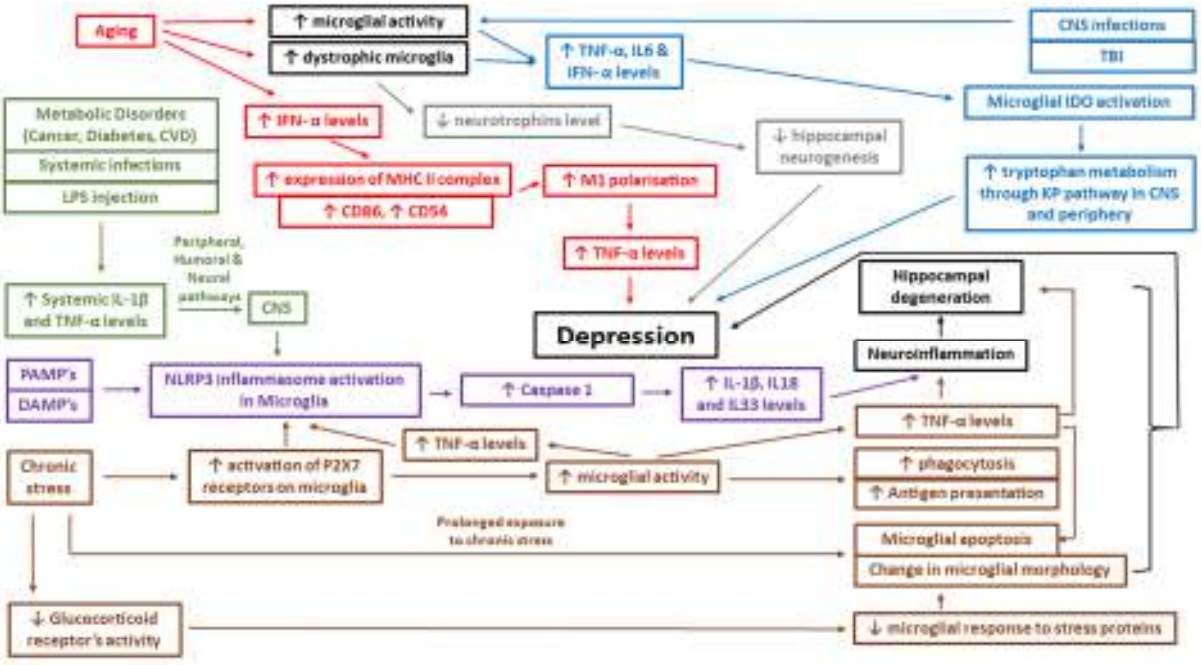

Fig. 1.4 Different hypotheses for depression converge together [101]. **Legend:** As seen in the figure, microglia can cause depression through different molecular pathways. These mechanistic pathways are sometimes interrelated making the whole mechanistic link between microglial action and depression complex. Red boxes

indicate 'neuroinflammatory pathway', gray boxes indicate 'altered neurotrophin levels pathway' and 'impaired hippocampal neurogenesis pathway', blue boxes indicate 'altered brain tryptophan metabolism pathway', green boxes indicate 'stimulation of peripheral immune system pathway', brown boxes indicate 'psychological/chronic stress and reduced immunity pathway' and purple boxes indicate 'Inflammasome pathway'. Black boxes are part of more than one pathways. IFN: interferon, TNF: tumor necrosis factor, IL: interleukin, P2X7: two-transmembrane ATP-gated ionotropic purinoreceptor, CVD: cardiovascular disease, LPS: lipopolysaccharide, PAMP's: pathogen-associated molecular patterns, DAMP's: damage-associated molecular patterns, CNS: central nervous system, TBI: traumatic brain injury, IDO: indoleamine 2, 3-dioxygenase enzyme, KP: kynurenine pathway.

The multifactorial etiology of depression makes it difficult to treat. Conventional anti-depressive drugs target monoamine neurotransmission, and includes selective serotonin reuptake inhibitors (SSRIs) and selective noradrenaline reuptake inhibitors (SNRIs) [172-174], tricyclic antidepressants [175], and monoamine oxidase inhibitors [176]. Evidence also suggests that SSRIs and SNRIs elicit anti-inflammatory effects [177, 178]. However, some of these drugs have tolerability issues [96] and nearly one thirds of patients fail to respond [179]. Therefore, more emphasis is being given to investigate the effects of anti-inflammatory treatments. Both selective cyclooxygenase (COX)-2 and non-selective COX inhibitor non-steroidal anti-inflammatory drugs (NSAIDs) have been investigated as possible adjuncts in the treatment of depression with antidepressants [180-193]. Their anti-inflammatory properties are primarily due to the inhibition of prostaglandin synthesis. NSAIDs are indeed widely used to control inflammation and are increasingly discussed for their clinical relevance in the treatment of depression [194, 195]. The results, however, varied from study to study. While some studies reported positive effects of NSAIDs as antidepressants [180, 184, 186, 187], some reported no effects [181, 182, 188], and others reported NSAIDs to reduce the antidepressant effect of SSRIs [183, 189]. The benefits of NSAIDs as antidepressants, however, are not without any side effects. Gastric ulceration and damage to kidneys have been reported after long-term use of NSAIDs [196, 197]. Similarly, there are case reports of neurological symptoms including ataxia, vertigo, dizziness, recurrent falls, nystagmus, headache, encephalopathy, seizures, aseptic meningitis and disorientation after an overdose of NSAIDs. Others reported NSAIDs to reduce the antidepressant effect of SSRIs [168, 174]. Owing to these mixed results of the effect of pharmacological anti-inflammatory treatments for depression other non-pharmacological interventions such as the use of fish oil, physical exercise and environmental enrichment [99, 198, 199] are trialed. The trials showed that these non-pharmacological treatments induce neuroplastic changes through the modulation of neuroimmune pathways, in turn, improving brain functions and alleviating psychiatric conditions like anxiety and depression. These have been discussed in the following sections.

Clinical symptoms, pathophysiology and current treatments for cognitive disorders

Cognitive disorders are defined as a state of difficulty in remembering, learning new things, concentrating, or making decisions that affect everyday life. Cognitive impairment ranges from mild to severe and is not a standalone brain disorder and occurring concurrently with other psychiatric illnesses such as depression and dementia. Indeed, dementia has been classified under cognitive disorders in the DSM-

V [63, 200]. A patient with cognitive impairment can have one or more of the following specific symptoms: problem with acquiring and remembering additional information, executive dysfunction, memory deficit, impaired reasoning and handling of complex tasks, poor judgment, reduced visuospatial abilities, impaired language functions and changes in personality, behavior, or comportment [201]. Cognitive impairment is also common during aging-associated chronic neuroinflammation-induced neurodegenerative disorders such as AD and PD [201-204]. Therefore, cognitive disorders possibly share similar pathophysiology as depressive disorders. For example, over or under-expression and morphological alterations of glial cells [124-126, 205-207], elevated levels of brain pro-inflammatory cytokines TNF-α, IL-1β and IL-6 [135, 136, 203, 204, 208-212] and development of antigenic proteins such as of tau [213, 214] and beta-amyloid (Aβ) [215-217] are some of the immune-related aetiologies associated with cognitive deficit.

Cognitive impairment has a significant physical, psychological, social, and economic impact on caretakers, families, and society, and no cure is available currently. Since cognitive impairment has been shown to comorbid with other psychiatric states, such as anxiety and depression, the improvement in cognition has been reported after anti-anxiety and anti-depressive treatments. Indeed, the use of anti-inflammatory agents has been proposed to treat cognitive disorders. For example, NSAIDs could be used to reduce neuroinflammation and treat cognitive impairment, however, this is accompanied by harmful side effects as mentioned before. As such, non-pharmacological anti-inflammatory treatments are being investigated extensively for treating neurocognitive disorders. Omega-3 long-chain polyunsaturated fatty acids (omega-3 PUFA), such as docosahexaenoic acid (DHA), have been shown to improve brain functions and reduce the cognitive deficit during Alzheimer's dementia [218, 219]. More recent evidence suggests that omega-3 PUFA are effective in the control of AD only if given in the early stages of disease progression [220, 221]. Similarly, physical exercise has been shown to elicit beneficial effects on cognitive impairment [222] by inhibiting the production of proinflammatory cytokines [223, 224]. External environmental input, such as psychosocial support and cognitive behavioral therapy can also help improve cognitive impairment.

Evidence from rodent studies for the effects of aging on affective-like behaviors

Rodent studies have shown that aging has significant effects on anxiety- and depressive-like behaviors. A significant increase in anxiety-like behavior during normal aging, in particular, late-middle age onwards has been reported [225-228]. No effects of age on anxiety-like behavior was shown until middle age [229]. Similarly, a study has shown no significant change in anxiety-like behavior in C57BL/6 mice until the late-middle age [230]. The findings from the rodent-based studies on the effects of aging on depressive-like behavior, however, provide a scrambled understanding, with some suggesting no effects [229, 230] while others suggesting a significant effect of aging on depressive-like behavior [227, 231]. Moreover, evidence suggests that the sex of the mice may also determine the onset of anxiety- and depressive-like behaviors during aging [225].

Evidence from rodent studies for the effects of aging on spatial learning and memory

Research in rodents has shown that aging significantly influences spatial learning and memory. Evidence suggests that the impairment in spatial learning and memory becomes significant late-middle age onwards in mice [225, 232-236]. However, this process of decline in spatial learning and memory can start as early as eight months of age (middle age) in C57BL/6 mice [227]. There are also evidences to suggest that this age-related deficit in spatial learning and memory is associated with the onset of oxidative stress, and reduced neurogenesis and synaptic plasticity in the aging hippocampus, which in turn, results in hippocampal kindling [237-241]. Furthermore, chronic stress can also accelerate the impairment of spatial memory during normal aging [242].

Aging-associated changes in hippocampal gene expression

Gene expression is the fundamental process whereby genotype expresses itself through the phenotype regulating the differentiation, development, and functioning of cells and tissues [243]. The genetic make-up has a close connection with the neural circuitry. Antibody-based protein profiling has shown that there is a high variability of gene expression in distinct brain regions [244-247]. This has been attributed to the significant differences in the cell composition of the various anatomical brain substructures [248]. Evidence suggests a correlation between the gene expression and the tasks (e.g. motor functions, cognitive abilities) performed by distinct brain substructures, such as the cerebellum, amygdala, and hippocampus [249]. The specific subset of transcripts present in particular cell types and involved in the cell's life support, development and specific functioning, called transcriptome, changes in the brain throughout the lifespan corresponding to the developmental prenatal and postnatal stages [243]. Studies have shown the important role of complex gene expression in mediating neuronal processes such as the formation of synapses, dendritic branching, synapse maturation, and synapse elimination, as well as higher-end mental functioning [250, 251]. Interestingly, these anatomical changes in the brain are accompanied by dynamic changes at the molecular level, involving a change in expression of several genes throughout the lifespan [162, 252-256].

Conventionally, changes in candidate gene expression in the brain have been linked to psychiatric disorders associated with early developmental stages and aging, such as autism and age-related neurodegenerative disorders [160-165]. However, with recent advances in mRNA profiling techniques, it has become possible to perform genome-wide gene expression analysis. There is evidence now for the role of the expression of several genes in the pathophysiology of schizophrenia [257-260], bipolar disorder [261-264] and depression [166-169]. It is therefore now established that any change in behavior and memory are directly correlated with the change in the expression of genes in distinct brain regions. Hence, of late, modulation of gene expression in the brain is being considered as the stepping stone to the development of the next generation of treatments for neuroinflammatory and neurodegenerative disorders, commonly associated with aging populations across the globe.

Aging-associated changes in brain glial cell expression

Glial cells are the primary immune effector cells in the brain and regulate neuroimmune homeostasis by expressing cytokines (e.g., IL-1, IL-6, IL-10, interferons α and β, TNF α and β) and chemokines (e.g., Ccl2, and Ccl5), thereby mediating immune reactivity in the brain. In constituent levels, microglia and astrocytes are important for brain development and repair activities. For example, microglia assist with neuronal

migration during brain development, repairing damaged neurons, filling of voids left by degenerative neurons, recycling of neurotransmitters, regulation of ionic balance, buffering pH and phagocytoses of dead cells and pathogens [265-267]. Similarly, astrocytes provide mechanical and trophic support to neurons [268], mediate neural development [269, 270], function [271, 272] and protection [273, 274], regulate neurogenesis, neurotransmission and synaptic plasticity [275-279], maintain ion balance [280], releasing growth factors [281-283], form the BBB [284] and secrete apolipoprotein E [285]. The normal expression of glial cells is essential for maintaining neurophysiological and neuroimmune homeostasis in the healthy brain.

Evidence suggests that glial cell phenotype changes with age, they overexpress proinflammatory cytokines, become increasingly dysfunctional, release neurotoxic mediators, and lose neuroprotective properties [42, 43, 286]. Recently a study demonstrated that glial cells showed an increase in the expression of proinflammatory cytokines TNF-α, IL-1β, and IL-6 in the hippocampus, hypothalamus, and cerebral cortex of senescence-accelerated mice [287]. Similarly, an increase in the number of immunopositive microglia and astrocytes in the brain during old age has been reported in rodents, which was subsequently found to be related to cognitive impairment [44, 288, 289]. The enhanced expression of proinflammatory cytokines results in neuroinflammation, which becomes chronic in the presence of persistent stimuli, such as protein byproducts of cellular senescence, and other genetic and acquired environmental risks [290]. Chronic neuroinflammation eventually leads to degenerative changes in the brain with subsequent development of cognitive deficit, and sickness and depressive-like behavior. This also predisposes the brain to the development of age-related neurodegenerative disorders, such as AD and PD [291, 292]. One of our recently published review discusses the role of microglia in the loss of neuroplasticity during depression [101]. Also, figure 1.5 that we published in another study [290] shows the role that cytokines play in the pathophysiology of neuroinflammation and subsequent onset of psychiatric conditions.

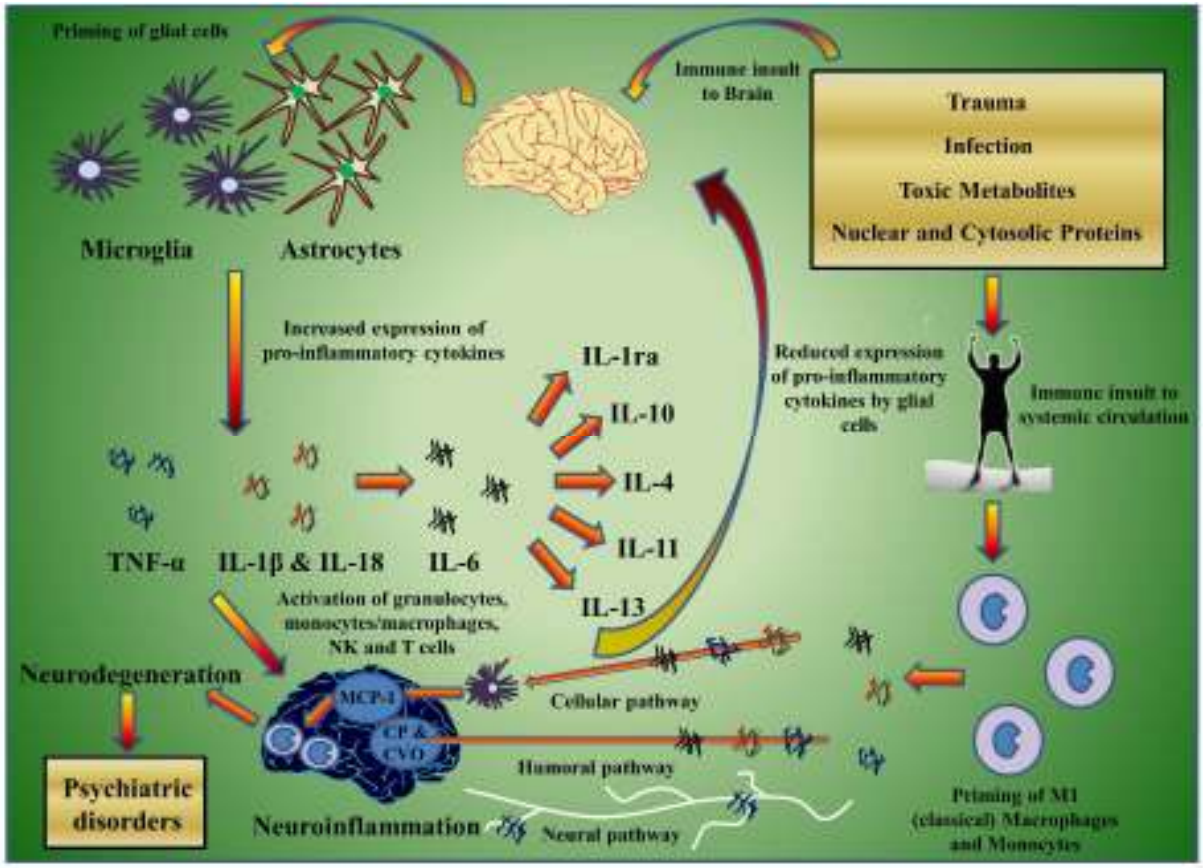

Fig 1.5 Cytokines hypothesis of neuroinflammation [290]. Implications in comorbidity of systemic illnesses with psychiatric disorders. **Legend:** Pro-inflammatory cytokines can migrate between systemic circulation and brain in both directions which could explain the comorbidity of systemic illnesses with psychiatric disorders. There are three pathways for the transport of pro-inflammatory cytokines from systemic circulation to brain as described by Capuron and Miller [293]: Cellular, Humoral and Neural. Moreover, PAMPs and DAMPs from trauma, infection and metabolic waste can prime glial cells to express pro-inflammatory cytokines TNF-α, IL-1β and IL-6. When expressed, these cytokines activate granulocytes, monocytes/macrophages, Natural Killer and T cells and together contribute to the pathophysiology of neuroinflammation. Chronic neuroinflammation could result in neurodegeneration and associated psychiatric disorders. These pro-inflammatory cytokines also stimulate production and expression of anti-inflammatory cytokine by glial cells that function as negative feedback to reduce the expression of pro-inflammatory cytokines, subsiding the neuroinflammation. MCP-1: Monocyte chemoattractant protein-1, CP: Choroid plexus, CVO: Circumventricular organ

Aging-associated changes in T cell-mediated immunity

T cells are important for the maintenance of neuroimmune bidirectional communication and homeostasis [294]. The regulation of immune response function of $CD4^+$ T cells reduces and cytotoxic function of $CD8^+$ T cells increases during old age [295, 296]. Furthermore, with increasing age, there is a reduction in the number of naïve T cells [297, 298], which, in turn, reduces the ability to establish adaptive immunity in response to new antigens. It has also been shown that early activated T cell numbers decline and memory T cell numbers gradually increase during aging [299]. The early

T cell activation markers function to downregulate the activation and proliferation of effector T cells, which is essential in maintaining tolerance to self-antigen and prevent autoimmune disorders [300, 301]. Aging, therefore, appears to have adverse effects on the T cell phenotype with $CD8^+$ cells slowly increasing with age. Indeed, it has been reported that $CD4^+$ to $CD8^+$ T cell ratio becomes inverted (less than 1) during old age [299].

Whether T cells have encountered a cognate antigen or not; they can be subdivided into naïve (T_N), central memory (T_{CM}) or effector memory (T_{EM}) T cells. T_N cells on recognition of a cognate antigen respond by acquiring activated phenotypes and upregulation of surface markers, such as $CD25^+$ and $CD69^+$. T_N cells are then further differentiated into T_{CM} or T_{EM} cells, the two cells differing in the absence or presence of immediate effector function and the expression of homing receptors that allow cells to migrate to secondary lymphoid organs versus nonlymphoid tissues [302, 303]. Aging also enhances the differentiation of T_N cells into T_{CM} or T_{EM} cells. T_{CM} cells have little or no effector function but mediate reactive memory where they migrate to secondary lymphoid organs and some of them proliferate and differentiate to effector cells in response to antigenic stimulation. It has been shown that the migrated T_{CM} cells produce IL-2 in the lymph nodes which function to differentiate T_N cells into T_{CM} and T_{EM} cells when the T_N cells are stimulated by an antigen, thus helping the body fight off infections [304]. IL-2 also protects against autoimmune diseases and helps in developing tolerance and immunity to self-antigens by promoting the differentiation of progenitor T cells into early activated T cells [304]. T_{EM} cells, on the other hand, have lower proliferation potential than T_{CM} cells and mediate protective memory where they migrate to inflamed peripheral tissues and display immediate effector function [302, 303]. T_{EM} cells produce IFN-γ in non-lymphoid tissues that activate macrophages and induce expression of MHC II molecule resulting in lytic activity [305]. The overexpression of IFNγ has been associated with age-related psychiatric, autoimmune and autoinflammatory diseases [306]. The $CD4^+CD25^+$ cells regulate IL-2 homeostasis, thereby modulating CD8+ T_{EM} differentiation [300]. T_{CM} and T_{EM} cells also differ in the expression of chemokine receptors, adhesion, and costimulatory molecules [302], further adding to the heterogeneity of memory T cells. 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 [307]. However, $CD4^+$ T_N cells in aged mice show reduced expansion, produce fewer cytokines, particularly IL-2, and differentiate slowly compared to $CD4^+$ T_N cells in young mice [308]. Moreover, aging has adverse effects on the $CD8^+$ T cells receptor repertoire diversity [309]. Age, however, appears to have positive effects on the early T cell activation markers, since a significant increase in $CD4^+CD25^+$ cells has been reported in the spleen cells of C57BL/6 aged mice [310].

How external environment influences brain functions?

Evidence suggests that internal and external environment greatly influences the cellular and molecular mechanisms associated with neuroplasticity and thus contributes to age-related psychiatric disorders that are characterized by the cognitive deficit, memory loss and behavioral impairment [31]. Hence, both extrinsic (e.g. environmental, experiences) and intrinsic (e.g. genetics, neurotransmitters, hormones) factors may play a role in impaired brain functions during aging [56, 311, 312]. In addition, external environmental factors also modulate immune functions in the brain, which are then manifested as changes in behavior, mood, and cognition [294, 313]. Two of the key external environment interventions, PE and EE, which have

shown to slow down neuronal aging [22, 23] and improve cognition, memory, behavior and motor coordination in pre-clinical rodent models of dementia, depression, AD, PD and HD [314-316] are discussed in the following sections. See Figure 1.6 for the representative images of PE, EE and the combination of PE and EE provided to laboratory rodents.

Fig. 1.6 Representative images of the (a) physical Exercise (PE), (b) Environmental Enrichment (EE) and (C) PE+EE paradigms.

The effects of PE on neurobiology, behavior, and memory

PE has significant effects on neurobiology and behavior in rodents, improving neuroplasticity, psychosocial functions and responsiveness to adverse situations [317, 318]. PE has been shown to regulate several neurobiological mechanisms, including monoamine metabolism, HPA axis activity, expression of neurotrophic factors, hippocampal neurogenesis, and thereby improving cognitive performances [4, 6, 319-321]. Similarly, short-term PE improved age-related decline in neurogenesis and impaired retention memory [319], as well as anxiety- and depressive-like behavior in rodents [3]. In another study, enhanced memory acquisition, memory retention, and reversal-learning were reported in rodents provided with voluntary PE on a running wheel [321]. Our published review on this topic provides a more detailed summary of the role of PE on neurobiology, behavior, and memory [31].

The effects of PE on hippocampal gene expression

There is evidence to suggest that the change in behavior and memory is a measure of hippocampal integrity [322-326]. The presence of running wheels has been shown to modulate hippocampal gene expression and, in turn, enhance cognition in C57BL/6

mice [72, 73]. Moreover, it has been shown that PE has a considerable influence on the expression of hippocampal genes associated with immune functions and this can manifest into known anti-inflammatory and cell-proliferative effects of PE [327]. PE, when provided for short-term, also enhances the expression of hippocampal genes that encode for neurotrophic factors (e.g., Bdnf, Fgf, Ngf), which has been attributed to the enhanced neurogenic effects of PE during aging. [327-330]. This evidence suggests that PE provided for a few weeks can improve hippocampal integrity by modulating the hippocampal gene expression, however, if this correlates to a change in brain function is not yet known.

The effects of PE on neuroimmune functions

PE can induce several immune-cytological changes, for example, in a clinical trial, PE has been shown to increase the concentration of neutrophilic granulocytes, lymphocytes, monocytes, the blood mononuclear cell subsets (CD3+, CD4+, CD8+, CDl6+, CDl9+ and CDl4+), the NK cell activity and plasma IL-6 [331, 332]. It has been shown to decrease the levels of TNF-α both in serum and CSF [223, 224], induces neurogenesis [333] and increases the leukocyte blood levels [334, 335]. However, other studies have also shown a significant increase in the circulating leukocyte, neutrophil and monocyte count, followed by the marked release of proinflammatory cytokines (TNF-α, IL-6, and IL-1), as well as an increase in the expression of brain glial cells post-exercise [332]. Similarly, PE has been shown to improve the functions of neutrophils and macrophages (e.g., phagocytoses, antigen presentation) [331, 336]. Other immune mechanisms that have been shown to be affected by PE include increased attraction of macrophages into the CNS, upregulation of MKP-1 and modulation of hippocampal T cells [6, 337]. This suggests that PE may exert a significant influence on the expression of immune components within the brain during normal aging. Our published review provides a more detailed summary of the role of PE on neuroimmune functions [31].

The effects of EE on neurobiology, behavior, and memory

Rodents raised in an environment enriched with toys, novel objects and accessories have been shown to possess higher visuospatial attention and spatial memory and enhanced sensory, cognitive and motor abilities [22, 31, 338-342]. This enhancement in brain functions has, however, been attributed to the improved hippocampal integrity and increase in the levels of neurotrophins in the hippocampus after EE [343, 344]. Moreover, EE shows a direct relevance to psychiatric disorders, for example, EE has been shown to improve behavior, memory and underlying molecular biology in the rodent models of AD [345, 346], PD [347], HD [348, 349], MS [350], stroke [351], traumatic brain injury [352, 353], schizophrenia [354], anxiety [28] and depression [355]. Our published review provides a more detailed summary of the role of EE on neurobiology, behavior, and memory [31].

The effects of EE on hippocampal gene expression

EE has also been shown to modulate gene expression. Recent evidence has started pointing to the role of EE in modifying the aminergic pathways and expression of associated genes, subsequently ameliorating behavioral, memory and cognitive deficits. In one such recent study on 5-HT1AR KO mice, EE in the absence of exercise showed anxiolytic effects [356]. Similar EE treatment, when provided to the 5-HTT KO mice, ameliorated the abnormal innate anxiety but did not rescue the depressive-like behavior [357]. The enriched housing also enhanced the gene expression of the

hippocampal AMPA GluR1 receptor subunit in LPS induced immune-challenged rats [358], thus suggesting that EE improves brain plasticity and helps to negate the adverse effects of repeated immune challenge through modulation of glutamatergic gene expression. EE has been reported to improve learning and memory by enhancing hippocampal LTP, which is dependent on the mGluR5 signaling [359, 360]. Similarly, EE has been shown to reverse the loss of dopamine D2 receptor (D2R) with aging, in turn improving lifespan, locomotor activity and behavior [361].

EE can also modulate the expression of several immune and growth genes profiles, for example, mRNA for C1q, leukotriene A4 hydroxylase, MHC class I and II antigens and prostaglandin D synthetase, were upregulated in the hippocampus following EE in a rodent study [362]. EE has been shown to regulate neuronal growth, structure, and functions by modulating the expression of genes in the cerebral cortex and hippocampus [363]. Likewise, the learning and problem-solving abilities improved when the expression of NGF mRNA in the visual cortex and hippocampus increased in an enriched environment [364]. In the same way, EE improved brain functions after stroke by increasing the nerve growth factor-induced gene A expression in the hippocampus [365]. In another study, EE differentially modified exon-specific BDNF gene expression in the hippocampus [366]. With evidence pointing at the upregulation of IGF-1 receptor gene in an enriched environment [362] and the circulating IGF-1 playing a role in the EE induced positive effects on the brain [367-370], the mechanism through which EE ameliorates age-related functional deficits is becoming clearer. Together, this suggests that genes may have a role to play in the effects of EE on brain functions and the underlying neurobiology and molecular biology, however the exact mechanism through which EE modifies gene expression is yet to be elucidated.

The effects of EE on neuroimmune functions

Environmental enrichment with novel objects and accessories has been reported to modify cytokine levels and T lymphocyte subsets within the brain. Expression of IL-1β, IL-6, IFN- α and TNF-α, as well as chemokines Ccl2, Ccl3 and Cxcl2 reduced within the hippocampus after EE [11, 371]. EE has also been reported to modulate cellular immune factors such as $CD4^+$ and $CD8^+$ T lymphocytes, and cytokines IL-2 and IL-1β after stressed pregnancies in adolescent rats [17]. After EE, a significant increase in both astrocyte (GFAP+) and microglia (Iba1+) antigen expression within the dentate gyrus, but not in the CA1 and CA3 regions of the hippocampus has also been reported [371]. Our published review provides a more detailed summary of the role of EE on neuroimmune functions [31].

Association between PE and EE

As discussed above, aging has been shown to adversely affect neurobiology, behavior, memory, neuroimmune mechanisms and hippocampal gene expression. Conversely, both PE and EE elicit beneficial effects and reverse the age-related changes in the brain. However, many studies, in particular, those published during the last two decades, have included running wheels as one of the methods of EE [28, 372-378]. The combination of PE and EE has been reported to produce beneficial effects in the brain of rodents of different ages. These effects include improved neurobiology, as well as behavior, cognition, and memory [25-27, 374, 375, 379, 380]. Since both PE and EE alone are also established modulators of neurobiology, behavior, and memory [3, 4, 321, 379, 381-386], the association between PE and EE is not yet clear. Hence, the possibility that the combination of PE and EE has greater effects on

neurobiology and brain functions than PE and EE alone cannot be ruled out. Our published review provides a more detailed summary of the role of PE+EE in the modulation of behavior and underlying neuroimmune mechanisms [31].

Duration of EE is an important factor in behavioral and physiological outcomes

Amongst several factors, EE protocols in the past also differed in the duration for which EE treatment was provided to rodents of different ages [28, 372, 375, 378, 382, 383, 387]. The treatment duration ranged from a few weeks to several months in rodents ranging from young to old age. Both short- and long-term EE has been shown to improve behavior, spatial learning and memory, cognition, as well as alter underlying neurobiology and molecular biology in caged rodents [22, 342, 374, 375, 388, 389]. It is, therefore, unclear if the duration of EE has had differential effects on brain functions, and underlying neurobiology and molecular biology and require further investigation.

The rationale for the use of rodent models to investigate the effects of normal aging

Pre-clinical studies are required to inform translational outcomes. Prior to initiating this research, an extensive literature review revealed that a large number of human studies investigating the effects of aerobic exercise and enriching stimuli on the brain showed varied neurocognitive, affective-like behavior (anxiety and depression), neurobiological and molecular outcomes. This was similar to the results obtained during preclinical trials in rodents provided with PE and EE [390-424]. The effects of EE in rodent studies are also comparable to the effects of activities in humans that stimulate attention and engagement to the external environment, such as cognitive stimulation [411, 412], cognitive training [413], cognitive rehabilitation [414] and cognitive remediation [415]. Similar activities in humans have been shown to improve cognition and quality of life. The previous literature provided a strong foundation to build upon the rationale for the use of further experimental rodent models investigating the effects of PE or cognitive stimulation on healthy brain aging. This also ensured that results from rodent models, including from our study, could be translated to human trials investigating the use of PE and cognitive stimulation for the prevention or treatment of psychiatric disorders that come with advancing age.

Research question

It is evident from the above-mentioned introduction that the direct association between normal aging and brain anatomy and functions may depend on several factors, including the strain of mice, as well as external environmental conditions and interventions. Hence, before we investigate the differential effects of PE, EE and their combination on aging, it is important to investigate and analyze the effects of normal aging on brain function and underlying molecular biology in C57BL/6 mice. This investigation allowed us to understand the effects of normal aging on brain functions and underlying immune functions and hippocampal gene expression across the life span. Hence, we first addressed the research question “Does normal aging have significant effects on brain functions, the underlying neuroimmune mechanisms, and hippocampal gene expression?”

Hypotheses

1. Normal aging will result in increased anxiety- and depressive-like behaviors, and decreased locomotion, spatial learning, and memory in male and female C57BL/6 mice, and this will be accompanied by underlying changes in hippocampal gene expression.
2. Alterations in brain functions during normal aging in C57BL/6 mice will be accompanied by underlying changes in neuroimmune functions, i.e., alterations in brain glial cell expression, and cervical lymph node T cell number and phenotype.

Aims

1. To investigate the effects of normal aging on locomotion, anxiety- and depressive-like behaviors, spatial learning and memory, and hippocampal gene expression in early (4 months), middle (9 months) and late-middle age (14 months) male and female C57BL/6 mice.
2. To investigate the effects of normal aging on brain immunopositive glial cell count, and T cell count and phenotype in the cervical lymph nodes, in early (4 months), middle (9 months) and late-middle age (14 months) male and female C57BL/6 mice.

Chapter 2: Effects of aging on the motor, cognitive and affective behaviors, neuroimmune responses and hippocampal gene expression

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Research report

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

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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.

Statement of Authorship

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Principal author

Name of Principal Author (Candidate)	Gaurav Singhal		
Contribution to the Paper	Planning and conducting experiments, behavioral and molecular analysis, data collection, organization, analysis and interpretation, writing and editing manuscript.		
Overall percentage (%)	80%		
Certification:	In this paper, the original research that I have conducted during the period of my Higher Degree by Research candidature has been reported. It is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.		
Signature		Date	24/05/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

1. the candidate's stated contribution to the publication is accurate (as detailed above);
2. permission is granted for the candidate in include the publication in the thesis; and
3. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution

Name of Co-Author	Julie Morgan		
Contribution to the Paper	Assistance with planning and conducting mouse husbandry and laboratory techniques throughout the research period.		
Signature		Date	27/5/19

Name of Co-Author	Dr. Magdalene C Jawahar		
Contribution to the Paper	Designing methodology/experimental protocol, teaching High-throughput qPCR technique, supervising at every step during the research, editing and revision of manuscript drafts.		
Signature		Date	21/11/19

Name of Co-Author	Dr. Frances Corrigan		
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Signature		Date	25/11/2019

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Contribution to the Paper	Conception of the project, designing methodology/experimental protocol, teaching behavioral testing in mice techniques, supervising at every step during the research, editing and revision of manuscript drafts.		
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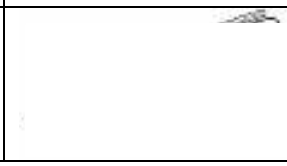
Contribution to the Paper	Teaching Fluorescent Activated Cell Sorting technique, guiding at every step during the research, editing and revision of manuscript drafts.		
Signature		Date	24/05/2019

Name of Co-Author	Dr. James Breen		
Contribution to the Paper	Helped with the statistical analysis of gene expression data using R package multcomp.		
Signature		Date	29|5|2019

Name of Co-Author	Dr. Stephen M Pederson		
Contribution to the Paper	Helped with the statistical analysis of gene expression data using R package multcomp.		
Signature		Date	27/05/2019

Name of Co-Author	Mr. Jim Manavis		
Contribution to the Paper	Helped with Immunohistochemistry techniques.		
Signature		Date	25|5|19

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Contribution to the Paper	Conception of the project, supervising every aspect of the research, editing and revision of manuscript drafts.		
Signature		Date	26/11/2019

Preamble to chapter 2

The evidence cited in chapter 1 clearly suggests that aging could have significant effects on neurobehavioral functions and associated underlying molecular biology, however, some of the evidence also suggested the contrary. Moreover, the rate of normal aging, and subsequently its effect on brain functions, is also dependent on both intrinsic (e.g. genotype) and extrinsic (e.g. environmental) factors, which creates uncertainty about the validity of findings reported in some past studies. Hence, before we test the effects of PE and EE on brain functions during normal aging, it was essential to elucidate the effects of normal aging on brain functions in a fully controlled external environment, i.e., ambient temperature and humidity, *ad libitum* standard laboratory food and water and absence of all external interventions. It is expected that under such controlled environmental conditions, the close to true effects of normal aging on brain functions and underlying molecular biology could be revealed.

Hence, C57BL/6 mice housed under controlled environmental conditions were tested for the effects of normal aging on brain functions, and changes in neuroimmune functions and hippocampal gene expression at 4 (early age), 9 (middle age) and 14 (late-middle age) months of age. This was done to answer the following research question: “Does normal aging has significant effects on brain functions, the underlying neuroimmune mechanisms, and hippocampal gene expression?”

We hypothesized that:

1. Normal aging will result in an increase in anxiety- and depressive-like behaviors, and decrease in locomotion, spatial learning, and memory in male and female C57BL/6 mice, and this will be accompanied by underlying changes in hippocampal gene expression.
2. The alteration in brain functions during normal aging in male and female C57BL/6 mice will be accompanied by underlying changes in neuroimmune functions, i.e., increase in the number of microglia and astrocytes in the brain, and decrease in T helper and increase in T cytotoxic cell subsets in the cervical lymph nodes.

Following aims were set forth at the start of the study:

1. To investigate the effects of normal aging on locomotion, anxiety- and depressive-like behaviors, spatial learning and memory, and hippocampal genes expression in early (4 months), middle (9 months) and late-middle age (14 months) male and female C57BL/6 mice.
2. To investigate the effects of normal aging on immunopositive glial cells count in the brain, and T cells count and phenotype in the cervical lymph nodes, in early (4 months), middle (9 months) and late-middle age (14 months) male and female C57BL/6 mice.

Mice were tested for locomotion in the Home Cage and Open Field, anxiety-like behavior in the Elevated Zero Maze and Open Field, depressive-like behavior in the Forced Swim Test, and spatial learning and memory in the Barnes Maze. The changes in neuroimmune functions and hippocampal gene expression were analyzed using immunohistochemistry and fluorescent activated cell sorting analysis, and high-throughput qPCR, respectively. All findings are reported and discussed systematically. The included chapter is the same as the manuscript accepted for publication in the journal Behavioral Brain Research.

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

Introduction

Aging is a known risk factor for degenerative changes in various brain regions [48, 49], which, in turn, also results in functional loss, such as progressive decline in learning, memory, and cognitive and motor functions [59-61, 277, 425-428]. Studies in the past have shown a significant increase in anxiety [225-228] and depressive-like [227, 231] behaviors, and decline in spatial learning and memory [225, 232-235] and locomotion [226, 227] 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 [429-433].

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 [124, 289, 291, 434]. The dysfunctional glial cells mediate immune reactivity and inflammation by 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 [101, 287]. The chronic pro-inflammatory environment may predispose the brain to neurodegenerative disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD) [43, 44, 435].

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 [101, 124, 275, 436, 437]. The decline in dopamine and serotonin levels during normal aging has been shown to result in impaired cognitive and motor performances [277, 278], and dysregulated synaptic plasticity and neurogenesis [276]. This has also been shown to alter hippocampal synaptic plasticity in aging rodents [438]. 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 [322, 324-326, 439-442]. 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) [276-278]. Aging has also been shown to impair growth hormones and glucose metabolism which may result in cognitive impairment and white matter lesions [276, 277, 443].

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 [444, 445]. Similarly, the expression of the gene that encodes the transcription factor CREB1 has been shown to decrease in the hippocampus of aging rats [446]. 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 [290, 447, 448]. 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 [434]. 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 [433, 449]. 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 [22, 31, 327, 345, 347, 358-360, 362-366, 383, 386, 388, 450-455].

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 [225], 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.

Methods

A. 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-hour 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 37cm x 20.5 cm x 13.5cm 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.

B. 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 2.1).

Table 2.1 Mouse numbers for behavioral and molecular analysis.

Age	Behavioral analysis n (males: females)	FACS and RT-qPCR n (males: females)	IHC n (males: females)
4M	19 (10:9)	8 (4:4)	7 (4:3)
9M	15 (9:6)	7 (4:3)	6 (3:3)
14M	12 (6:6)	6 (3:3)	6 (3:3)

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

The cages had the following dimensions: 48.5cm x 15.5 cm x 12cm. 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 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.

C. Behavioral analysis

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

Locomotor and Exploratory Behaviour

Home cage activity: The baseline locomotor activity of mice was analyzed in home cages with 2-day old bedding under basal non-stressful conditions according to previously published protocols [19]. The total distance covered over five minutes was recorded as a measure of baseline locomotor activity.

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 lux) square arena of 40cm x 40cm, with clear 35cm high walls, according to previously published protocols [19, 456, 457]. The floor was divided into inner (26.6cm x 26.6 cm) and outer (13.4cm x 13.4cm) zones. Total distance traveled over 5 minutes in Open Field was measured as an indication of locomotor activity and exploratory behavior.

Anxiety-like Behaviour

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 [458]. 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 lux) area during the behavioral testing. The mouse was placed on the open quadrant and allowed to explore for 5 minutes according to published protocols [459]. 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 [460].

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 [19, 456, 457].

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 lux) area and the procedures were carried out according to published protocols [19, 461, 462]. 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 minutes 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 [463]. On day 5, the position of escape box was changed from the original training position (in the NW quadrant) to the probe trial position (in the 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 [463]. The latency to find the location of the escape box in the NE quadrant was recorded to assess cognitive flexibility [463]. Since the decreased drive to explore is a key factor underlying many aspects of reduced behavioral performance, including cognitive capacity during aging

[226], we also statistically analyzed the distances traveled by mice while finding the location of the escape box on training days and during the 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. [464]. This included nose pokes and head deflections over a hole. An increased number of entry errors were considered indices of spatial learning and memory impairment.

Depression-like Behaviour

Forced-swim test: Duration of immobility (when mice were floating with no movement of limbs in any direction) in a 4L 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 lux) area was recorded using a ceiling-mounted camera as a measure of despair and depressive-like behavior [460, 465, 466]. One trial of six minutes was conducted per animal to minimize distress associated with repeated testing.

D. 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 [467]. 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.

Peripheral T cell immunophenotyping

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 $2x10^6$ 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 10µL 0.5mg/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 minutes at room temperature with the respective mAbs (as shown in Table 2.2) after which they were washed twice before resuspension in 300µL 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 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.

Table 2.2 Monoclonal antibodies used for FACS.

mAb	Clone	Fluorochromes	BD Biosciences catalog no.	Conc. (mg/mL)	Antigen Distribution/Function
CD3	145-2C11	FITC	553061	$1.0x10^3$	T-cell identification marker
CD45	30-F11	V500	561487	$2.0x10^3$	Nucleated hematopoietic cell lineage marker; common leukocyte antigen
CD4	GK1.5	APC-H7	560181	$2.0x10^4$	T helper cell co-receptor for MHC II-restricted antigen induced T-cell activation
CD8	53-6.7	PerCP-Cy5.5	551162	$2.0x10^4$	Cytotoxic T-cell Co-receptor for MHC I restricted antigen induced T-cell activation
CD25	3C7	PE	561065	$2.0x10^3$	Early T-cell activation marker
CD44	IM7	PerCy7	560569	$5.0x10^4$	Activation marker for effector or memory T-cells; attachment and rolling
CD62L	MEL-14	V450	560507	$5.0x10^5$	T-cell homing receptor; transmigration
CD69	H1.2F3	APC	560689	$2.0x10^3$	Early T-cell activation marker

Glial cell immunophenotyping

Immunohistochemistry

Brains preserved in formalin were cut into five 3mm 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 150µm apart. Each section was 5µm 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 mins. Antigen retrieval was performed by heating at close to boiling point for 10 mins in citrate buffer, and slides were then allowed to cool below 40°C before further processing. The appropriate primary antibody (IBA1 for microglia, 1: 10000; GFAP for astrocytes 1: 40000; 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 minutes. The formed immune complex was then further amplified by incubating slides with a biotin-binding protein, streptavidin-peroxidase conjugate, for 60 minutes. 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 the 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.

Gene expression analysis

Real-time quantitative PCR

Aging has been shown to influence gene expression and gene-gene interactions in the hippocampus regulating neuronal growth, structure, and functions [229]. 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 [468, 469]. 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 [160-165]. 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. 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 2.3) 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 the hippocampus tissues stored in RNA later (Ambion, Life Technologies) using the 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 20ng 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 X 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).

Table 2.3 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

E. 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 [470]. 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 [471, 472].

Results

A. 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 2.1).

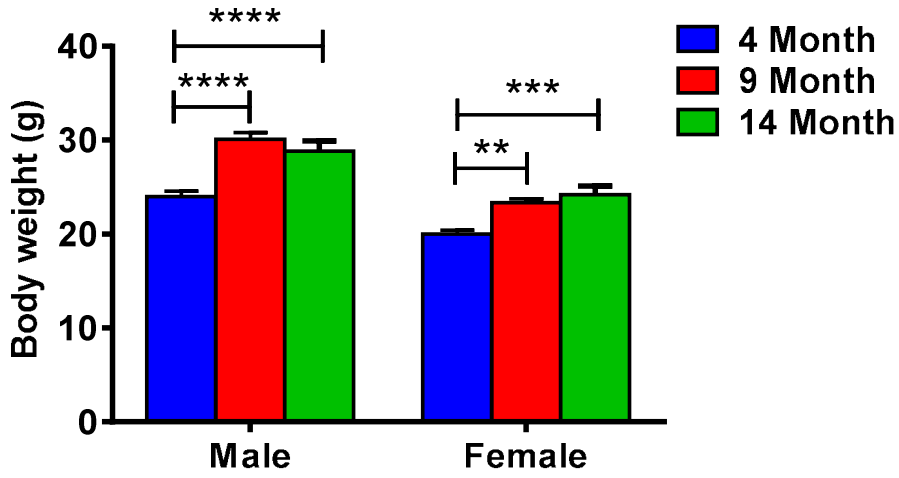

Fig. 2.1 Bodyweights of male and female mice. Two-way ANOVA, n = 6-10 per group. All data represented as mean ± SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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 2.2). 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.

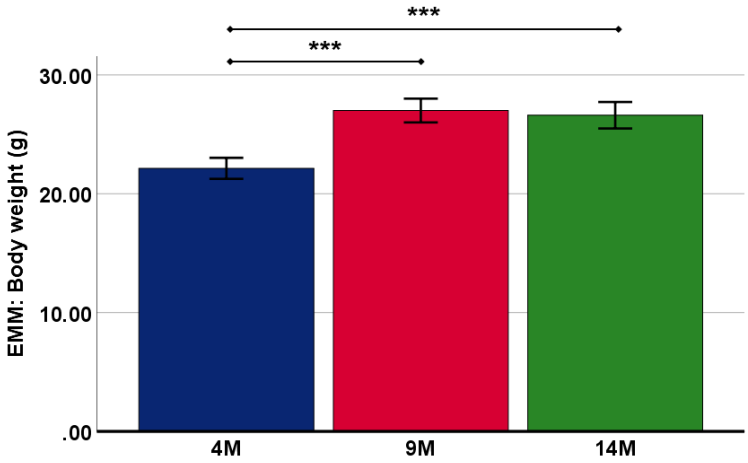

Fig. 2.2 Bodyweights of mice with sex as a covariate. Sex used as a covariate at the following value: 1.48. All data represented as estimated marginal means (EMM) ± 2 SE, n = 12-19 per group. *** $p < .001$.

B. Behavior

Locomotor activity

Baseline locomotor activity was measured in the home cage (Fig 2.3). 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 ANOVA which showed that aging significantly affects baseline locomotion in the Home Cage ($F_{(2, 43)} = 3.84$, $p = 0.029$). On posthoc analysis, significant differences were noted between the 4 month and 14-month age groups (11.9 ± 0.7 vs. 8.8 ± 0.9).

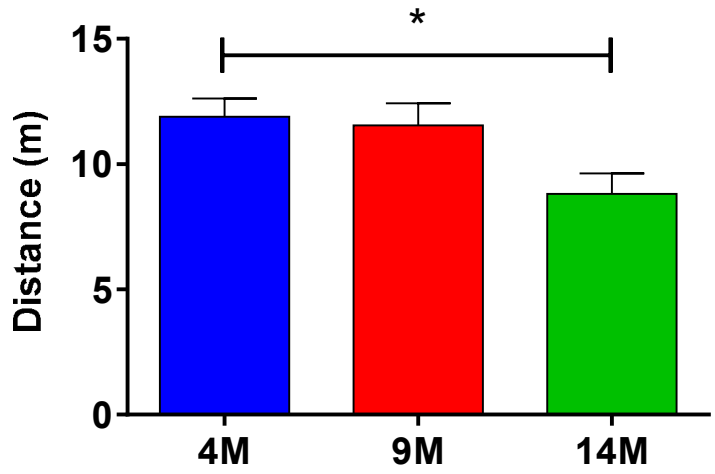

Fig. 2.3 Locomotor activity in Home Cage. Distance traveled. All data represented as mean ± SEM, n = 12-19 per group. * $p < .05$.

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 ($F_{(1, 40)} = 1.18$, $p = .283$).

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 quadrants 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 2.4B). 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, $p = 0.009$).

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 2.4C). 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, $p = 0.002$).

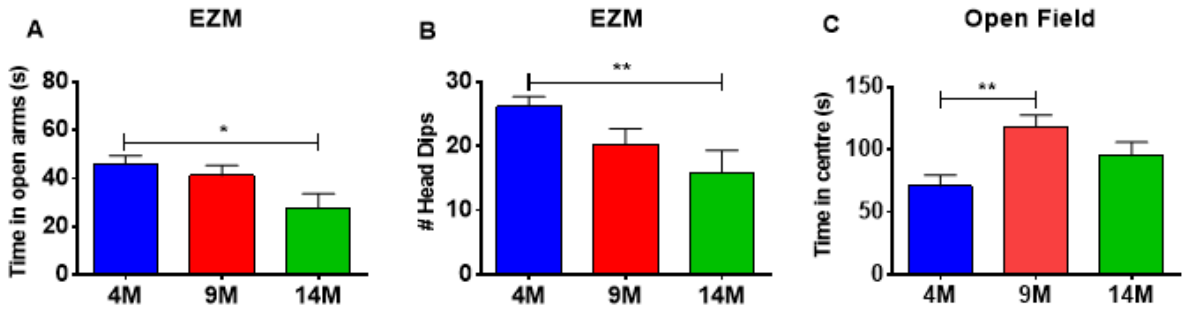

Fig. 2.4 Anxiety-like behavior in EZM and Open Field. (A) EZM: Time in open arms, (B) EZM: Number of head dips and (C) Open Field: Time in inner zone. All data represented as mean ± SEM, n = 12-19 per group. * $p < 0.05$, ** $p < 0.01$.

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 2.5).

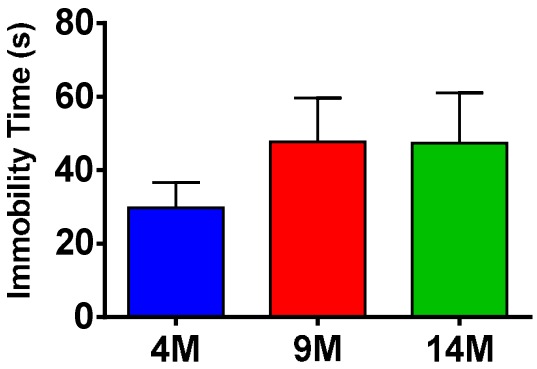

Fig. 2.5 Depressive-like behavior in FST. Immobility time. All data represented as mean ± SEM, n = 12-19 per group.

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 during 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-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 training (Fig 2.6A). 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 training. Also, although not significant at $p < .05$, a trend at $p < .1$ of 14-month 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 Branes 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 the distance traveled during four days of training in Barnes maze ($F_{(2, 43)} = 1.23$, $p = .303$; Fig 2.6B).

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 2.6C).

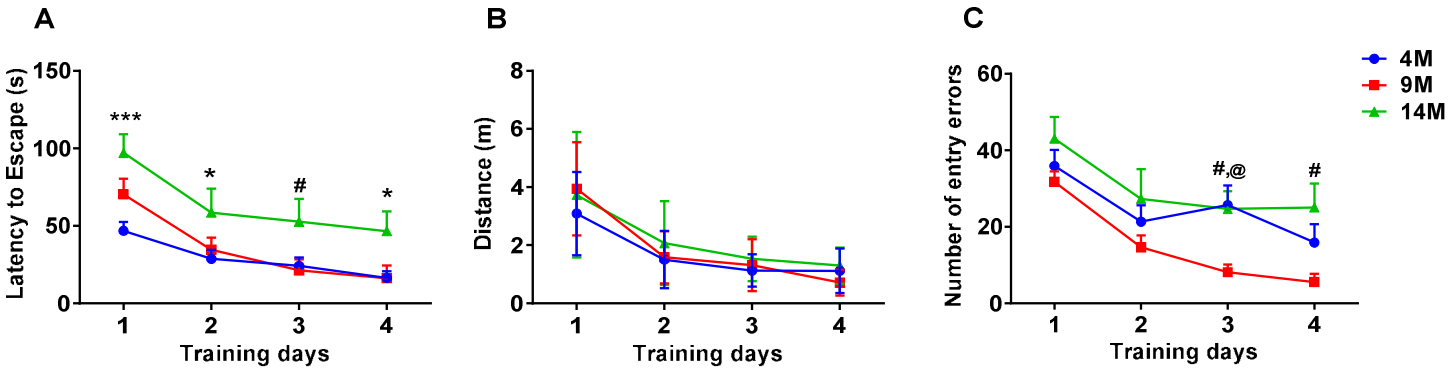

Fig. 2.6 Spatial learning in Barnes maze. (A) Latency to find the escape box, (B) Distance traveled, and (C) Entry errors over 4 days of training. All data represented as mean ± SEM, n = 12-19 per group. * 4M vs. 14M, @ 4M vs. 9M, # 9M vs. 14M. *, #, @ $p < .05$, *** $p < .001$.

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 2.7A). 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 2.7B). 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, $p = .028$).

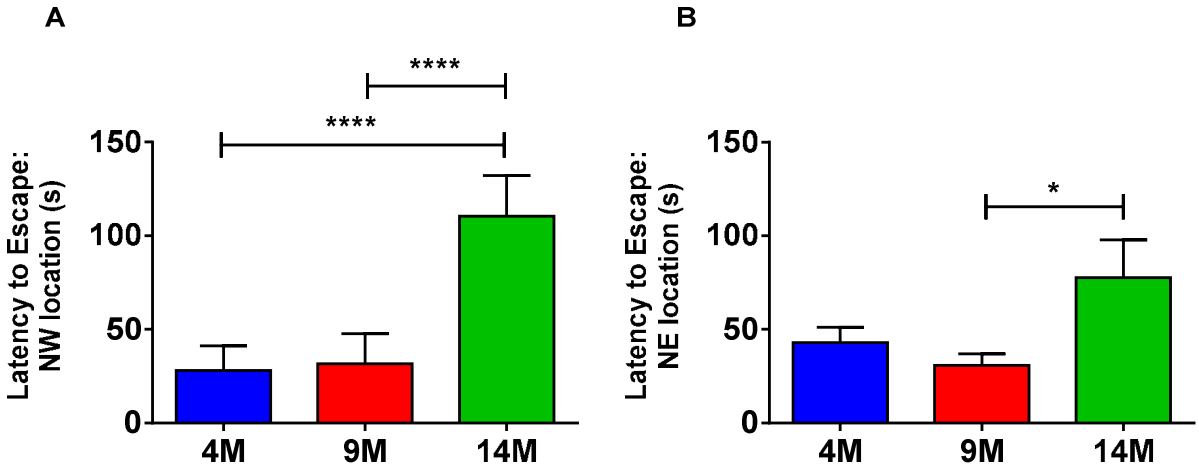

Fig. 2.7 Spatial memory in Barnes maze: Latency to escape. During probe trial. Latency to escape in (A) NW location and (B) NE location. All data represented as mean ± SEM, n = 12-19 per group. * $p < 0.05$, **** $p < 0.0001$.
For the analysis of distance traveled during probe trial, we conducted a two-way ANOVA that showed non-significant interaction 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 2.8A). 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 2.8B).

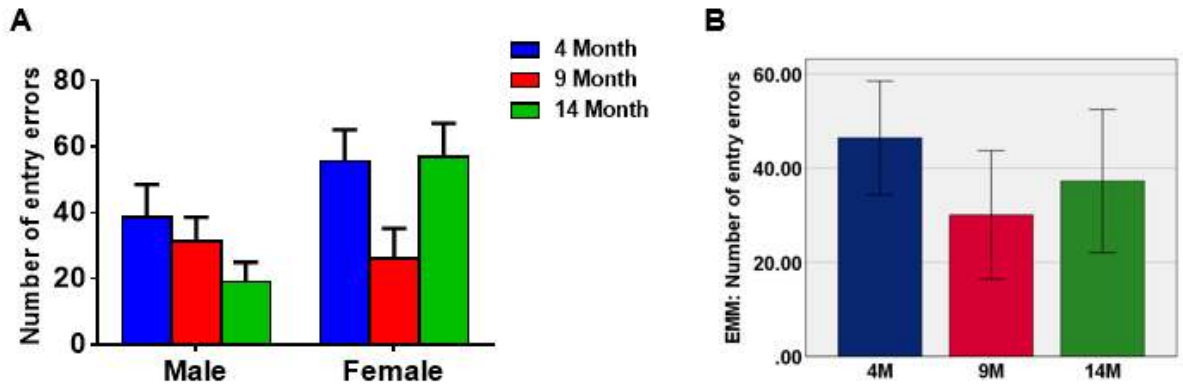

Fig. 2.8 Spatial memory in Barnes maze: Entry errors. During probe trial. (A) Sex comparisons, and (B) Comparison with sex as a covariate. Sex was used as a covariate at the following value: 1.4565. All data represented as mean ± SEM, n = 6-

10 per group, and (B) all data represented as estimated marginal means (EMM) ± 2 SE, n = 12-19 per group.

C. Molecular analyses

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 2.9), with decrease in the number of immunopositive microglia from 4 months to 9 months (71.6 ± 4.9 vs 51.7 ± 5.3, $p = 0.036$) followed by an increase from 9 months to 14 months of age (51.7 ± 5.3 vs 78.7 ± 5.3, $p = 0.007$).

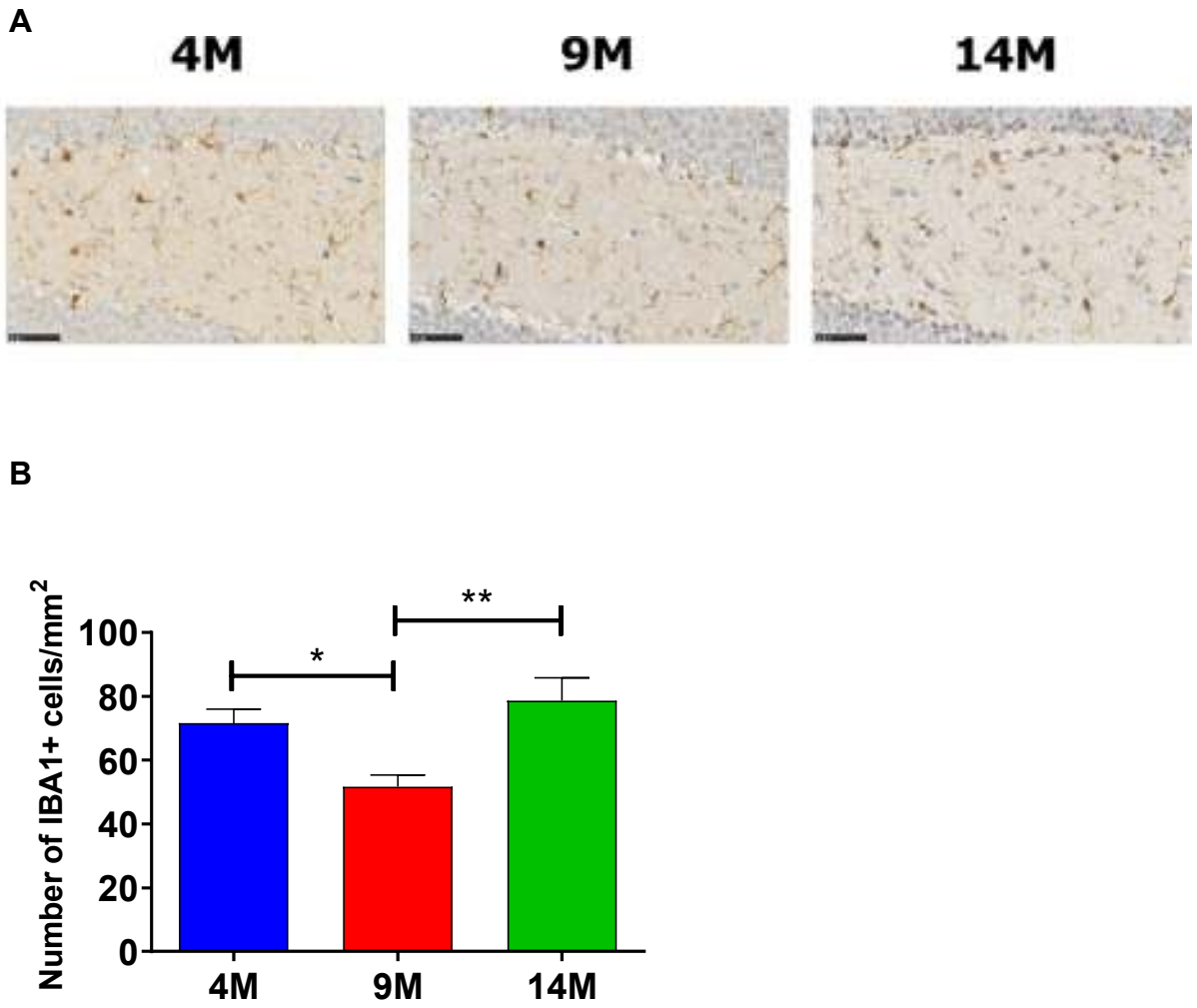

Fig. 2.9 IBA1$^+$ microglia in the dentate gyrus. (A) Representative immunohistochemical images, and (B) Number of IBA1$^+$ cells/mm^2. Black scale represents 50µm length. One-way ANOVA, all data represented as Mean ± SEM, n = 6-7 per group. * $p<0.05$, ** $p<0.01$.

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 2.10).

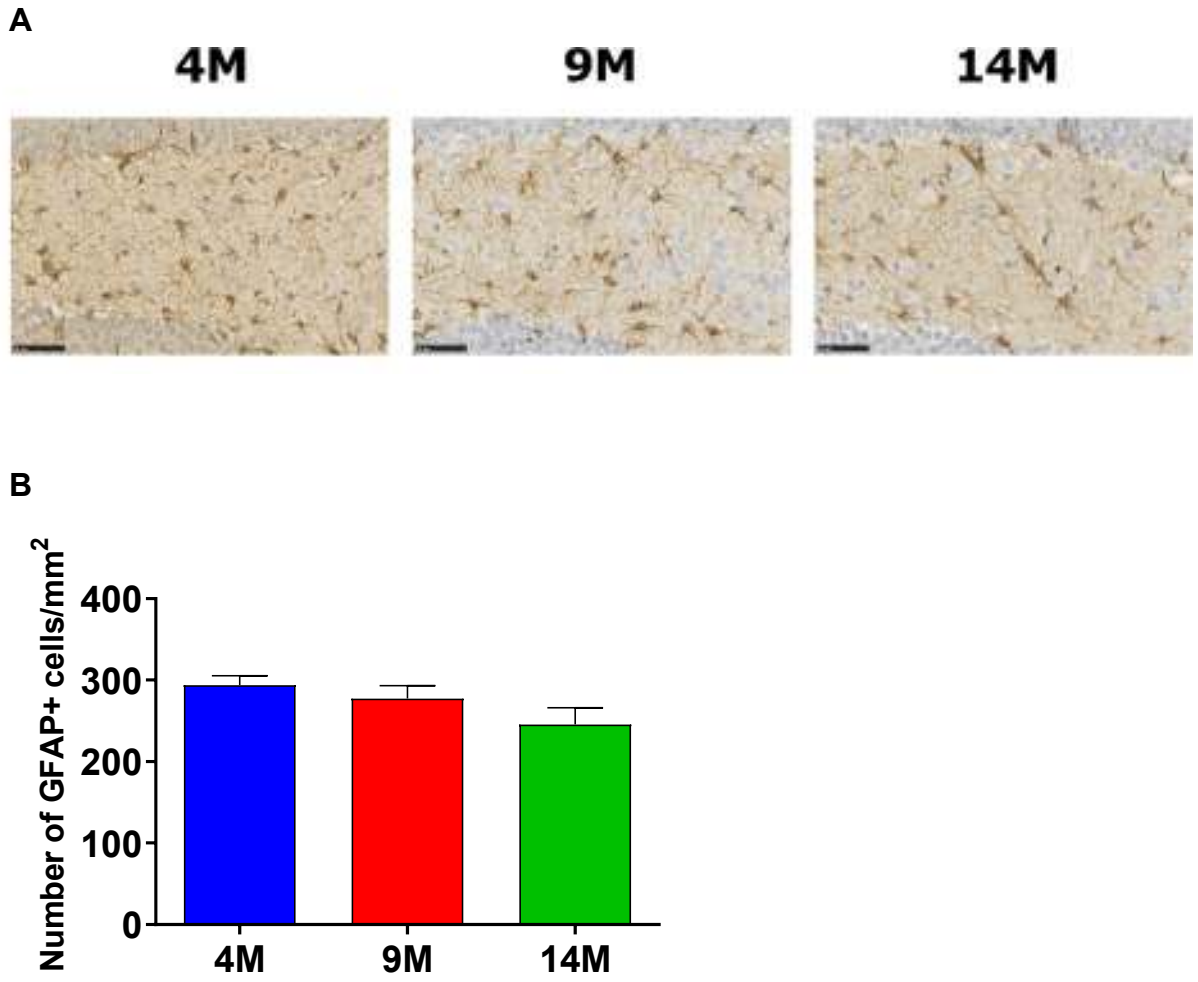

Fig. 2.10 GFAP$^+$ astrocytes in the dentate gyrus. (A) Representative immunohistochemical images, and (B) Number of GFAP$^+$ cells/mm^2. Black scale represents 50µm length. One-way ANOVA, all data represented as mean ± SEM, n = 6 per group.

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 2.11A). 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 2.11C). 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 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 2.4.

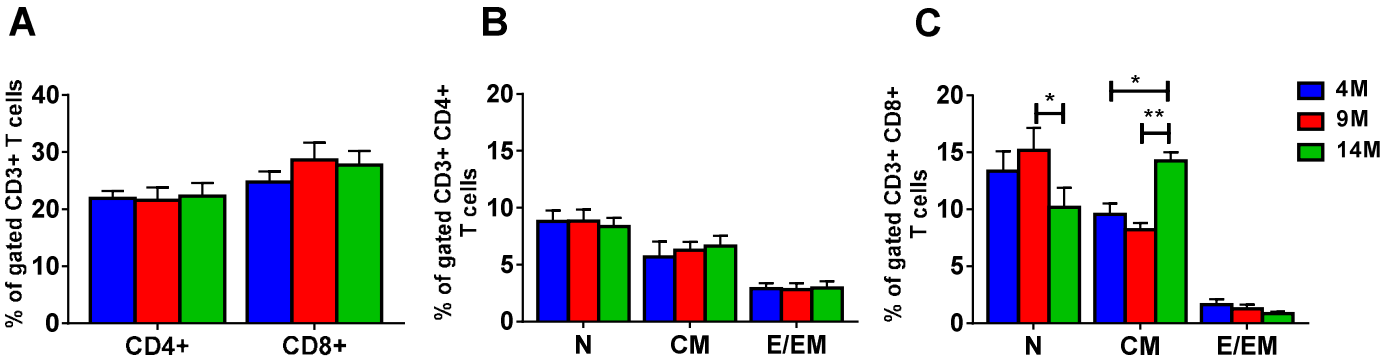

Fig. 2.11 T-cell subset composition. Proportions of the gated (A) $CD4^+$ and $CD8^+$ T, (B) $CD4^+$ T_N, T_{CM} and T_{EM}, and (C) $CD8^+$ T_N, T_{CM} and T_{EM} cells. All data represented as estimated marginal means of the proportion of T cells, n = 5-8 per group. * p<0.05, ** p<0.01. N: naïve, CM: central memory, EM: effector memory.

Table 2.4 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

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

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 2.2) 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 2.5). 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.

Table 2.5 Differentially expressed genes. 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	↓

Legend: 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 the upregulation of gene expression. Negative Z-score indicates the downregulation of gene expression. Adj. p: adjusted p-value.

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.

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 [473, 474]. A highly significant increase in body weight has been reported in C57BL/6 mice from 2 to 18 months of age [234]. 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 [226]. 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.

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 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 [226]. 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 minutes [227]. 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 lux, 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 lux of light [233]. 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 [475].

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 [225-228], but not in 5 and 11-month-old [225, 229] 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 not male mice [225], 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 [230]. 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.

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 [229, 230] while others are suggesting that aging has a significant effect on depressive-like behavior [227, 231]. 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 [229, 230]. 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 [227]. 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 [231]. 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 [476]. 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 [477, 478].

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 indicates impaired spatial learning, memory, and cognitive flexibility.

As described previously [464], 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) [225]. 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 [232]. 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 [233]. 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 [234]. Evidence suggests that while the spatial learning and memory functions significantly decline from 12 months of age [235], this process starts as early as 8 months of age in C57BL/6 mice [227]. During our study, however, while impaired cognition was observed at 14 months, no significant impairment in cognition was observed at 9 months of age.

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 [101]. During aging, there is an increase in the expression of cellular senescence proteins. This results in an increased expression of reactive microglia

with age [101], 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 [289, 479], depression [480], and neurodegenerative diseases such as AD [44]. 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 [268], mediate neural development [269, 270], function [271, 272] and protection [273, 274], as well as neurotransmission and synaptic plasticity [275-278]. 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 [434]. 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.

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 [299]. 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 [297, 298], 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 [302, 303]. 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 [307]. Similarly, the adverse effects of aging on the $CD8^+$ T cells receptor repertoire diversity has also been reported in another study [309]. Our results, therefore, suggest that normal aging has limited effects on the T cell number and diversity until the late-middle age.

Normal aging significantly affects the expression of hippocampal genes

The significant differences were observed 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 [481-483]. 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 [444] and brain pathology [445]. Conversely, the expression of the *Bdnf* gene within hippocampus has been shown to increase after external interventions, such as physical activity [454] and environmental enrichment [388]. 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 [484, 485]. 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 [486-489], 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 [490-492], 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 [290]. 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 [290]. 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 [446, 493-501]. 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 [498, 499, 502-507], 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 [447, 448, 508-515]. 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.

Research summary

In this chapter, we investigated the effects of normal aging on the brain functions and underlying neuroimmune mechanisms and hippocampal gene expression over the life span of C57BL/6 mice. The mice were housed in controlled environmental conditions and were provided with no external interventions. Once of the desired age, i.e., 4, 9 or 14 months, they were tested for various behaviors using a standardized behavioral battery. Following this, the mice were sacrificed, and blood and brain were collected and analyzed for changes in neuroimmune functions and hippocampal gene expression using immunohistochemistry and FACS, and high-throughput qPCR, respectively.

Aging was found to regulate the locomotor activity, cognition- and anxiety-like behaviors, neuroimmune response, and hippocampal gene expression but not depressive-like behavior. Late-middle age (14 months) mice showed a significant reduction in locomotion but increase in anxiety-like behavior. Similarly, 14-month mice showed significantly impaired spatial learning and memory. Also, microglia and $CD8^+$ T memory cell count increased towards late-middle age, as well as aging had a significant effect on the expression of 43 genes of interest in the hippocampus.

Unlike previous reports, our research suggests that aging alone is not the predictor for depressive-like behavior, and internal or external factors could have played an important role in the onset of depression that has been reported previously. Our findings can help researchers developing therapies for treating depression in old age people.

Chapter 3: Short-term environmental enrichment, and not physical exercise, alleviate cognitive decline and anxiety from middle age onwards without affecting hippocampal gene expression

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Short-term environmental enrichment, and not physical exercise, alleviate cognitive decline and anxiety from middle age onwards without affecting hippocampal gene expression

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Abstract

Physical exercise (PE) and environmental enrichment (EE) have consistently been shown to modulate behavior and neurobiological mechanisms. The current literature lacks evidence to confirm the relationship between PE and EE, if any, and whether short-term treatment with PE, EE, or PE+EE could be considered to correct age-related behavioral deficits. Three-, 8-, and 13-month-old C57BL/6 mice were assigned to either PE, EE, or PE+EE treatment groups (n = 12-16/group) for 4 weeks before behavioral testing and were compared to controls. Differential effects of the treatments on various behaviors and hippocampal gene expression were measured using an established behavioral battery and high-throughput qPCR respectively. Short-term EE enhanced locomotor activity at 9 and 14 months of age, whereas the combination of PE and EE reduced locomotor activity in the home cage at 14 months. Short-term EE also was found to reverse the age-related increase in anxiety at 9 months and spatial memory deficits at 14 months of age. Conversely, short-term PE induced spatial learning impairment and depressive-like behavior at four months but showed no effects in 9- and 14-month-old mice. PE and PE+EE, but not EE, modified the expression of several hippocampal genes at 9 months of age compared with control mice. In conclusion, short-term EE may help to alleviate age-related cognitive decline and increase in anxiety, without altering hippocampal gene expression. On the contrary, PE is detrimental at a young age for both affective-like behaviors and spatial learning and memory but showed no effects at middle and late middle age despite hippocampal gene expression alterations.

Keywords Environmental enrichment · Exercise · Depression · Anxiety · Cognition · Brain · Behavior · Aging · Gene

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Contribution to the Paper	Planning and conducting experiments, behavioral and molecular analysis, data collection, organization, analysis and interpretation, writing and editing manuscript.		
Overall percentage (%)	80%		
Certification:	In this paper, the original research that I have conducted during the period of my Higher Degree by Research candidature has been reported. It is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.		
Signature		Date	24/05/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

1. the candidate's stated contribution to the publication is accurate (as detailed above);
2. permission is granted for the candidate in include the publication in the thesis; and
3. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution

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Preamble to chapter 3

In chapter 2, we were able to deduce the effects of normal aging on brain functions, and underlying changes in the expression of hippocampal genes over the life span of C57BL/6 mice. We observed that normal aging significantly impairs all brain functions except for depressive-like behavior. Also, aging significantly changed the expression of hippocampal genes, which complemented the behavioral changes.

In chapter 1, we provided evidence in support of the beneficial effects of PE and EE on brain functions, as well as on the expression of genes in the hippocampus. Also, it was mentioned that PE has been used as the method of EE in many past studies. Since PE is a strong modulator of behavior and memory, and hippocampal gene expression, the inclusion of PE has created uncertainty on the efficacy of other enrichment objects used during EE studies. Hence, deducing the differential effects of PE and EE could help in formulating better techniques for the enrichment of the external environment for animals in current research. The results may also be translated to clinical trials since a lot of efforts are being placed currently on the use of physical exercise and cognitive training for treating the mental disorders that are commonly observed during old age.

As such, we investigated the effects of short-term (4 weeks) PE, EE and the combination of PE and EE on locomotion, anxiety- and depressive-like behaviors, spatial learning and memory and the 43 genes of interest in the hippocampus over the life span of C57BL/6 mice. For this, we used standardized behavioral testing techniques and high-throughput qPCR. The following two questions were addressed during this research.

1. Does short-term PE and EE differ in effects that they have on brain functions and hippocampal gene expression during normal aging?
2. Does the combination of short-term PE and EE elicit greater effects than individual effects of short-term PE and EE on brain functions and hippocampal gene expression during normal aging?

We hypothesized that:
1. Short-term PE and EE of 4 weeks will reverse the aging-associated impairment in brain functions, and hippocampal genes expression in C57BL/6 in early (4 months), middle (9 months) and late-middle age (14 months) male and female C57BL/6 mice compared to age-matched control mice.
2. The combination of short-term PE and EE will have greater beneficial effects than PE and EE alone on aging-associated impairment in brain functions, and hippocampal genes expression in early (4 months), middle (9 months) and late-middle age (14 months) male and female C57BL/6 mice compared to age-matched control mice.

Following aims were set forth before the start of the second set of experiments:
1. To investigate the effects of short-term PE, EE and the combination of PE and EE on anxiety- and depressive-like behaviors, spatial learning and memory, and hippocampal genes expression in early (4 months), middle (9 months) and late-middle age (14 months) male and female C57BL/6 mice.

Mice were tested for locomotion in the Home Cage and Open Field, anxiety-like behavior in the Elevated Zero Maze and Open Field, depressive-like behavior in the Forced Swim Test, and spatial learning and memory in the Barnes Maze. The changes in hippocampal gene expression were analyzed using high-throughput qPCR. All findings are reported and discussed systematically. The included chapter is the same as the manuscript published in the journal Cognitive and Behavioral Neuroscience.

Short-term environmental enrichment, and not physical exercise, alleviate cognitive decline and anxiety from middle age onwards without affecting hippocampal gene expression

Introduction

In the past, there have been numerous studies vastly covering the effects of physical exercise (PE) and environmental enrichment (EE) on affective and cognitive behaviors, locomotion and spatial learning and memory at various age points, but a comparative study over the life span has never been undertaken. PE, which broadly includes forced running on a treadmill or voluntary running on wheels in cages for rodents, has been shown to improve locomotion [387], enhance spatial memory acquisition, retention, and retrieval [319-321, 516-519], augment cognitive abilities [520-523] and alleviate anxiety- and depressive-like behaviors [524-528]. PE has also been shown to alter molecular biologies, such as monoamine metabolism [320, 529] and neurotrophin levels in the hippocampus [424, 518, 522, 530, 531]. Evidence suggests that improvement in hippocampal plasticity and neurogenesis is a prerequisite for change in brain functions in response to PE [319, 516, 521, 523].

In rodents, the external environment can be enriched with a variety of toys and novel objects, and this has been shown to elicit a considerable influence on neuronal plasticity and brain functions during aging [31]. Such rodents exhibited higher locomotion [385, 387], improved visuospatial attention performances and recognition memory [379, 383], enhanced cognition [25], and decreased anxiety- and depressive-like behaviors [532, 533] in preclinical trials. Also, EE has been shown to enhance neurogenesis and neuronal survival [381], increase synaptic plasticity and synaptogenesis [381, 382], and improve neurotrophin [381, 383], monoamine [450, 533] and glucocorticoid [386] metabolism in the hippocampus of rodents.

Many studies in rodents investigated the effects of either PE or EE, and not their combination, on behavior, memory, neurobiology and underlying molecular biology [319, 383-385, 387, 450, 520, 529, 534]. Most studies on EE in recent times, however, have also utilized running wheels as a tool of enrichment [28, 372-378]. The rationale behind the inclusion of running wheels in the EE paradigms is still unclear, and this may have been a source of bias in such behavioral studies on EE. Nevertheless, like PE and EE, the combination of PE and EE (PE+EE) has also been shown to improve cognition, spatial learning and memory [25-27, 374, 375, 535], and alleviate anxiety- and depressive-like behaviors [28, 323, 376, 377, 535]. Neurobiological and molecular changes in the brain, for example, enhanced hippocampal neurogenesis [375] and dendritic growth [374], improved neurotransmission [375] and HPA axis reactivity [17, 373] and increased BDNF levels in the brain [378] have also been reported after PE+EE.

The abovementioned functional change in the brain after PE, EE and PE+EE may also involve change in gene expression, particularly in the hippocampus since the effects of EE and PE on the brain result primarily from a change in hippocampal integrity [343, 344, 536]. Indeed, it has been reported that the external environment influences gene expression and gene-gene interactions, thereby suggesting that modulation of the external environment could be used as a tool to modify gene expression in the brain

[537]. Independent studies have shown that after PE, EE and PE+EE, genes associated with immune functions, aminergic pathways, neurotrophin metabolism, HPA axis activity, transcription factors, and mitochondrial function express differentially in the brain, thereby improving neural functions and biology [327, 358-360, 362-366, 383, 386, 450, 451, 535]. Any change in gene expression, therefore, can alter underlying neurobiology and may manifest into observable behavioral changes. Indeed, enhanced hippocampal neurogenesis and improved affective-like behaviors after EE have been shown to be the outcomes of an increase in the expression of Ngf and Bdnf [364, 383], and 5-ht1a receptor [450] mRNA in the hippocampus of rats. Similarly, PE has been shown to regulate the *Sirt1* and *Tnf* activity, thereby playing a key role in cellular energy metabolism, gene transcription, immune response and, consequently, in cell survival [451]. The differential effects of PE, EE, and PE+EE on hippocampal gene expression across life-span, however, remain unexplored.

The changes in behavior, memory, neurobiology, cellular and molecular functions, and hippocampal gene expression after PE, EE, and PE+EE may differ. Perhaps, the age and sex of an animal also influence behavioral and neurobiological responses after PE, EE, and PE+EE as has been reported previously [26, 27, 379, 538, 539]. It also seems probable that when PE and EE are combined, this could potentiate the individual effects of both treatments. These research questions remain unanswered in the absence of a direct comparison between the three treatments. This study, therefore, examined the differential effects of short-term PE, EE and the combination of PE and EE on the locomotor activity, affective-like behaviors, and spatial and retention memory at three time-points over the lifespan of healthy male and female C57BL/6 mice. The change in the expression of 43 genes of interest in the hippocampus, which were selected after extensive literature review and included 12 immune (e.g. *Il1b, Tnf, Aif1, Gfap*), 9 monoamine (e.g., *Htr1aa, Grin2a, Tph2*), 4 growth factors (e.g., *Bdnf, Ngf, Igf1*), 5 transcription factor (e.g., *Creb1*, *Smad2*, *Foxo3*), 3 HPA axis (*Nr3c1, Crhr1, Crh*) and 10 mitochondrial (e.g. *Sirt1, Gpx1, Prkaa1*) function-related genes has also been investigated in response to the abovementioned treatments. We tested the hypothesis that short-term PE and EE of 4 weeks will reverse the aging-associated impairment in brain functions, and hippocampal genes expression in early (4 months), middle (9 months) and late-middle age (14 months) male and female C57BL/6 mice compared to age-matched control mice, and that the combination of short-term PE and EE will have additive beneficial effects than PE and EE alone. Together, this allowed us to investigate the potential underlying mechanisms whereby these three treatments can enhance neuronal function and ensure healthy aging.

Methods

A. Animals

Wild-type (C57BL/6) mice (n=158; 80 males and 78 females), parental substrain Nhsd (derived from a colony from the National Institutes of Health), were bred in-house and housed in same-sex groups of 4-5 in individually ventilated cages (IVCs) in the Laboratory Animal Services facility at the University of Adelaide. In the time-period of the experiments, the C57BL/6Nhsd line has inbred between 9-13 generations. The strain of the mice is important as it has been reported that C57BL/6 mice are more reactive and alert to the external environmental stimuli [385]. All mice were given *ad libitum* access to standard laboratory food and water and were maintained under

controlled conditions of temperature (21±1 ºC), humidity (55 %) and a 12:12-hour dark-light cycle. This study was approved by the University of Adelaide Animal Ethics Committee (M-216-12), and all guidelines as prescribed for handling the experimental animals were followed during the study.

B. Experimental design

Once of the desired age (3, 8 and 13 months), mice that showed no signs of injury and sickness were distributed equally into three treatment (Physical exercise (PE), Environmental Enrichment (EE) and PE+EE) and control groups (Fig 3.1). Mice were randomly paired (males and females paired separately) and transferred to open top cages on the morning of Monday, week 1 (2 mice per cage unless fighting necessitated separation). Control mice received no intervention and were kept in cages with the following dimensions: 48.5cm x 15.5 cm x 12cm. PE, EE, and PE +EE mice were kept in plexiglass cages with dimensions: 37cm x 20.5 cm x 13.5cm, as these had more breadth and depth to provide extra space for running wheel and the objects associated with EE. Mice were inspected daily but handled only once a week while transferring them to clean cages every Friday morning, starting Friday of 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 is 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. Only the EE mice received nesting material (paper shreds) during the experiments.

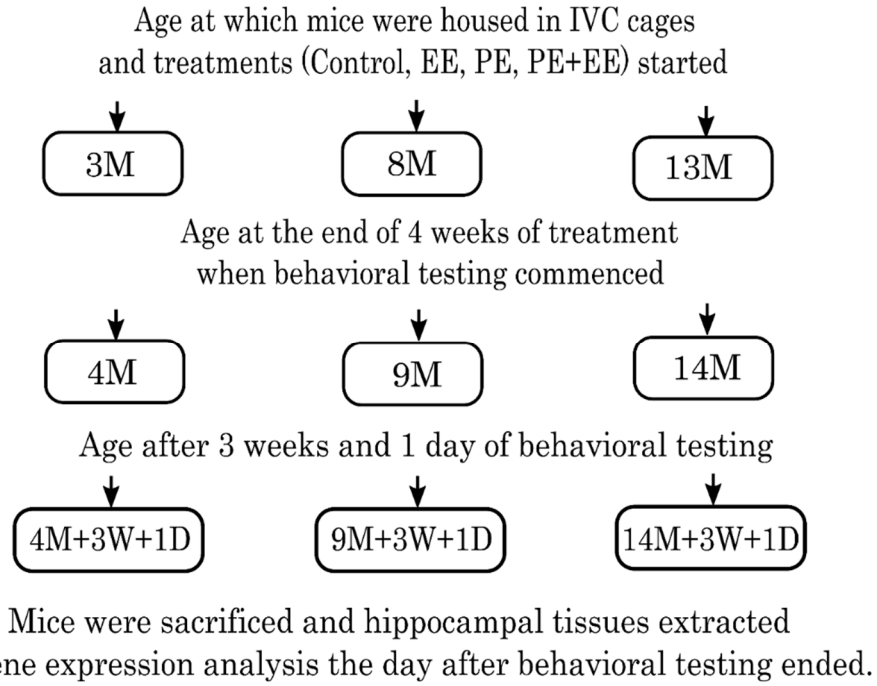

Fig. 3.1 Schematic representation of the experimental design. Four weeks of treatments are considered equivalent to one month of treatments for the sake of simplicity. **Legend:** IVC: Individually ventilated cage, PE: Physical exercise, EE: Environmental Enrichment, M: Month, W: Week.

The groups assigned as PE and PE+EE were provided with a running wheel for four weeks before behavioral testing, i.e., starting when the mice were 3, 8, and 13 months

of age. Wheels were made of metal and had the following dimensions: 12 cm in diameter and 5.5 cm wide. Running wheels remained in cages throughout the behavioral testing period. Wheel revolutions were counted using an automated counter on the wheels every Friday during change of cages. At the same time, wheels were also cleaned using F10SC veterinary disinfectant. Final readings of the wheel revolutions were taken the day after the behavioral testing ended. The total number of revolutions was then multiplied by the circumference of the wheels, i.e., 37.7 cm, to obtain the total distance traveled. Since the mice were kept in pairs, the total distance traveled for one cage reflects the distance traveled by a pair of mice. Distance traveled was then converted into kilometers for statistical analysis.

The group assigned as EE, and PE+EE were provided with a variety of non-toxic objects (toys, non-chewable novel plastic, and wooden objects, two types of houses - house on the cage floor and house suspended from the open-top roof, colored plastic balls of different texture, hanging toys, ladder and tunnels) and extra bedding as per previously published protocols [345, 348, 374] during the same time period (see table 3.1 for the EE protocol that we followed over 8 weeks). The objects and bedding were changed once every week to maintain novelty (during change of cages on Fridays to minimize handling stress to mice). The objects remained in the cages throughout the behavioral testing period. Fig. 1.6 shows the representative images of the PE, EE and PE+EE protocols for mice in cages utilized during our experiments.
A priori power analysis of the experimental design using the program G*Power indicated a 98% chance of detecting a large effect size and a 62% chance of detecting a medium effect size (defined by Cohen, 1992, as 0.4 and 0.25 of a population standard deviation between the means respectively [540]) between the groups significant at the 5% level (ANOVA: fixed effects, main effects and interactions). We observed that the power of our analyses would be 0.8 at the moderate effect size of 0.3 with n=158, which we deemed acceptable for research.

Table 3.1 Enrichment protocol over eight weeks.

Week	Objects used for EE
1 (Monday to Friday)	• House on the cage floor • 2 Toys (One ball + A toy with rough texture) • Straight tunnel • A couple of round and irregular stones (made of hard plastic) • Shredded paper as nesting material
2 (Friday to Friday)	• House on the cage floor • Three toys (One ball + a hanging toy + novel object) • U shaped tunnel • Increase in the number of irregular stones (4-5) • Shredded paper as nesting material
3 (Friday to Friday)	• House suspended from the open-top cage roof • Three toys (A different ball + a different hanging toy + novel object) • Y shaped tunnel (one arm closed) • Ladder • Shredded paper as nesting material
4 (Friday to Friday)	• House suspended from the open-top cage roof • Three toys (A different hanging toy + 2 novel objects) • Y shaped tunnel (all arms open) • Slope • Shredded paper as nesting material
5 (Friday to Friday)	• House on the cage floor • A straight and a U tunnel

	• A different ball + 2 novel objects • Shredded paper as nesting material
6 (Friday to Friday)	• House suspended from the open-top cage roof • U tunnel and a ladder • A couple of circular and irregular stones • A different ball + a different hanging toy + novel object • Shredded paper as nesting material
7 (Friday to Friday)	• House on the cage floor • Y shaped tunnel (one arm closed) and a slope • Few irregular stones • Three novel objects • Shredded paper as nesting material
8 (Friday to Tuesday)	• House suspended from the open-top cage roof • Y shaped tunnel (one arm closed), slope and a ladder • A different hanging toy + 2 novel objects • Shredded paper as nesting material
Mice were sacrificed for blood and brain collection on Tuesday morning of week 8.	

Legend: The first week consisted of only five days as the EE objects were changed every Friday morning during change of cages.

C. Behavioral analysis

After four weeks of exposure to PE and/or EE mice underwent a behavioral test battery following established procedures in the laboratory starting at 4, 9 and 14 months of age (four weeks of treatments are considered equivalent to one month of treatments for the sake of simplicity), 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 in order of the least to most stressful tests. All trials were recorded by a ceiling-mounted camera and analyzed using Any-maze software ver. 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.

Locomotor and Exploratory Behaviour

Home cage activity: The general locomotor activity and exploratory behavior of mice were analyzed in home cages with 2-day old bedding under basal non-stressful conditions according to previously published protocols [19]. The total distance covered over five minutes was recorded as a measure of locomotor activity. Running wheels were removed during the Home cage test since wheels occupied significant space in the cage and may have affected the home cage activity results. However, mice assigned as EE and PE+EE were left with two random EE objects to keep the environment enriched during the Home cage test.

Open Field: Locomotor activity and exploratory behavior of mice under more anxiety-inducing conditions were measured in the Open Field where mice were individually tested in a brightly lit (approximately 600 lumens) square arena of 40cm x 40cm, with clear 35cm high walls, according to previously published protocols [19, 541]. The floor was divided into inner (26.6cm x 26.6 cm) and outer (13.4cm x 13.4cm) zones. Total distance traveled in Open Field over five minutes was measured as an indication of locomotor activity and exploratory behavior.

Anxiety-like Behaviour

Elevated zero maze: The elevated zero maze is a round maze, 105 cm in diameter, with a 5 cm wide platform 40 cm above the floor and divided into four equal quadrants [458]. Two quadrants have 15 cm high walls (closed), and two are open in alternate order. The EZM was placed in a brightly lit (approximately 600 lumens) area during the behavioral testing. The mouse was placed on the open quadrant and allowed to explore for 5 minutes according to published protocols [459]. The time spent by the mouse in closed and open quadrants and the number of head dips were recorded as a measure of anxiety in mice. Increased time spent in closed as opposed to open quadrants and lower number of head dips is considered indices of anxiety-like behaviors [460].

Open Field: Time spent by mice in the inner and outer zones of the Open Field was recorded as a measure of anxiety-like behavior. Less time spent in the inner zone is an index of anxiety-like behavior [19, 541].

Spatial learning and retention memory

Barnes Maze: Change in spatial learning and retention memory was measured using the Barnes Maze, a circular grey platform 91 cm in diameter and elevated 90 cm above the ground, with 20 holes on the perimeter, one with a hidden escape box and the rest with false boxes. 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 procedures were carried out according to published protocols [19, 461, 462]. Time to find the location of the escape box over four days of training was recorded. The mice were placed in the centre of the maze and were allowed to locate the escape box for three minutes. Three trials separated by 15 minutes were performed for each mouse on a day. Latency to find the location of the escape box was used to assess the spatial learning and visual memory of the mouse in the new environment [463].

On day 5, the position of escape box was changed from the original training position (in the NW quadrant) to the probe trial position (in the NE quadrant – at 90 degrees). Time 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 [463].

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. [464]. This included nose pokes and head deflections over a hole. The increase in the number of entry errors were considered indices of spatial learning and memory impairment.

Depressive-like Behaviour

Forced swim test: Duration of immobility (when mice were floating with no movement of limbs in any direction) over 6 minutes in a 4L circular cylinder, 20 cm in diameter and 45 cm high and filled 2/3rd with water (at 23-24 degree Celsius) was recorded using a ceiling-mounted camera as a measure of despair and depression-like behavior [460, 465, 466].

D. Molecular analysis

Gene expression analysis using Real-time quantitative PCR

Mice were lethally injected intraperitoneally with pentobarbital (60 mg/kg IP) a day after forced swim test. Brains were extracted, dissected and hippocampal tissues were stored in RNAlater (Ambion, Life Technologies) at -80 degrees C until further processing.

Quantification of the levels of mRNA of 43 genes of interest (GOI) across various cytokines, monoamines, neurotrophins, and other genes 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 RNALater 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 20ng of each cDNA sample mixed with pooled TaqMan assays (47 assays, including 4 endogenous reference genes, listed in Table 2.3) and PreAmp Master Mix (Fluidigm Inc., USA). Real-time quantitative PCR was performed for each TaqMan assay for each sample in a 96.96 dynamic array nanofluidic chip (Fluidigm Inc., USA). A total of 47 X 96 (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).

E. Statistical Analysis

In 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. Further analyses were carried out in SPSS statistics version 25.0 (IBM Corporation). The three-way interactions between age, treatments, and sex for body weight, distance traveled on running wheels, locomotion, anxiety- and depressive-like behaviors, and spatial learning and memory were determined using three-way ANOVA's. Following this, the analyses of simple two-way interaction effects between age and treatments for males and females, simple main effects of the treatments and simple comparisons between the groups were conducted. If the three-way interaction was significant, then two-way ANCOVA was used to control for sex differences while determining the effects of age and treatment on the dependent variable. If the three-way interaction was non-significant, the two-way interaction effect of age and treatment on the dependent variable was determined using a two-way ANOVA. Post hoc analysis was conducted using Tukey's multiple comparison test. Results were presented as Mean ± SE. 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 control and treatment groups, and adjusted for multiple comparisons using the R package multcomp [470]. Genes were identified as differentially expressed between control and EE groups when the adjusted p values

were <0.05 with Z scores indicating the direction of expression change. GeneMANIA was used to identify the molecular pathways modulated by the differentially expressed genes at a false discovery rate (FDR) < .05 [471]. We were not able to analyze the sex differences for gene expression due to the relatively smaller sample size of gene expression data compared to behavioral data. This also limits us from performing the correlation analysis between behavioral and gene expression data.

Results

A. Body weights

Body weights of mice at the start and end of experiments were statistically analyzed using a two-way and three-way ANOVA, respectively. Body weights were measured in grams and are presented as a mean ± standard error unless stated otherwise. No statistically significant interaction between age and sex was observed for body weights at the start of the experiment ($F_{(2, 152)} = 1.001$, $p = .370$). There was, however, a statistically significant main effect of age on starting body weights ($F_{(2, 152)} = 110.741$, $p < .001$). The simple pairwise comparison revealed that the mean body weight of 4-month mice was significantly lower than 9- and 14-month-old mice at the start of experiments (21.3 ± .3 vs. 26.2 ± .3 and 26.7 ± .3; $p < .001$). The analysis of body weights taken at the end of experiments using a three-way ANOVA showed a non-significant interaction between age, treatment, and sex ($F_{(6, 134)} = 1.808$, $p = .102$). A two-way ANOVA also revealed a non-significant interaction between age and treatment ($F_{(6, 146)} = .837$, $p = .543$), as well as the main effect of treatment ($F_{(3, 146)} = .904$, $p = .441$), but the main effect of age was significant ($F_{(2, 146)} = 37.438$, $p < .001$). Body weights of 4 months mice were significantly lower than both 9- and 14-months old mice in all experimental groups (Table 3.2; $p < .001$).

Table 3.2 Mouse body weights at the start and end of the experiment.

Age	Start of the experiment					End of the experiment			
	C	PE	EE	PE+EE		C	PE	EE	PE+EE
4	21.9 ± 0.57	21.2 ± 1	21.1 ± 0.36	20.5 ± 0.79		23.8 ± 0.51	22.7 ± 0.72	22.8 ± 0.37	22.8 ± 0.72
9	27.4 ± 1	26.2 ± 0.83	25.8 ± 1.1	25.4 ± 0.53		27.7 ± 0.85	26.1 ± 0.36	26.5 ± 1.14	26.1 ± 0.60
14	26.5 ± 0.98	28.3 ± 1.09	26.7 ± 0.56	26.5 ± 0.70		27.3 ± 1.1	28.9 ± 0.67	26.8 ± 0.47	27.3 ± 0.67

Legend: All data represented as mean ± SEM, n = 11-19 per group.

B. Distance traveled on wheels during the experimental period

Three-way ANOVA found a statistically significant three-way interaction between age, treatment, and sex for distance traveled on running wheels ($F_{(2, 62)} = 3.96$, $p = .024$). However, the simple two-way interactions between age and treatment for both males and females were non-significant ($F_{(2, 62)} = 1.666$, $p = .197$, $F_{(2, 62)} = 2.832$, $p = .067$). Hence, a two-way ANOVA was conducted to analyze the two-way interaction effect of age and treatment on distance traveled on running wheels, but the result was still non-significant ($F_{(2, 68)} = .459$, $p = .634$; Fig 3.2).

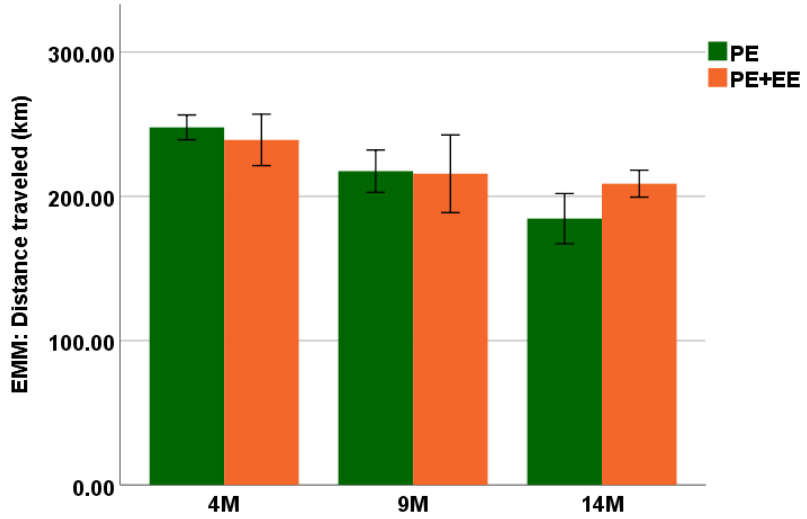

Fig. 3.2 Distance traveled on wheels during the experimental period. Separate cohorts of C57BL/6 mice exposed to PE and PE+EE conditions were compared using a Two-way ANOVA. All data represented as estimated marginal means (EMM) ± 2 SE, n = 11-14 per group.

C. Behavior

Locomotor activity

- **Home Cage: Distance traveled**

Baseline locomotor activity was measured in the home-cage following one-month exposure to either control conditions, PE, EE, or PE+EE at 4, 9 and 14 months of age. Distance traveled was measured in meters and is presented as mean ± standard error, unless stated otherwise. Three-way ANOVA found a significant three-way interaction between age, treatment and sex ($F_{(6, 133)} = 2.581$, $p = .021$). Further analysis revealed a significant simple two-way interaction between age and treatment for both males ($F_{(6, 133)} = 2.320$, $p = .037$) and females ($F_{(6, 133)} = 4.018$, $p = .001$). There was a significant simple main effect of treatment for males at 14 month ($F_{(3, 133)} = 6.901$, $p < .001$), and females at 4 ($F_{(3, 133)} = 5.604$, $p = .001$), 9 ($F_{(3, 133)} = 4.198$, $p = .007$) and 14 ($F_{(3, 133)} = 4.163$, $p = .007$) months. Hence, simple pairwise comparisons were conducted for males and females with a Bonferroni adjustment applied (Fig 3.3 A). The 14-month male PE+EE mice were significantly more mobile in home cage than 14-month male control mice (14.5 ± 1.2 vs. 8.2 ± 1.2; $p = .001$) and PE mice (14.5 ± 1.2 vs. 8.6 ± 1.1; $p = .002$). Conversely, PE and EE treatments, and not PE+EE, significantly improved the baseline locomotion compared to age matched controls in 14-month female mice (14.5 ± 1.2 and 15.7 ± 1.2 vs. 9.4 ± 1.2; $p = .013$ and .001 respectively). EE also improved the baseline locomotion of female mice significantly at 9 month compared to age matched controls (18.1 ± 1.2 vs.12.4 ± 1.2; $p = .003$) and 4 month EE mice (18.1 ± 1.2 vs. and 12.2 ± 1.2; $p = .001$). Female PE mice at 4 month, however, showed significantly reduced baseline locomtion than 4-month female control, EE and PE+EE mice (7.7 ± 1.2 vs. 12.2 ± .9, 12.2 ± 1.2 and 12.5 ± 1.2; $p = .016$, .036 and .021 respectively), as well as 9- and 14-month female PE mice (7.7 ± 1.2 vs. 15.2 ± 1 and 14.5 ± 1.2; $p < .001$ for both).

Following above analysis, we controlled baseline locomotion for sex differences using two-way ANCOVA and found a significant two-way interaction between age and treatment ($F_{(6, 144)} = 3.384$, $p = 0.004$; Fig 3.3 B). An analysis of simple main effects

for age and treatment with statistical significance receiving a Bonferroni adjustment and being accepted at the p<0.0125 level for age and p<0.0167 for treatment was conducted. EE mice were significantly more mobile than control mice at 9 months (16.0 ± .876 vs. 11.7 ± .785; p = .003) and 14 months (13.8 ± .876 vs. 8.8 ± .876; p < .001), and PE+EE mice showed significantly higher locomotion than controls at 14 months (14.0 ± .915 vs. 8.8 ± .876; p < .001). Also, EE and PE mice at 9 months showed significantly higher locomotion than 4-month treatment matched cohorts (16.0 ± .876 vs. 11.9 ± .876; p = .004 and 12.7 ± .784 vs. 9.1 ± .915; p = .009).

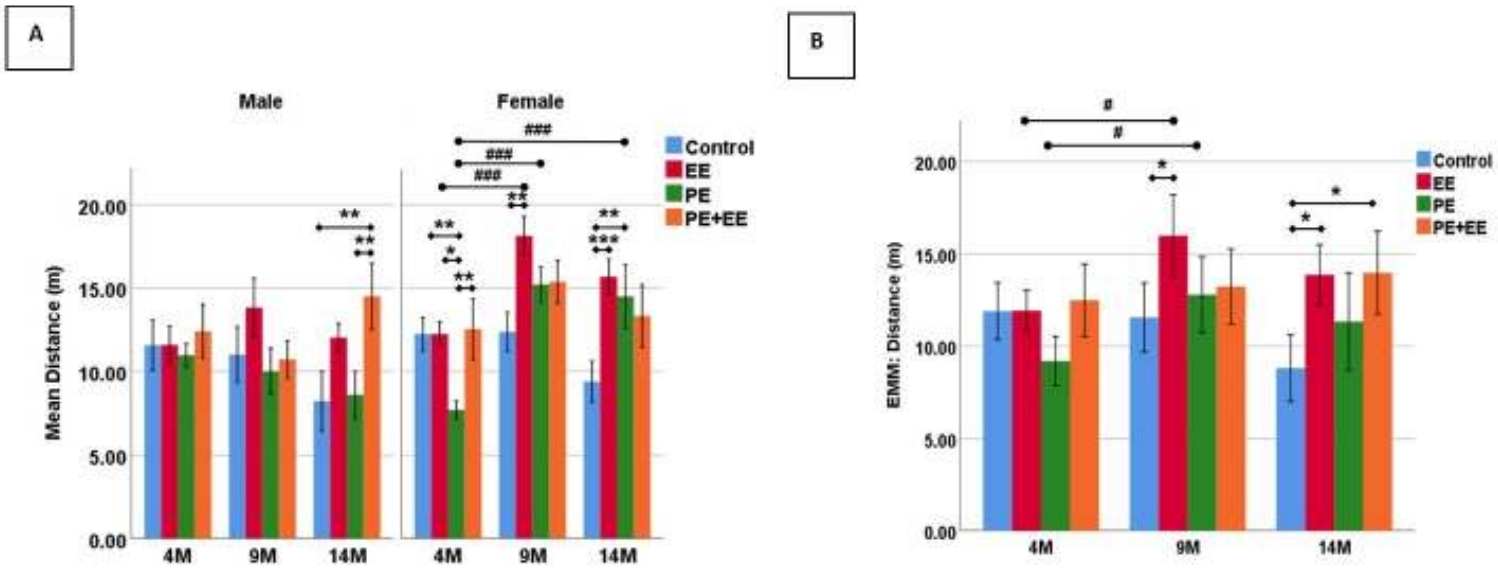

Fig. 3.3 Locomotor activity in Home Cage. Distance traveled. (A) Sex comparisons, and (B) Comparisons with sex as a covariate. All mice were exposed to PE, EE, PE+EE or control conditions at 4, 9 and 14 months of age. (A) Three-way ANOVA, all data represented as Mean ± SE, n = 6-10 per group. (B) Two-way ANCOVA with sex as a covariate at the following value: 1.4904, data represented as estimated marginal means (EMM) ± 2 SE, n = 11-19 per group. * represents a significant difference between a treatment and age-matched control or between two treatments at one age point. # represents a significant difference between the matched treatments over two age points. *,# p < .05, ** p < .01, ***,### p < .001.

- **Open Field: Distance traveled**

Mice were again tested for locomotor activity in the open field test one day after the home cage. Distance traveled was measured in meters and are presented as mean ± standard error unless stated otherwise. A three-way ANOVA found non-significant interaction between age, treatment and sex for locomotion in the open field ($F_{(6, 132)}$ = 1.694, p = .127). The simple two-way interactions between age and treatment were also non significant for both male and female mice ($F_{(6, 132)}$ = 1.344, p = .242 and $F_{(6, 132)}$ = 1.209, p = .306). Hence, no corrections were made for sex differences, and further analysis was carried out using a two-way ANOVA. No significant main effects of interaction and age were noted ($F_{(6,144)}$ = .782, p = .586 and $F_{(2,144)}$ = .245, p = .783); however, the main effect of treatment was found to be significant ($F_{(3,144)}$ = 5.807, p = .001; Fig 3.4). At 14 months, PE+EE treated mice traveled significantly less distance than controls (10.5 ± 1.2 vs. 14.7 ± 0.9 m; p = .0216). At nine months, both PE and PE+EE treated mice showed significantly lower locomotion than EE treated

mice (12.7 ± 1.1 vs. 15.8 ± 0.8 m; p<0.05; p = .0228 and 10.6 ± 0.7 vs. 15.8 ± 0.8 m; p = .0002).

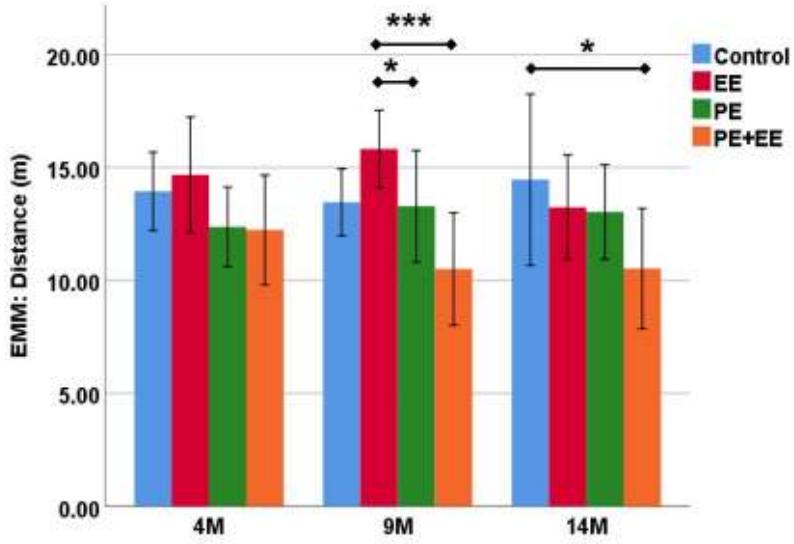

Fig. 3.4 Locomotor activity in Open Field. Distance traveled. All mice were exposed to PE, EE, PE+EE or control conditions at 4, 9 and 14 months of age. Two-way ANOVA, all data represented as estimated marginal means (EMM) ± 2 SE, n = 11-19 per group. * represents a significant difference between a treatment and age-matched control or between two treatments at one age point. * p < .05, *** p < .001.

Anxiety-like behavior

- **EZM: Time in open arms**

Time spent in the open quadrants of EZM is a measure of anxiety-like behavior, with less anxious mice spending more time in the open quadrants. Time was measured in seconds and is presented as a mean ± standard error unless stated otherwise. A three-way ANOVA showed statistical significance for the three-way interaction between age, treatment, and sex for time in open arms ($F_{(6, 118)} = 2.477$, p = .027). Further analysis showed a statistically significant simple two-way interaction between age and treatment for males ($F_{(6, 118)} = 3.165$, p = .006), but not for females ($F_{(6, 118)} = 1.599$, p = .153). There was a significant simple main effect of treatment for males at 9 month ($F_{(3, 118)} = 3.680$, p = .014) and females at 4 month ($F_{(3, 118)} = 5.211$, p = .002). Hence, simple pairwise comparisons were run for males and females with a Bonferroni adjustment applied (Fig 3.5 A). 9-month male EE mice were found to spend significantly more time in the open arms of EZM than 4 and 14 month male EE mice (64.3 ± 8 vs. 36.8 ± 8 and 14.6 ± 7.3; p = .049 and < .001 respectively), and 9 month male PE mice (64.3 ± 8 vs. 30.7 ± 6.8; p = .010). The time in open arms was significantly low for 14-month male PE+EE mice compared to 4- and 9-month male PE+EE mice (9.3 ± 7.3 vs. 42.7 ± 8 and 48.3 ± 8; p = .007 and .001 respectively). For females, 4 month control mice spent significantly more time in the open arms of EZM than age-matched PE (52 ± 6.3 vs. 9 ± 8.9; p = .001) and 14-month control mice (52 ± 6.3 vs. 23.9 ± 7.3; p = .013).

Using a two-way ANCOVA, we then controlled time in open arms in the EZM for sex differences and found a non-significant two-way interaction effect between age and treatment ($F_{(6, 129)} = 2.152$; p = .052). An analysis of the simple main effects for age and treatment with statistical significance receiving a Bonferroni adjustment and being

accepted at the p < 0.0125 level for age and p < 0.0167 for treatments showed non-significant main effect of treatment ($F_{(3, 129)}$ = 1.736, p = .163) but significant main effect of age ($F_{(2, 129)}$ = 16.965, p < 0.0001; Fig 3.5 B). The EE mice at 9 months, and PE+EE mice at 4 and 9 months spent significantly more time in open arms than 14-month EE (56.7 ± 5.6 vs. 19.7 ± 5.6; p = .049) and PE+EE (38.0 ± 5.6 and 45.2 ± 5.6; p = .011 and <.001) mice respectively. Also, 9-month EE mice were found to spend significantly more time in the open arms than 9-month PE mice (56.7 ± 5.6 vs. 31.6 ± 5.1; p = .007).

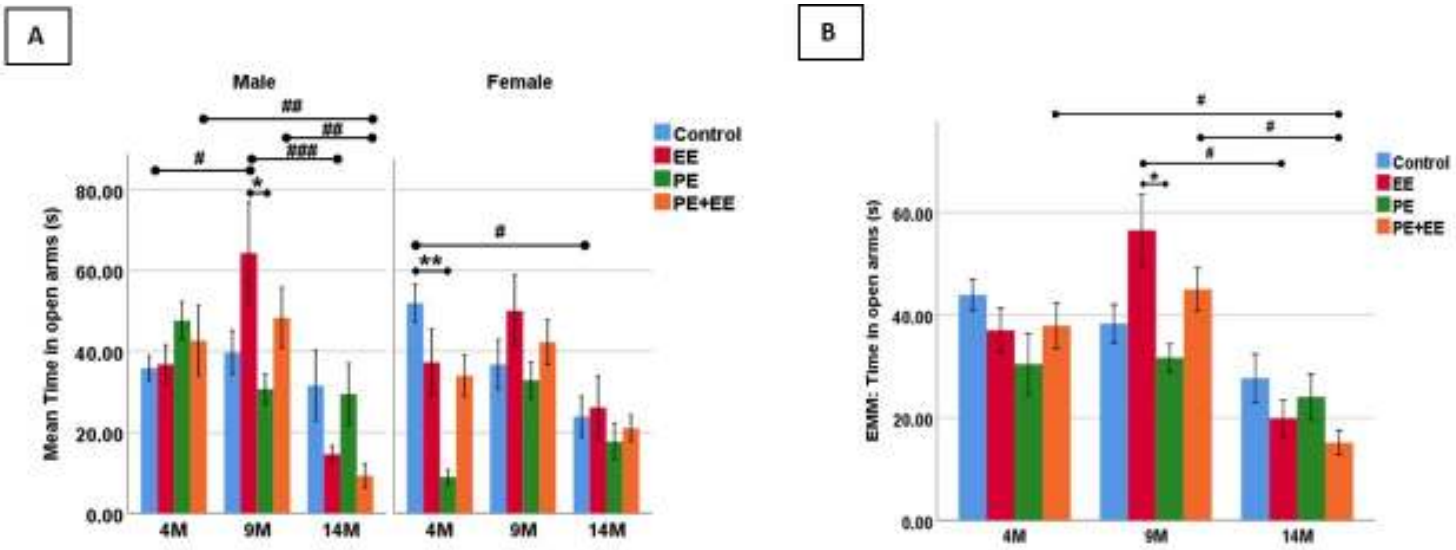

Fig. 3.5 Anxiety-like behavior in EZM: Time in open arms. (A) Sex comparisons, and (B) Comparisons with sex as a covariate. All mice were exposed to PE, EE, PE+EE or control conditions at 4, 9 and 14 months of age. (A) Three-way ANOVA, all data represented as Mean ± SE, n = 6-10 per group. (B) Two-way ANCOVA with sex as a covariate at the following value: 1.4904, data represented as estimated marginal means (EMM) ± 2 SE, n = 11-19 per group. * represents a significant difference between a treatment and age-matched control or between two treatments at one age point. # represents a significant difference between the matched treatments over two age points. *, # p < .05, **, ## p < .01, ### p < .001.

- **EZM: Number of Head dips**

The number of head dips along the open quadrants in EZM is a measure of anxiety-like behavior, with less anxious mice showing more number of head dips. Data for head dips are presented as a mean ± standard error unless stated otherwise. The three-way ANOVA showed a non-significant three-way interaction between age, treatment, and sex for head dips in the elevated zero maze ($F_{(6, 132)}$ = 1.830, p = 0.098). Two-way interactions between age and treatment for both males and females were also non-significant ($F_{(6, 132)}$ = 2.083, p = 0.059 and $F_{(6, 132)}$ = .332, p = 0.919). Hence, no corrections were made for sex differences. Follow up analysis with a two-way ANOVA showed that the main effect of interaction between age and treatments on the number of head dips in EZM was non-significant ($F_{(6,144)}$ = .453; p = .842), but the main effects of treatment and age were significant ($F_{(3,144)}$ = 5.155; p = .002 and $F_{(2,144)}$ = 7.367; p = .001; Fig 3.6). At 4 months, EE mice showed a significantly higher number of head dips than PE+EE mice (33.1 ± 2.6 vs. 19.8 ± 1.7; p = .0099). Also, the 4-month controls and EE treated mice showed a significantly higher number of head

dips than 14-month controls and EE treated mice respectively (25.2 ± 1.7 vs. 15.9 ± 3.4; p = .0429 and 33.1 ± 2.6 vs. 21.1 ± 4.4; p = .0143).

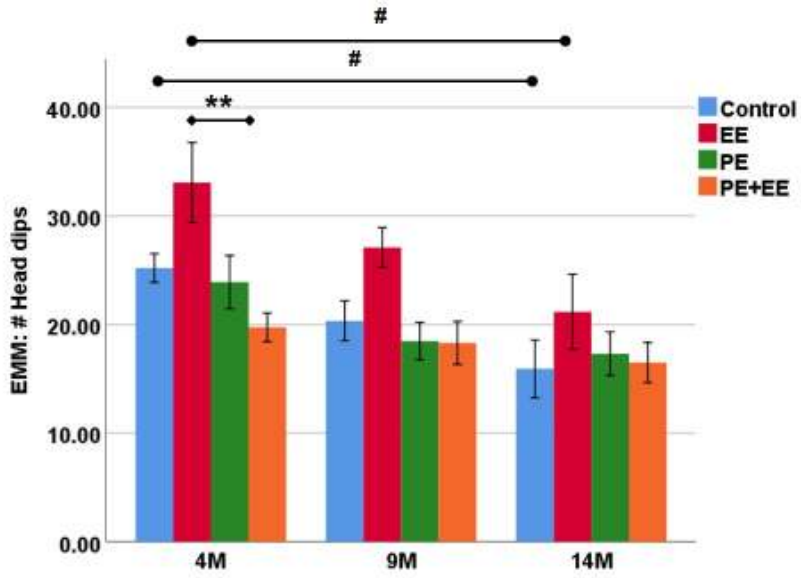

Fig. 3.6 Anxiety-like behavior in EZM: Number of head dips. All mice were exposed to PE, EE, PE+EE or control conditions at 4, 9 and 14 months of age. Two-way ANOVA, all data represented as estimated marginal means (EMM) ± 2 SE, n = 11-19 per group. * represents a significant difference between a treatment and age-matched control or between two treatments at one age point. # represents a significant difference between the matched treatments over two age points. # p < .05, ** p < .01.

- **Open Field: Time spent in the inner zone**

Time spent in the inner zone of the open field is a measure of anxiety-like behavior, with more anxious mice spending less time in the inner zone. Time was measured in seconds and is presented as mean ± standard error unless stated otherwise. The three-way ANOVA showed a non-significant three-way interaction between age, treatment and sex ($F_{(6, 134)} = 0.368$, p = .152). Similarly, the two-way interactions between age and treatment for both males and females were also non-significant ($F_{(6, 134)} = 1.195$, p = .313 and $F_{(6, 134)} = .202$, p = 0.976). Hence, no corrections were made for sex differences. Following this, a two-way ANOVA was utilized to examine the effects of age and treatments on time spent in the inner zone of open field and found that the main effects of interaction and treatment were non significant ($F_{(6,146)} = 1.048$; p = .397 and $F_{(3,146)} = 2.540$; p = .059), but the main effect of age was significant ($F_{(2,146)} = 8.805$; p < .001; Fig 3.7). Post hoc analysis using Tukey's multiple comparison test showed that 9-month-old control mice spent significantly more time in the inner zone than 4-month control mice (80.2 ± 16.2 vs. 57.9 ± 8.1; p = .0178). Similarly, 9-month PE mice showed significantly higher time in the inner zone than 4- and 14-month PE mice (127.9 ± 15.9 vs. 75.5 ± 15.5 and 76.0 ± 9.3; p = .0124 and .0124 respectively).

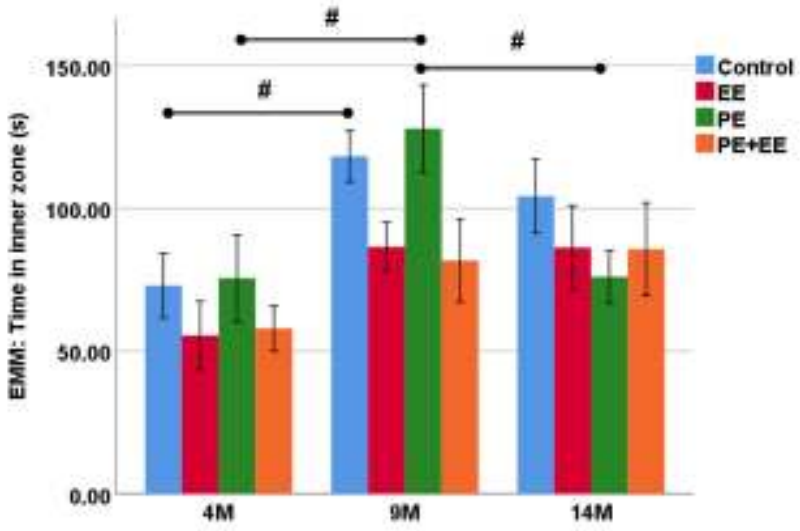

Fig. 3.7 Anxiety-like behavior in Open Field. Time in inner zone. All mice were exposed to PE, EE, PE+EE or control conditions at 4, 9 and 14 months of age. Two-way ANOVA, all data represented as estimated marginal means (EMM) ± 2 SE, n = 11-19 per group. # represents a significant difference between the matched treatments over two age points. # $p < .05$.

Depressive-like behavior

- **FST: Immobility time**

Mice exposed to PE, EE, PE+EE, or control conditions were tested for depressive-like behavior in the Forced Swim Test. Time was measured in seconds and is presented as a mean ± standard error unless stated otherwise. The three-way ANOVA showed a non-significant three-way interaction between age, treatment, and sex for immobility time in FST ($F_{(6, 131)} = 0.627$, $p = .708$). The two-way interactions between age and treatment for immobility time in FST were also non significant for both male and female mice ($F_{(6, 131)} = 1.005$, $p = .425$ and $F_{(6, 131)} = 0.658$, $p = .684$). As such, no corrections were made for sex. Further analysis with the two-way ANOVA to determine the effects of age and treatments on immobility time in FST showed non-significant main effects of interaction and age ($F_{(6, 143)} = 1.052$, $p = .395$ and $F_{(2, 143)} = 0.816$, $p = .444$), but the main effect of treatment was significant ($F_{(3,143)} = 5.673$; $p = .001$; Fig 3.8). Post hoc analysis using Tukey's multiple comparison test showed that at 4 months, PE mice were immobile for a significantly longer time than both controls and EE treated mice (82.6 ± 17.0 vs. 29.7 ± 6.9 and 22.1 ± 6.9; p = .0171 and .0171).

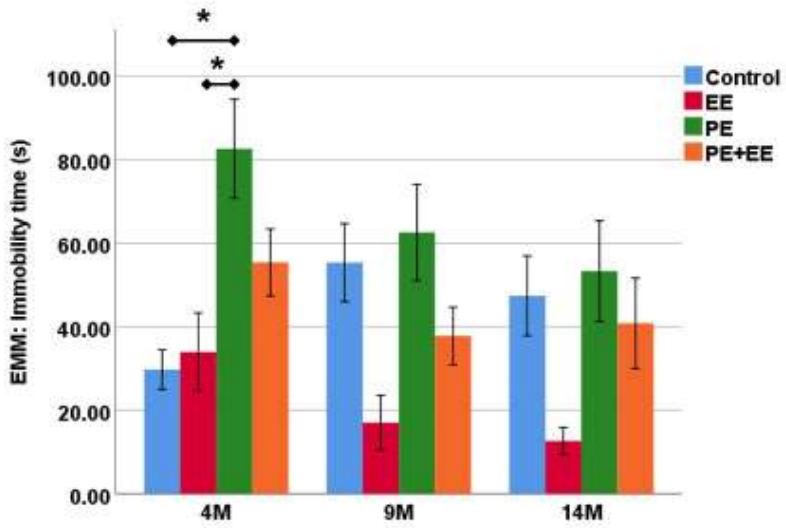

Fig. 3.8 Depressive-like behavior in FST. Immobility time. All mice were exposed to PE, EE, PE+EE or control conditions at 4, 9 and 14 months of age. Two-way ANOVA, all data represented as estimated marginal means (EMM) ± 2 SE, n – 12-14 per group. * represents a significant difference between a treatment and age-matched control or between two treatments at one age point. * $p < .05$.

Spatial learning and memory

- **Spatial learning over 4 days of training in Barnes maze**

Spatial learning at 4, 9 and 14 months in separate cohorts of mice exposed to PE, EE, PE+EE or control conditions was tested by measuring the latency to find the escape box and entry errors over 4 days of training in Barnes maze. Latency to escape was measured in seconds and is presented as a mean ± standard error unless stated otherwise. Data were analyzed using three-way ANOVA, which showed a three-way interaction between age, treatment, and sex for spatial learning over four days of training ($F_{(6,\ 536)} = 2.600$, $p = .017$). Post hoc analysis using Tukey's multiple comparison test showed significant differences between groups at 4 and 14 months for females and 14 months for males (Table 3.3, Fig 3.9).

Table 3.3 Multiple sex comparisons for latency to escape over four days of training in the Barnes maze

	Days	Tukey's multiple comparisons test	Mean comparison	adj. p
4M	**Day1**	PE Females vs. PE+EE Females	84.3 ± 14.6 vs. 31.9 ± 7.5	0.0408
	Day2	Control Female*s* vs. PE Females	29.0 ± 9.2 vs. 77.8 ± 12.0	0.0353
		PE Females vs. EE Females	77.8 ± 12.0 vs. 24.8 ± 9.9	0.0353
	Day3	Control Female*s* vs. PE Females	30.6 ± 9.7 ± 81.9 ± 16.1	0.0154
		PE Females vs. EE Females	81.9 ± 16.1 vs. 18.2 ± 4.0	0.0065
		PE Females vs. PE + EE Females	81.9 ± 16.1 vs. 21.7 ± 9.6	0.0099
	Day4	Control Female*s* vs. PE Females	20.0 ± 7.1 vs. 74.0 ± 17.1	0.0141
		PE Females vs. EE Females	74.0 ± 17.1 vs. 22.1 ± 5.4	0.0372
9M	No significant differences between any of the groups were noted.			
14M	**Day 3**	PE Males vs. EE Males	98.0 ± 19.7 vs. 29.1 ± 9.1	0.0014
		EE Males vs. PE+EE Males	29.1 ± 9.1 vs. 81.3 ± 21.3	.0.0335

		Control Females vs. PE Females	33.9 ± 9.5 vs. 91.6 ± 30.3	0.0151
		PE Females vs. EE Females	91.6 ± 30.3 vs. 40.6 ± 17.8	0.0341
		PE Females vs. PE+EE Females	91.6 ± 30.3 vs. 32.4 ± 12.8	0.0141
	Day 4	PE Males vs. EE Males	84.4 ± 21.8 vs. 33.3 ± 7.8	0.0351
		Control Females vs. PE Females	32.8 ± 15.5 vs. 85.6 ± 25.5	0.0325
		PE Females vs. EE Females	85.6 ± 25.5 vs. 28.2 ± 14.1	0.0189
		PE Females vs. PE+EE Females	85.6 ± 25.5 vs. 33.7 ± 8.2	0.0325

Legend: All data represented as mean ± SEM, n = 5-10 per group.

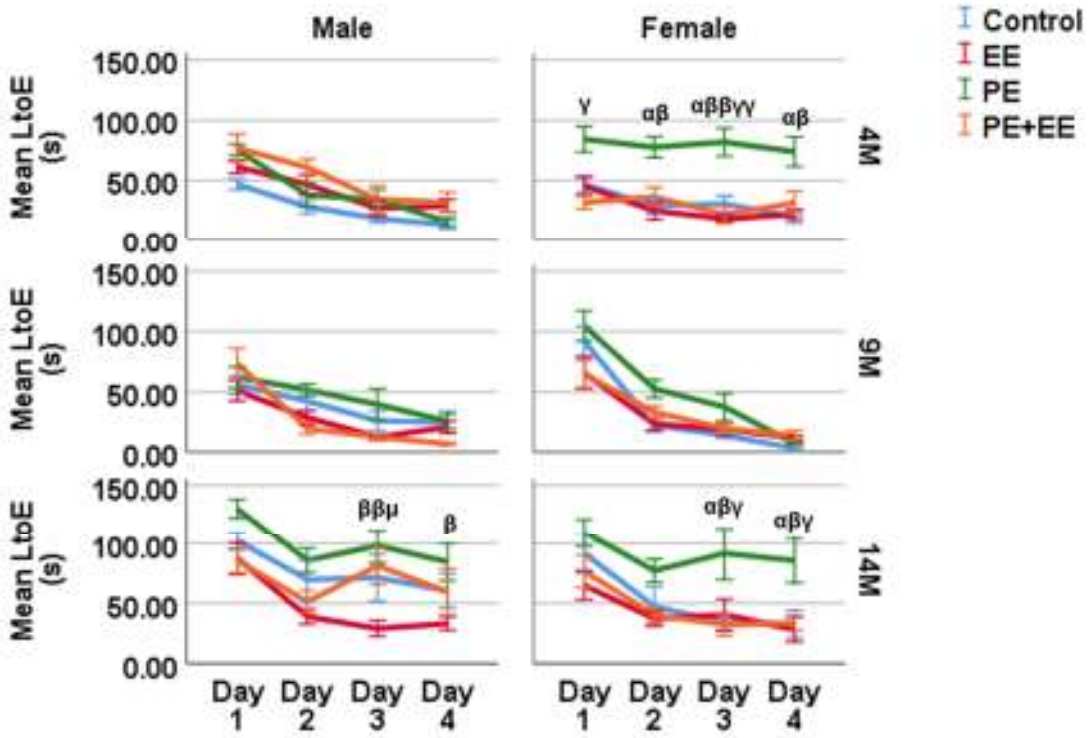

Fig. 3.9 Spatial learning in Barnes maze: Sex comparisons for latency to escape. Over four days of training. All mice were exposed to PE, EE, PE+EE or control conditions. Three-way ANOVA, all data represented as mean ± SE, n = 5-10 per group. α: Control vs. PE, β: PE vs. EE, γ: PE vs. PE+EE, μ: EE vs. PE+EE. α, β, γ, μ: $p < .05$, ββ, γγ: $p < .01$. **Legend:** LtoE: latency to find the escape box.

Three-way ANOVA also showed a significant three-way interaction between age, treatment, and sex for entry errors over 4 days of training in Barnes maze ($F_{(6, 536)} = 4.391$, $p < .001$). However, post hoc analysis with Tukey's multiple comparison tests showed that none of the male and female groups differed from controls on all 4 days of training, except for female 4-month PE+EE mice, which showed significantly lower entry errors than 4-month female control mice on day 3 (9.6 ± 2.8 vs. 33.4 ± 8.8; $p = .0149$). Also, 9- and 14-month male PE mice showed significantly higher entry errors than age-matched male PE+EE (43.7 ± 4.1 vs. 20.8 ± 4.7; $p = .0334$) mice on day 1 and EE mice on day 2 (49.0 ± 9.3 vs. 18.3 ± 5.5; $p = .0013$) respectively (Fig 3.10).

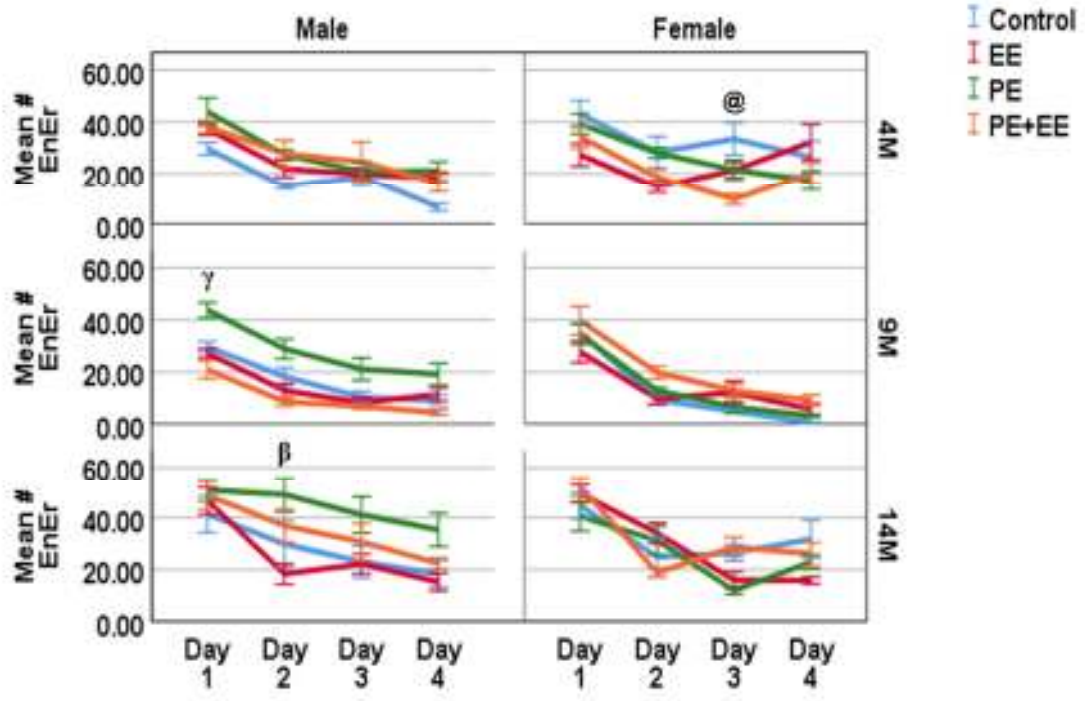

Fig. 3.10 Spatial learning in Barnes maze: Sex comparisons for entry errors. Over four days of training. All mice were exposed to PE, EE, PE+EE or control conditions. Three-way ANOVA, all data represented as mean ± SE, n = 5-10 per group. @: Control vs. PE+EE, β: PE vs. EE, γ: PE vs. PE+EE. @, β, γ: $p < .05$. **Legend:** EnEr: entry errors.

The sex differences for latency to escape and entry errors over 4 days of training in Barnes maze were controlled using a two-way ANCOVA. We observed that the two-way interaction was significant for latency to escape ($F_{(6, 583)} = 3.191$, $p = .004$), but non-significant for entry errors ($F_{(6, 583)} = .504$, $p = .805$). Post hoc analysis using Tukey's multiple comparisons for latency to escape showed significant differences between groups at 4 and 14 months (Table 3.4, Fig 3.11).

Table 3.4 Multiple comparisons with sex as a covariate for latency to escape over four days of training in the Barnes maze. Sex used as a covariate at the following value: 1.4937.

	Days	Tukey's multiple comparisons test	Mean comparison	adj. p
4M	**Day1**	Control vs. PE	46.8 ± 5.7 vs. 80.3 ± 8.2	0.0498
	Day3	Control vs. PE	28.6 ± 5.9 vs. 59.7 ± 10.4	0.0282
		PE vs. EE	59.7 ± 10.4 vs. 35.7 ± 8.0	0.0368
9M	No significant differences between any of the groups were noted.			
14M	**Day 1**	PE vs. EE	120.8 ± 9.7 vs. 82.3 ± 11.6	0.0255
		PE vs. PE+EE	120.8 ± 9.7 vs. 81.1 ± 11.2	0.0192
	Day 2	PE vs. EE	81.8 ± 9.5 vs. 41.0 ± 5.4	0.0182

		PE vs. PE+EE	81.8 ± 9.5 vs. 45.8 ± 7.5	0.0369
	Day 3	Control vs. PE	57.1 ± 15.3 vs. 95.0 ± 16.8	0.0234
		PE vs. EE	95.0 ± 16.8 vs. 37.0 ± 10.4	0.0002
		PE vs. PE+EE	95.0 ± 16.8 vs. 56.9 ± 14.0	0.0228
	Day 4	Control vs. PE	50.5 ± 13.2 vs. 85.0 ± 15.9	0.0490
		PE vs. EE	85.0 ± 15.9 vs. 31.4 ± 8.4	0.0006
		PE vs. PE+EE	85.0 ± 15.9 vs. 46.6 ± 13.7	0.0216

Legend: All data represented as mean ± SEM, n = 11-19 per group.

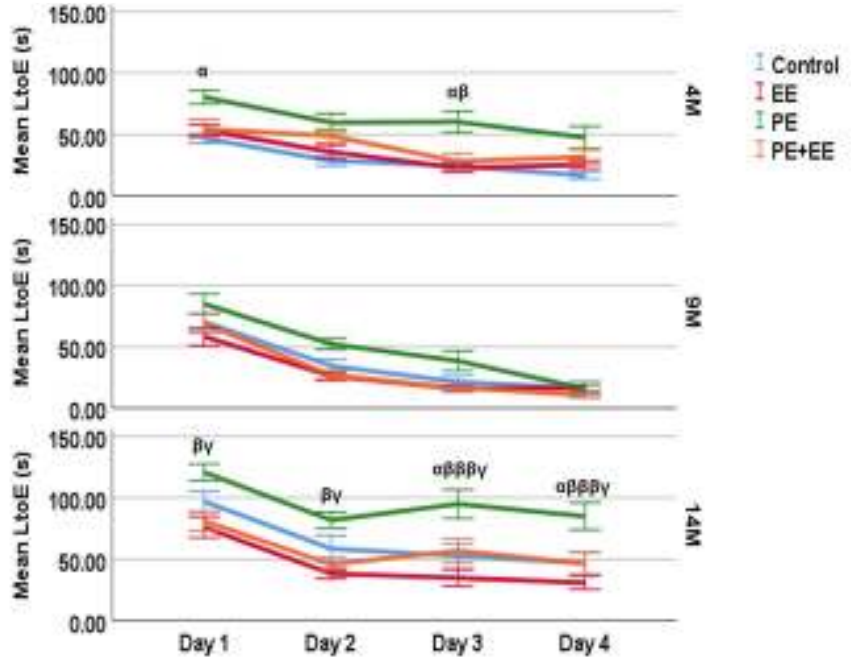

Fig. 3.11 Spatial learning in Barnes maze with sex as a covariate. Latency to escape over four days of training. All mice were exposed to PE, EE, PE+EE or control conditions. Sex was used as a covariate at the following value: 1.4937. Two-way ANOVA, all data represented as mean ± SE, n = 11-19 per group. α: Control vs. PE, β: PE vs. EE, γ: PE vs. PE+EE. α, β, γ: $p < .05$, βββ: $p < .001$. **Legend:** LtoE: latency to find the escape box.

- **Retention of spatial memory in Barnes maze probe trial on day 5**

After 4 days of training on Barnes maze, mice were tested for retention of spatial memory in a probe trial on day 5. A three-way ANOVA showed non-significant three-way interaction between age, treatment and sex for both, latency to escape ($F_{(6, 126)} = 0.270$, $p = .950$) and entry errors ($F_{(6, 134)} = 0.739$, $p = .619$). The two-way interactions between age and treatment for latency to escape and entry errors during the probe trial were also non significant for both male ($F_{(6, 126)} = 0.446$, $p = .846$ and $F_{(6, 134)} = 1.627$, $p = .144$) and female ($F_{(6, 126)} = 1.431$, $p = .208$ and $F_{(6, 134)} = 0.935$, $p = .472$) mice. Therefore, the data was analyzed again without controlling for sex differences using a two-way ANOVA.

No significant main effects of interaction and treatment were noted for escape latency, but the main effect of age was significant ($F_{(2,139)} = 23.11$; $p < .0001$; Fig 3.12 A). Post-

hoc analysis showed that at 14 months controls and PE mice had significantly higher latency to find the NW quadrant location of the escape box than EE mice (110.5 ± 21.6 and 107.5 ± 20.4 vs. 21.0 ± 8.3; p = .0391 and .0406). Similarly, 14 month control, PE and PE+EE mice showed significantly higher latency compared to 4 and 9 month control (110.5 ± 21.6 vs. 3.7 ± 0.7 and 4.1 ± 1.0; p = .0002 and < .0001), 9 month PE (107.5 ± 20.4 vs. 6.2 ± 1.0; p < .0001) and 9 month PE+EE (73.4 ± 21.1 vs 7.7 ± 1.4; p = .0140) mice respectively. Also, 4 months PE mice showed significantly higher latency to find the NW quadrant location of the escape box than 9 months PE mice (68.9 ± 24.3 vs. 6.2 ± 1.0; p = .0163).

Using two-way ANOVA, we also noted a significant main effect of age for entry errors ($F_{(2,146)}$ = 8.365; p = .0004; Fig 3.12 B). Again, this was because of the 14-month-old EE and PE mice showing significantly higher number of entry errors than 4- and 9-month EE (69.8 ± 10.6 vs. 40.8 ± 11.8 and 30.7 ± 7.3; p = .0396 and .0054) and PE (63.9 ± 13.8 vs. 26.5 ± 6.2 and 29.3 ± 5.5; p = .0085 and .0085) cohorts.

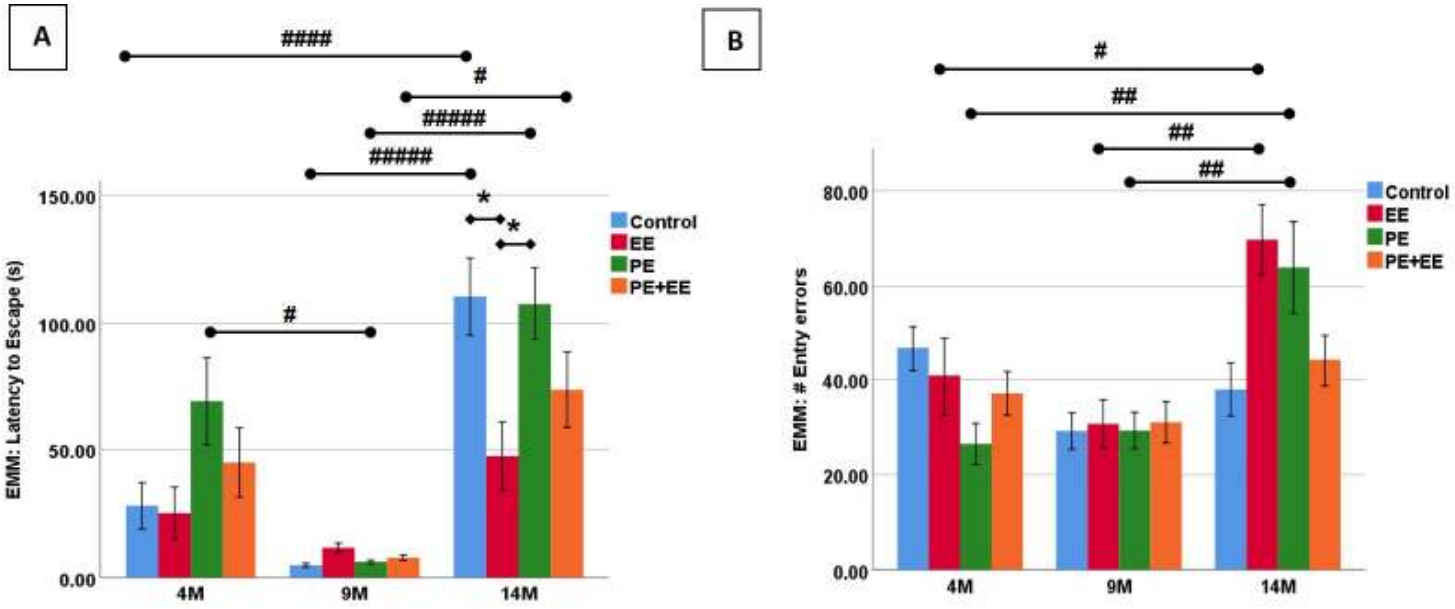

Fig. 3.12 Spatial memory in Barnes maze. (A) Latency to escape, and (B) Entry errors during probe trial. All mice were exposed to PE, EE, PE+EE or control conditions. Two-way ANOVA, all data represented as estimated marginal means (EMM) ± 2 SE, n = 11-19 per group. * represents a significant difference between a treatment and age-matched control or two different treatments at one age point. # represents a significant difference between the matched treatments over two age points. $^{*,\#}$ p < .05, $^{\#\#}$ p < .01, $^{\#\#\#}$ p < .001, $^{\#\#\#\#}$ p < .0001.

D. Molecular analyses

Changes in the expression of genes of interest in the hippocampus of C57BL/6 mice exposed to short-term PE, EE or PE+EE

The differential effects of PE, EE, and PE+EE on hippocampal gene expression were investigated with high-throughput qPCR (43 target genes, Table 2.3). No effects of any of the treatments on hippocampal gene expression, with an adjusted p-value <0.05 for significance, were observed at 4 and 14 months of age. However, at 9 months, several genes were differentially expressed amongst the groups. Three genes, i.e., *Creb1*, *Smad2,* and *Il1r1* differentially expressed during PE vs. control, PE

vs. EE, PE+EE vs. control, and PE+EE vs. EE comparisons. Similarly, genes *Sirt1* and *Il12a* were found to be differentially expressed on comparing PE with control, and PE with EE groups. The gene *Prkaa2* was found to be differentially regulated during PE vs. control, and PE vs. EE group comparisons. Other genes that were differentially expressed during single comparisons only were *Actb*, *Ifng*, *Htr1a*, *Tnfrsf1a,* and *Foxo3* (PE vs. control), and *Htr2a*, *Cs,* and *Prkaa1* (PE+EE vs. EE). None of the genes differentially expressed during the remaining two comparisons, i.e., EE vs. C and PE vs. PE+EE groups. The differentially expressed genes between the mice groups at 9 months of age are listed in Table 3.5.

Table 3.5 Differentially expressed genes at 9 months of age. Listed in order of increasing adjusted p values.

Groups compared	Gene	Std. Error	Z	p values	adjusted p	Direction of gene expression change
PE - C						
1	*Creb1*	0.191433	4.497535	6.87E-06	0.0005	↑
2	*Smad2*	0.1758727	3.857107	0.0001	0.0042	↑
3	*Il1r1*	0.4474172	3.496435	0.0005	0.0116	↑
4	*Sirt1*	0.1908514	3.328333	0.0009	0.0162	↑
5	*Il12a*	0.1965468	3.18867	0.0014	0.0212	↑
6	*Actb*	0.1791941	3.060445	0.0022	0.0273	↑
7	*Prkaa2*	0.2794528	3.006629	0.0026	0.0279	↑
8	*Ifng*	0.8951038	-2.940542	0.0033	0.0303	↓
9	*Htr1a*	0.500134	2.88788	0.0039	0.0319	↑
10	*Tnfrsf1a*	0.2850217	-2.829359	0.0047	0.0330	↓
11	*Foxo3*	0.6820933	2.813408	0.0049	0.0330	↑
PE+EE - C						
1	*Creb1*	0.1995607	6.09142	1.12E-09	8.28E-08	↑
2	*Il1r1*	0.4730396	4.058302	4.94E-05	0.0018	↑
3	*Smad2*	0.1874809	3.235324	0.0012	0.0225	↑
PE - EE						
1	*Creb1*	0.1995809	4.9507104	7.39E-07	0.0001	↑
2	*Smad2*	0.1836931	4.3623256	1.29E-05	0.0011	↑
3	*Sirt1*	0.1993378	3.4757951	0.0005	0.0162	↑
4	*Prkaa2*	0.2918789	3.3122057	0.0009	0.0257	↑
5	*Il12a*	0.2052864	3.2643984	0.0011	0.0287	↑
6	*Il1r1*	0.4669941	3.2230505	0.0013	0.0296	↑
PE+EE - EE						
1	*Creb1*	0.2073895	6.474284	9.53E-11	4.23E-08	↑
2	*Smad2*	0.1948359	3.7443337	0.0002	0.0081	↑
3	*Il1r1*	0.4915971	3.7846389	0.0002	0.0081	↑
4	*Htr2a*	0.3305805	3.5094451	0.0004	0.0161	↑
5	*Prkaa2*	0.3095843	3.1811221	0.0015	0.0310	↑
6	*Cs*	0.4501019	3.0650659	0.0022	0.0409	↑
7	*Prkaa1*	0.1962215	3.0301516	0.0024	0.0434	↑

Legend: C: control, PE: Physical exercise, EE: Environmental enrichment; data presented were analyzed with a linear mixed-effects model of the treatment groups.

Coefficients of the model fit and standard errors are detailed, with test statistics (Z-score) and adjusted p-value calculated after correcting for multiple tests.

Discussion

In our study, none of the treatments, i.e., PE, EE, and PE+EE, affected body weight significantly during experiments. Similarly, distance traveled on running wheel during the experiment was not significantly different between the PE and PE+EE mice at all three age points, i.e., early (4 months), middle (9 months) and late-middle (14 months) age. While short-term EE improved locomotor activity, reversed the age-related decline in spatial memory and reduced anxiety-like behavior, short-term PE had no effects on spatial memory and affective-like behaviors in middle and late-middle age mice. In early age mice, short-term PE reduced spatial learning and increased depressive-like behavior, particularly affecting female mice. These results on the effects of PE are interesting, knowing that short-term PE alone and in combination with EE modified the expression of many hippocampal genes of interest in middle-age mice. EE alone showed no effects on the expression of genes of interest in the hippocampus of mice at all ages. Main findings of our research are discussed below pointwise.

Short-term PE, EE, and combined PE and EE do not affect the mean body weight of C57BL/6 mice.

There is early evidence to suggest that PE reduces body weight by mobilizing fats, both within rodents [542-544] and humans [545, 546]. The lack of significant changes in body weight of both male and female mice within our study may be attributable to the short duration of the treatment protocols or the young baseline age of mice with minimal body fat, to begin with. Our reasoning is consistent with a previous study that reported a decrease in body weight only in response to continuous exercise and not intermittent exercise [545]. Similarly, EE of 6 weeks that included a running wheel in 10-week old mice resulted in no change in body weight [28]. We did not investigate the body composition of mice or food intake in the study and recommend this be investigated in similar future studies to get more clarity on the effects of external interventions on body weight. We also theorize that a lengthier exposure to voluntary PE on mice older than late-middle age may show observable changes in body weights.

EE improves baseline locomotion from middle age onwards. PE+EE, although beneficial at late-middle age, reduces locomotion under stress.

Short-term EE under non-threatening environmental conditions of the home cage induced increased locomotion at 9 and 14 months of age while PE+EE resulted in increased baseline locomotion at 14 months only. The effects of EE on baseline locomotion is primarily driven by a significant increase in the baseline locomotion of female mice and not male mice at 9 and 14 months. On the contrary, the effects of PE+EE at 14 months were driven by the enhanced baseline locomotion of male mice. Furthermore, 4-month-old female PE mice showed significantly reduced baseline locomotion, however, this seems to be compensated by the baseline locomotion of the 4-month male PE mice, thereby resulting in an overall non-significant difference between 4-month PE and age-matched control PE mice.

Under more threatening environmental conditions in the open field, PE+EE mice were less mobile at 14 months of age compared to controls. This may be due to the greater anxiety-inducing environment of the open field when compared with the home cage.

While not significantly different to age-matched controls, EE mice outperformed PE and PE+EE mice in exploring the open field at 9 months of age. We observed no sex differences in open field locomotor activity. Our results for locomotor activity comply with the previously published findings. For example, voluntary wheel running of 6 weeks reduced the locomotor activity of 8-weeks-old spontaneously hypertensive male rats in the Open Field test [534]. EE without running wheel for 39 days has been shown to enhance locomotion in young male Sprague-Dawley rats in the Open Field test [384]. Similarly, EE without running wheel for 11 weeks enhanced play behavior in home conditions in newly weaned male mice [547]. Overall, our result suggests that EE primarily enhances the locomotor activity of female C57BL/6 mice, but the effects of PE+EE on locomotor activity depends on the presence or absence of stressful conditions. These results contrast with the previously reported findings in male rodents; however, this can be attributed to the effect of different rodent species and strain used [385]. Further research into differentiating the effects of PE, EE, and PE+EE on locomotion after a longer duration of treatment in C57BL/6 mice is required.

EE, PE, and PE+EE did not affect anxiety-like behavior compared to age-matched controls at all ages but had an effect across the lifespan.

While none of the treatments were different to controls at all ages for anxiety-like behavior, we found age effects within treatments. Short-term EE and PE mice appeared to exhibit less anxiety-like behavior at 9 months compared to their 4- and 14-months cohorts as evident by significantly more time spent in the open quadrants of EZM and inner zone of Open Field respectively. However, sex influenced anxiety-like behavior. We found that EE mice were different due to the significantly less anxiety-like behavior of 9-month male EE mice compared to 4- and 14-months male EE mice. Similarly, PE was found to be anxiogenic for females compared to age-matched controls at 4 months. Indeed, PE, when added to EE at 4 months, increased anxiety, as evident by significantly fewer head dips by 4-month PE+EE mice compared to 4-month EE mice in the EZM. Furthermore, the observed significant higher anxiety-like behavior of 14-month PE+EE mice compared to 9-month counterparts, as evident by differences between the treatment matched age cohorts for time in open arms of EZM, was primarily due to higher anxiety-like behavior in male PE+EE mice. The 14-month controls and EE mice showed significantly less head dips in the EZM than their cohorts at 4 months, suggesting significantly higher anxiety-like behavior at late-middle age compared to early age cohorts in untreated and EE mice during our experiments. Together, our findings point towards EE being anxiolytic at young and middle age, PE being anxiogenic for females at young age and EE when combined with PE may induce anxiety at late-middle age in male C57BL/6 mice.

PE and EE, as well as the combination of PE and EE, are established anxiolytic interventions [28, 323, 548-550]. Indeed, both PE and EE seem to positively affect the ventral hippocampus, a region involved in anxiety response [550, 551]. Short-term PE+EE has shown to reduce anxiety-like behavior at a young age in female transgenic models of AD [550, 551], male C3H/eB mice [28], as well as in healthy male Wistar rats [323]. This contradicts our above-reported findings. Similarly, our results also challenge the previously reported findings on PE. For example, voluntary wheel running of 4 weeks when provided to 12-14 weeks old single house C57BL/6 mice reduced anxiety-like behavior and impulsiveness [524]. Similarly, 8 weeks old male C57BL/6 mice, which underwent voluntary wheel running of 2 weeks showed reduced anxiety-like behavior [526]. The discrepancy in our findings for PE and PE+EE effects

at a young age could be attributed to differences in cages, running wheel dimensions, or handling time during the experiment and may require further validation. However, there are also evidence suggesting that physical exercise is anxiogenic in young male C57BL/6 mice [552] and have no effects on anxiety-like behavior in young male Sprague–Dawley rats [62]. In the former study, however, mice were single-housed, and social isolation is an established etiology for the onset of psychological stress and subsequent anxiety-like behavior in rodents [541].

Short-term PE induces depressive-like behavior at an early age.
EE mice showed the least depressive-like behavior among all groups at 4, 9, and 14 months of age, although results were not significant when compared to controls. EE has been shown to attenuate depressive-like behavior associated with prenatal chronic stress [377] but not with early life stress [376]. We house mice in controlled environmental conditions where chances of stress were minimal. Hence, it is possible that the non-significant results for EE and PE+EE in FST could be due to the short duration of the treatment, and hence longer treatment with EE could be more effective in the treatment of depressive-like behavior. Like EE, PE+EE mice were not significantly different to control mice for depressive-like behavior at all ages. PE, however, acted as an inducer of depressive-like behavior at 4 months of age, as evidenced by significantly higher immobility time of 4-month PE mice compared to both 4-month-old controls and EE mice in the FST. However, research has shown that moderate PE alleviates depressive-like behavior in rodents [402, 525, 553]. Indeed, our finding contradicts another similar research where voluntary wheel running of 3-4 weeks produced an antidepressant-like behavioral response in the FST in 8-10 weeks old wild-type mice [525]. No sex differences were observed for depressive-like behavior. The longer immobility time in FST could represent a faster adaptation of young PE mice to an acute stressor, thereby switching from active to passive behavior to conserve energy for better survival [554]. However, in the absence of similar adaptation by PE mice at middle and late-middle age, the longer immobility time of young PE mice in FST during our experiments is indicative of depressive-like behavior and not coping strategy, since we find no reason for PE mice losing their capability to adapt to an acute stressor after young age. The discrepancy in our findings for PE in FST from past studies could be due to differences in cages, running wheel dimensions, or handling time during the experiment and may require further validation.

Short-term PE adversely affects spatial learning of female mice at early and late-middle age. Short-term EE improves retention of spatial learning at late-middle age.
The observed impaired spatial learning in 4- and 14-month-old PE mice was primarily driven by 4- and 14-month female PE, showing significantly higher latency to escape than age-matched female controls. At 14 months, PE mice also showed significantly lower spatial learning than EE and PE+EE mice, with contribution from both PE sexes but more significantly from female PE mice. The analysis of entry errors also shows a trend for male and female PE mice low on spatial learning. Overall, this suggests that PE at a young age and late-middle age may affect spatial learning of female C57BL/6 mice adversely.

Furthermore, analyzing the escape latency of controls in the probe trial of the Barnes maze revealed that while retention of spatial learning improved from 4 to 9 month, it deteriorated after that. Interestingly, EE reversed the age-related decline in spatial

memory at 14 months, but without any significant sex differences. EE mice, however, also showed a significantly higher number of entry errors in locating the escape box at 14 months compared to 4- and 9-month EE mice. This low escape latency with a high number of entry errors could indicate higher exploration activity and enhanced cognition in searching the escape box with visual input. These findings on EE that we report comply with the previously published literature. For example, short-term EE without running wheels has been shown to improve spatial memory of the young male Wistar and Sprague-Dawley rats [381, 383]. Similarly, short-term EE with running wheel has been shown to improve spatial memory by promoting hippocampal neurogenesis, dendritic arborization, and spine density in the brain of Wistar rats [374, 375]. There is also evidence suggesting that EE from weaning to 4 months of age in rats accelerate the acquisition of spatial information in naïve male Wistar rats [555]. Since spatial memory is the function of the dorsal hippocampus [323, 556], it seems that there is a substantial improvement in the integrity of dorsal hippocampus after EE.

Unlike EE, PE showed detrimental effects on the spatial learning and retention of spatial memory on a Barnes maze test. However, PE has widely been accepted as a modulator of brain functions and shown to improve spatial memory in young, middle age and old age male rodents [31, 517, 519-521, 557-559]. Notably, all of these studies except for two utilized only male animals for research. Among the two, one balanced research for gender [521] and the other used only female animals [519] but both reported improvement in the spatial memory after PE. Lack of research on female mice in past studies may be a reason for the discrepancy that we report for spatial learning and memory after PE in this study. The species and strain of rodents could also have a significant influence on the rate of spatial memory acquisition. Moreover, PE in short-term may have served as a stressor at an early age for female C57BL/6 mice. At late-middle age, the adverse effects of PE were seen on both male and female mice, which could be attributed to the altered physiological response of the body to acute physical strain after middle age. This hypothesis, however, needs validation through future research. Other factors, such as the design of our study, cage dimensions, the presence of one wheel per cage, handling frequency, and mouse-mouse interactions, may have played a role as well.

PE, PE+EE elicit differential gene expression at nine months, but EE has no effect on gene expression at all ages.

There is evidence to suggest that the change in behavior and memory is a measure of hippocampal integrity [322-326]. Both PE and EE have a considerable influence on the expression of hippocampal genes associated with immune functions, aminergic pathways, neurotrophin metabolism, HPA axis, transcription factors, and mitochondrial function, and this can manifest into known anti-inflammatory, cell-proliferative and neuroprotective effects of the two treatments [327, 358-360, 362-366, 383, 386, 450, 451]. In this context, we analyzed the differential expression of hippocampal genes of interest in response to short-term PE, EE, and combined PE and EE. We found no significant differential expression after all treatments at 4 and 14 months of age, however, at 9 months, a set of genes expressed differentially after PE and PE+EE treatments, whereas EE showed no effect.

The absence of treatment effects on the expression of hippocampal genes during early and late-middle age was unexpected. However, this could be attributed to genes being more responsive to the external environment during middle age than young and late-

middle age. In middle age mice, we observed 11, 3, 6, and 7 differentially expressed genes during PE vs. controls, PE+EE vs. controls, PE vs. EE and PE+EE vs. EE comparisons, respectively. None of the genes differentially expressed during EE vs. C and PE vs. PE+EE comparisons (see Table 5 for the list of differentially expressed genes between the mice groups at nine months of age). Surprisingly, the differential expression of hippocampal genes after PE at 9 months did not manifest into significant behavioral changes. Mice were tested on a behavioral battery immediately after 4-weeks of treatments. It is possible that the effects of differentially expressed genes on behavior could have appeared at a later time point. Moreover, PE of longer duration may be required for observable behavioral changes that could be correlated to differentially expressed genes. Also, the genes that differentially expressed after PE at 9 months elicit neuroprotective effects, which could have allowed 9-month old PE mice to adapt to behavior similar to the age-matched control mice. All these hypotheses need to be tested for validity in future research.

We performed pathway analysis for the differentially expressed genes using GeneMANIA to understand the molecular effects PE might have had on mice. Genes that were differentially expressed during simple comparisons between groups were mapped in GeneMANIA (Fig 3.13) and molecular processes statistically significant at FDR < 0.01 were noted (Table 3.6).

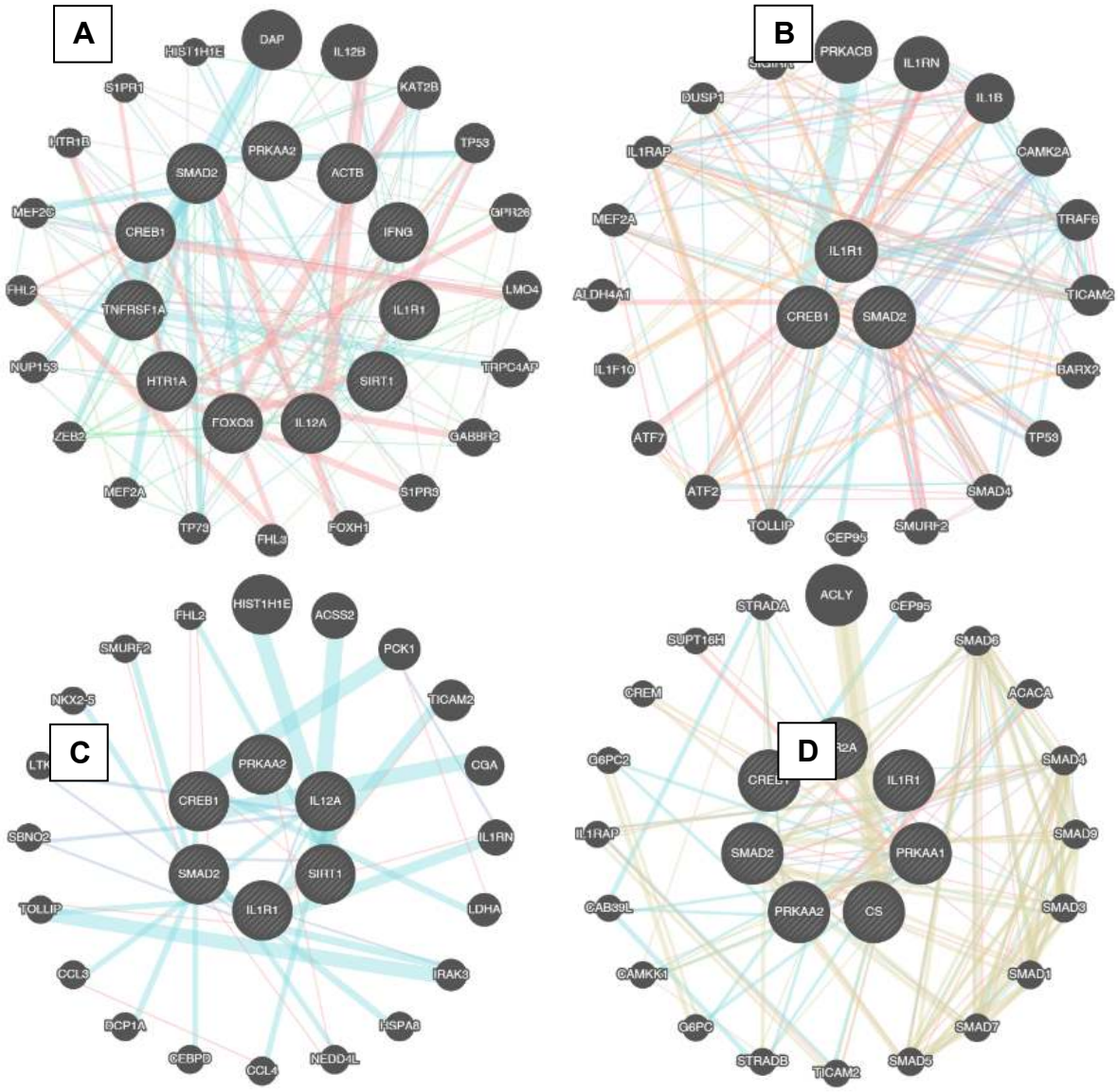

Fig. 3.13 Gene interaction and function prediction network obtained using GeneMANIA for (A) PE vs. C, (B) PE+EE vs. C, (C) PE vs. EE and (D) PE+EE vs. EE. The colors represent significantly modulated molecular pathways.

Table 3.6 Differentially expressed genes and the associated statistically significant key molecular processes as observed during group comparisons.

Groups compared	Differentially expressed genes	Total links with the 20 most related genes	Statistically significant key molecular processes (FDR < 0.01)
PE vs. C	9 genes upregulated: *Creb1*, *Smad2*, *Il1r1*, *Sirt1*, *Il12a*, *Actb*, *Prkaa2*, *Htr1a* and *Foxo3.* 2 genes down-regulated: *Ifng* and *Tnfrsf1a*	140	Cell-type-specific apoptotic processes, G-protein coupled receptor activity, cellular response to external stimuli and regulation of innate, and adaptive immune response.
PE+EE vs. C	3 genes upregulated: *Creb1*, *Il1r1*, *Smad2*	213	Immune and transcription pathways, such as pattern recognition receptors signaling, transcription factor binding, and regulation of innate immune response.

PE vs. EE	6 genes upregulated: *Creb1*, *Smad2*, *Sirt1*, *Prkaa2*, *Il12a*, *Il1r1*	39	IL-1 mediated signaling, cell apoptotic process, regulation of transforming growth factor-beta (TGFβ) receptor signaling, and cytokine receptor binding.
PE+EE vs. EE	7 genes upregulated: *Creb1, Smad2, Il1r1, Htr2a, Prkaa1, Prkaa2, Cs*	165	SMAD protein complex assembly and binding, regulation of TGFβ receptor signaling, and positive regulation of cell morphogenesis and differentiation.
EE vs. C	None of the genes differentially expressed.		
PE+EE vs. PE	None of the genes differentially expressed.		

Three of the genes, i.e., *Creb1*, *Smad2,* and *Il1r1* were upregulated in all groups with PE when compared to groups without PE (Table 3.6), suggestive of the effects of PE and not EE on these genes. *Creb1* encodes for the transcription factor CREB (cyclic adenosine monophosphate response element-binding protein), which is involved in the regulation of genes important for neuronal regulation and survival of proteins such as brain-derived neurotrophic factor. The expression of *Creb1* has been reported to increase in the hippocampus, temporal cortex, and nucleus accumbens of animal models of major depressive disorder and related disorders after chronic treatment with antidepressant drugs [493, 560, 561]. *Smad2* mediates TGF-β signals controlling a wide variety of cellular processes, including cell differentiation, proliferation, apoptosis, developmental fate specification, and differentiation of hippocampal granule neurons [505-507]. Similarly, IL-1R1 signaling mediates innate and acquired immune responses [502], associated with protection or aggravation of the disease states in the CNS, infections, and cancers [503, 562]. Knocking out these genes have shown to result in apoptotic cell death and affects brain functions and neurobiology adversely [505, 563, 564]. The upregulation of these three genes in all groups after PE at nine months suggests the neuroprotective role of PE, which has also been reported earlier [565].

Two of the genes, i.e., *Sirt1* and *Il12a,* were upregulated in PE mice compared to control and EE mice. These genes were not differentially expressed in PE+EE mice. This suggests that the addition of EE to PE reduces the expression of these two genes. *Sirt1* gene is a mitochondrial gene that encodes for SIRT1 protein, which influences energy metabolism and is important for neuronal plasticity, cognitive functions, as well as protection against aging-associated neuronal degeneration and cognitive decline [500, 501]. PE has been shown to enhance *Sirt1* activity [451]. *Il12a* gene encodes cytokine IL 12a, which act as a growth factor for activated T and NK cells, enhance the lytic activity of NK cells, and stimulate the production of IFN-γ by resting peripheral blood mononuclear cells. Upregulation of these two genes after PE is again suggestive of the neuroprotective role of PE at nine months of age.

Other genes that were upregulated in response to PE or PE+EE when compared to control or EE mice are *Foxo3*, Cs, *Actb*, *Prkaa1*, *Prkaa2*, *Htr1a,* and *Htr2a*. *Foxo3* activates genes that preserve cellular quiescence, prevents premature differentiation, and controls oxygen metabolism during aging [566]. *Foxo3* maintains the neural stem cells (NSC) pool by preventing the premature proliferation of progenitors and preserving the relative quiescence of NSC. Indeed *Foxo3* KO mice were found to have fewer NSC in vivo than wild-type counterparts [567]. Interestingly, a study has shown

that *Foxo3* deficient mice are significantly less immobile in FST [568], suggesting that *Foxo3* regulates the behavioral manifestation of depression. A review validated this further by reporting that Forkhead box O transcription factor influences the development of major depressive disorder by regulating neuronal morphology, synaptogenesis, and adult hippocampal neurogenesis [569]. We, however, observed no significant change in depressive-like behavior after PE at middle age. This is because none of the groups, including controls, showed the development of depressive-like behavior at middle age during our experiments.

Mitochondrial genes Cs, *Actb*, *Prkaa1,* and *Prkaa2* were also upregulated in the hippocampus of 9-month-old PE and PE+EE mice when compared to control and EE mice. A study has indeed shown that PE stimulates the expression of mitochondrial genes in the hippocampus [327], thus suggesting that there can be a possible link between mitochondrial function and behavioral changes. Moreover, during aging, there is an increase in dysfunctional brain mitochondria, increased oxidation products, and diminished mitochondrial functional activity, resulting in long-term behavioral changes. Moderate exercise has been reported to reverse mitochondrial dysfunction and lower content of oxidation products and improve related neurological performances [570]. Cs encode citrate synthase, an enzyme of the tricarboxylic acid cycle that plays a key role in regulating energy generation of mitochondrial respiration. *Actb* encodes for protein beta (β)-actin that forms the structural framework inside developing neurons. PE has been previously reported to upregulate the *Cs [571]* and *Actb* expression [572]. *Prkaa*1 and *Prkaa*2 genes encode proteins that are the catalytic subunit of the AMP-activated protein kinase (AMPK). AMPK maintains energy homeostasis within cells. Enhanced expression of *Prkaa*1 and *Prkaa* 2 gene has been reported after short-term PE in mice [573]. Together, the upregulation of these mitochondrial genes after PE suggests that short-term PE play a key role in cellular energy metabolism and neurogenesis during middle age.

The upregulated *Htr1a* and *Htr2a* genes encode serotonin 1A and 1B receptors which mediate inhibitory neurotransmission. Inactivation of *Htr1a* and *Htr2a* genes in mice results in behavior consistent with an increased anxiety and stress response. For example, allelic variation in 5-HT1A and 5-HT2A receptor expression has been shown to be associated with anxiety- and depression-related personality traits [499]. Studies have shown that PE alters levels of serotonin in the brain of rodents [574]. Upregulation of *Htr1a* and *Htr2a* genes, therefore, suggests improved serotonin neurotransmission in the hippocampus after short-term PE at middle age.

The downregulated immune genes Ifng and *Tnfrsf1a* encode IFN-γ and members of the TNF receptor family of membrane-bound and soluble proteins that interact with the ligand TNF-α. Both IFN-γ and TNF-α regulate complex injury promoting proinflammatory and apoptotic and protective cell proliferation and survival roles in the CNS depending on dose, disease phase, and cell development stage [447, 514]. Moreover, evidence suggests that IFN-γ is involved in modulation of CNS plasticity and the activity of related brain monoamine systems, thereby also regulates memory function and anxiety and depressive states [575, 576]. Similarly, TNF-α has been shown to play a role in the pathophysiology of affective-like behavior [448, 515]. The downregulation of Ifng and *Tnfrsf1a* again suggests that PE at nine months is neuroprotective.

Given the effects of PE on gene expression, as mentioned above, the observed changes in gene expression appear primarily due to the effects of PE and not EE during PE+EE vs. C comparison. It is, however, interesting to find other genes, for example, *Prkaa2*, *Ifng,* and *Htr1a* that were differentially expressed after PE lost significance after PE+EE. Also, finding the same genes being upregulated in 9-month PE mice when compared to age-matched controls and EE mice suggests that the EE might have no or minimal effects on the hippocampal gene expression.

A short note on the effects of short-term EE on the hippocampal gene expression

EE did not alter the expression of any GOI. This contrasts with previously published literature. For example, a study reported that EE upregulates the *Htr5B* gene expression and reverses the depression-like state caused by early life seizure experiences [355]. Likewise, EE in the absence of exercise showed anxiolytic effects in 5-HT1AR KO mice [356]. EE has also been shown to modulate the expression of 5-HT receptors in the hippocampus of R6/1 transgenic mice enriched for four weeks, from 8 to 12 weeks of age [577]. However, the authors also noted gender effects, and the *Htr2a* gene differentially expressed only in females. Hence, the observed change in the behavior despite no GOI being expressed differentially after EE could be attributed to gender effects that we could not measure due to low sample size for gene expression analysis. While our behavioral results suggest that EE elicits age-dependent effects on anxiety-like behavior and could be anti-depressive in long-term, longer exposure of EE may be required to elicit significant changes in hippocampal gene expression. We also propose that study confounders such as the age of mice, and differences in the delivery of enrichment such as box dimensions, number/type of toys and how frequently they were changed may have a role to play for the reported change in behavior. Since aging is a known factor for the differential expression of genes in the hippocampus, it is possible that the effects of short-term EE on hippocampal gene expression could be more significant in mice older than late-middle age. Also, we analyzed 43 genes only, which leaves scope for future studies to investigate if other genes are responsible for the change in behavior after EE that we have reported in this study.

Past studies that have reported gene modulation effects of EE also used running wheels to enrich the environment of rodents [58, 69]. Moreover, the absence of EE effects on hippocampal gene expression could be due to factors such as differences in the delivery of enrichment such as box dimensions, number/type of toys (i.e., quantity, quality and complexity of EE) and how frequently they were changed (i.e., the novelty of EE). A study indeed reported that EE, in the absence of running wheels, has limited transcriptomic effects genome-wide [68]. More research with long-term EE using a wide variety of objects may be required to understand the effects of EE on hippocampal gene expression fully. Also, if the inclusion of running wheels affects gene expression results in EE studies needs to be investigated further, especially when the presence of running wheel alone has been shown to modulate hippocampal gene expression in C57BL/6 mice [72, 73].

Research Summary

In this chapter, we investigated the effects of PE, EE and the combination of PE and EE on brain functions and changes in the expression of 43 hippocampal genes of interest over the life span of C57BL/6 mice. The mice were housed in controlled environmental conditions and once of the desired age, i.e., 3, 8 and 13 months, they were provided with 4 weeks of PE, EE or the combination of PE and EE. At the end of short-term treatments, mice were tested for various behaviors using a standardized behavioral battery. Following this, the mice were sacrificed, their brains were extracted, dissected and the hippocampal tissues were collected for the analysis of changes in hippocampal gene expression using high-throughput qPCR.

The results from the research suggest that short-term EE can help to alleviate age-induced impairment in brain functions after middle age. Interestingly, short-term PE can induce depression at an early age and may not be an effective modulator of brain functions until late middle age. This was despite PE and PE+EE, and not EE, modulating the expression of hippocampal genes at middle age.

Overall, our results are important since PE has been considered beneficial at all ages. However, we have shown that the short-term bout of PE may be detrimental at an early age. Perhaps continuous PE at an early age may be beneficial, and this could be tested in future research. On the contrary, short-term EE, although showed no effects on hippocampal gene expression, could be beneficial to alleviate age-related brain disorders. The rationale that we could provide for our results is that while both PE and EE enhance hippocampal neurogenesis, they do it via dissociable pathways. EE increases the likelihood of survival of new cells and PE increases the level of proliferation of progenitor cells. Future research can validate our reasoning.

The research we conducted here is one of its kind where we investigated the differential effects of PE, EE and their combination on behavior, memory and hippocampal gene expression over the lifespan. Further research with a longer duration of EE and PE and their combination would be useful.

Chapter 4: Short-term environmental enrichment is a stronger modulator of brain glial cells and cervical lymph node T cell subtypes than exercise or combined exercise and enrichment

Statement of Authorship

Title of Paper	Short-term environmental enrichment is a stronger modulator of brain glial cells and cervical lymph node T cell subtypes than exercise or combined exercise and enrichment
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Principal author

Name of Principal Author (Candidate)	Gaurav Singhal		
Contribution to the Paper	Planning and conducting experiments, behavioral and molecular analysis, data collection, organization, analysis and interpretation, writing and editing manuscript.		
Overall percentage (%)	80%		
Certification:	In this paper, the original research that I have conducted during the period of my Higher Degree by Research candidature has been reported. It is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.		
Signature		Date	24/05/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

1. the candidate's stated contribution to the publication is accurate (as detailed above);
2. permission is granted for the candidate in include the publication in the thesis; and
3. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution

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Contribution to the Paper	Assistance with planning and conducting mouse husbandry and laboratory techniques throughout the research period.		
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Preamble to chapter 4

In chapter 1, with the help of available evidence we mentioned that both PE and EE modulate neuroimmune functions, which could be responsible for the observable changes in brain functions. In chapter 2, we were able to show that microglia and $CD8^+$ T memory cell counts increase towards late-middle age. Depending on the phenotype, both microglia and T cells can elicit either neuroprotective or neuroinflammatory effects. Hence, the changes in brain glial cells count or peripheral T cell count, and phenotype could be the underlying factors for the findings that we reported in chapter 3, i.e., the effects of PE, EE and the combination of PE and EE on locomotion, behavior, and memory. Hence, deducing the differential effects of PE and EE on brain glial cells and peripheral T cells could provide an explanation for the observed functional changes. It may also help in formulating non-pharmacological techniques to improve neuroimmune homeostasis during old age via the modulation of glial cells and T cell expression.

Hence, during this study, we investigated the change in neuroimmune functions in C57BL/6 mice that underwent behavioral testing in chapter 3. For this, we sacrificed all the mice after the behavioral testing and utilized immunohistochemistry to analyze the changes in immunopositive microglia and astrocytes count in the dentate gyrus, and fluorescent activated cell sorting to analyze the changes in the total number of T cells, $CD4^+$ and $CD8^+$ T cell subpopulations (Naïve or T_N, Central memory or T_{CM} and Effector memory or T_{EM}), and early activated T cell phenotype CD25 in the cervical lymph nodes. During this research, the following two questions were addressed.

1. Does short-term PE and EE differ in effects that they have on brain immunopositive glial cells count, and T cells count and phenotype in the cervical lymph nodes during normal aging?
2. Does the combination of short-term PE and EE elicit greater effects than individual effects of short-term PE and EE on brain immunopositive glial cells count, and T cells count and phenotype count in the cervical lymph nodes during normal aging?

We hypothesized that:

1. Short-term PE and EE of 4 weeks will reverse the aging-associated impairment in immunopositive glial cells count in the brain, and T cells count and phenotype in the cervical lymph nodes in early (4 months), middle (9 months) and late-middle age (14 months) male and female C57BL/6 mice compared to age-matched control mice.
2. The combination of short-term PE and EE will have greater beneficial effects than PE and EE alone on aging-associated impairment in immunopositive glial cells count in the brain, and T cells count and phenotype count in the cervical lymph nodes in early (4 months), middle (9 months) and late-middle age (14 months) male and female C57BL/6 mice compared to age-matched control mice.

Following aim was set forth before the research commenced:

1. To investigate the effects of short-term PE, EE and the combination of PE and EE on immunopositive glial cells count in the brain, and T cells and phenotype count in the cervical lymph nodes in early (4 months), middle (9 months) and late-middle age (14 months) male and female C57BL/6 mice.

Mice were tested for change in glial cells count in the dentate gyrus, and total T cells and $CD4^+$ and $CD8^+$ T cell subpopulations count in the cervical lymph nodes using immunohistochemistry and fluorescent activated cell sorting laboratory techniques, respectively. All findings are reported and discussed systematically. The included chapter is the same as the manuscript accepted for publication in the journal Cellular and Molecular Neurobiology.

Short-term environmental enrichment is a stronger modulator of brain glial cells and cervical lymph node T cell subtypes than exercise or combined exercise and enrichment

Introduction

The detrimental effects of aging on the neuroimmune system are well documented [298, 578]. During normal aging, the immune system becomes increasingly sensitized to both intrinsic and extrinsic pathogenic factors and shows a constant functional decline, impacting health and survival. Studies have shown that microglia and astrocytes, which are essential for the innate neuroimmune and tissue repair processes and the maintenance of neurobiological homeostasis [579, 580], play a role in the onset of inflammatory and degenerative changes in the brain during aging [124, 125]. The glial cells of the brain become increasingly dysfunctional, lose neuroprotective properties, and secrete significantly higher levels of pro-inflammatory cytokines (e.g., TNF-α, IL-1β) and neurotoxic substances (e.g., reactive oxygen species, nitric oxide) during aging, which contributes to chronic neuroinflammation and subsequent neurobiological damage, cognitive dysfunction, memory deficit and mood disorders [581, 582]. The increased activation of hippocampal IBA^+ microglia and $GFAP^+$ astrocyte cells with the increased expression of IL-1β during aging has been reported to be responsible for neuroinflammation associated impaired neurogenesis and loss of brain function [583].

Circulating T cells, which are important for cell-mediated immunity, as well as for the healthy functioning of the brain and the maintenance of neuroimmune bidirectional communication and homeostasis [294], undergo a major transformation during aging. Evidence suggests that there is an aging-induced reduction in the thymic output that results in a low number of naïve T cells [298]. As a result, while the proliferation of T cells continues during aging, there is a reduction in the ability to establish immunological memory in response to new antigens. Furthermore, there is a gradual decline in the number of early T cell activation markers and increase in the number of memory T cells, suggestive of the adverse effects of aging on cell-mediated immunity [299]. The significant reduction in the antigen presentation capacity of the helper $CD4^+$ T cells and antigen elimination function of the cytotoxic $CD8^+$ T cells has also been reported during normal aging [295, 296]. Collectively, the alteration in peripheral T cell subsets during aging can predispose the brain for neuroinflammatory and neurodegenerative diseases.

The abovementioned dysregulated innate and adaptive immunity makes the aging brain prone to increased occurrence of lymphoproliferative disorders, the formation of neurotoxic amyloid-beta and tau proteins, and expression of pro-inflammatory cytokines and acute-phase proteins. The resultant exacerbated neuroinflammation, in turn, may act as an etiology for neurodegenerative disorders during old age, including Alzheimer's disease (AD) and dementia [584]. For this reason, several pharmacological and non-pharmacological treatments have been explored in recent times to influence the aging-associated neuroimmune changes in the brain. Evidence suggests that non-pharmacological treatments, such as physical exercise (PE) and species-specific enrichment of the external environment (environmental enrichment, EE), exert a considerable influence on behavior and neuroimmune mechanisms [31,

585], and therefore could be utilized to modulate the neuroimmune functions associated with aging-related brain disorders. Indeed, in animal studies, these treatments (i.e., PE and EE) can be studied separately and in combination.

There are substantial evidence suggesting that hippocampus, particularly the dentate gyrus region, play a vital role in the regulation of behavior and memory in response to external stimulus [322-325]. Both PE and EE have been shown to enhance hippocampal neurogenesis, improve cognition, memory and motor coordination, and alleviate mood disorders in pre-clinical rodent models of psychiatric disorders [3, 22, 319-321, 345, 347, 383, 455, 521]. Research has also shown an association between the beneficial effects of PE and EE on brain functions and the altered expression of peripheral T- cells [547, 586] and brain glial cells [371, 587, 588]. Together, this suggests that PE and EE modulate brain functions by also altering the expression of T- cells in the periphery and glial cells in the dentate gyrus region of the hippocampus in the brain.

The paradigms of PE and EE function either at the physical or cognitive level. In PE, rodents are provided with voluntary or involuntary access to run on a wheel or treadmill respectively as compared with EE where mice voluntarily engage with cognitive stimuli including a range of objects of different sizes, shapes, and composition (e.g., plastic tubes, toys, ropes, ladders, tunnels, house, ramps, and platforms). It is, however, important to note that running wheels have also been used as an EE tool in a large number of studies [22, 345, 371, 373-375, 589, 590]. Since immune alterations in the brain during aging can affect behavior, mood, and cognition [306, 313], this, therefore, also suggests that short-term PE, EE, and their combination could be useful as immunotherapy for age-related brain disorders.

The reported neuroimmune outcomes after short-term PE and EE, however, have been inconsistent across past studies. For instance, EE of 8 to 16 weeks has been shown to reduce the levels of cytokines IFN-γ, TNF-α, IL-2, and IL-10 and the percentage of $CD8^+$ cells in the supernatants of activated spleen cells, and improve macrophage chemotactic activity and phagocytosis, and basal lymphocyte proliferation and chemotactic activity in mice [547, 586, 591]. Short-term PE alone has been shown to improve neuroimmune functions in rodents by enhancing NK cell activity and T suppressor cell activity [10, 28]. Furthermore, the combination of short-term PE and EE (PE+EE) has been shown to increase astrocyte and microglia antigen expression in the dentate gyrus, and decrease the expression of proinflammatory cytokines TNF-α and IL-1β in the hippocampus of Sprague Dawley rats [371]. These disparate immune outcomes, therefore, create uncertainty regarding the utility of short-term PE and EE as external non-pharmacological treatments in psychiatric studies. Also, the neuroimmune outcomes may vary across the life span. For example, a preclinical study in rodents has shown that while short-term EE increases the astrocyte number and size, as well as GFAP levels, in the hippocampus and corpus callosum of young male Wistar rats, it decreases the same in old rats [342].

The currently available literature, therefore, lacks evidence that can establish the differential neuroimmune effects of short-term PE, EE, and their combination across the lifespan. In our recently published study [592], we found that the short-term EE of 4 weeks in the absence of PE improved locomotion, and reversed age-related anxiety and cognitive deficit, but showed no significant effects on depressive-like behavior.

Short-term PE and PE+EE, however, were found to be ineffective. We, therefore, wanted to investigate whether the observed changes in behavior are related to underlying immune changes. Hence, all mice were sacrificed the day after behavioral testing ended for the analyses of change in numbers of astrocytes and microglia in the dentate gyrus and T cell subset proportions from cervical lymph nodes. C57BL/6 mice were selected for the study since they have been characterized well in terms of humoral and cellular neuroimmune responses to environmental factors [593]. The dentate gyrus region of the hippocampus was selected for molecular analysis due to its known role in the regulation of behavior and memory as mentioned previously. Since the migration of leucocytes from brain to cervical lymph nodes is established [594] and it has been suggested that T cells from the cervical lymph nodes are good representative of the status of cellular immunity in the brain [595], we selected cervical lymph nodes for the analysis of T cell-mediated immunity in the brain of mice for our research.

Methods

A. Animals

Wild-type (C57BL/6) mice (n=158; 80 males and 78 females), parental substrain Nhsd (derived from a colony from the National Institutes of Health, Bethesda, Maryland, USA), were bred in-house in the laboratory animal services (LAS) facility at the University of Adelaide and housed in same-sex groups of 4-5 in individually ventilated cages (IVCs) under controlled conditions of temperature (21±1 ^{o}C), humidity (55%), and a 12-12-hour dark-light cycle. During experiments, the C57BL/6Nhsd line was inbred for 9-13 generations.

B. Experimental design

Early age (3m), middle-age (8m) or late-middle age (13m), mice that showed no signs of injury and sickness, hence not challenged immunologically, were randomly allocated into four groups, i.e., Control, Physical Exercise (PE), Environmental Enrichment (EE) and PE+EE, as shown in Figures 1.6 and 4.1. Mice were then randomly paired (males and females paired separately) and transferred to open top cages on the morning of Monday, week 1 (2 mice per cage unless fighting necessitated separation). Each group had 11-19 (50 % male and 50 % female) mice per age group. Control mice received no treatment and were kept in cages with the following dimensions: 48.5cm x 15.5 cm x 12cm. PE, EE, and PE+EE mice were kept in plexiglass cages with dimensions: 37cm x 20.5 cm x 13.5cm, as these cages provided more breadth and depth to provide extra space for the objects associated with EE and running wheels.

The groups assigned as PE and PE+EE were provided with a running wheel for 4 weeks before behavioral testing, i.e., starting at 3, 8, and 13 months of age and ending at 4, 9, and 14 months of age, respectively. Similarly, the groups assigned as EE, and PE+EE were provided with a variety of non-toxic novel objects (houses, colored balls, toys, hanging toys, ladders, and tunnels) and extra bedding as per previously published protocols [345, 348, 374] for 4 weeks. The running wheels and EE objects remained in the cages throughout the three weeks of the behavioral testing period that followed four weeks of treatments (Fig 4.1). ***The ages of the mice at the end of behavioral testing (3, 8, and 13 months + 7 weeks, respectively) are referred to***

as 5, 10 and 15 months throughout this result chapter for the sake of simplicity and as advised by the reviewer.

Mice were inspected daily but handled only once a week while transferring them to clean cages every Friday morning to minimize handling stress, starting Friday of week 1. At the same time, mice were weighed on a digital weighing scale and wheel revolutions were counted using an automated counter on the wheels, the analyses of which have been published [592]. Also, the wheels were cleaned with F10SC veterinary disinfectant and the EE objects were changed to maintain novelty for mice. Mice were monitored for dominancy throughout the experiments, and those found to be highly aggressive were segregated to prevent dominance effects on neuroimmune mechanisms. Only the EE mice received nesting material (paper shreds) during the experiments. Mice underwent 3 weeks and 1 day of behavioral testing post four weeks of treatment (Fig 4.1).

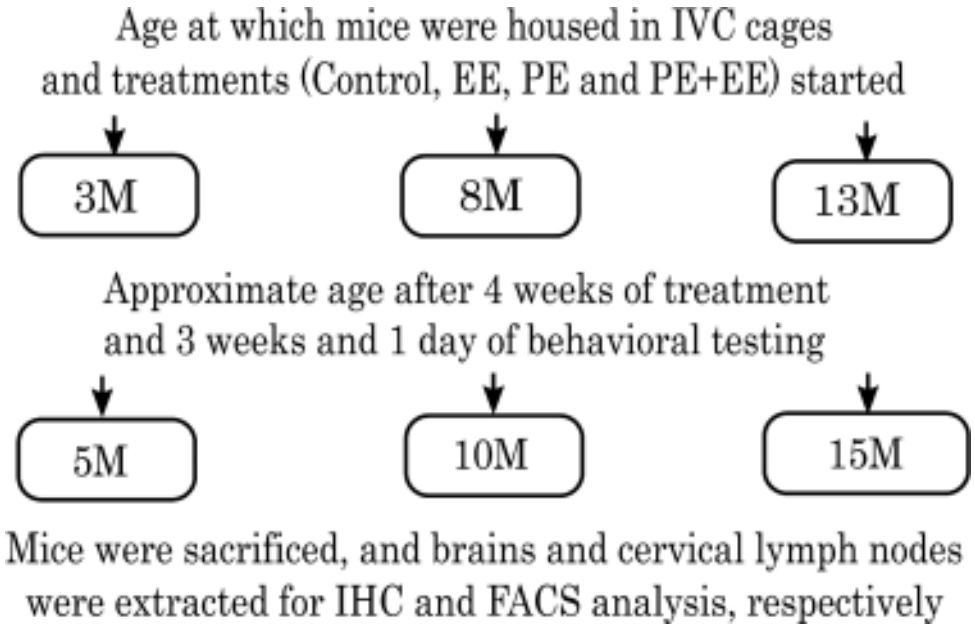


Fig. 4.1 Schematic representation of the experimental design. **Legend:** M: months, W: weeks, IHC: Immunohistochemistry, FACS: Fluorescence-activated cell sorting.

C. Molecular analyses

After seven weeks of exposure to treatments during which all mice also underwent identical behavioral testing in the last three weeks, mice were euthanized with a lethal dose of pentobarbital (60 mg/kg IP), and blood was collected through cardiac puncture [467]. Mice used for immunohistochemistry were perfused with 10% neutral buffered formalin via transcardiac injection, with the brains rapidly removed and preserved in 10% formalin. Mice utilized for FACS have had their draining cervical lymph nodes of the brain removed and collected in Roswell Park Memorial Institute (RPMI+) medium. It is important to note that the behavioral testing ended with the forced swim test a day before tissue collection. For full behavioral testing protocol and data analyses, see our recently published paper [592]. While both male and female mice were used to represent findings from a population comprising of both sexes in approximately equal numbers, it is important to note that we could not perform sex analysis due to low n for both males and females when taken separately.

Analysis of the number of immunopositive glial cell markers (IBA1 and GFAP) in the dentate gyrus

Following collection in 10% formalin, brains were cut into five 3mm coronal slices and following overnight treatment with increasing concentrations and exposure of ethanol, xylene, and paraffin baths; the sliced brain samples were embedded in paraffin wax. The hippocampus was then serially sectioned, with six sections 150µm apart. Each section was 5µm thick.

For immunohistochemistry, 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 mins. Antigen retrieval was performed by heating at close to boiling point for 10 mins in citrate buffer, and slides were then allowed to cool below 40°C before further processing. The appropriate primary antibody (IBA1 for microglia, 1: 10000; GFAP for astrocytes 1: 40000; 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 minutes. The formed immune complex was then further amplified by incubating slides with a biotin-binding protein, streptavidin-peroxidase conjugate, for 60 minutes. 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 using the 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 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.

Peripheral T cell immunophenotyping using fluorescence-activated cell sorting (FACS)

For the analysis of T cell mediated immunity, we extracted the cervical lymph nodes and total number of T cells, $CD4^+$ and $CD8^+$ T cell subpopulations (Naïve or T_N, Central memory or T_{CM} and Effector memory or T_{EM}), and early activated T cell phenotype CD25 were characterized using FACS.

Cervical lymph nodes were retrieved one day after behavioral testing ended and collected in the RPMI+ medium. Lymph nodes were passed through a 0.1µ sieve (BD) using RPMI+ and the end of a 1mL syringe and centrifuged to separate cells from tissue debris. Retrieved lymph node cells were counted on a hemocytometer and re-suspended in PBS to a final concentration of $2x10^6$ 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 10µL 0.5mg/mL Fc block. Eight color staining panel was used to immunophenotype $CD4^+$ and $CD8^+$ T cells. Unstained cells were used to exclude 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 binding). Cells were incubated for 30 minutes at room temperature with the respective mAbs (as shown in table 2.2) after which they were washed twice before resuspension in 300µL 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 gated. Percentages of $CD3^+$ $CD4^+$ or $CD3^+$ $CD8^+$ gated cells were used to calculate total cell numbers in combination with cell counts. Further gating on $CD44^+$ and $CD62L^+$ cell populations in the FCS Express software-enabled identification and estimation of T cell subsets (T_N, T_{CM}, and T_{EM}).

D. Statistical Analysis

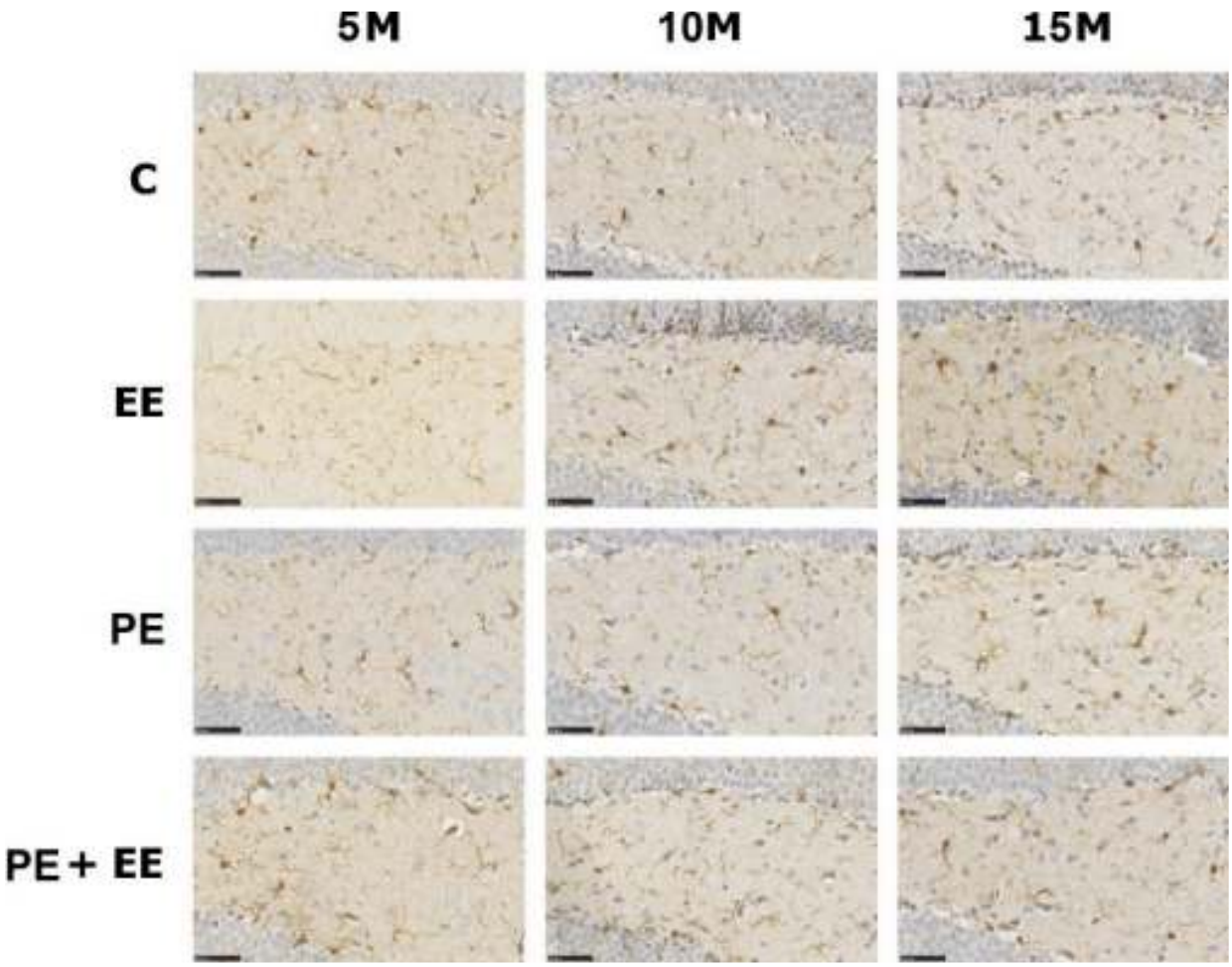
Statistical analyses were conducted using GraphPad Prism version 7.02 (GraphPad Software Inc.). All data outliers were removed using the ROUT method, and normality of data distribution was determined by visual inspection of histograms. Comparisons between the treatments (PE, EE, PE+EE) and controls were performed using two-way ANOVA. The multiple comparisons posthoc Holm-Sidak's test was used to confirm significant interactions between groups. Results are presented as mean ± SEM. Differences are considered statistically significant when $p < 0.05$.

Results

A. Alteration in microglia and astrocyte number in the dentate gyrus of C57BL/6 mice exposed to PE, EE or PE+EE at 5, 10 and 15 months of age

Immunopositive ($IBA1^+$) microglia were counted in the dentate gyrus of the hippocampus and analyzed statistically using two-way ANOVA with posthoc Holm-Sidak's multiple comparison test (Fig 4.2). A significant interaction effect ($F_{(6, 56)} = 2.936$; $p < 0.05$) with significant main effects of treatment ($F_{(3, 56)} = 6.749$; $p<0.001$) and age ($F_{(2, 56)} = 8.947$; $p < 0.001$) were noted. EE-treated mice showed a significantly higher number of $IBA1^+$ microglia than control mice at both 5 (90.3 ± 2.9 vs. 71.6 ± 4.4; $p < 0.05$) and 10-months (75.4 ± 4.2 vs. 51.7 ± 3.6; $p < 0.01$) of age. Also, PE and PE+EE treated mice showed a significantly higher number of $IBA1^+$ microglia than control mice at 10-months (78.0 ± 2.3 and 75.8 ± 4.2 vs. 51.7 ± 3.6; $p < 0.01$). Post-hoc analysis for the effect of age revealed that 10-month controls had a significantly lower number of $IBA1^+$ microglia in the dentate gyrus than both 5-month (51.7 ± 3.6 vs. 71.6 ± 4.4; $p < 0.01$) and 15-month old (51.7 ± 3.6 vs. 78.7 ± 7.1; $p < 0.001$) control mice. Likewise, 15-month PE+EE treated mice had a significantly higher number of $IBA1^+$ microglia in the dentate gyrus than both 5-month (95.1 ± 8.8 vs. 70.1 ± 0.6; $p < 0.01$) and 10-month (95.1 ± 8.8 vs. 75.8 ± 2.4; $p < 0.05$) PE+EE mice.

A


5M
10M
15M
C
EE
PE
PE+ EE

B

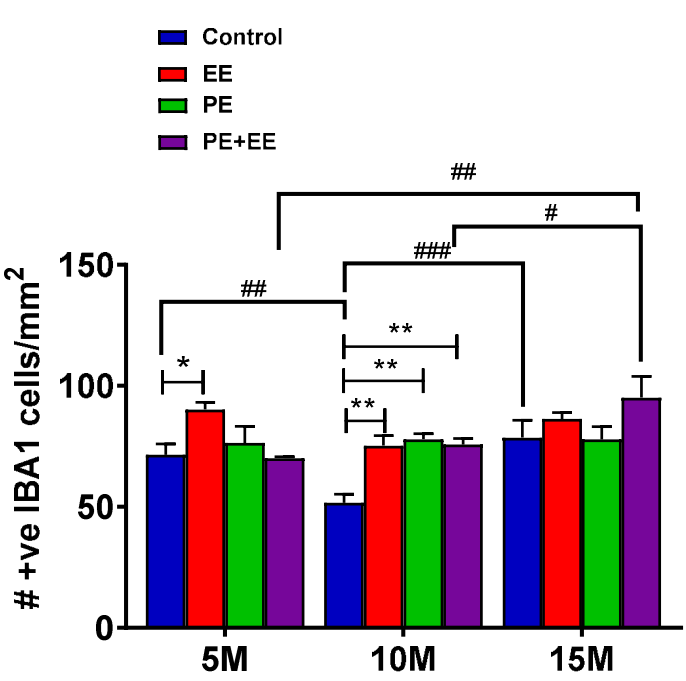

Control
EE
PE
PE+EE
+ve IBA1 cells/mm2
150
100
50
0
5M
10M
15M
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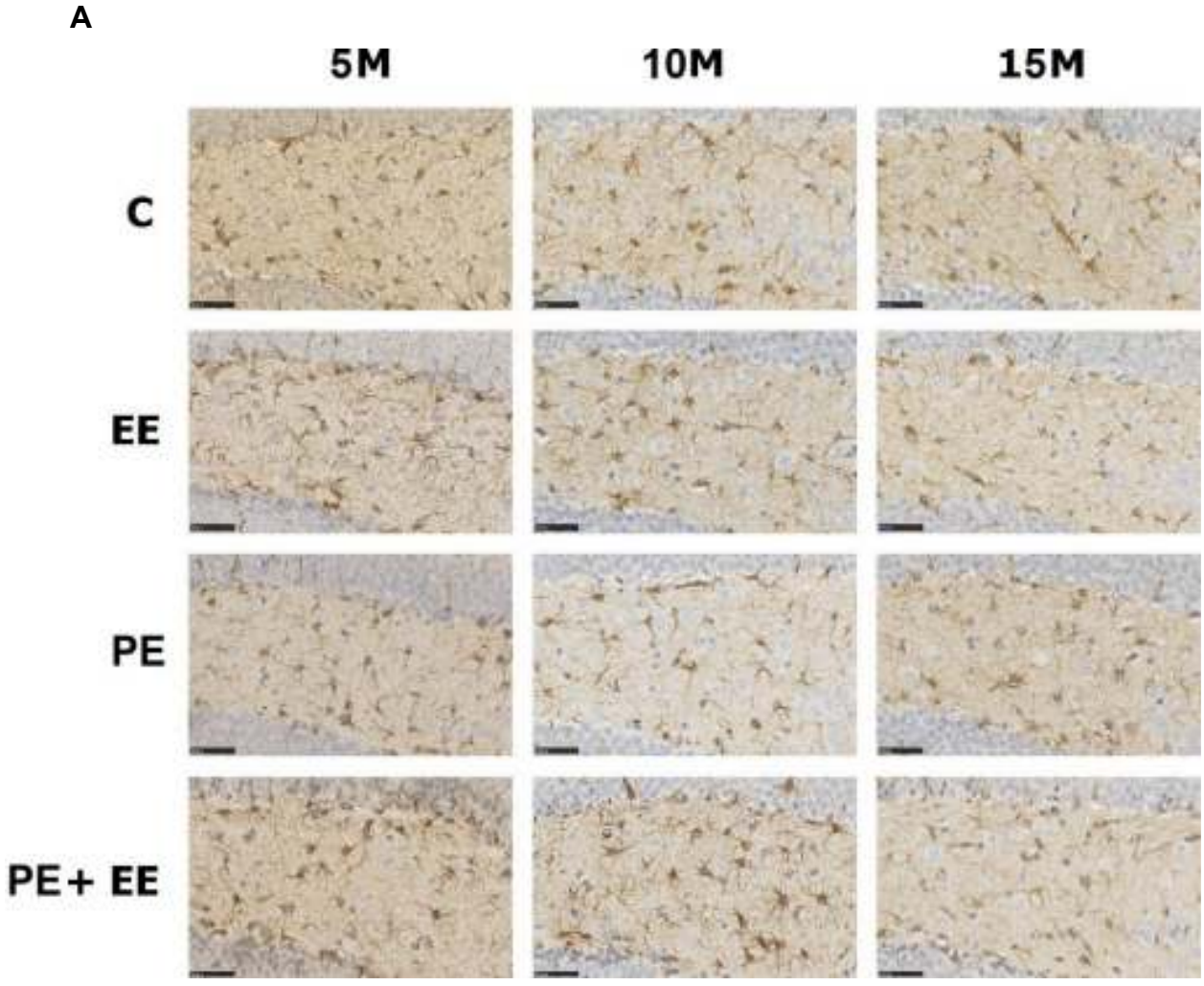
Fig. 4.2 IBA1$^+$ microglia in the dentate gyrus. (A) Representative immunohistochemical images, and (B) Number of IBA1$^+$ cells/mm^2. Black scale represents 50µm length. Two-way ANOVA, all data represented as mean ± SEM, n = 4-8 per group. * represents a significant difference between a treatment and age-matched control or between two treatments at one age point. $^{\#}$ represents a significant difference between the matched treatments over two age points. $^{*,\#}$ $p < 0.05$, $^{**,\#\#}$ $p<0.01$, $^{\#\#\#}$ $p<0.001$.

Immunopositive (GFAP$^+$) astrocytes were counted in the dentate gyrus of the hippocampus and analyzed statistically using two-way ANOVA with posthoc Holm-Sidak's multiple comparison test (Fig 4.3). Non-significant interaction effect and the main effect of treatment were noted; however, the main effect of age was found to be significant ($F_{(2, 56)} = 12.73$; $p < 0.0001$). This was because, at 5 months of age, PE-treated mice had a significantly higher number of GFAP$^+$ astrocytes in the dentate gyrus than their cohort at 15 months of age (311.8 ± 13.4 vs. 237.9 ± 11.7; $p < 0.05$).



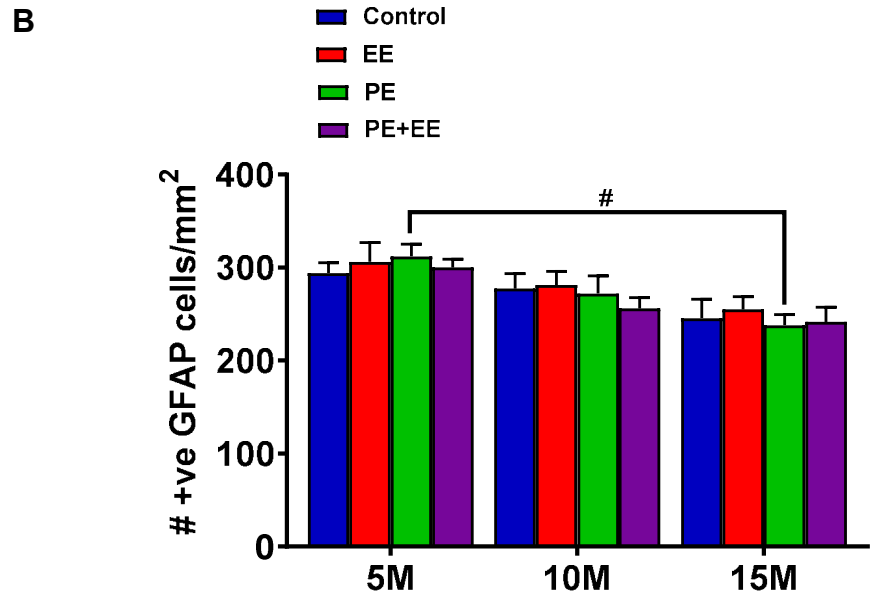


Fig. 4.3 GFAP$^+$ astrocytes in the dentate gyrus. (A) Representative immunohistochemical images, and (B) Number of GFAP$^+$ cells/mm^2. Black scale represents 50µm length. Two-way ANOVA, all data represented as mean ± SEM, n = 4-8 per group. $^{\#}$ represents a significant difference between the matched treatments over two age points. $^{\#}$ $p < 0.05$.

B. T cell immunophenotyping of cervical lymph node cells exposed to PE, EE or PE+EE at 5, 10 and 15 months of age

CD4$^+$ (helper T) and CD8$^+$ (cytotoxic T) total cell numbers indicate the treatment-specific immune response. Representative images obtained during the FACS data analysis using FCS Express software are shown in figures 4.4 and 4.5. Statistical data and group comparisons for T cell subsets (T_N, $T_{CM,}$ and T_{EM}) are displayed in Table 4.1, which are supported by figures 4.6 and 4.7, A-F.

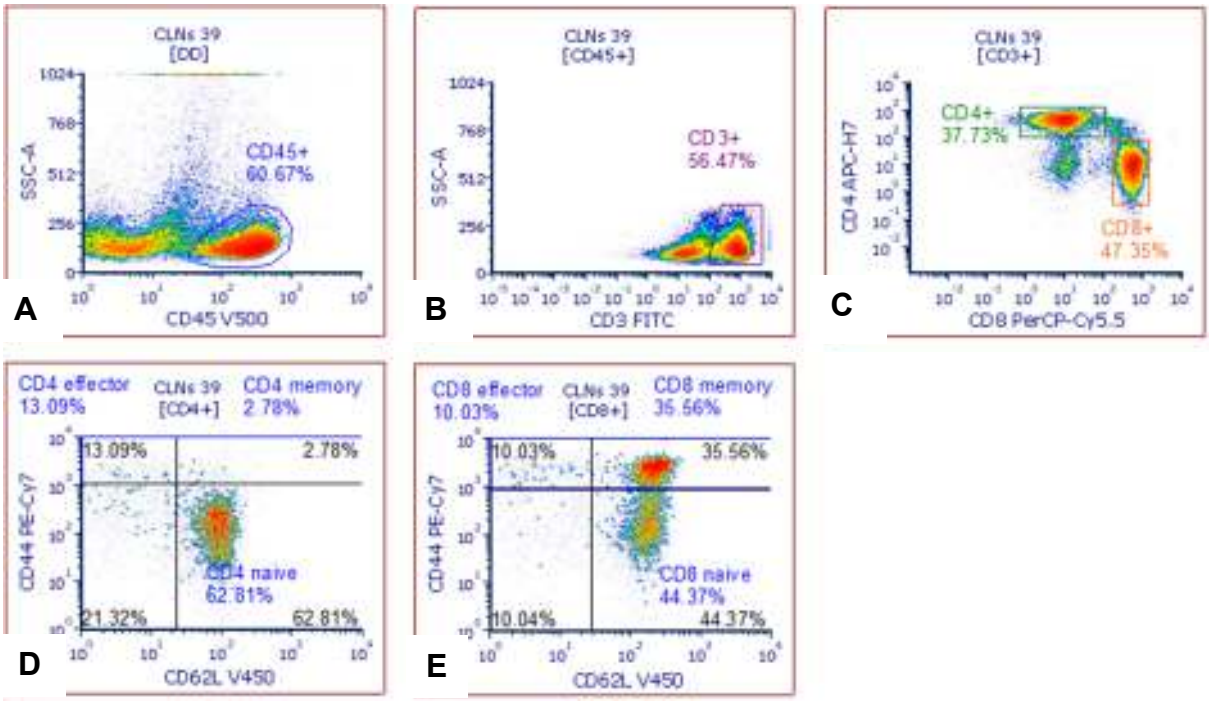


Fig. 4.4 Representative density plots of the gated T cells. (A) $CD45^+$ T, (B) $CD3^+$ T, (C) $CD3^+$ $CD4^+$ and $CD8^+$ T, (D) $CD4^+$ T_N, T_C and T_{EM}, and (E) $CD8^+$ T_N, T_C and T_{EM} cells. CD3+ T cells were derived from the gated CD45+ cells, CD4+ and CD8+ T cells were distinguished from the total CD3+ gated cells and further gating on CD44+ and CD62L+ cell populations enabled the identification of T cell subsets, i.e., Naïve (T_N), Central memory (T_{CM}) and Effector memory (T_{EM}) T cells.

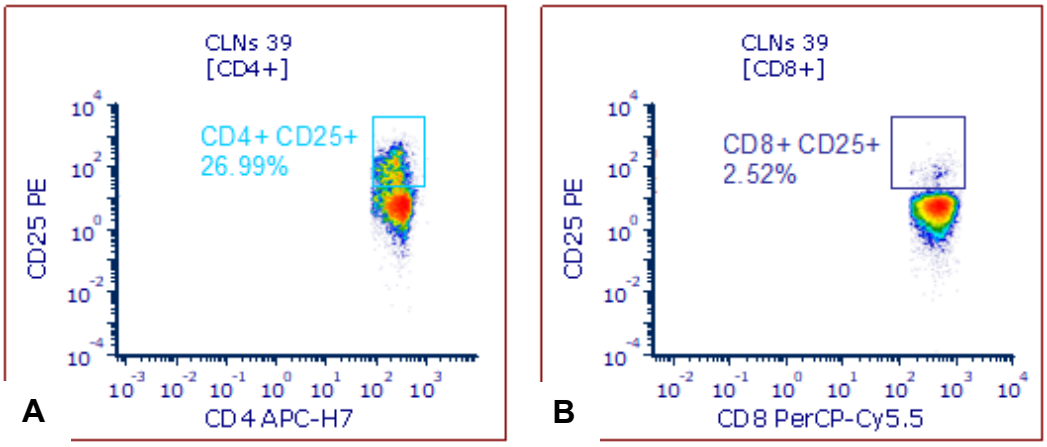


Fig. 4.5 Representative density plots of the early activated $CD25^+$ marker. (A) $CD4^+$ $CD25^+$ T and (B) $CD8^+$ $CD25^+$ T cell subpopulations.

Table 4.1 Multiple comparisons for T cell subsets percentage in the cervical lymph nodes of C57BL/6 mice after short-term PE, EE, and PE+EE treatments.

T cell phenotype	Interaction effect	Main effect of age	Main effect of treatment	Vs. Control (%)	Between age groups (%)	Between treatment groups (%)
$CD4^+$ cells (Fig 4.6A)	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**
$CD4^+$ T_N cells (Fig 4.6B)	**NS**	**S** ($F_{(2, 60)} = 7.445$; $p < 0.01$)	**S** ($F_{(3, 60)} = 3.024$; $p < 0.05$)	5m PE+EE > 5m C (14.6 ± 1.4 vs. 9.7 ± 1.2; $p < 0.01$)	5m PE+EE > 10m PE+EE (14.6 ± 1.4 vs. 10.1 ± 0.7; $p < 0.01$) 5m PE+EE >15m PE+EE (14.6 ± 1.4 vs. 9.2 ± 0.7; $p < 0.01$)	**NS**
$CD4^+$ T_{CM} cells (Fig 4.6C)	**S** ($F_{(6, 62)} = 3.497$; $p < 0.01$)	**S** ($F_{(2, 62)} = 9.335$; $p < 0.001$)	**NS**	5m EE > 5m C (11.1 ± 1.0 vs. 5.7 ± 1.4; $p < 0.01$) 5m PE > 5m C (10.3 ± 0.7 vs. 5.7 ± 1.4; $p < 0.05$)	5m EE > 10m EE (11.1 ± 1.0 vs. 6.2 ± 0.4; $p < 0.01$) 5m EE > 15m EE (11.1 ± 1.0 vs. 5.6 ± 0.5; $p < 0.01$) 5mPE > 10m PE (10.3 ± 0.7 vs. 4.7 ± 1.2; $p < 0.01$) 5m PE > 15m PE (10.3 ± 0.7 vs. 4.6 ± 1.4; $p < 0.01$)	5m EE > 5m PE+EE (11.1 ± 1.0 vs. 6.4 ± 0.5; $p < 0.05$) 5m PE > 5m PE+EE (10.3 ± 0.7 vs. 6.4 ± 0.5; $p < 0.05$)
$CD4^+$ T_{EM} cells Fig (4.6D)	**S** ($F_{(6, 58)} = 3.399$; $p < 0.01$)	**S** ($F_{(2, 58)} = 4.931$; $p < 0.05$)	**S** ($F_{(3, 58)} = 3.324$; $p < 0.05$)	5m EE < 5m C (0.7 ± 0.3 vs. 3.5 ± 0.7; $p < 0.01$) 5m PE < 5m C (0.8 ± 0.4 vs. 3.5 ± 0.7; $p < 0.01$)	5m EE < 15m EE (0.7 ± 0.3 vs. 2.7 ± 0.2; $p < 0.05$) 5m PE < 10m PE (0.8 ± 0.4 vs. 3.6 ± 0.6; $p < 0.01$) 5m PE < 15m PE (0.8 ± 0.4 vs. 3.4 ± 0.3; $p < 0.01$	5m EE < 5m PE+EE (0.7 ± 0.3 vs. 3.1 ± 0.4; $p < 0.01$) 5m PE < 5m PE+EE (0.8 ± 0.4 vs. 3.1 ± 0.4; $p < 0.01$)
$CD4^+$ $CD25^+$ cells (Fig 4.6E)	**S** ($F_{(6, 61)} = 3.235$; $p < 0.01$)	**NS**	**NS**	5m PE > 5m C (22.7 ± 2.4 vs. 10.7 ± 2.7; $p < 0.01$)	5m PE > 10m PE (22.7 ± 2.4 vs. 13.0 ± 3.4; $p < 0.05$) 5m PE > 15m PE (22.7 ± 2.4 vs. 12.1 ± 3.4; $p < 0.05$)	**NS**
$CD8^+$ cells (Fig 4.7A)	**S** ($F_{(6, 61)} = 2.286$; $p < 0.05$)	**S** ($F_{(2, 61)} = 5.321$; $p < 0.01$)	**S** ($F_{(3, 61)} = 6.713$; $p < 0.001$)	15m EE > 15m C (37.4 ± 2.3 vs. 25.7 ± 2.9; $p < 0.05$)	15m EE > 5m EE (37.4 ± 2.3 vs. 20.6 ± 1.8; $p < 0.05$) 15m EE > 10m EE (37.4 ± 2.3 vs. 26.8 ± 3.8; $p < 0.01$)	5m PE+EE > 5m EE (34.3 ± 1.8 vs. 20.6 ± 1.8; $p < 0.01$) 5m PE+EE > 5m PE (34.3 ± 1.8 vs. 20.7 ± 0.7; $p < 0.01$)
$CD8^+$ T_N cells (Fig 4.7B)	**NS**	**NS**	**S** ($F_{(3, 61)} = 8.369$; $p < 0.0001$).	5m PE+EE > 5m C (20.2 ± 0.8 vs. 13.4 ± 1.8; $p < 0.05$) 15m EE > 15m C (18.4 ± 1.8 vs. 10.2 ± 1.7; $p < 0.05$)	5m EE < 15m EE (10.7 ± 1.1 vs. 18.4 ± 1.8; $p < 0.05$)	5m PE+EE > 5m EE (20.2 ± 0.8 vs. 10.7 ± 1.1; $p < 0.01$)

				15m PE+EE > 15m C (17.6 ± 1.3 vs. 10.2 ± 1.7; $p < 0.05$)		5m PE+EE > 5m PE groups (20.2 ± 0.8 vs. 10.4 ± 1.0; $p < 0.01$)
$CD8^+$ T_{CM} cells (Fig 4.7C)	**NS**	**S** ($F_{(2, 60)} = 9.552$; $p < 0.001$)	**NS**	**NS**	15m C > 10m C (12.8 ± 1.6 vs. 8.2 ± 0.6; $p < 0.05$) 15m EE > 10m EE (12.8 ± 2.1 vs. 8.4 ± 0.7; $p < 0.05$)	**NS**
$CD8^+$ T_{EM} cells (Fig 4.7D)	**S** ($F_{(6, 59)} = 2.455$; $p < 0.05$; Fig 6D)	**S** ($F_{(2, 59)} = 3.173$; $p < 0.05$)	**NS**	**NS**	10m PE > 5m PE (1.6 ± 0.5 vs. 0.2 ± 0.1; $p<0.05$) 15m PE > 5m PE (2.0 ± 0.6 vs. 0.2 ± 0.1; $p < 0.05$)	**NS**
$CD8^+$ $CD25^+$ cells (Fig 4.7E)	**NS**	**S** ($F_{(2, 62)} = 4.2$; $p < 0.05$)	**S** ($F_{(3, 62)} = 3.839$; $p < 0.05$)	**NS**	5m PE+EE < 10m PE+EE (19.4 ± 3.6 vs. 33.2 ± 1.3; $p < 0.05$) 5m PE+EE < 15m PE+EE (19.4 ± 3.6 vs. 33.7 ± 1.6; $p < 0.05$)	10m PE+EE > 10m PE (33.2 ± 1.3 vs. 17.2 ± 6.0; $p < 0.05$)

Legend: S: significant, NS: not significant, m: month, T_N: naïve T cells, T_{CM}: central memory T cells, T_{EM}: effector memory T cells.

The interaction effect was non-significant for the proportion of the gated $CD4^+$ T cells (Fig 4.6 A). However, the interaction effects were significant for $CD4^+$ T_{CM} (Table 4.1; Fig 4.6 C) and T_{EM} (Table 4.1; Fig 4.6 D) cells. In addition, we observed significant main effects of (i) age for the T_N (Table 4.1; Fig 4.6 B), T_{CM} (Table 4.1; Fig 4.6 C) and T_{EM} (Table 4.1; Fig 4.6 D) subsets, and (ii) treatment for the T_N (Table 4.1; Fig 4.6 B) and T_{EM} (Table 4.1; Fig 4.6 D) subsets of $CD4^+$ cells.

Post-hoc analysis revealed significantly higher expression of $CD4^+$ T_N cells in 5-month PE+EE mice compared to 5-month controls mice, and 10-month and 15-month PE+EE mice (Table 4.1; Fig 4.6 B). Similarly, the proportion of $CD4^+$ T_{CM} cells were significantly higher in 5-month EE and PE mice compared to 5-month control mice (Table 4.1; Fig 4.6 C), 5-month PE+EE mice (Table 4.1; Fig 4.6 C), and respective EE and PE mice at 10 (Table 4.1; Fig 4.6 C) and 15 months (Table 4.1; Fig 4.6 C). The results were, however, different for $CD4^+$ T_{EM} cells. The 5-month control and PE+EE mice expressed significantly higher $CD4^+$ T_{EM} cells in the cervical lymph nodes than similar-aged EE and PE mice (Table 4.1; Fig 4.6 D). Furthermore, the expression of $CD4^+$ T_{EM} cells in the EE group at 5 months was significantly lower than the EE group at 15 months (Table 4.1; Fig 4.6 D). Likewise, the PE group at 5 months showed significantly lower expression of $CD4^+$ T_{EM} cells than PE groups at both 10 and 15 months (Table 4.1; Fig 4.6 D).

We observed a significant interaction effect for $CD4^+$ $CD25^+$ T cells (Table 4.1; Fig 4.6 E). The 5-month PE group showed a significantly higher proportion of $CD4^+$ $CD25^+$ T_{EM} cells than 10 and 15-month PE groups (Table 4.1). Additionally, we observed that 5-month-old PE mice had significantly more $CD4^+$ $CD25^+$ cells when compared to the same age controls (Table 4.1).

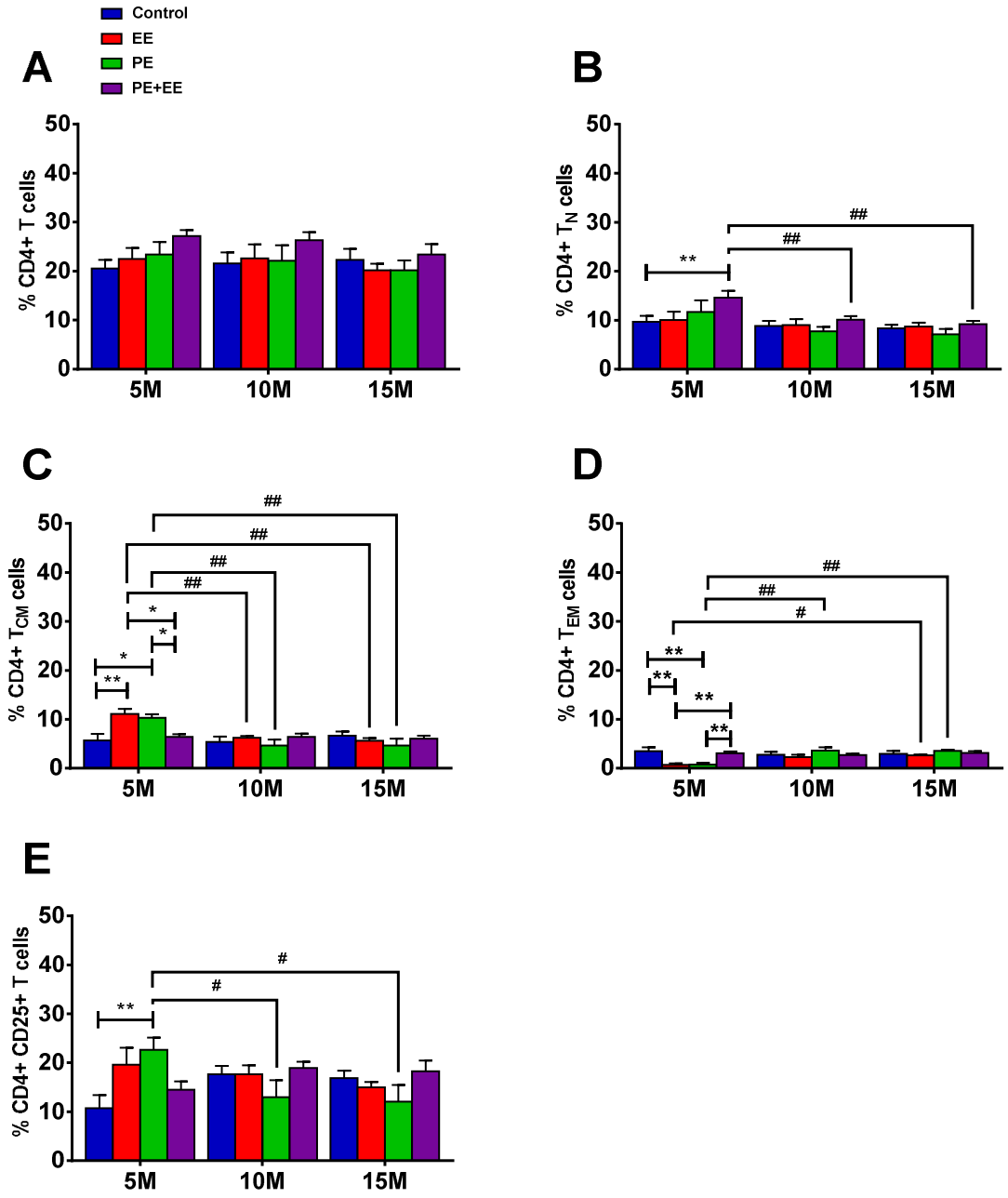


Fig. 4.6 $CD4^+$ T-cell subset composition. Proportions of the gated (A) $CD4^+$ T, (B) $CD4^+$ T_N, (C) $CD4^+$ T_{CM}, (D) $CD4^+$ T_{EM} cells, and (E) $CD4^+$ $CD25^+$ T cells. Two-way ANOVA, all data represented as mean ± SEM of the proportion of $CD4^+$ T cells, n = 4-8 per group. * represents a significant difference between a treatment and age-matched control or between two treatments at one age point. # represents a significant difference between the matched treatments over two age points. * and # $p<0.05$, ** and ## $p<0.01$. **Legend:** M: month, EE: environmental enrichment, PE: physical exercise, N: naïve, CM: central memory, EM: effector memory.

Two-way ANOVA found significant interaction effect, as well as significant main effects of age and treatments for the proportion of $CD8^+$ T cells (Table 4.1; Fig 4.7 A), with a proportion of $CD8^+$ T cells significantly higher in (i) 4-month PE+EE group compared to the same age EE and PE groups, (ii) 14-month EE group compared to the 14-month controls group, and (iii) 14-month EE group compared to the 4 and 9-month EE groups.

Furthermore, we observed the significant interaction effect for $CD8^+$ T_{EM} cells (Table 4.1; Fig 4.7 D), significant main effects of age for $CD8^+$ TC_M (Table 4.1; Fig 4.7 C) and T_{EM} (Table 4.1; Fig 4.7 D) cells and significant main effect of treatment for $CD8^+$ T_N cells (Table 4.1; Fig 4.7 B).

On post hoc analysis, the 5-month PE+EE group had a significantly higher proportion of $CD8^+$ T_N cells compared to 5-month controls, EE, and PE groups (Table 4.1; Fig 4.7 B). Similarly, the 15-month EE and PE+EE groups showed a significantly higher proportion of $CD8^+$ T_N cells than the 15-month controls group (Table 4.1; Fig 4.7 B). Furthermore, the 5-month EE group had significantly less proportion of $CD8^+$ T_N cells compared to the 15-month EE group (Table 4.1; Fig 4.7 B). The proportion of $CD8^+$ T_{CM} cells was significantly higher in control and EE groups at 15-month than at 10-month (Table 4.1; Fig 4.7 C). In the case of $CD8^+$ T_{EM} cells, both 10 and 15-month PE groups showed a greater proportion of cells than the 5-month PE group (Table 4.1; Fig 4.7 D).

We found a non-significant interaction effect but significant main effects of both age and treatment for $CD8^+$ $CD25^+$ T cells (Table 4.1; Fig 4.7 E). The posthoc analyses revealed that 5-month PE+ EE group had a significantly lower proportion of $CD8^+$ $CD25^+$ T cells than both 10 and 15-month PE+EE groups (Table 4.1; Fig 4.7 E). Additionally, the 10-month PE+EE group showed a significantly higher proportion of $CD8^+$ $CD25^+$ T cells than the 10-month PE group (Table 4.1).

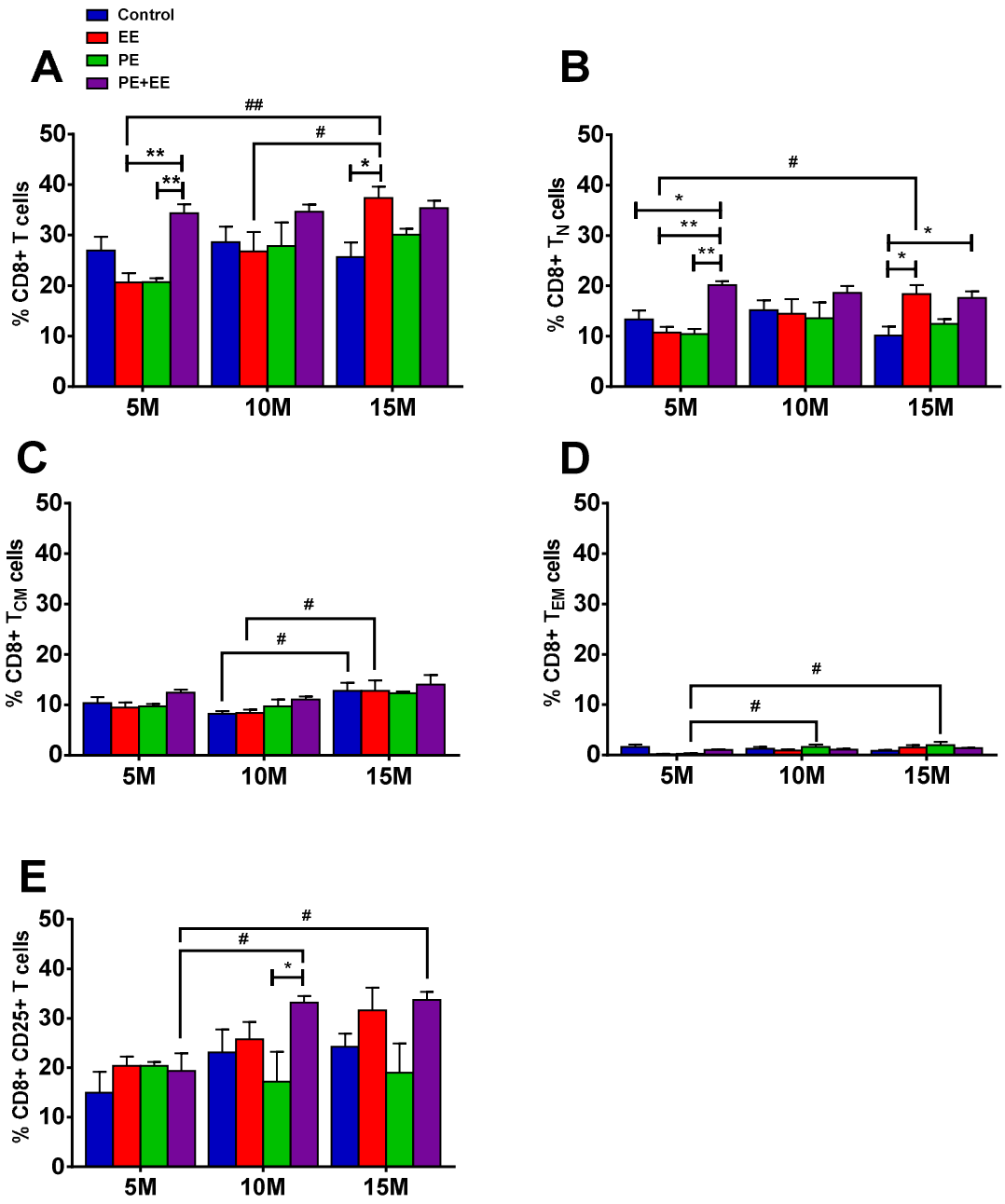


Fig. 4.7 CD8⁺ T-cell subset composition. Proportions of the gated (A) CD8⁺ T, (B) CD8⁺ T_N, (C) CD8⁺ T_{CM}, (D) CD8⁺ T_{EM} cells, and (E) CD8⁺ CD25⁺ T cells. Two-way ANOVA, all data represented as mean ± SEM of the proportion of CD8⁺ T cells, n = 4-8 per group. * represents a significant difference between a treatment and age-matched control or between two treatments at one age point. # represents a significant difference between the matched treatments over two age points. * and # $p<0.05$, ** and ## $p<0.01$, **** $p<0.0001$. **Legend:** M: month, EE: environmental enrichment, PE: physical exercise, N: naïve, CM: central memory, EM: effector memory.

Discussion

In this study, we evaluated the effects of short-term cognitive stimuli and physical activity treatments alone or in combination at three different ages, i.e., 5, 10 and 15 months, on microglia and astrocytes numbers in the dentate gyrus and change in the proportion of peripheral CD4⁺ and CD8⁺ T cells, their subsets (T_N, T_{CM}, T_{EM}), and

$CD25^+$ T cells. We observed that while all three treatments reversed the aging-related decrease in the number of immunopositive microglial cells at middle age, none of them showed any effects on the number of immunopositive astroglial cells at all ages. Additionally, all three treatments were found to modulate the proportion of peripheral T cell subsets during normal aging, especially at early and late-middle age.

Only short-term EE enhanced microglial number at middle age, reversing the effects of aging. None of the treatments affected astroglia number

We observed a significant increase in the number of immunopositive $IBA1^+$ microglia after short-term EE at both 5 and 10 months and after short-term PE and PE+EE at 10 months. None of the treatments significantly differed from controls at 15 months. We noticed that the significant increase observed after EE, PE and PE+EE at 10 months could, in turn, be the result of unusual reduction in the number of IBA1+ microglia in 10-month-old control mice. Indeed, 10-month controls also showed a significantly lower number of $IBA1^+$ microglial cells than 5 and 15-month controls. These results are, however, in line with previously published findings where short-term EE significantly increased microglia antigen ($IBA1^+$) expression within the dentate gyrus of adult male Sprague–Dawley rats (Williamson et al. 2012). In addition, we observed that short-term PE+EE mice had significantly more $IBA1^+$ microglial cells in the dentate gyrus at 15 months compared to 5- and 10-months PE+EE mice.

It must, however, be noted that the phenotype of microglia determines the functional state of microglia. Quiescent microglia within the CNS lack phenotypical markers required for antigen presentation but assist in neuronal migration and repair, recycling of neurotransmitters, regulating ion balance and buffering pH, and maintaining neuronal homeostasis (Singhal and Baune 2017). When activated in the presence of pathogens or stress proteins, microglia 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 cell subsets essential to mounting an innate immune response (reactive microglia). The number of reactive microglia increases in areas of neurodegeneration in aging rodents (Singhal and Baune 2017), which has, in turn, been associated with aging-related cognitive and memory impairment (Rozovsky et al. 1998; Sugaya et al. 1996), depression (Norden and Godbout 2013) and neurodegenerative diseases such as AD (Mrak and Griffin 2005). The immunohistochemical stain that we used stained both quiescent and reactive microglia equally, hence, differentiating the two types of microglia was not possible. Also, we investigated mice that aged from early to late middle age, and not old age, in a relatively stress-free environment, thereby reducing the likelihood of pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) accelerating the microglia-related process. As such, an increase in microglial number during our work is indicative of the effects of normal aging with other external confounders significantly filtered out, and could primarily be due to an increase in the number of quiescent and not reactive microglia. Future research investigating the change in the proportion of two phenotypes after the three treatments is required. This is important especially when the available evidence suggest that altered microglial phenotype modulates affective-like and cognitive behaviors in young and adult rodents (Biscaro et al. 2012; Li et al. 2014; Moraes et al. 2015; Wang et al. 2018). Nonetheless, the depletion in microglial number has also been shown to protect mice for neuroinflammation-associated learning and memory impairment in postoperative young mice (Feng et al. 2017). Likewise, elimination of microglia has shown to improve

cognition in six months old C56BL/6 mice following cranial irradiation (Acharya et al. 2016). These evidences suggest that change is microglial number may also result in change in behavior.

The overexpression of microglia can result in a significant increase in the production and expression of proinflammatory cytokines (e.g. TNF-α, IL-1β) and neurotoxic substances (e.g. reactive oxygen species, nitric oxide) (Singhal and Baune 2017), subsequently leading to cognitive dysfunction and psychiatric illnesses, such as depression (Patel 2013) and Alzheimer's disease (AD) (Mrak 2012). However, the increase in the number of microglia may not corroborate fully with the change in microglial functions in the absence of an analysis of microglial activation state. Moreover, we observed no significant change in the microglia number at 15 months after PE and EE. Interestingly, the combination of PE and EE also showed no effects on the increase in microglia number when compared with controls at all three age points. The beneficial effects of the short-term EE on locomotor activity, anxiety-like behavior and spatial memory at late-middle age that we have reported recently (Singhal et al. 2019) could possibly be due to an increase in the number of quiescent microglia. These results, however, do not explain the reported spatial learning impairment and depressive-like behavior at an early age after short-term PE. Yet, these results are interesting given the microglia are considered the primary immune effector cells in the brain and hence any change in microglial number could be associated with microglial dysfunctioning.

None of the short-term treatments differed significantly from controls for $GFAP^+$ astroglial cells at any of the ages. However, 15-month PE mice showed significantly lower expression of $GFAP^+$ astroglial cells than 5-month PE mice. It has been reported that during normal aging of the brain, astrocytes reduce in number but their phenotype changes to those of reactive astrocytes, which are the hallmarks of neuroinflammatory disorders (Clarke et al. 2018). These astrocytes affect several neural functions, such as neurotransmission, synaptic plasticity, and neurogenesis, dysregulation of which, in turn, has been associated with the development of psychiatric symptoms (Clarke et al. 2018). Indeed, the association between microglia and astrocytes during old age results in impairment of several vital neuroprotective functions, in particular, the formation and maturation of synapses, neuroinflammation, and neurodegeneration (Dilger and Johnson 2008; Moranis et al. 2012; Wang et al. 2011). As such, we hypothesized a decrease in the number of immunoreactive astrocytes within the dentate gyrus during normal aging. Our results suggest that normal aging until late middle age has no significant effects on the number of $GFAP^+$ astroglial cells in the dentate gyrus. Similarly, no effects of short-term PE, EE, and PE+EE compared to controls were observed on $GFAP^+$ astroglial number in the dentate gyrus. Furthermore, although the $GFAP^+$ astroglial cells number reduced in all 15-month treatment groups when compared to 5-month treatment cohorts, the reduction was significant only in case of short-term PE mice, thereby suggesting that the short-term PE might influence astrocyte-related processes during old age more than short-term EE. Perhaps, the longer duration of PE, EE, and PE+EE treatments may be required to observe a significant change in the $GFAP^+$ astroglial cells number in the dentate gyrus.

Together, the results for microglia and astrocytes suggest that any short-term change in behavior or memory may not be accompanied by a concurrent change in glial cells

expression and other immune and non-immune factors may be responsible for the reported functional changes (Singhal et al. 2019).

Short-term PE and EE did not affect the cervical lymph nodes $CD4^+$ T cell subsets until late-middle age. Only short-term PE+EE enhanced $CD4^+$ T_N cells proportion at an early age

Ours is the first study to investigate the change in the proportion of T cell subsets in the cervical lymph nodes in response to short-term EE. The CSF drains to the cervical lymph nodes through the Cribroid plate and nasal mucosa, and hence, the analysis of T cells from the cervical lymph nodes provides a good measure of the T cell-mediated immunity in the brain (Engelhardt et al. 2016; Goldmann et al. 2006). There were no significant differences found in the overall proportion of $CD4^+$ T cells after short-term treatments at all ages suggesting no effects of the short-term physical exercise or enrichment on helper T cells. These findings are similar to a male CS mouse study whereby no effect of EE when compared with controls was found on the . proportion of CD4+ T cells (Marashi et al. 2003). However, this is in contrast to a significant reduction in peripheral blood CD4+ helper T cell proportion in comparison with controls in rats which underwent short-term (4 weeks) PE using a swimming protocol (Kaufman et al. 1994). Similarly, in another rodent study, the combination of PE+EE was seen to affect T helper cells. In this case, the reduction in CD4+ T helper cell proportions as found in adolescent rat offspring in response to gestational stress was reversed with a short term PE+EE intervention (Laviola et al. 2004). However, moderate endurance training on a treadmill has shown to increase the percentage of CD4+ T cells in the Thymus of adult rats, suggesting that the different type of exercise may elicit differnet effects on T cell subsets is different immune locations (Ferry et al. 1992). It is important to note that differences in genotypes and species across studies mentioned, could also contribute to differences in reported findings of peripheral lymphocyte phenotypes.

Further subset analyses of the proportion of $CD4^+$ T cells were carried out. The estimation of the T cell subsets is important in measuring the cell-mediated immune response (Golubovskaya and Wu 2016; Lanzavecchia and Sallusto 2000; Sallusto et al. 1999; Seder and Ahmed 2003).

We observed a significantly higher proportion of T_N cells in the 5-month PE+EE mice compared to 5-month controls and PE+EE mice at 10 and 15 months. Moreover, the proportion of T_{CM} cells was significantly higher in 5-month EE and PE mice when compared to 5-month control mice, and 10- and 15-month mice that received the same treatments. Interestingly, the proportions of T_{EM} in 5-month PE and EE groups were significantly lower than the same age controls as well as older mice receiving the same treatments. Overall, this suggests that while the combination of short-term PE+EE increased the proportion of $CD4^+$ T_N cells, PE or EE alone did not. On the other hand, short-term PE and EE in isolation not only induced an increase in the proportion of $CD4^+$ T_{CM} cells but also reduced the proportion $CD4^+$ T_{EM} cells at an early age.

Naïve T cells become activated, proliferate, differentiate into memory and effector cells, and show upregulation of surface marker $CD25^+$ on encountering a cognate antigen. Studies investigating T cell receptor responses indicate $CD4^+$ T_N cells proliferate and convert into memory cells at a lower rate after an initial lag period upon antigen stimulation, in comparison to $CD8^+$ T_N cells (Foulds et al. 2002; Jelley-Gibbs et al. 2000). Similarly, the effects of aging and subsequently of PE on T cell substes in Spleen and Thymus has been demonstrated by another study (Woods et al. 2003).

The authors reported significantly higher percentage of splenic memory cells and a lower percentage of näive cells in both the $CD4^+$ and $CD8^+$ T cell subsets in 18-month old BALB/c mice, which was reversed by moderate exercise training of 4-months on a treadmill. No effects of PE was observed on the T cell subsets in Thymus. In contrast, in our study, exposure to either physical or environmental enrichment appears to induce an earlier expansion of CD4+ T_N to $CD4^+$ T_{CM} cells in the cervical lymph nodes, however it must be noted that mice during our study aged only until middle age. Also, the lower proportions of CD4+ T_{EM} cells in 5-month PE and EE can be explained by the fact that mice during our experiments were raised in a relatively stress-free environment also devoid of PAMPs. However, aging can also result in the production of DAMPs, which can act as cognate antigens. This might explain the higher proportions of CD4+ T_{EM} cells at 10 and 15 months.

The controlled environment could also be the reason for no differences noted in the proportion of $CD4^+$ $CD25^+$ cells except for 5-month PE group showing a significantly higher proportion of $CD4^+$ $CD25^+$ cells than the 5-month controls and the older mice receiving the same treatment. $CD25^+$ is the alpha chain of the IL-2 receptor and is up regulated following T cell receptor ligation acquiring high affinity and making cells sensitive for IL-2, also acting as early T cell activation marker. IL-2 controls the differentiation and homeostasis of T cells, thereby regulating cell-mediated immune response. $CD4^+$ $CD25^+$ cells may be associated with the 'activation', 'priming' or 'memory' state of $CD4^+$ cells (Sakaguchi 2000). Also, since $CD4^+$ $CD25^+$ cells have been reported to suppress proliferation of effector T cells (Sakaguchi et al. 2008; Wing and Sakaguchi 2008), this suggests that short-term PE might help to develop immunological self-tolerance at an early age.

Short-term EE and PE+EE increase the proportion of peripheral $CD8^+$ T_N cells in young and late middle age mice, respectively. Only PE+EE increases the proportion of early activated $CD8^+$ T cells across all ages

We observed that the proportion of peripheral $CD8^+$ T cells at the late-middle age increased significantly after short-term EE. However, no change in the proportion of $CD8^+$ T cells was noted after both short-term PE and PE+EE at all ages. This was contrary to the hypothesis that short-term PE will improve neuroimmune functions during normal aging by reducing the proportion of $CD8^+$ T cells in C57BL/6 mice in a controlled environment, as has been reported in the spleens of rodents trained in PE using a swimming protocol over 4 weeks [10]. Similarly, the reduced anxiety-like behavior and improvement in spatial memory at middle and late middle age respectively after short-term EE [592] is not explained by $CD8^+$ T cell results.

CD8+ T_N cells require significantly less time of antigen exposure for activation than CD4+ T_N cells. This, in turn, translates into the less time required by the CD8+ T_N cells to activate, divide and differentiate, and have a faster rate of division than CD4+ T_N cells (Seder and Ahmed 2003). Furthermore, CD4+ effector T cells are more susceptible to apoptosis at the end of the effector phase than CD8+ effector T cells. This provides a better opportunity to CD8+ effector T cells to develop into stable long-term resting memory cells than CD4+ effector cells. Also, unlike CD4+ memory cells, both CD8+ memory cell subsets (T_{CM} and T_{EM}) are equally efficient in producing effector functions on restimulation with antigen. These differences makes CD8+ T cells more potent than CD4+ T cells in dispersing cell mediated immune functions.

Further subset analyses of the proportion of $CD8^+$ T cells revealed an increase in proportion of $CD8^+$ T_N but not T_{CM} and T_{EM} cells after short-term EE at late-middle age. However, a human study has shown that aging is associated with a decrease in $CD8^+$ T_N cells in donor cervical lymph nodes [307]. This suggests that EE improves basal cytotoxic immunity during the late-middle-age reversing the age-related effects. This, possibly also explains the reported improvement in brain functions after short-term EE [592]. In contrast, we observed that the combination of short-term PE and EE also increased the proportion of $CD8^+$ T_N cells, but only at an early age and not at middle and late-middle age. These results are interesting and show the influence that the inclusion of a running wheel in EE paradigms can have on cell-mediated immunity. Furthermore, the proportion of $CD8^+$ T_{CM} cells in the cervical lymph nodes of control mice significantly increased from middle to late-middle age. This can again be explained by the fact that there is an increase in the production of DAMPs during aging, which may act as cognate antigens stimulating the conversion of the peripheral $CD8^+$ T_N cells to $CD8^+$ T_{CM} cells. The latter have little or no effector function but mediate reactive memory where they migrate to secondary lymphoid organs, and some of them proliferate and differentiate into effector cells in response to antigenic stimulation, thus helping the body to resolve inflammation [304]. The proportion of $CD8^+$ T_{EM} cells in the cervical lymph nodes of 5-month PE mice were significantly lower compared to older PE groups. Since the $CD8^+$ T_{EM} cells are important mediators of protective memory as mentioned before, the lower number of $CD8^+$ T_{EM} cells after PE at 5 months suggests that perhaps the cell-mediated immunity gets compromised after short-term PE at an early age, which may have a role to play in the reported development of depressive-like behavior in young mice [592].

The combination of short-term PE with EE significantly improved the proportion of early activated $CD8^+$ $CD25^+$ cells T cell from middle age onwards when compared to early age PE+EE mice, suggesting that PE+EE might enhance CD8+ T cells responsiveness if provided for long-term [605]. Further research into investigating the effects of long-term PE, EE, and their combination on aging-associated changes in T cell subset proportions is required.

Research Summary

In this chapter, we investigated the effects of PE, EE and the combination of PE and EE on immunopositive brain glial cells count in the dentate gyrus, and total number of T cells, $CD4^+$ and $CD8^+$ T cell subpopulations and early activated T cell phenotype CD25 in the cervical lymph nodes over the life span of C57BL/6 mice.

The results from the research suggest that all three treatments reversed the effects of aging on microglia at middle age, however, only PE decreased the number of immunopositive astroglia cells at 14 months compared to 4-month PE mice. We also observed an increase in the proportion of peripheral CD8+ T_N cells at late-middle age after EE, which is suggestive of enhanced cytotoxic immunity after EE in a controlled environment.

Together, it suggests that short-term EE is a stronger modulator of the neuroimmune functions compared to short-term PE and PE+EE until late-middle age. This could be a reason for the improvement of brain functions after EE that we have reported in the previous chapter. In addition, PE suppresses CD8+ T_{EM} activity at an early age, which is also suggestive of possible immunosuppressive action. This could be an explanation for depressive-like behavior after short-term PE in early age mice as reported in the previous chapter. Again, the research we conducted is one of its kind where we investigated the differential effects of PE, EE and their combination of brain glial cells and peripheral T cells count. More research in the future using bigger sample size and long-term treatments may be required to develop a better understanding.

Chapter 5: The effects of short-term and long-term environmental enrichment on locomotion, mood-like behavior, cognition, and hippocampal gene expression

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Research report

The effects of short-term and long-term environmental enrichment on locomotion, mood-like behavior, cognition and hippocampal gene expression

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ABSTRACT

Background: Environmental enrichment (EE) has been shown to modulate behavior and hippocampal gene expression; however, the currently available literature does not explain the differential effects that may relate to the duration of EE.

Aim: To investigate the differential effects of short- and long-term EE on locomotion, anxiety-, depressive- and cognition-like behaviors, and hippocampal gene expression under physiological conditions.

Methods: We assigned either short-term or long-term intervention with respective controls to healthy C57BL/6 mice (n = 12–16/group). The short-term EE group received EE for four weeks starting at eight months of age, while the long-term EE group received EE for six months starting at three months of age. Differential effects of the duration of EE on various behaviors and hippocampal gene expression at nine months of age were measured using an established behavioral battery and high-throughput RT-qPCR, respectively.

Results: Both short-term and long-term EE significantly enhanced locomotion in the home cage and reduced depressive-like behavior in the forced-swim test. Long-term EE, however, reduced locomotion in the open-field test. Additionally, short-term EE reduced the mean body weight and showed anxiolytic effects in the elevated-zero maze (EZM), while these effects were lost after long-term EE. There were no effects of either short-term or long-term EE on the expression of 43 hippocampal genes of interest tested at adjusted $p < 0.05$.

Conclusion: Both short and long-term EE are equally beneficial for baseline locomotor activity and depressive-like behavior. However, long-term EE affects locomotion adversely in a threatening environment and is anxiogenic.

Statement of authorship

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Principal author

Name of Principal Author (Candidate)	Gaurav Singhal		
Contribution to the Paper	Planning and conducting experiments, behavioral and molecular analysis, data collection, organization, analysis and interpretation, writing and editing manuscript.		
Overall percentage (%)	80%		
Certification:	In this paper, the original research that I have conducted during the period of my Higher Degree by Research candidature has been reported. It is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.		
Signature		Date	24/05/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

1. the candidate's stated contribution to the publication is accurate (as detailed above);
2. permission is granted for the candidate in include the publication in the thesis; and
3. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution

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Contribution to the Paper	Assistance with planning and conducting mouse husbandry and laboratory techniques throughout the research period.		
Signature		Date	27/5/19

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Contribution to the Paper	Conception of the project, designing methodology/experimental protocol, teaching behavioral testing in mice techniques, supervising at every step during the research, editing and revision of manuscript drafts.		
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Contribution to the Paper	Conception of the project, supervising every aspect of the research, editing and revision of manuscript drafts.		
Signature		Date	26/11/2019

Preamble to chapter 5

In chapters 3 and 4, we have reported that short-term EE is a stronger modulator of brain functions than PE and PE+EE at late-middle age despite not modifying the expression of hippocampal genes of interest in C57BL/6 mice. Conversely, PE, although it did not show significant effects on the brain functions, modified the expression of hippocampal genes at middle age. It was also clear that the beneficial effects of EE start at middle age and become significant by late-middle age. We were intrigued by the results and this forced us to think if long-term EE could have stronger effects on brain functions than short-term EE in middle-age mice and if this will be accompanied by changes in the hippocampal gene expression. In chapter 1, with supporting evidence, we have also mentioned that past studies used different durations of EE for rodents without providing any rationale for using them. It is possible that the duration of EE may have significant differential effects on brain functions and hippocampal gene expression. We believe that elucidating the differential effects of the duration of EE could also help to formulate better techniques for the enrichment of the external environment for animals in ongoing research.

As such, we investigated the effects of short-term (4 weeks) and long-term (6 months) EE on locomotion, anxiety- and depressive-like behaviors, spatial learning and memory and the 43 genes of interest in the hippocampus in middle age (9 months) C57BL/6 mice. For this, we used standardized behavioral testing techniques and high-throughput qPCR. During this research, we addressed the research question ‘Does duration of EE differentially affects brain functions and hippocampal genes expression in middle age C57BL/6 mice'?’

We hypothesized that long-term EE would show greater effects than short-term EE on brain functions, and hippocampal genes expression in middle age (9 months) male and female C57BL/6 mice compared to age-matched control mice. Before the study commenced, we set forth our aim ‘to investigate the effects of short-term and long-term EE on brain functions, and hippocampal genes expression in middle age (9 months) male and female C57BL/6 mice'.

At the end of treatments, mice were tested for locomotion in the Home Cage and Open Field, anxiety-like behavior in the Elevated Zero Maze and Open Field, depressive-like behavior in the Forced Swim Test, and spatial learning and memory in the Barnes Maze. The changes in hippocampal gene expression were analyzed using high-throughput qPCR. All findings are reported and discussed systematically. The included chapter is the same as the manuscript published in the journal Behavioral Brain Research.

The effects of short-term and long-term environmental enrichment on locomotion, mood-like behavior, cognition, and hippocampal gene expression

Introduction

Environmental Enrichment (EE) is the concept of customizing the environment of captive animals to enhance their physical and psychological well-being by providing stimuli meeting their species-specific needs [1]. Rats and mice raised in an enriched environment have shown enhanced sensory, cognitive and motor abilities [2]. EE also has beneficial effects on experience-dependent cellular plasticity and finds direct clinical relevance to neurological and psychiatric disorders through epidemiological studies [3]. In addition, rodent models of Huntington's disease [4, 5], Alzheimer's disease [6, 7], Parkinson's disease [8], stroke [9], traumatic brain injury [10, 11], multiple sclerosis [12], schizophrenia [13], anxiety [14] and depression [15] have shown improvement in behavioural, cellular and molecular deficits after EE. Of clinical relevance, the effects of EE in rodent studies is comparable to activities in humans that stimulate attention and engagement to the external environment, such as cognitive stimulation [16, 17], cognitive training [18], cognitive rehabilitation [19] and cognitive remediation [20], and in turn improves cognition, memory and quality of life.

In rodents, the environment is enriched with a variety of objects, that may include running wheels, puzzles (mazes, plastic tubes in different configurations) and accessories (toys, ropes, ladders, tunnels, hanging objects, house, ramps, and platforms) to stimulate their attention and engagement in the environment [1, 2]. Since EE is a technique of enriching the environment relative to standard-housed controls, past studies differed significantly in EE protocols, duration of EE treatment and frequency of change of EE to maintain novelty for rodents in cages. The dissimilarities in EE protocols across studies has created uncertainty regarding the viability of EE as an adjunct therapy with other approaches for treating neurological and psychiatric disorders.

Past studies on EE have provided EE to rodents at different ages and for different time intervals reporting varied outcomes. A study that investigated changes in the behavior of C57BL/6 mice after enriching their environment for ten months starting at ten months of age reported significant improvement of learning parameters, exploratory behavior and locomotor activity in enriched mice [21]. The authors attributed the beneficial effects of EE on behavior to a fivefold increase in hippocampal neurogenesis after long-term EE. Ickes et al. who raised rats in enriched environmental conditions from 2 months to 12 months of age found an increase in NGF and BDNF levels in various brain regions, thereby establishing the enhanced expression of neurotrophins in the brain as the reason for the increase in neurogenesis after long-term EE [22]. In another study, EE from weaning to 23 months of age in male Wistar-derived rats reduced their aging-related acquisition and memory deficit [23]. The authors, however, also noted a decrease in the number and size of astrocytes in the hippocampus and corpus callosum, which was in contrast to hypertrophied astrocytes shown by rats kept in standard housing conditions [23], suggestive of neuroprotective effects of long-term EE during old age.

Similarly, EE, when provided for short-term, has also shown varied outcomes across studies. In one of the studies, short-term EE when provided to 21 days old Wistar rats until they turned three months of age improved their spatial abilities and enhanced dendritic arborization and density of dendritic spines in layer-III parietal pyramidal neurons [24]. Interestingly, Segovia et al. reported EE of 8 weeks improved spatial learning in 2 months but not in 25 months old male Wistar rats when tested on the Morris water maze [25]. The authors also observed mixed neurobiological and molecular results, with short-term EE enhanced neurogenesis in the dentate gyrus of both age groups but increased basal extracellular concentrations of neurotransmitters glutamate and GABA in aged rats only and not in young rats [25]. The effects of short-term EE on neurobiology became clearer when a study that investigated the effects of ten days of EE on the brain architecture of streptozotocin-induced type 1 diabetic C57BL/6 mice reported enhanced proliferation, survival and dendritic arborization of newborn neurons, recovered dendritic tree length and spine density of pyramidal CA1 neurons, and increased vascular network in the dentate gyrus [26].

The evidence mentioned above utilized either short-term or long-term EE techniques, however, there are also studies that while not explicitly suggesting differential effects of the duration of EE, have shown varying effects of short-term vs. long-term EE on brain functions. In one such example, one-month-old Long-Evans female rats when housed in enriched conditions and tested at either 3 months, 12 months or 24 months of age, showed improved spatial memory at all ages, but improved attention performance only in aged rats [27]. Likewise, 24 hours of continuous complex enrichment for ten weeks significantly reduced age-related spatial memory decline in 23-month-old male C57BL/6 mice. However such improvement was not observed after 5 min of daily handling or 3 hours of daily basic enrichment [28]. On the contrary, 3-month-old male Wistar rats exposed to 2 hours of EE daily for the first 15 consecutive days, and then 3 hours of EE once per week during the next 18 months, showed reduced recognition memory decline and increased synaptic plasticity and hippocampal neurogenesis [29]. These disparate findings, therefore, raise more questions than answers on the validity of the use of the different duration of EE in research.

EE, therefore, has been known to enhance cognition, visual-spatial attention, spatial memory, and motor coordination, and ameliorate anxiety- and depressive-like behaviors in rodents [27, 28, 30, 31]. However, the differential effects of the duration of treatment with EE has received less attention and not yet been elucidated. In addition, while physical exercise in itself is a known inducer of neurogenesis [32, 33] and modulator of behavioral functions [34-36], adding a running wheel to the enriched environment of rodents in several studies mentioned above has created bias and could be confounding to the results of the effect of EE on behavioral and underlying molecular biology. Nevertheless, some investigators have also explicitly explored the effects of physical activity-independent EE on brain functions. For example, short-term EE of 2, 3 and 6 weeks in the absence of running wheel has been shown to enhance cognition, NGF concentration, neurogenesis and synaptogenesis in young male Wistar rats [37].

Within the present study, we investigated the effects of duration of EE treatment on various brain functions and underlying changes in hippocampal gene expression. The 3-month and 8-month-old C57BL/6 mice were provided with 6 months or 4 weeks of EE (running wheel not included) respectively and then analyzed for changes in anxiety-

and depressive-like behavior, spatial learning and memory and locomotor activity using an established behavioral battery. This was followed by the differential analysis of the 43 target hippocampal genes of interest (GOI) expression associated with immune-, monoaminergic-, growth factors- and HPA axis-pathways. We observed that both short and long-term EE enhance locomotion and reduce depressive-like behavior under baseline circumstances. Interestingly, long-term EE could have adverse effects on locomotion in a threatening environment and may reverse the beneficial effects of short-term EE on anxiety-like behavior. Remarkably, these effects were observed without any change in the expression of GOI in the hippocampus.

Methods

A. Animals

C57BL/6 mice, parental substrain 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. In the time-period of the experiments, the C57BL/6Nhsd line has inbred between 9-13 generations. A total of 58 mice (31 males and 27 females) housed in same-sex groups of 4-5 in individually ventilated cages (IVCs) were used in this study. All mice were given *ad libitum* access to standard laboratory food and water and maintained under controlled conditions of temperature (21±1 ^{o}C), humidity, and a 12-12 hour dark-light cycle. The ethics approval for performing experiments on C57BL/6 mice was received from the University of Adelaide Animal Ethics Committee (M216-12), and all guidelines as prescribed for handling the experimental animals were followed during the study.

B. Experimental design

Mice for short-term EE experiments were housed in IVCs until 7 months of age. Long-term EE experiment mice were housed in IVCs from birth to 12 weeks of age. Once of the desired age, mice that showed no signs of injury and sickness were randomly paired (males and females paired separately), distributed equally into treatment and control groups, and transferred to open top cages (Fig. 5.1). The groups had 12-16 (50 % male and 50 % female) mice. Mice were weighed one at a time on a digital weighing scale while also changing the used cages with clean ones every Friday.

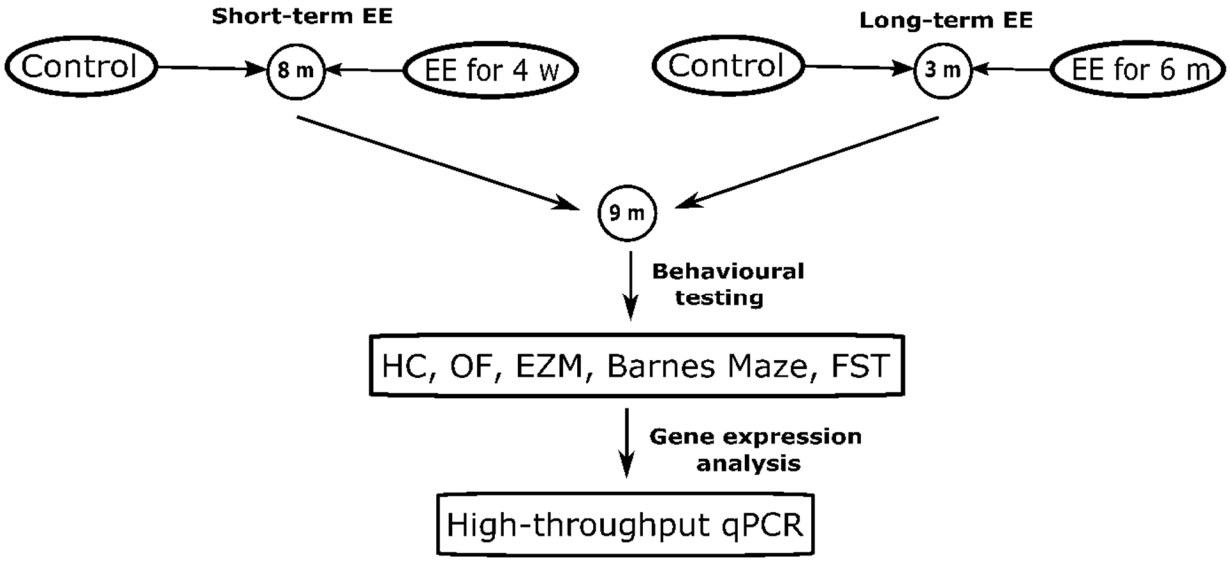

Fig. 5.1 Schematic representation of the experimental design. **Legend:** EE: Environmental Enrichment, HC: Home Cage, OF: Open Field, EZM: Elevated-zero maze, FST: Forced-swim test, m: months, w: weeks.

Control group
Control mice were kept in same-sex pairs in cages with the following dimensions: 48.5cm x 15.5 cm x 12cm. They received no intervention and were given *ad libitum* access to standard laboratory food and water.

EE groups
EE mice were kept in same sex pairs plexiglass cages with dimensions: 37cm x 20.5 cm x 13.5cm, as these had more breadth and depth to provide extra space for the objects associated with EE. The group assigned as short-term EE were provided with a variety of non-toxic plastic and wooden objects (house, colored balls, toys, ladder, and tunnels) and extra bedding as per previously published protocols [4, 24, 38] for 1 month before behavioral testing, i.e., starting at 8 months of age and ending at 9 months of age. The objects were changed once every week to maintain novelty. Similarly, the group assigned as long-term EE were provided with a variety of non-toxic novel objects and extra bedding for 6 months starting at 3 months of age, with objects changed once every week to maintain novelty. The objects remained in the cages throughout the behavioral testing period. Fig. 1.6 shows a representative image of the EE protocol for mice in cages. All EE mice were given *ad libitum* standard laboratory food and water.

C. Behavioral analysis

At 9 months of age mice undertook a behavioral battery following established procedures in the laboratory and as per the below schedule:
Week 1: Home cage, open field
Week 2: Elevated-zero maze
Week 3: Barnes maze
Week 4: Forced-swim test
These tests were planned in order of the least to most stressful. All trials were recorded by a ceiling-mounted camera and analyzed using ANY-maze software ver. 4.70 from Stoelting, USA. F10SC veterinary disinfectant was used to wipe the testing areas between mice to remove any olfactory traces.

Locomotor and Exploratory Behaviour
Home cage activity: Mice were individually tested for general locomotor activity in home cages with two-day-old bedding under basal non-stressful conditions according to previously published protocols [39]. The total distance covered over a five-minute period was recorded as a measure of baseline locomotor activity.

Open Field: Mice were further individually tested for general locomotor activity in the more stressful open field (a brightly lit square arena, 40cm x 40cm, with clear 35cm high walls) according to published protocols [39-41]. The floor was divided into inner (26.6cm x 26.6 cm) and outer (13.4cm x 13.4cm) zones. Total distance traveled over a period of 5 minutes was measured as an indicator of locomotor activity.

Anxiety-like Behaviour

Elevated-zero maze: The elevated-zero maze is a round maze, 105 cm in diameter, with a 5 cm wide platform 40 cm above the floor and divided into four equal quadrants [42]. Two arms have 15 cm high walls (closed), and two are open in alternate order. The mouse was placed on the open arm and allowed to explore for 5 minutes according to published protocols [43]. The time spent by the mouse in the open arms and the number of head dips were recorded as a measure of anxiety in mice. Anxious mice tend to avoid exposure to open arms, spending more time in the closed arms [44].

Open Field: In the Open Field, time spent by mice in the inner and outer zones was also recorded as a measure of anxiety-like behavior. Anxious mouse tends to spend less time in the inner zone [39-41].

Depression-like Behaviour

Forced-swim test: Duration of immobility (when mice were floating with no movement of limbs in any direction) was measured over six minutes in a 4L circular container, 20 cm in diameter and 45 cm high and filled 2/3rd with water at 23-24 degree Celsius, as a measure of despair and depression [44-46]. One trial of 6 mins was conducted per animal to minimize distress associated with repeated testing.

Spatial learning and memory

Barnes Maze: The Barnes maze 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. The false boxes look the same as the target escape box to the mouse but are too small to enter and hence remove visual cues that might be observed through an open hole. Barnes maze procedures were carried out according to published protocols [39, 47, 48]. The mice were placed in the centre of the maze and were allowed to locate the escape box for three minutes. Three trials separated by 15 minutes were performed for each mouse on a day. Latency to locate the escape box over four days of training was used as a measure of spatial learning and visual memory of the mouse. On the fifth day, in a probe trial, the Barnes maze was moved by 90 degrees and mice were allowed to locate the new location of escape box again. Latency to find the new location was used to assess cognitive flexibility [49].

We also calculated the entry errors, i.e., searches of any holes that did not contain the hidden escape box beneath it. This included nose pokes and head deflections over a hole. This analysis was carried out following the protocol published by Nithianantharajah et al. [50].

D. Molecular analyses

Gene expression analysis using Real-time quantitative PCR

The day after the forced swim test, six mice/group (3 male and 3 female) were terminally anesthetized with a lethal intraperitoneal dose of pentobarbital (60 mg/kg IP), brains were extracted, dissected and hippocampal tissues were stored in RNA later (Ambion, Life Technologies) at -80 degrees C until further processing.

Quantification of the levels of mRNA of 43 genes of interest (GOI) across various cytokines, monoamines, neurotrophins and other genes (Supplementary Table 1) was performed using TaqMan assays (Life Technologies, ThermoFisher, Australia) in the high-throughput qPCR system BioMarkHD^{TM} (Fluidigm Inc., USA). Briefly, total RNA

was extracted from HC tissues stored in RNALater 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 20ng of each cDNA sample mixed with pooled TaqMan assays (47 assays, including 4 endogenous reference genes, listed in Table 2.3) and PreAmp Master Mix (Fluidigm Inc., USA). Real-time quantitative PCR was performed for each TaqMan assay for each sample in a 96.96 dynamic array nanofluidic chip (Fluidigm Inc., USA). A total of 47 X 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).

E. Statistical Analysis

Statistical analyses of 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. Comparisons between the treatments (short-term and long-term EE) and respective controls were performed using two-way ANOVA. The multiple comparisons posthoc Holm-Sidak's test was used to confirm significant interactions between groups. Results were presented as mean ± SEM. Differences were considered statistically significant when $p<0.05$.

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 control and treatment groups and adjusted for multiple comparisons using the R package multcomp [51]. Genes were identified as differentially expressed between control and EE groups when the adjusted p values were <0.05 with Z scores indicating the direction of expression change. GeneMANIA (Multiple Association Network Integration Algorithm) prediction server was used to identify the molecular pathways modulated by the differentially expressed genes at false discovery rate (FDR) <0.05 [52].

Results

A. Body weights

Bodyweight of mice was measured once every week from the day of placing them in standard versus enriched cages until they were sacrificed for tissue collection at the end of behavioral testing (Fig 5.2). A two-way ANOVA found the statistically significant main effect of EE treatment ($F_{(1, 55)} = 28.8$; $p< 0.0001$). The posthoc analysis with Holm-Sidak's multiple comparison tests showed mean body weights of the short-term controls was statistically significantly higher than the mean bodyweight of the short-term EE mice (29.7 ± 0.6 vs. 27.0 ± 1.1; $p<0.05$). While the trend was the same for the long-term EE mice ($p = 0.08$), the difference for mean body weight between the controls and treatment groups was found to be not statistically significant in the posthoc analysis.

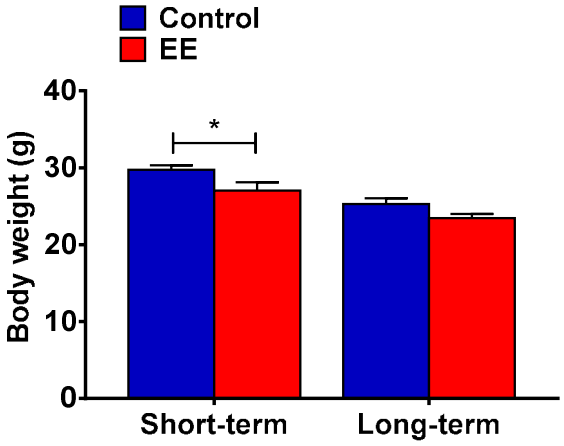

Fig. 5.2 Bodyweights of the short- and long-term EE mice. All data represented as mean ± SEM, n = 12-16 per group. * p< 0.05.

B. Behavior

Locomotor activity

Locomotor activity was measured in the home cage (Fig 5.3 A) and open field (Fig 5.3 B). EE objects were removed while testing baseline locomotion in the home cage to facilitate free movement for the mice. A two-way ANOVA found the statistically significant main effect of the EE treatment ($F_{(1, 53)} = 16.3$; $p<0.001$), but the main effect of the duration of treatment was not statistically significant. Indeed, mice treated with both short-term EE (16.0 ± 1.0 vs. 11.6 ± 0.9; $p<0.01$) and long-term EE (14.8 ± 3.0 vs. 12.5 ± 2.4; $p<0.05$) showed statistically significantly higher locomotor activity than their controls. During the analysis of the effects of the duration of EE on the locomotor activity of different sexes, we found that the female mice that received short-term EE traveled significantly more distance than the short-term female controls in the home cage (18.1 ± 0.9 vs. 12.4 ± 0.9; $p<0.05$; Fig 5.4 A).

Within the more stressful environment of the open field, there was no main effect of EE treatment compared to controls, although the two EE groups were statistically significantly different from each other ($F_{(1, 54)} = 13.05$; $p<0.001$). Short-term EE treated mice traveled statistically significant further than long-term EE treated mice in the open field (15.8 ± 0.8 vs. 11.0 ± 0.8; $p<0.001$). However, this was due to female short-term EE mice traveling significantly more distance than the female long-term EE mice in the open field (15.6 ± 1.4 vs. 10.0 ± 1.5; $p<0.05$; Fig 5.4 B).

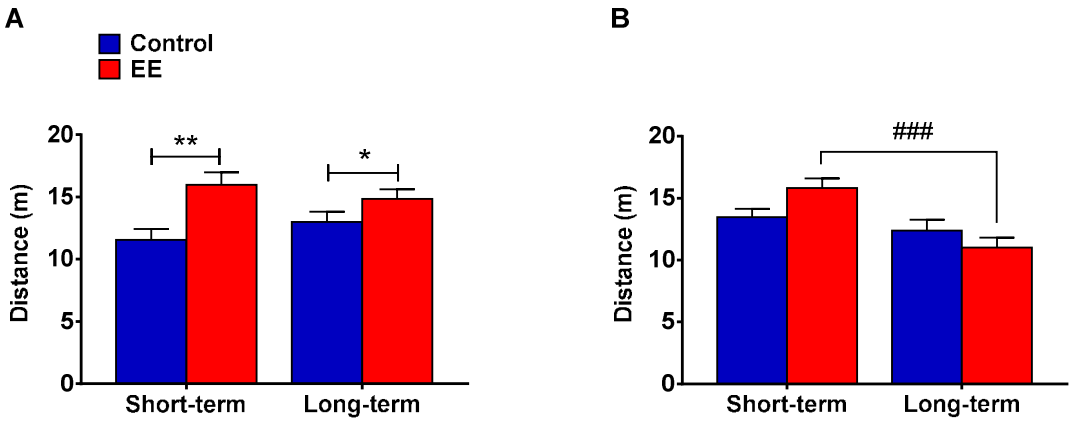

Fig. 5.3 Locomotor activity in Home Cage and open Field. Distance traveled in (A) Home Cage, and (B) Open Field. All data represented as mean ± SEM, n = 12-16 per group. * p< 0.05, ** p<0.01, ### p<0.001.

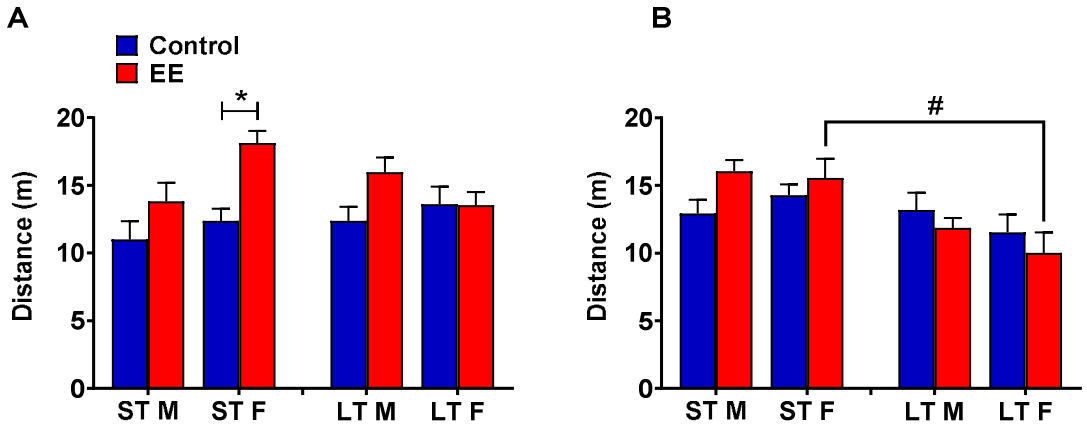

Fig. 5.4 Sex comparisons for locomotor activity. Distance traveled in (A) Home Cage, and (B) Open Field. All data represented as mean ± SEM, n = 6-9 per group. * and # p< 0.05. **Legend:** ST: short-term, LT: long-term, M: males, F: females.

Anxiety-like behavior

Time spent in the open arms of EZM was recorded as a measure of anxiety-like behavior (Fig 5.5 A). Two-way ANOVA showed a statistically significant interaction effect ($F_{(1, 49)}$ = 6.552; p<0.05), with short-term EE treated mice spending statistically significantly more time in the open arms of EZM than long-term EE treated mice (56.5 ± 8.8 vs. 26.3 ± 6.2; p<0.01), however, there was no significant difference between either EE group and their control. This was supported by analysis of the number of head dips in the open arms where a statistically significant interaction was observed ($F_{(1, 53)}$ = 7.098; p<0.05), with short-term EE animals having a higher number of head dips than long-term EE treated mice (29.0 ± 2.8 vs. 20.4 ± 2.3; p<0.05) (Fig 5.5 B). Furthermore, short-term EE mice also had a statistically significantly higher number of head dips than their controls (29.0 ± 2.8 vs. 20.2 ± 2.4; p<0.05). No sexually dimorphic effects of the duration of EE on anxiety-like behavior were noted (p>0.05).

In the Open Field, time spent in the inner zone was taken as the measure of anxiety-like behavior. A two-way ANOVA found the statistically significant main effect of the EE treatment ($F_{(1, 53)}$ = 6.291; p<0.05), as well as the main effect of the duration of treatment ($F_{(1, 53)}$ = 9.249; p<0.01) (Fig. 5.5 C). On posthoc analysis, we observed that

long-term EE spent significantly less time in the inner zone of the open field than long-term controls (150.8 ± 13.74 vs. 112.1 ± 12.06; p<0.05).

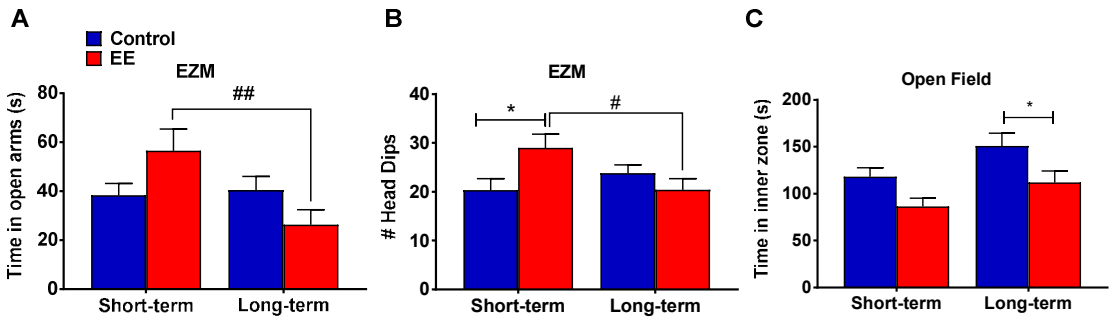

Fig. 5.5 Anxiety-like behavior in EZM and Open Field. (A) EZM: Time spent in the open arms, (B) EZM: Number of head dips, and (C) Open Field: Time spent in the inner zone. All data represented as mean ± SEM, n = 12-16 per group. $^{\#,*}$ p<0.05, $^{\#\#}$ p<0.01.

Spatial memory and Cognition

Spatial memory and cognition were tested on the Barnes maze by measuring the latency to find the escape box over four days of training and probe trial on the fifth day. Data were analyzed using two-way ANOVA, with no statistically significant effects of EE noted on the days of training (Fig 5.6 A and B). This was confirmed with a probe trial on day 5, where no significant differences were seen in identifying the new location of the escape box (Fig 5.6 C).

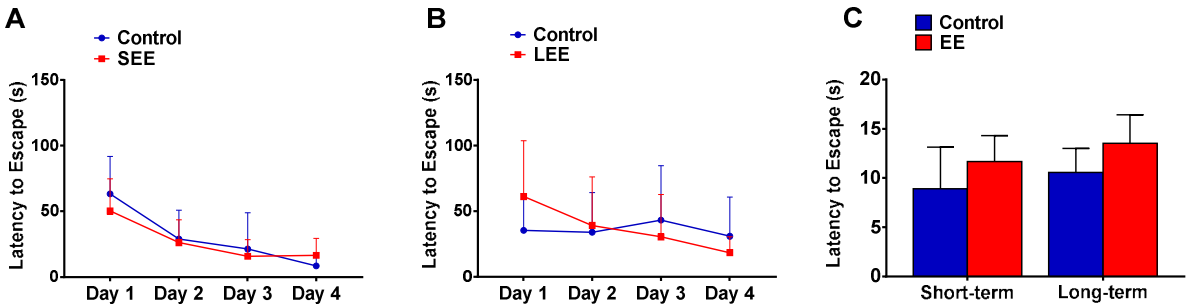

Fig. 5.6 Spatial learning in Barnes maze: Latency to escape. Over four days of training of (A) SEE and (B) and LEE mice, and (C) probe trial. All data represented as mean ± SEM, n = 12-16 per group.

Similarly, no significant differences were observed on the days of training and probe trial in the analysis of the number of entry errors made in order to locate the escape box (Fig 5.7 A-C). Furthermore, no sexually dimorphic effects of the duration of EE on cognition-like behavior in Barnes maze were noted (p>0.05).

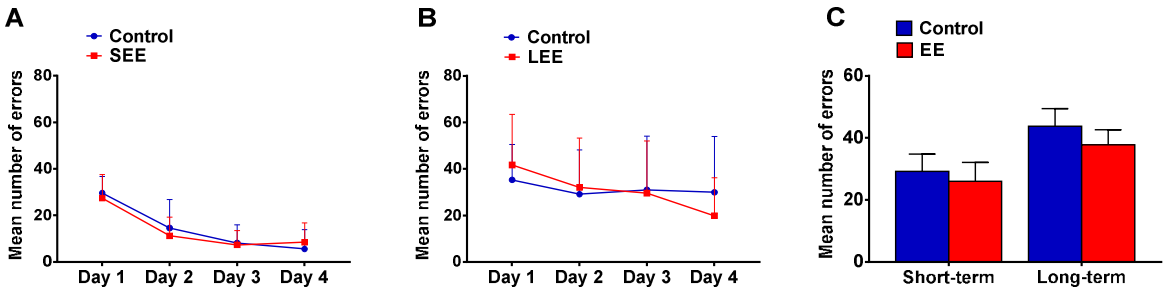

Fig. 5.7 Spatial learning in Barnes maze: Entry errors. (A) SEE and (B), and LEE male and female C57BL/6 mice, and (C) probe trial. All data represented as mean ± SEM, n = 12-16 per group.

Depressive-like behavior

Immobility time in forced swim test is taken as a measure of depressive-like behavior in mice. Two-way ANOVA found no main effect of the duration of treatment. However, it showed statistically significant main effect of treatment ($F_{(1, 52)} = 15.33$; $p<0.001$; Fig 5.8). There was a statistically significant reduction in the immobility time of mice treated with short-term EE compared to their controls in FST (17.0 ± 9.4 vs. 55.4 ± 13.5; $p<0.05$). Similarly, long-term EE also attenuated the immobility time of mice in FST when compared to their controls (12.7 ± 4.0 vs. 53.0 ± 9.8; $p<0.05$). This suggests EE reduces depressive-like behavior irrespective of the duration of enrichment.

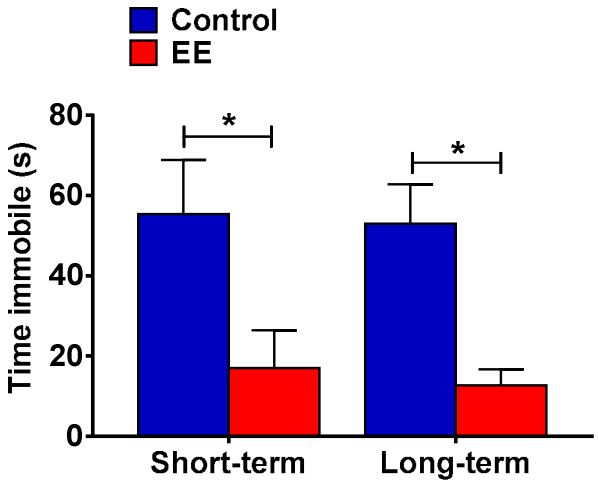

Fig. 5.8 Depressive-like behavior in FST. Immobility time. All data represented as mean ± SEM, n = 12-16 per group. * $p<0.05$.

We also noted the sexually dimorphic effects of the duration of EE on depressive-like behavior in C57BL/6 mice (Fig 5.9). Both short-term and long-term male enriched mice showed significantly lower immobility time than the respective male controls (81.6 ± 20.8 vs. 4.1 ± 2.3; $p<0.01$ and 65.6 ± 10.9 vs. 11.7 ± 5.9; $p<0.05$). While we also noted a very similar trend for female mice, the difference between female EE mice and respective controls were not statistically significant.

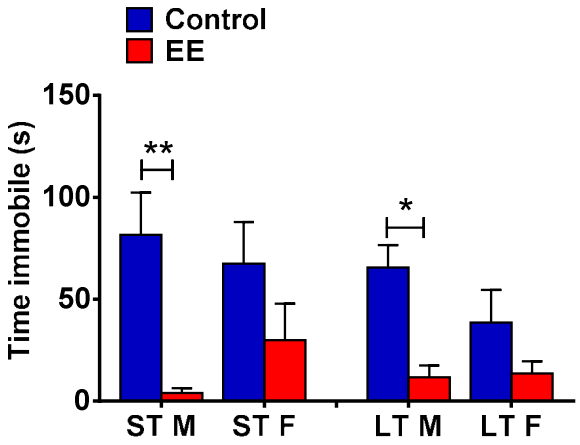

Fig. 5.9 Sex comparisons for depressive-like behavior in FST. Immobility time. All data represented as mean ± SEM, n = 6-9 per group. * p<0.05, * p<0.01. **Legend:** ST: short-term, LT: long-term, M: males, F: females.

C. Molecular analyses

Changes in the expression of hippocampal genes of interest in the of C57BL/6 mice exposed to short or long-term EE

High-throughput qPCR was used to analyze changes in the expression of 43 target hippocampal genes (Table 2.3) in response to both short-term and long-term EE. Differential gene expression analysis of the 43 genes between Control and each treatment group (i.e., SEE and LEE) did not identify genes with an adjusted p-value <0.05 for significance. For the full list of gene expression results, see Supplementary Table 5.1, 5.2, 5.3 and 5.4 where the results are sorted in the order of increasing adjusted p values.

Discussion

Environmental enrichment in rodents has been studied extensively in recent decades for its beneficial effects on behavior as well as changes in cellular and molecular processes. This has enabled a greater understanding of the underlying neurobiological mechanisms that drive altered behaviour. In our study, we investigated the effect of duration of EE on these parameters in healthy 9-month-old C57BL/6 mice, with animals assigned to receive either short-term EE treatment of 4 weeks or long-term EE treatment of 6 months. Regardless of the duration, EE animals showed increased baseline locomotor activity in the home cage and decreased depressive-like behavior in the FST. However, only short-term EE reduced anxiety-like behavior compared to controls, with an increased number of head dips into the open arm, and neither treatment showed any effect on cognition in the Barnes Maze. Interestingly, long-term EE reversed the beneficial effects of short-term EE on locomotion in the threatening environment of the open-field test, as well as on anxiety-like behavior in the EZM. We also found that short-term EE of 4 weeks caused a significant decrease in the body weight of mice when compared to short-term controls. We will discuss these effects pointwise below.

Short-term EE causes a significant decrease in mean body weight

Short-term EE was observed to cause a statistically significant decrease in the body weight of mice when compared to standard-housed controls. A similar trend was seen for long-term EE group, whose mean body weight was lower, but not significantly different than the long-term controls. We observed that our results on the effects of long-term EE on body weight are similar to previously published findings. A study has reported a non-significant reduction in the mean body weight of 1-month old C3H/eB mice after 2.5 months of EE [14]. However, in another study, no significant change in the mean body weights of 3-week-old mice was observed after three months of EE [53]. Taken together, this suggests short-term EE treatment can induce a significant decrease in mean body weight. However, this may become non-significant after long-term EE treatment.

Long-term EE affects baseline locomotion in a threatening environment

Both short-term and long-term EE treatments enhanced baseline locomotor activity of the mice in a home cage. However, no significant differences in locomotor behavior relative to controls were noted in the more challenging environment of the open field. This validates the previously published findings where EE of 2-2.5 months when provided since weaning day (day 22 ± 1), caused a significant increase in the baseline locomotor activity of mice in the home cage [54, 55]. Similar to our results, no significant change in the locomotor behavior of mice was observed by researchers in the challenging environment of the open field after 6 and 12 months of EE treatment, which they attributed to locomotor habituation [56]. Interestingly, long-term EE mice traveled significantly shorter distance than short-term EE mice in the open field, thus suggesting that locomotor activity in a threatening environment is dependent on the duration of EE, with short-term EE leading to an increase in locomotion relative to the long-term EE group.

Our results are also suggestive of the sexually dimorphic effects of the duration of EE on locomotion, i.e., short-term EE significantly enhanced the locomotion of the middle-aged female C57BL/6 mice but had no significant effects on the locomotion of age matched male mice. On the other hand, long-term EE has no significant effects on the locomotion of both middle-aged male or female C57BL/6 mice.

However, it is to be noted that the home cage test was conducted in cages of different sizes for controls and EE mice as described for housing in the methods section. It is, therefore, possible that differences in distance traveled may be due to differences in home cage size during testing and not due to the effects of EE. The home cages for controls mice provided less space to mice to move compared to home cages for EE mice. This could be a confounding variable during the home cage experiment and possibly a reason why EE mice traveled more than controls mice. A further trial using similar dimension cages for both controls and EE mice may be needed to validate our findings.

While short-term EE is anxiolytic, long-term EE is anxiogenic in middle age

In regards to anxiety-like behavior, we observed that only short-term EE treatment improves anxiety-like behavior, with these mice showing increased interest in the open arm of EZM with a significantly higher number of head dips than short-term controls. This again complies with the previously published findings whereby short-term EE of 2 weeks eliminated stress-induced anxiety phenotype in 7-week-old male Wistar rats

[57]. Interestingly, the effects of short-term EE on anxiety-like behavior in middle-aged mice has not previously been investigated.

Although not significant in the EZM, long-term EE of 6 months showed anxiogenic effects. Similarly, in the Open field, LEE mice spent significantly less time in the inner zone, hence showed significantly higher anxiety-like behavior compared to LEE controls mice. This contrasts previously published findings of 6, 11 and 12 months of EE showing anxiolytic effects in C57BL/6 mice [56, 58]. However, both the above studies also used running wheels to enrich the environment of mice. Physical exercise has been shown to reduce anxiety-like behavior in some rodent studies [59, 60], although there is also evidence to suggest that physical exercise could be anxiogenic [61] or have no effects on anxiety-like behavior [62].

Nonetheless, we observed significant differences between the short-term and long-term EE mice, with the latter showing more anxiety-like behavior than the former. This suggests that long-term EE reverses the beneficial effect of short-term EE on anxiety-like behavior during middle age. This effect could be attributed to overdependence of mice on EE after long-term exposure, resulting in increased anxiety-like behavior in the threatening environment of EZM which is devoid of all enrichment.

EE reduces depressive-like behavior but does not affect spatial learning and memory irrespective of the duration

Both short-term and long-term EE significantly reduced the depressive-like behavior, consistent with all previous findings [15, 63, 64]. Interestingly, the difference was statistically significant only for male mice, suggestive of EE, irrespective of duration, affects male mice more than female mice while ameliorating depressive-like behavior. We found no change in cognitive functionality after both short-term and long-term EE when tested on Barnes Maze over four days of training. This may be because naïve early-middle-age mice were exposed to EE, as opposed to aged mice. Previous research, on the other hand, showed improved cognition and spatial memory in aged mice after EE. For example, exposure of aged (21-23 months) C57BL/6 mice to 24-hour EE for ten weeks improved spatial learning and cognitive abilities [28]. Likewise, EE mitigated cognitive deficits in aged mouse models of Alzheimer's disease [6, 65].

Both short- and long-term EE do not alter the expression of hippocampal GOI in middle aged C57BL/6 mice

The external environment has been shown to influence gene expression and gene-gene interactions, thereby suggesting that modulation of the external environment could be used as a tool to modify gene expression in the brain [66]. EE has been shown to modulate the expression of genes in the hippocampus that regulate neuronal growth, structure, and functions [67].

In this context, we analyzed the change in the expression of hippocampal genes associated with immune, monoamine, transcription, growth metabolism and HPA-axis activity in response to short and long-term EE. We found no significant differences in expression of the 43 genes of interest after both short-term and long-term EE at adjusted $p<0.05$. This could be due to the absence of running wheels in our EE paradigms or differences in the delivery of enrichment such as box dimensions, number/type of toys (i.e., quantity, quality and complexity of EE) and how frequently they were changed (i.e., the novelty of EE). Indeed, a study has reported that long-

term home-cage EE, in the absence of running wheels, has limited transcriptomic effects genome-wide [68]. Also, studies that reported upregulation in the expression of genes associated with synaptic plasticity, transcription regulation and T cell differentiation after both short-term and long-term EE also used running wheel to enrich the environment of rodents [58, 69]. More research into individual actions of running wheels and EE with a variety of toys and other accessories on gene expression may be required to confirm if running wheels confounds gene expression results in EE studies.

However, we observed that a set of genes differentially expressed at non-adjusted p<0.05. These genes were *Uqcrc1, Tnfrsf1a, Htr2a, Il1r1, Gfap, Foxo3, Sod1, Ifng, Gpx1, Crhr1, Sod2, Il1b*. This suggests that the duration of EE might have mild effects on the expression of hippocampal genes associated with mitochondrial electron transport chain, cellular respiration, proton transport, oxidative phosphorylation, expression of 5-HT (serotonin) receptors and regulation of inflammatory response. It is possible that the age of the mice (9-month) and absence of running wheels during our experiments might have affected our gene expression results. For instance, differential expression of the genes involved in inflammatory response, neuronal structure and signaling, neuropeptide metabolism, amyloid precursor protein processing, and neuronal apoptosis has been reported in the hippocampus of 18 to 24 months old mice, contributing to age-related memory deficits and neurodegenerative diseases [70, 71]. However, EE reversed the effects of aging on hippocampal gene expression, correcting learning and memory deficits [71]. The presence of running wheels has also been shown to modulate hippocampal gene expression and enhance cognition in C57BL/6 mice [72, 73].

Supplementary Tables

ST 5.1 Gene expression in SEE compared to SC

Gene	Estimate	Std. Error	Z	p values	adj. p
Uqcrc1	-1.022691336	0.441241011	-2.317761293	0.020462298	0.430434548
Igf1	-0.481189613	0.253870779	-1.895411573	0.058037897	0.623907389
Tnf	0.74478957	0.402886056	1.848635758	0.064510426	0.652693719
Tnfrsf1a	-0.435335572	0.253778127	-1.715418022	0.086268614	0.676121068
Htr2a	-0.574788775	0.338098589	-1.700062623	0.089119147	0.676121068
Crhr1	-0.513310076	0.339705537	-1.511044184	0.130777194	0.676121068
Htr1b	-0.414140992	0.299472637	-1.382900942	0.166695242	0.697432747
Gpx1	-0.539375111	0.392868	-1.372916884	0.169778187	0.697432747
Nlrp3	-0.261085237	0.190904342	-1.367623356	0.171430002	0.697432747
Il1b	0.536856079	0.407748002	1.316636933	0.187960353	0.697432747
Smad2	-0.196751653	0.1512426	-1.300901021	0.193292337	0.697432747
Sod2	-0.181517055	0.151774454	-1.195965792	0.231709909	0.751964232
Cat	-0.190750802	0.164306063	-1.160948039	0.245663031	0.757653367
Ifng	-0.638487874	0.566332396	-1.127408352	0.259569869	0.757653367
Prkaa1	-0.335804375	0.298264182	-1.125862222	0.260223842	0.757653367
Il10	0.89640027	0.83238599	1.076904561	0.281522905	0.757653367

Prkaa2	-0.275535057	0.258672553	-1.06518861	0.286790587	0.757653367
Cs	-0.23806843	0.23158049	-1.02801592	0.303942339	0.757653367
Tnfrsf1b	-0.436088771	0.432680889	-1.007876202	0.313513898	0.763893198
Sod1	-0.186788846	0.201000554	-0.929295179	0.352736133	0.784658903
Creb1	-0.203431437	0.27994494	-0.726683744	0.467419714	0.883474625
Slc6a3	-1.316093153	1.887728927	-0.697183337	0.485688066	0.904224588
Foxo3	-0.180019335	0.291439064	-0.617691166	0.536778936	0.91749036
Sirt1	-0.13025532	0.215479168	-0.604491475	0.545516935	0.91749036
Aif1	0.108519634	0.185246245	0.585812866	0.558001277	0.919379235
Grin2a	0.204453578	0.371493632	0.550355539	0.582075538	0.929823737
Il1r1	0.184482414	0.345169071	0.534469711	0.5930166	0.935769313
Tph2	0.229670736	0.461830448	0.497305315	0.618973764	0.938742034
Ntrk1	-0.537335289	1.094259779	-0.491049109	0.623391714	0.938742034
Il6	0.173021325	0.353593536	0.489322648	0.62461329	0.938742034
Ngf	0.104634139	0.215722274	0.485040961	0.62764729	0.938742034
Nr3c1	-0.073863422	0.166313085	-0.444122735	0.65695385	0.94753292
Foxp3	0.225669919	0.66038533	0.34172461	0.73255815	0.978008579
Crh	-0.082586173	0.25668498	-0.321741354	0.747648646	0.978008579
Gfap	-0.10816359	0.349401093	-0.309568552	0.756889074	0.978008579
Bdnf	-0.071346867	0.255828254	-0.278885799	0.780332467	0.978008579
Smad3	0.090647171	0.363884623	0.249109649	0.803275966	0.978008579
Htr1a	-0.052021495	0.280588946	-0.185401083	0.852914459	0.978008579
Gria2	0.043395494	0.290200312	0.149536346	0.881130432	0.981106744
Slc6a4	0.087681254	0.823549754	0.106467464	0.915211469	0.981106744
Il12a	0.013429633	0.21985466	0.061084139	0.951292201	0.981106744
Actb	-0.007202867	0.180426188	-0.039921407	0.968155784	0.984861819
Gria1	0.006219086	0.272619665	0.022812316	0.981799984	0.984861819

Legend: 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 SEE and SC. Z: Z-score, is the number of standard deviations from the mean of all the values within the same gene. Positive Z-score indicates the upregulation of gene expression in SEE compared to SC. Negative Z-score indicates the downregulation of gene expression in SEE compared to SC. adj. p: adjusted p-value.

ST 5.2 Gene expression in LEE compared to LC

Gene	Estimate	Std. Error	Z	p value	adj. p
Foxo3	-0.671129753	0.306112438	-2.192428891	0.028348552	0.446232204
Htr2a	-0.701246575	0.355038093	-1.975130526	0.048253337	0.623907389
Il1r1	-0.701718707	0.362473505	-1.935917238	0.052877838	0.623907389
Creb1	-0.467388461	0.294007111	-1.58971822	0.111898335	0.676121068
Prkaa2	-0.422359952	0.271658293	-1.554747133	0.120006305	0.676121068
Grin2a	-0.603404485	0.389961857	-1.547342321	0.121780722	0.676121068
Prkaa1	-0.444770657	0.313225406	-1.419969926	0.155616437	0.697432747

Sod1	0.282610836	0.211089831	1.33881786	0.180629975	0.697432747
Ifng	0.721573289	0.544114566	1.326142201	0.1847926	0.697432747
Gria2	-0.399488787	0.304737572	-1.31092725	0.189882343	0.697432747
Cs	-0.309060295	0.243248953	-1.270551389	0.20388829	0.715689507
Smad3	-0.46722859	0.382043103	-1.222973497	0.221339707	0.746479011
Bdnf	0.296102854	0.268679015	1.102069152	0.270431609	0.757653367
Tnf	-0.464036445	0.430703311	-1.077392333	0.281305028	0.757653367
Sirt1	-0.241155798	0.226323444	-1.065536092	0.286633402	0.757653367
Igf1	-0.277041653	0.266652819	-1.03896015	0.29882327	0.757653367
Aif1	0.195256377	0.194458126	1.004104999	0.315328006	0.763893198
Il12a	0.222252305	0.230933831	0.962406868	0.335845265	0.784658903
Tnfrsf1b	-0.431295442	0.454457297	-0.949034034	0.342603303	0.784658903
Actb	-0.179079509	0.189489906	-0.945060941	0.344627764	0.784658903
Smad2	-0.138054881	0.158763556	-0.869562794	0.384539378	0.787390154
Tph2	0.394772944	0.49558413	0.796581085	0.425694366	0.841602655
Htr1a	-0.226438339	0.294675489	-0.768432895	0.442230043	0.860331117
Sod2	0.110021588	0.159321858	0.690561793	0.489840965	0.904224588
Gria1	-0.185638243	0.28628413	-0.648440566	0.51670004	0.91749036
Crh	0.171845629	0.269613985	0.63737654	0.523879606	0.91749036
Htr1b	-0.200207639	0.314516895	-0.636556072	0.524414046	0.91749036
Slc6a3	-1.216446453	2.032089897	-0.598618424	0.549427367	0.91749036
Tnfrsf1a	0.152922988	0.266467896	0.573888978	0.566042932	0.919379235
Nlrp3	0.101518458	0.200397588	0.50658523	0.612445874	0.938742034
Slc6a4	0.395497081	0.858302226	0.460789998	0.644949278	0.94753292
Ntrk1	-0.554613765	1.227043893	-0.451991789	0.651274898	0.94753292
Foxp3	-0.227296071	0.701257201	-0.324126541	0.745842234	0.978008579
Ngf	0.058474642	0.226551432	0.258107582	0.79632388	0.978008579
Uqcrc1	0.117072112	0.46322781	0.252731182	0.80047595	0.978008579
Gfap	-0.07977336	0.367008361	-0.217361151	0.827926904	0.978008579
Il6	-0.079665384	0.379436535	-0.209957073	0.833701177	0.978008579
Gpx1	0.085218263	0.412619078	0.20653011	0.836376848	0.978008579
Crhr1	-0.072627746	0.356815761	-0.203544108	0.838709774	0.978008579
Il10	-0.085583631	1.361420818	-0.062863466	0.949875227	0.981106744
Il1b	0.024360528	0.428181203	0.056893035	0.954630403	0.981106744
Nr3c1	-0.009506464	0.174644328	-0.054433285	0.956589961	0.981106744
Cat	-0.003272578	0.172476636	-0.018974034	0.984861819	0.984861819

Legend: 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 LEE and LC. Z: Z-score, is the number of standard deviations from the mean of all the values within the same gene. Positive Z-score indicates the upregulation of gene expression in LEE compared to LC. Negative Z-score indicates the downregulation of gene expression in LEE compared to LC. adj. p: adjusted p-value.

ST 5.3 Gene expression in SEE compared to LEE

Gene	Estimate	Std. Error	Z	p values	adj. p
Il1b	1.110985541	0.436315509	2.546289366	0.01088749	0.430434548
Grin2a	0.695687925	0.391679802	1.776164922	0.075705738	0.676121068
Gfap	0.658524975	0.371741748	1.771458223	0.076484534	0.676121068
Cat	-0.309774455	0.176314658	-1.756941025	0.078927862	0.676121068
Tnf	0.713605841	0.430703311	1.656838532	0.097552124	0.676121068
Actb	0.314500968	0.19280825	1.631159286	0.102856714	0.676121068
Foxp3	1.134924553	0.701257201	1.618414117	0.105573382	0.676121068
Igf1	-0.427862365	0.269545615	-1.587346781	0.112434125	0.676121068
Htr1a	0.467828013	0.297168517	1.57428525	0.115421518	0.676121068
Gria2	0.472427743	0.306958061	1.539062834	0.123788957	0.676121068
Smad3	0.518622433	0.384213518	1.349828698	0.177070937	0.697432747
Slc6a3	-2.594853539	1.997941425	-1.29876357	0.19402508	0.697432747
Il12a	-0.254345444	0.233959589	-1.087134086	0.276977556	0.757653367
Tnfrsf1b	0.496064113	0.459182322	1.080320581	0.279999448	0.757653367
Gria1	0.25929047	0.288451609	0.898904573	0.368703492	0.784658903
Crhr1	0.324222524	0.362121342	0.895342214	0.370604181	0.784658903
Aif1	0.173242	0.198785289	0.871503123	0.383479501	0.787390154
Nlrp3	-0.152410492	0.204856918	-0.743985088	0.456885497	0.873158949
Htr1b	0.216940527	0.317307083	0.683692673	0.494169251	0.904224588
Ngf	-0.149497313	0.230626293	-0.648223197	0.516840601	0.91749036
Gpx1	0.259338539	0.419602956	0.618056988	0.536537774	0.91749036
Prkaa1	-0.172946459	0.315722342	-0.547780235	0.583842811	0.929823737
Htr2a	0.17746101	0.35765381	0.496180958	0.619766745	0.938742034
Uqcrc1	0.215794679	0.473190606	0.456041765	0.648359945	0.94753292
Il6	0.166359043	0.379436535	0.438437071	0.661069479	0.94753292
Bdnf	0.11568355	0.271048843	0.426799647	0.669525264	0.951721863
Sirt1	-0.080279807	0.229936618	-0.349138855	0.726985068	0.978008579
Sod2	0.046030664	0.162867154	0.282627057	0.777462737	0.978008579
Il1r1	0.096736153	0.36525626	0.264844612	0.791129168	0.978008579
Nr3c1	-0.046846015	0.177996122	-0.263185594	0.792407534	0.978008579
Tph2	-0.129508969	0.49558413	-0.261325901	0.793841194	0.978008579
Cs	-0.059907856	0.246222376	-0.24330793	0.807766862	0.978008579
Prkaa2	0.061678996	0.273949946	0.225146954	0.821864955	0.978008579
Slc6a4	0.187534154	0.864585077	0.216906535	0.828281184	0.978008579
Crh	0.051431852	0.272701519	0.188601268	0.850405339	0.978008579
Foxo3	0.057815361	0.309428631	0.186845545	0.851781736	0.978008579
Sod1	0.037932303	0.214898006	0.17651305	0.859890897	0.979478373
Tnfrsf1a	-0.045091941	0.271814598	-0.165892271	0.86824173	0.981106744
Il10	-0.101518561	0.899581145	-0.112850921	0.910148747	0.981106744
Ntrk1	-0.113689723	1.227043893	-0.092653346	0.926178962	0.981106744
Creb1	0.017570066	0.296600078	0.059238238	0.952762354	0.981106744
Ifng	0.029618719	0.566332396	0.052299178	0.958290308	0.981106744

Smad2	-0.004704724	0.162296429	-0.028988466	0.97687379	0.984861819

Legend: 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 SEE and LEE. Z: Z-score, is the number of standard deviations from the mean of all the values within the same gene. Positive Z-score indicates the upregulation of gene expression in SEE compared to LEE. Negative Z-score indicates the downregulation of gene expression in SEE compared to LEE. adj. p: adjusted p-value.

ST 5.4 Gene expression in SC compared to LC

Gene	Estimate	Std. Error	Z	p values	adj. p
Uqcrc1	1.355558127	0.430539527	3.148510281	0.00164105	0.282260515
Sod1	0.507331984	0.196923809	2.576285658	0.00998681	0.430434548
Ifng	1.389679881	0.544114566	2.554020728	0.010648692	0.430434548
Il1r1	-0.789464968	0.342223018	-2.306872791	0.021061913	0.430434548
Gpx1	0.883931913	0.385399959	2.293544386	0.021816683	0.430434548
Crhr1	0.764904855	0.334044118	2.289831828	0.022031067	0.430434548
Sod2	0.337569308	0.147963609	2.281434678	0.022522738	0.430434548
Tnfrsf1a	0.54316662	0.248042943	2.189808803	0.028538106	0.446232204
Gfap	0.686915205	0.344360761	1.99475458	0.046069653	0.623907389
Bdnf	0.483133271	0.253316073	1.907234963	0.056490168	0.623907389
Foxo3	-0.433295056	0.287915743	-1.504937009	0.132340272	0.676121068
Il1b	0.59848999	0.399031768	1.499855493	0.133651839	0.676121068
Htr1b	0.43087388	0.296514676	1.453128345	0.146188122	0.697432747
Aif1	0.259978743	0.180594969	1.439568025	0.149989651	0.697432747
Slc6a3	-2.495206839	1.923834689	-1.29699649	0.194632395	0.697432747
Tnf	-0.495220173	0.402886056	-1.229181715	0.219003683	0.746479011
Crh	0.305863654	0.253402371	1.207027594	0.227421517	0.751964232
Tnfrsf1b	0.500857442	0.427663164	1.171149365	0.241538744	0.757653367
Nlrp3	0.210193203	0.186110999	1.129396992	0.258730401	0.757653367
Htr1a	0.293411168	0.277947249	1.055636165	0.291134472	0.757653367
Foxp3	0.681958564	0.66038533	1.032667646	0.30175946	0.757653367
Prkaa1	-0.281912741	0.295619824	-0.953632736	0.340269579	0.784658903
Ngf	-0.195656809	0.21136027	-0.925702873	0.354600414	0.784658903
Sirt1	-0.191180285	0.211619292	-0.903416148	0.366305073	0.784658903
Igf1	-0.223714404	0.250797248	-0.892012996	0.372385967	0.784658903
Creb1	-0.246386958	0.27719622	-0.888853963	0.37408157	0.784658903
Il10	-1.083502462	1.317985904	-0.822089568	0.411025923	0.831723043
Actb	0.142624326	0.176875697	0.806353436	0.420039087	0.840078175
Cat	-0.122296231	0.160180566	-0.763489815	0.445171334	0.860331117
Slc6a4	0.495349981	0.816951377	0.606339613	0.544289259	0.91749036
Cs	-0.130899722	0.228416545	-0.573074606	0.56659418	0.919379235
Smad2	0.053992047	0.147445109	0.366184049	0.714227744	0.978008579
Prkaa2	-0.085145899	0.256244307	-0.332284062	0.739674773	0.978008579

Grin2a	-0.112170138	0.369681892	-0.303423404	0.761567212	0.978008579
Gria1	0.067433141	0.270325275	0.249451855	0.803011278	0.978008579
Il6	-0.086327667	0.353593536	-0.244143792	0.807119457	0.978008579
Il12a	-0.045522771	0.216631984	-0.210138736	0.833559393	0.978008579
Htr2a	0.05100321	0.335330368	0.152098392	0.879109333	0.981106744
Ntrk1	-0.130968198	1.094259779	-0.119686568	0.904731441	0.981106744
Smad3	-0.039253327	0.361592207	-0.108556896	0.913553951	0.981106744
Nr3c1	0.017510943	0.162720816	0.107613414	0.914302356	0.981106744
Gria2	0.029543461	0.287850582	0.10263471	0.918252893	0.981106744
Tph2	0.035593238	0.461830448	0.077069925	0.938567918	0.981106744

Legend: 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 SC and LC. Z: Z-score, is the number of standard deviations from the mean of all the values within the same gene. Positive Z-score indicates the upregulation of gene expression in SC compared to LC. Negative Z-score indicates the downregulation of gene expression in SC compared to LC. adj. p: adjusted p-value.

Research summary

In this chapter, we investigated the effects of the duration of EE on brain functions and changes in the expression of 43 hippocampal genes of interest in middle age C57BL/6 mice. The mice were housed in controlled environmental conditions and once of the desired age, i.e., 3 or 8 months, they were provided with either 6 months or 4 weeks of EE respectively. At the end of short- and long-term treatments, mice were tested for various behaviors using a standardized behavioral battery. Following this, the mice were sacrificed, their brains were extracted, dissected and the hippocampal tissues were collected for the analysis of changes in hippocampal gene expression using high-throughput qPCR.

We hypothesized long-term EE to be beneficial and elicit stronger effects on brain functions and hippocampal gene expression than short-term EE. However, the long-term EE did not show stronger effects on brain functions than short-term EE. Furthermore, like short-term EE, long-term EE also did not modify the expression of 43 hippocampal genes of interest. Interestingly, we observed that while short-term EE is anxiolytic, long-term EE could be anxiogenic during middle age.

The results are important since they suggest that short-term EE is better for modulating the brain functions than long-term EE. This, we find, could contrast with the effects of PE that we have reported in chapter 3. Unlike EE, PE in the long-term could be more beneficial than short-term PE. Again, this could be attributed to the hippocampal neurogenesis via dissociable pathways after PE and EE. EE increases the likelihood of survival of new cells and PE increases the level of proliferation of progenitor cells in the hippocampus. This research we conducted is one of the few where particularly the effects of the duration of the EE has been investigated. Our findings are exciting and add to the current understanding of the effects of EE on brain functions and hippocampal gene expression.

Chapter 6: Duration of environmental enrichment determines astrocyte numbers and cervical lymph node T lymphocyte proportions but not microglial numbers in middle-aged C57BL/6 mice

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Duration of Environmental Enrichment Determines Astrocyte Number and Cervical Lymph Node T Lymphocyte Proportions but Not the Microglial Number in Middle-Aged C57BL/6 Mice

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Environmental enrichment (EE) has been shown to modulate behavior and immunity. We recently reported that both short and long-term EE enhance baseline locomotion and alleviate depressive-like behavior, but only long-term EE affects locomotion adversely in a threatening environment and enhances anxiety-like behavior in middle-age mice. We have now investigated whether the observed changes in behavior after short- and long-term EE were associated with underlying immune changes. Hence, at the end of behavioral testing, mice were sacrificed, and brains and cervical lymph nodes were collected to investigate the differential effects of the duration of EE (short- and long-term) on the number of immunopositive glial cells in the dentate gyrus, CA1, CA2, and CA3 regions of the hippocampus and proportions of T cell subsets in the cervical lymph nodes using immunohistochemistry and flow cytometry, respectively. EE, regardless of duration, caused an increase in microglia number within the dentate gyrus, CA1 and CA3 hippocampal regions, but only long-term EE increased astrocytes number within the dentate gyrus and CA3 hippocampal regions. A significantly higher proportion of $CD8^+$ naive T cells was observed after long-term EE vs. short-term EE. No significant differences were observed in the proportion of central memory and effector memory T cells or early activated $CD25^+$ cells between any of the test groups. Our results suggest that EE, irrespective of duration, enhances the numbers of microglia, but long-term EE is required to modify astrocyte number and peripheral T cell proportions in middle-aged mice. Our findings provide new insights into the therapeutic effects of EE on various brain disorders, which may be at least partly mediated by glial and neuroimmune modulation.

Keywords: environmental enrichment, immune, brain, microglia, astrocytes, T cells

Statement of Authorship

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Contribution to the Paper	Planning and conducting experiments, behavioral and molecular analysis, data collection, organization, analysis and interpretation, writing and editing manuscript.		
Overall percentage (%)	80%		
Certification:	In this paper, the original research that I have conducted during the period of my Higher Degree by Research candidature has been reported. It is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.		
Signature		Date	24/05/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

1. the candidate's stated contribution to the publication is accurate (as detailed above);
2. permission is granted for the candidate in include the publication in the thesis; and
3. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution

Name of Co-Author	Julie Morgan
Contribution to the Paper	Assistance with planning and conducting mouse husbandry and laboratory techniques throughout the research period.

Signature		Date	27/5/19

Name of Co-Author	Dr. Magdalene C Jawahar		
Contribution to the Paper	Designing methodology/experimental protocol, supervising at every step during the research, editing and revision of manuscript drafts.		
Signature		Date	21/11/19

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Contribution to the Paper	Conception of the project, teaching immunohistochemistry techniques, designing methodology/experimental protocol, supervising at every step during the research, editing and revision of manuscript drafts.		
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Contribution to the Paper	Conception of the project, editing and revision of manuscript drafts.		
Signature		Date	27/05/2019

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Contribution to the Paper	Conception of the project, supervising every aspect of the research, editing and revision of manuscript drafts.		
Signature		Date	26/11/2019

Preamble to chapter 6

In chapter 4, we reported that short-term EE is a strong modulator of the neuroimmune functions and reverses the effects of aging on microglia and peripheral T cells at middle age, in turn, enhancing cytotoxic immunity in a controlled environment. This could be the reason for the observable changes in brain functions after EE. In chapter 5, we showed that long-term EE is not a stronger modulator of brain functions than short-term EE. Instead, long-term EE was found to be anxiogenic at middle age. Again, as mentioned in chapter 1, different duration of the EE has been used in past studies, so it was important to identify the underlying changes in brain glial cells and peripheral T cells after long-term EE and to compare them with the changes after short-term EE. We expected that, like in chapter 4, deducing the differential effects of the duration of EE on brain glial cells and peripheral T cells could provide an explanation for the observed functional changes after short- and long-term EE. This will also help to develop a better understanding of the EE paradigms.

Hence, during this study, we investigated the effects of short-term (4 weeks) and long-term (6 months) on changes in neuroimmune functions in middle age C57BL/6 mice. For this, we used immunohistochemistry to analyze the change in immunopositive microglia and astrocytes count in the dentate gyrus, and fluorescent activated cell sorting to analyze the changes in the total number of T cells, $CD4^+$ and $CD8^+$ T cell subpopulations (Naïve or T_N, Central memory or T_{CM} and Effector memory or T_{EM}), and early activated T cell phenotype CD25 in the cervical lymph nodes. We addressed the research question “Does duration of EE has any differential effect on brain immunopositive glial cells count, and peripheral T cells count and phenotype?” during this research. All our mice during this study were aged until middle age, and therefore, only the beneficial effects of glial cells were expected. We, therefore, hypothesized that any beneficial effects of the duration of treatment on brain functions would be accompanied by an increase in the number of $IBA1^+$ microglia and $GFAP^+$ astrocytes in the dentate gyrus, and that long-term EE will show greater effects than short-term EE on the T cells and subsets count in the cervical lymph nodes of middle-age male and female C57BL/6 mice compared to age-matched control mice. Hence, the aim of the study was “to investigate the effects of short- and long-term EE on brain immunopositive glial cells count, and T cells and phenotype count in the cervical lymph nodes of early (4 months), middle (9 months) and late-middle age (14 months) male and female C57BL/6 mice”.

Mice were tested for change in glial cells count in the dentate gyrus, and T cells and phenotype count in the cervical lymph nodes using immunohistochemistry and fluorescent activated cell sorting laboratory techniques, respectively. All findings are reported and discussed systematically. The included chapter is the same as the manuscript published in the journal Frontiers of Cellular Neuroscience.

Duration of environmental enrichment determines astrocyte numbers and cervical lymph node T lymphocyte proportions but not microglial numbers in middle-aged C57BL/6 mice

Introduction

Environmental enrichment in rodents has been studied extensively in the last decade for its beneficial effects on neuroimmune mechanisms that enhanced our understanding and approach in dealing with psychiatric disorders. EE promotes cognitive stimuli in the experimental animals with the use of running wheels, novel objects, puzzles (mazes, plastic tubes in different configurations) and accessories (toys, ropes, ladders, tunnels, hanging objects, house, ramps, and platforms) [31]. Brain functions including behavior, cognition, and locomotion, improve after EE [22, 338-340, 342, 374, 375, 379, 606]. EE has also shown beneficial effects in several rodent models of psychiatric and neurodegenerative disorders, such as Huntington's disease [348, 349], Alzheimer's disease (AD) [345, 346], Parkinson's disease [347], stroke [351], traumatic brain injury [352, 353], multiple sclerosis [350], schizophrenia [354], anxiety [28] and depression [355]. Extensive evidence suggests that the functional behavioral changes in the brain post EE correlate with the underlying immune changes [17, 342, 371, 587, 607-610]. However, the effects of the duration of EE, if any, on neuroimmune functions are still unknown.

Studies have shown EE induces a significant alteration within rodent brains in molecular configuration and function of immune factors. In particular, glial cells, T cell subsets, and pro- and anti-inflammatory cytokines undergo a significant change in molecular configuration post EE. Brain glial cells are also known to modulate neurobiology through the expression of pro- or anti-inflammatory factors [290, 291, 583, 611]. However, the reported neuroimmune outcomes post EE varied greatly amongst studies, which may be accounted for by confounders such as strain, gender, and age, as well as duration and timing of EE. For instance, long-term EE from weaning to 23 months of age reduced the number and size of astrocytes in the hippocampus and corpus callosum in male Wistar-derived rats, which was in contrast to hypertrophied astrocytes and memory deficit of rats kept in standard housing conditions [342]. Likewise, EE significantly increased both astrocyte ($GFAP^+$) and microglia ($IBA1^+$) antigen expression within the dentate gyrus, but not in the CA1, CA3, or cortex of adult male rats that were housed for 12 hours in an enriched environment [371]. In a separate study, juvenile Sprague-Dawley rats from stressed pregnancies showed an increase in the basal circulating levels of $CD4^+$ and $CD8^+$ T lymphocytes, increased levels of cytokine IL-2, and reduced levels of cytokine IL-1β when housed in short-term enriched conditions at an early age [17].

Furthermore, EE for rodents in large cages with toys and other accessories has shown beneficial effects on brain glial cell morphology and numbers in models of several neurodegenerative and psychiatric disorders such as AD [607], schizophrenia [612] and depression [608]. While abnormalities in brain glial cell morphology and functioning are the established etiologies of these psychiatric disorders [609, 610], the effects of different EE protocols and inclusion or exclusion of EE objects on glial cells may differ in different brain regions. For example, while treatment with EE led to a significant increase in the number of new astrocytes in layer 1 of the motor cortex, physical exercise using voluntary wheel running, a technique which has also been used as a

tool to enrich the environment of rodents extensively, induced the proliferation of microglia in superficial cortical layers of several brain regions [587]. Interestingly, physical exercise is, in itself, a known inducer of neurogenesis [319, 516]. This evidence suggests that the inclusion of running wheels in EE protocols may create a bias and could be confounding to the results on the effect of EE alone on immune functions. However, it must be noted that EE animals, although not as active in the absence of running wheels, do explore EE tools in their cages, which adds to additional physical activity for them compared to control (standard-housed) animals.

Hence, in the absence of direct assessment, the role of the duration of the EE (without running wheels) in the modulation of neuroimmune mechanisms remains unclear. In our recently published work, we reported an increase in baseline locomotion and a decrease in depressive-like behavior after both short and long-term EE in middle-aged mice. Furthermore, long-term EE affected locomotion adversely in a threatening environment and was found to be anxiogenic [613]. We, therefore, wanted to investigate if the observed changes in behavior in response to the different duration of EE were the result of underlying neuroimmune outcomes. Hence, all mice that underwent behavioral battery were sacrificed, and brains and cervical lymph nodes were collected for the analysis of change in numbers of astrocytes and microglia in the dentate gyrus regions of the hippocampus and T cell subset proportions from cervical lymph nodes. We believe the findings from this study will benefit future research targeting EE to enhance neural function through immunomodulation during middle age.

Methods

This study is a continuation of the behavioral study that we have published recently. Hence, animals and experimental designs are similar to what we have already reported. The behavioral battery is discussed in detail in the published article [613].

A. Animals

Wild-type C57BL/6 mice (n=58; 31 males and 27 females), parental substrain Nhsd (derived from the National Institutes of Health, Bethesda, Maryland, USA), were bred in-house in the laboratory animal services (LAS) facility at the University of Adelaide and housed in individually ventilated cages (IVCs) under controlled conditions of temperature (21±1 ^{o}C), humidity (55%) and a 12-12 hour dark-light cycle. During the experimental timeline, the C57BL/6Nhsd mouse line was inbred between 9-13 generations. Mice were provided *ad libitum* standard laboratory food and water. C57BL/6 mice were selected for the study since they have been characterized well in terms of humoral and cellular neuroimmune responses to environmental factors [593].

Ethics approval for performing experiments on C57BL/6 mice was received from the University of Adelaide Animal Ethics Committee (M216-12), and all guidelines as prescribed for handling the experimental animals were followed during the study.

B. Experimental design

Once the desired age (12 weeks or eight months of age) was reached, those mice without signs of injury or illness, hence not challenged immunologically, were randomly allocated into either treatment (short-term and long-term EE) or control groups (n=12-16). Mice were randomly paired (males and females paired separately) and transferred to open top cages (2 mice per cage). Control mice received no treatment and were kept in cages with the following dimensions: 48.5cm x 15.5 cm x 12cm. EE mice were

kept in plexiglass cages with dimensions: 37cm x 20.5 cm x 13.5cm, as these had more breadth and depth to provide extra space for the objects associated with EE.

The group assigned as short-term EE were provided with a variety of non-toxic novel objects (house, colored balls, toys, hanging toys, ladder, and tunnels) and extra bedding as per previously published protocols [314, 348, 374] for 4 weeks before behavioral testing, i.e., starting at 8 months of age and ending at 9 months of age. Four weeks of EE is considered equivalent to one month of EE for the sake of simplicity. The objects were changed once every week to maintain novelty (during change of cages on Fridays, starting Friday of week 1, to minimize handling stress to mice). Similarly, the group assigned as long-term EE were provided with a variety of non-toxic novel objects and extra bedding for six months starting at three months of age, with objects changed once every week to maintain novelty. At the time of change of cages, mice were weighed on a digital weighing scale, the analyses of which have been published [613]. The objects remained in the cages throughout the three weeks of the behavioral testing period that followed the short-term and long-term treatments (Fig 6.1). Mice in both groups also received nesting material (paper shreds) during the experiments. Fig. 1.6 shows a representative image of the EE protocol for mice in cages. Mice were monitored for dominancy throughout the experiments, and those found to be dominant were segregated to prevent dominance effects on neuroimmune mechanisms.

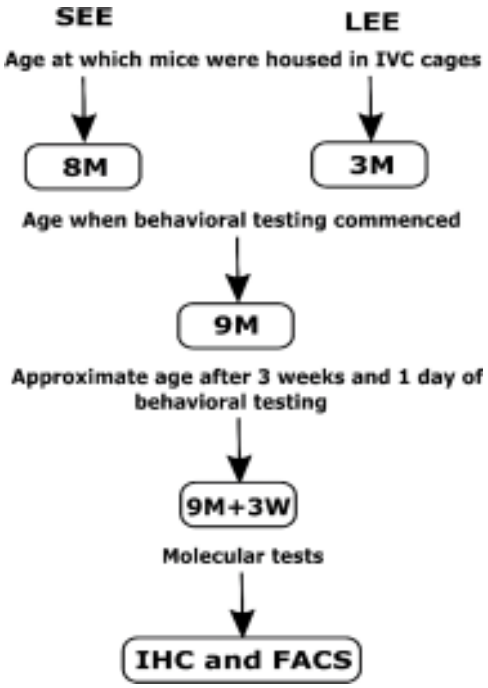

Fig. 6.1 Schematic representation of the experimental design. **Legend:** M: months, W: weeks, IHC: Immunohistochemistry, FACS: Fluorescence-activated cell sorting.

It is important to note that the behavioral testing ended with the forced swim test a day before tissue collection. For full behavioral testing protocol and data analyses, see the published paper [613]. We used both male and female mice in approximately equal numbers to represent findings from a population comprising of both sexes, however, it is important to note that we could not perform sex analysis due to low n for both males and females when taken separately (Table 6.1).

Table 6.1 Mouse numbers for behavioral and molecular analysis.

Treatment	Behavioral analysis n (males: females)	FACS n (males: females)	IHC n (males: females)
Short-term Control	15 (9:6)	7 (4:3)	6 (3:3)
Short-term EE	12 (6:6)	6 (3:3)	6 (3:3)
Long-term Control	16 (8:8)	7 (4:3)	6 (3:3)
Long-term EE	15 (8:7)	8 (4:4)	6 (3:3)

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

C. Molecular analysis

After completion of either short-term or long-term EE treatments and behavioral testing, mice were terminally anesthetized with a lethal intraperitoneal dose of pentobarbital (60 mg/kg IP), and blood was collected through cardiac puncture [467]. Animals utilized for immunohistochemistry were perfused via transcardiac injection with 10% neutral buffered formalin, with the brains rapidly removed and placed in 10% formalin until the further procedure. Animals utilized for FACS have their draining cervical lymph nodes of the brain removed and collected in Roswell Park Memorial Institute (RPMI+) medium.

Glial cell immunophenotyping using Immunohistochemistry

Brains preserved in 10% formalin were cut into five 3mm 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 serially sectioned, with six sections 150µm apart taken.

For immunohistochemistry, 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 mins. Antigen retrieval was performed by heating at close to boiling point for 10 mins in citrate buffer, and slides were then allowed to cool below 40°C before further processing. The appropriate primary antibody (IBA1 for microglia, 1: 10000; GFAP for astrocytes 1: 40000; Abcam, United Kingdom) was applied to the slides which were then left to incubate overnight, 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 minutes. The formed immune complex was then further amplified by incubating slides with a biotin-binding protein, streptavidin-peroxidase conjugate, for 60 minutes. 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 regions of the hippocampus were counted manually for statistical analysis. The hippocampus, in particular, the dentate gyrus region, has been shown to play a vital role in the regulation of behavior and memory in response to external stimulus and shows alterations after EE [24, 322-326, 371, 516, 538, 614]. Hence, we selected the hippocampus for the analysis of glial cells.

Freehand boxes were drawn to cover the entire dentate gyrus regions of the six stained sections followed by counting of the cells within the boxes. For each section, the count 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.

Peripheral T cell immunophenotyping using fluorescence-activated cell sorting (FACS)

FACS was applied for the detection of T cell proportions 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}), and early activated T cell phenotype ($CD25^+$).

Cervical lymph nodes collected in RPMI+ were passed through a 0.1µ sieve (BD) using additional 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 $2x10^6$ cells/ml. 250 µL of the cell suspension was then washed once with FACS buffer (PBS with 1% heat-inactivated bovine serum albumin) and blocked with 10µL 0.5mg/mL Fc block. An 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 binding). Cells were incubated for 30 minutes at room temperature with the respective mAbs (Table 2.2). Following this, the cells were washed twice before resuspension in 300µL 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 gated. $CD4^+$ or $CD8^+$ T cell subpopulations were then gated on $CD45^+$ $CD3^+$ T cells. Percentages of $CD4^+$ or $CD8^+$ gated T cells were used to calculate total cell numbers when multiplied with cell counts. Further gating on $CD44^+$ and $CD62L^+$ cell populations enabled the identification of naïve and memory T cells subsets (T_N, $T_{CM,}$ and T_{EM}).

D. Statistical Analysis

Statistical analyses 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. Comparisons between the treatments (short-term and long-term EE) and respective controls were performed using a fixed-effect model of two-way ANOVA. The two-way ANOVA statistical model was chosen to elucidate a two-way interaction effect between the two independent variables (treatment and duration) on the dependent variable (count of glial or T cells). The multiple comparisons post hoc Holm-Sidak's test was used to confirm significant interactions between groups. Results are presented as mean ± SEM. Differences are considered statistically significant when $p < .05$.

Results

A. Alteration in microglia and astrocyte activation number in the dentate gyrus of C57BL/6 mice exposed to short or long-term EE

Immunopositive microglia and astrocytes were counted in the dentate gyrus regions of the hippocampus and analyzed statistically using two-way ANOVA with post hoc Holm-Sidak's multiple comparison test.

Both short- and long-term EE induced a significant increase in the number of IBA1$^+$ microglia in the dentate gyrus

Figures 6.2 shows the representative immunohistochemical images of the number of IBA1$^+$ microglia in the dentate gyrus region of the hippocampus in short- and long-term control and EE groups. A non-significant interaction effect ($F_{(1, 20)} = .09$; $p = .7615$) and the main effects of duration ($F_{(1, 20)} = .15$; $p = .6994$) were noted for IBA1$^+$ microglia within the dentate gyrus. However, the main effect of treatment was significant ($F_{(1, 20)} = 53.1$; $p < .0001$). Both short-term and long-term EE treated mice showed significantly higher IBA1$^+$ cell numbers than their respective controls in the dentate gyrus (75.4 ± 4.2 vs. 51.7 ± 3.6; $p < .0001$ and 73.2 ± 2.1 vs. 51.5 ± 2.0; $p = .0002$, respectively; Fig 6.3).

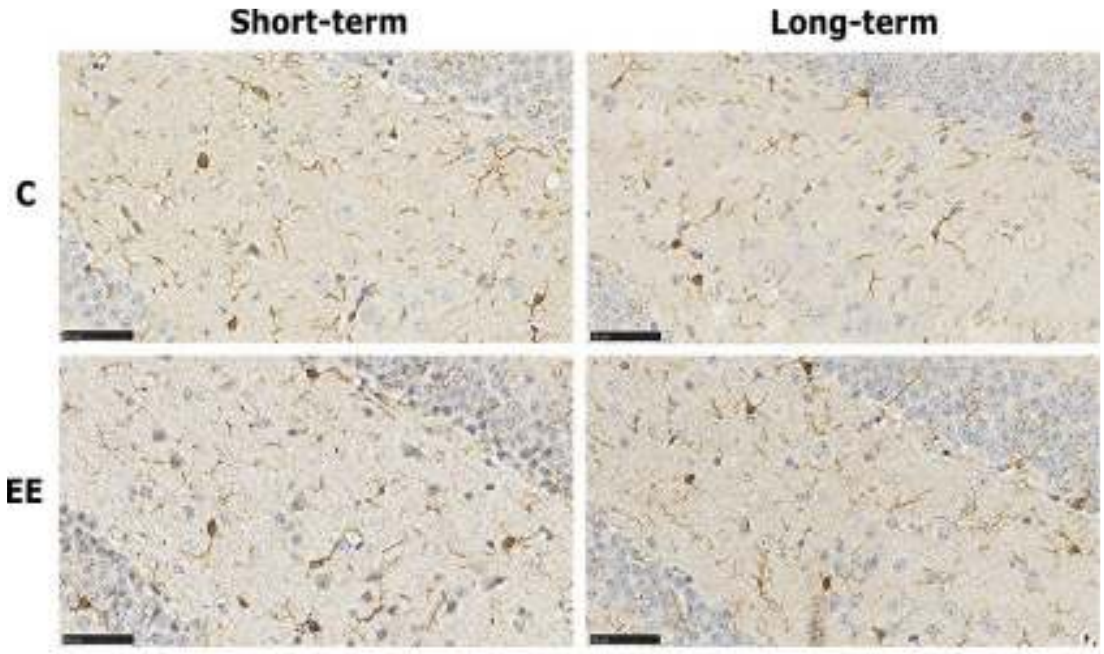

Fig. 6.2 IBA1+ microglia in the dentate gyrus. Representative immunohistochemical images in the dentate gyrus region of the hippocampus in short- and long-term control and EE groups (the black scale represents 50µm length).

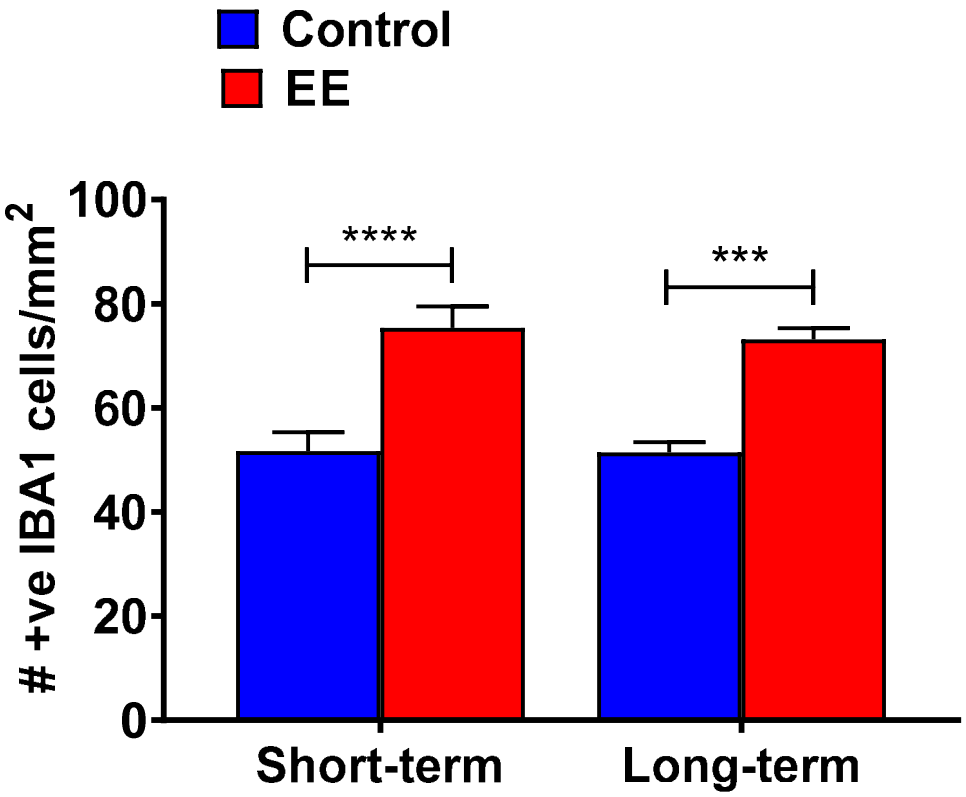

Fig. 6.3 Number of IBA1$^+$ microglia in the dentate gyrus. All data represented as mean ± SEM, n = 6 per group. *** p < .001, **** p < .0001.

Only long-term EE induced a significant increase in the number of GFAP$^+$ cells in the dentate gyrus regions of the hippocampus

Figures 6.4 shows the representative immunohistochemical images of the number of GFAP$^+$ astrocytes in the dentate gyrus region of the hippocampus in short- and long-term control and EE groups. The significant interaction effect ($F_{(1, 20)} = 4.36$; $p = .0497$) and the main effect of treatment ($F_{(1, 20)} = 5.56$; $p = .0286$) were noted, but the main effect of duration ($F_{(1, 20)} = .83$; $p = .3739$) was non-significant in the dentate gyrus. On post hoc analysis, long-term EE mice showed significantly greater number of GFAP$^+$ cells than long-term controls (297.1 ± 11.3 vs. 236.6 ± 11.8; p = .0102; Fig 6.5). Also, the number of GFAP$^+$ astrocytes was low in long-term controls when compared to short-term controls at the significance level of p < .1 (p = .0912).

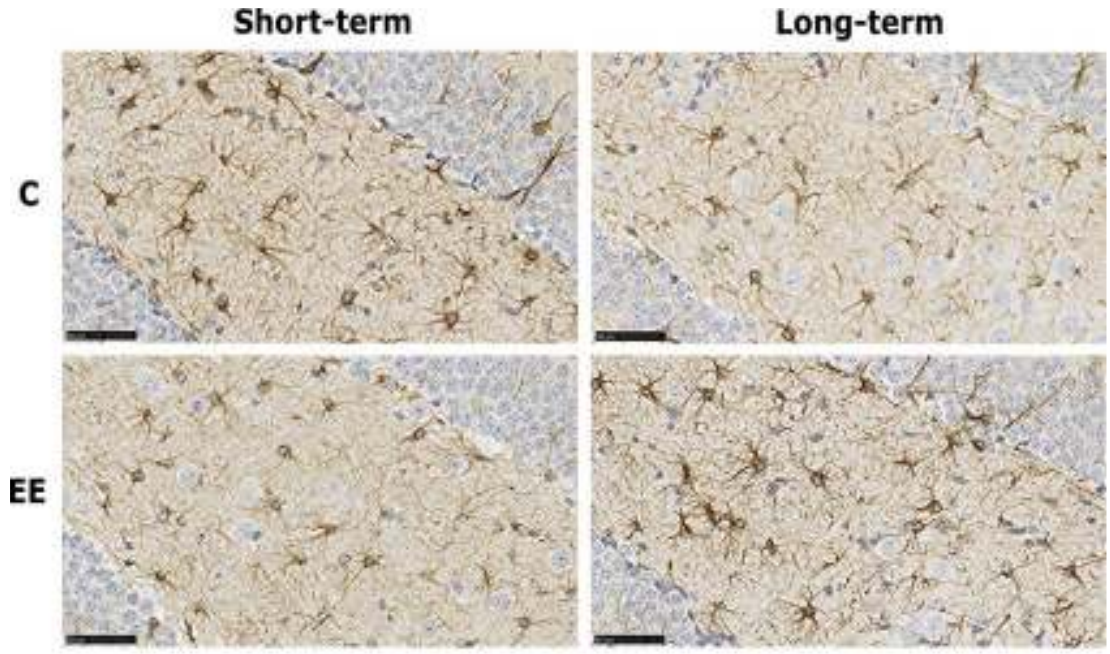

Fig. 6.4 GFAP+ astrocytes in the dentate gyrus. Representative immunohistochemical images in the dentate gyrus region of the hippocampus in short- and long-term control and EE groups (the black scale represents 50μm length).

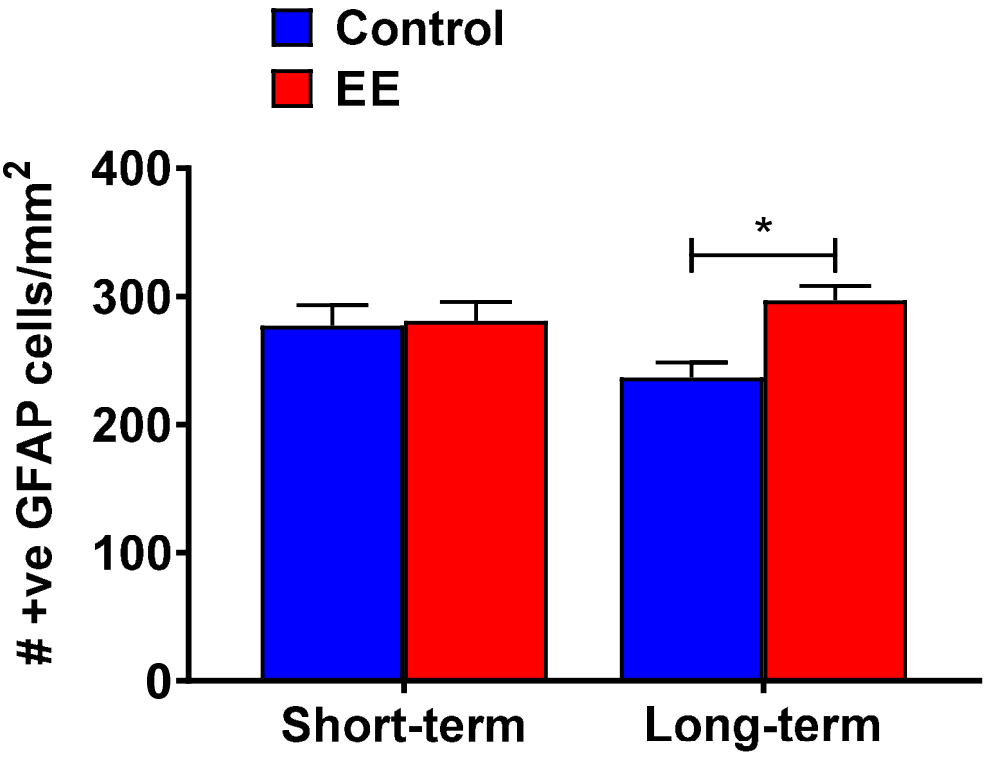

Fig. 6.5 Number of GFAP$^+$ astrocytes in the dentate gyrus. All data represented as mean ± SEM, n = 6 per group. * p < .05.

B. Altered T cell immunophenotype in C57BL/6 mice exposed to either short or long-term EE

CD4+ (T helper) and CD8+ (cytotoxic T) cell proportions were measured as an indication of cellular immune response to the duration of the EE treatment. Representative images obtained during the FACS data analysis using FCS Express software are shown in figure 6.6.

The change in the proportion of the gated $CD4^+$ T cells was found to be non-significant in both short-term and long-term EE groups when compared to respective controls, with $p > .05$ for the interaction effect, as well as for the main effects of treatment and duration (Fig 6.7A). Likewise, no significant interaction effect and the main effects of the treatment and duration were observed for T_N, T_{CM}, and T_{EM} $CD4^+$ T cells ($p > .05$; Fig 6.7B). However, two-way ANOVA, while still non-significant for the interaction effect and the main effect of treatment ($p > .05$), revealed significant main effect of the duration for the proportion of $CD8^+$ T cell ($F_{(1, 24)} = 11.78$; $p = .0022$). The proportion of CD8+ T cells was significantly higher in the long-term EE group when compared with the short-term EE group (39.4 ± 1.9 vs. 26.8 ± 3.8; $p = .0195$; Fig 6.7C). This was, in turn, the result of the long-term EE group showing a significantly higher proportion of the gated $CD8^+$ T_N cell than the short-term EE group (22.5 ± 1.0 vs. 14.5 ± 2.9; $p = .0016$; Fig 6.7D).

The proportions of the T cell early activation marker CD25 on both $CD4^+$ and $CD8^+$ T cells were found to be non-significantly different between either short-term or long-term EE groups when compared to respective controls ($p > 0.05$, data not shown).

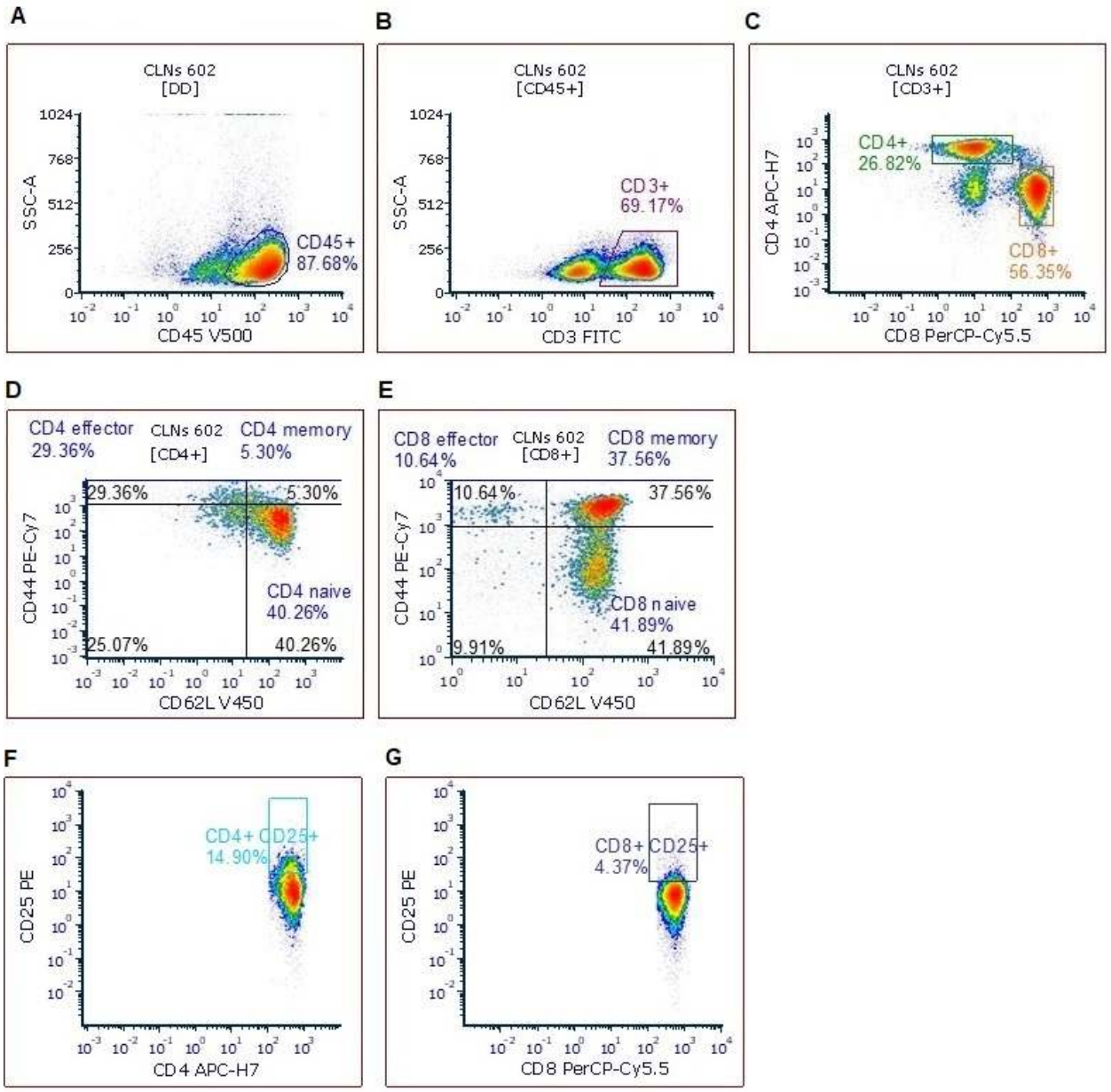

Fig. 6.6 A-E: Representative density plots of the subsets of T cells. (A) Gated CD45$^+$ cells, (B) CD3$^+$ cells derived from gated CD45+ cells, and (C) CD3$^+$ CD4$^+$ and CD3$^+$ CD8$^+$ T cells distinguished from total CD3$^+$ gated cells. Further gating on CD44$^+$ and CD62L$^+$ cell populations enabled the identification and estimation of (D) CD4$^+$ and (E) CD8$^+$ T cell subsets, i.e., Naïve (T_N), Central memory (T_{CM}) and Effector memory (T_{EM}) T cells. F-G: Representative of the density plots showing the proportion of early activation markers CD25$^+$ on (F) CD4+ and (G) CD8+ T cell subpopulations derived from the gated CD45+ T cells.

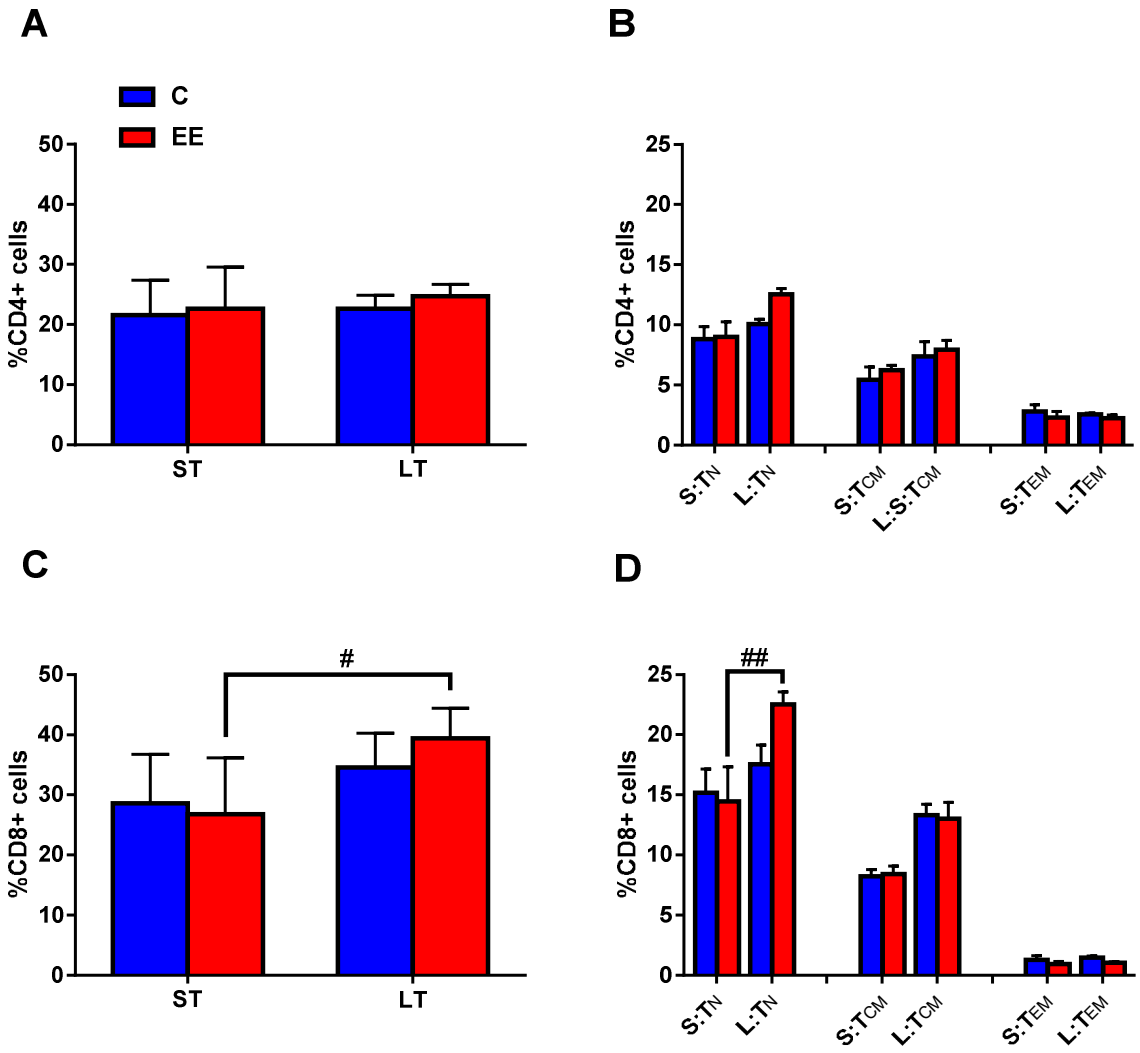

Fig. 6.7 T-cell subset composition. Proportions of the gated (A) $CD4^+$ and $CD8^+$ T, (B) CD4 T_N, T_{CM} and T_{EM}, and (C) T_N, T_{CM} and T_{EM} cells. All data represented as mean ± Sem of the proportion of $CD4^+$ or $CD8^+$ T cells, n = 6-8 per group. $^{\#\#}$ $p < 0.01$. **Legend:** S: short-term, L: long-term, N: naïve, CM: central memory, EM: effector memory.

Discussion

The objective of this study was to determine if the functional effects that we have reported in the recently published study [613] were associated with changes in brain glial cells number and peripheral T cell subset proportion. Hence, we investigated the effect of duration of EE on glial cell (microglia and astrocytes) numbers in the hippocampus, and T cell subsets and phenotype in the cervical lymph nodes of mice that underwent behavioral battery. There are a good number of evidence to suggest that EE alter the functions of the dentate gyrus region of the hippocampus, which regulates key brain functions, such as learning and memory [24, 371, 516, 538, 614]. EE, regardless of duration, increased the number of IBA^+ microglia within the dentate gyrus region of the hippocampus. Conversely, only the long-term EE enhanced $GFAP^+$ astrocyte number within the dentate gyrus region of the hippocampus when compared to control mice. Evaluation of the T cell population within the cervical lymph nodes revealed that both short-term and long-term EE did not affect the proportion of $CD4^+$ T cells and $CD4^+$ T cell subsets. However, long-term EE mice showed a significantly higher proportion of $CD8^+$ T cells compared to short-term mice, which we observed

was due to the significantly higher proportion of $CD8^+$ T_N cells in long-term EE mice. Both short-term and long-term EE showed no effect on the proportion of T cell early activation marker CD25.

Duration of EE affects astrocyte but not microglial numbers in the dentate gyrus at middle age

Astrocytes and microglia are the primary immune effector cells in the CNS and regulate the extensive bi-directional communication between the nervous and the immune systems in response to different immunological, physiological and psychological stressors, as well as external laboratory treatments. Indeed, in constitutive states, they are essential for the maintenance of neurobiological homeostasis and play a vital role in neuroprotection and neuroplasticity [17, 18]. Microglia are important for tissue maintenance and repair and maintaining homeostasis in the CNS [615], whilst astrocytes provide a structural framework to the blood-brain-barrier, regulate the transmission of electrical impulses within the brain, release glycogen and provide nutrients, such as lactate, to the nervous tissue, maintain extracellular ion balance, and repair damaged tissues within the brain [616-619]. Glial cells are not only altered in density and function during psychiatric disorders but also under improved environmental conditions, particularly in the hippocampus. As such, measuring the change in the number of the astrocytes and microglia in the dentate gyrus, the region widely associated with altered cognitive function and affect regulation, can provide satisfactory evidence pointing towards an association of EE on neuroimmune homeostasis. It must be noted that during old age, the dysregulated functioning of microglia and astrocytes leads to enhanced glial pro-inflammatory cytokine signaling, impaired neurobiology, and an increase in apoptotic cell death [583].

We investigated the effects of EE in middle-age mice that were sacrificed immediately after the behavioral testing. Since mice did not grow old and were housed in a controlled environment devoid of external stressors, we hypothesized an increase in the number of both immunopositive microglia and astrocyte with enhanced productive functions after EE. We indeed observed an increase in microglial numbers within the dentate gyrus region after both short- and long-term EE. The increase in the number of IBA^+ microglia in the dentate gyrus after EE is consistent with the previously published findings in 2 months old male Sprague-Dawley rats, however, unlike our results, the authors reported no change in the immunopositive microglia number within the CA1 and CA3 regions of the hippocampus [371]. An increase in the number of microglia in the dentate gyrus at middle age after EE indicates enhanced neuroprotection. In our published behavioral study, we have reported enhanced baseline locomotion and reduced depressive-like behavior after both short and long-term EE [613]. Taken together, we believe the reported improved behavioral outcomes after the EE could be associated with the increased number of microglia in the hippocampus, in particular, within the dentate gyrus of mice. However, since the differences were observed both in control and EE mice, we attribute this to the experimental variability and not to the effects of duration of EE or the starting age of EE treatment.

We observed no effects on the number of $GFAP^+$ astrocytes in any of the hippocampal regions after the short-term EE, but the number of $GFAP^+$ astrocytes increased significantly after the long-term EE in the dentate gyrus region of the hippocampus. It is, therefore, likely that astrocytes are less reactive to EE than microglia and a longer

duration of EE may be required to significantly alter astrocytes number in the brain. Our results contradict the previously reported findings of a significant increase in the number of astrocytes in 2-month-old C57BL/6 female mice after 7 weeks of EE, although the increase was shown in layer 1 of the motor cortex and not in the hippocampus [587]. Similarly, another study reported a significant increase in the number of $GFAP^+$ astrocyte within the dentate gyrus, but not in the CA3 region, of 2-month-old male Sprague-Dawley rats, housed for 12 hours in an enriched environment daily for seven weeks [371]. This possibly suggests that astrocytes are more reactive to EE at an early age but not at middle age. We believe ours is the first report of an increase in the number of $GFAP^+$ astrocytes in the dentate gyrus after long-term EE at middle age. Long-term EE of 3 months, from 5 to 8 months of age, in the presence of a running wheel, has also been shown to increase the number of astrocytes in a mouse model of Alzheimer's disease [620]. However, it must be noted that physical exercise alone can enhance the number of hippocampal $GFAP^+$ astrocytes [621]. The evidence mentioned above suggests that the age of the rodents, duration of EE, as well as the presence or absence of running wheels, may alter the effects of the duration of EE on astrocyte numbers within the hippocampus.

Interestingly, an increase in both microglia and astrocytes number in the dentate gyrus of the middle age long-term EE mice was also accompanied by an increase in anxiety-like behavior that we have reported in the recently published study [613]. We observed that this could be because of reduced activity of long-term EE mice in the challenging conditions of brightly lit open field and elevated zero maze after 5 months of habituation to an enriched environment in basal home conditions. Indeed, long-term EE mice showed a significant reduction in locomotion in the stressful environment of Open Field. We, therefore, believe that the increased anxiety of long-term EE mice may not necessarily be associated with an increase in microglia or astrocytes number in the dentate gyrus that we have reported in this study.

Only long-term EE alters peripheral T cell proportion at middle age

Ours is the first study to investigate the change in the proportion of T cell subsets in the cervical lymph nodes in response to different durations of EE. The analysis of the total T cell counts, including $CD4^+$ (helper T cells) and $CD8^+$ (cytotoxic T cell), helped us to determine the cellular immune response after short-term and long-term EE treatments. We observed that altered CD8+ (cytotoxic T) but not CD4+ (T helper) cell proportions were associated with the duration of the EE treatment.

Mature T_N cells proliferate and convert into T_{CM} or T_{EM} cells on the recognition of a cognate antigen. T_{CM} cells have little or no effector function but mediate reactive memory - proliferate and differentiate to effector cells in response to antigenic stimulation. T_{EM} cells, which are short-lived, migrate to the site of infection, eliminate the antigenic molecular patterns and undergo apoptosis once the antigens are eliminated. However, some T_{EM} cells also differentiate into memory cells that provide long-term adaptive immunity. Our results suggest that the duration of EE has no differential effects on the proportion of $CD4^+$ T cells and their subsets. However, a longer duration of EE may be beneficial by supporting an increase in the proportion of cytotoxic CD8+ T and $CD8^+$ T_N cells as found in middle age when compared with those with short term EE.

T_N cells also respond by acquiring activated phenotypes and upregulation of early T cell activation markers, such as CD25, which are important in the maintenance of tolerance to self-antigen and prevention of autoimmune disorders [622-624]. However, no differences in $CD4^+$ and $CD8^+$ early activation T cell markers were observed after both short- and long-term EE. Our results are in contrast to the known neuroprotective effects of EE [31, 625, 626]. Nonetheless, the enhanced cytotoxic immunity could be attributed to the observed decrease in locomotion in a threatening environment (open field) and increases in anxiety-like behavior in middle-aged C57BL/6 mice after long-term EE that we have reported in our recent publication [613].

The controlled environment, absence of pathogens and stressful stimuli, age and strain of the rodents, EE paradigm structure, size and traits of EE objects, and the frequency of changing the objects that we utilized during our study, may all have a role to play in the observed findings. Further research into the differential effect of the various EE objects on neuroimmune functions in older mice may be required. Furthermore, the results need to be followed up in old-age and psychiatric disease rodent models to further validate and extend the findings. Additionally, since running wheels have been used as an EE tool in past studies, the differential effects of long-term EE, long-term PE, and their combination, need to be explored.

Research Summary

In this chapter, we investigated the effects of short- and long-term EE on immunopositive brain glial cells count in the dentate gyrus and peripheral T cells count and phenotype in the cervical lymph nodes in middle age C57BL/6 mice to confirm if the observed functional results after short- and long-term EE were related with underlying neuroimmune changes. Following the behavioral testing that we have reported in chapter 5, half of the mice were sacrificed, their brains were extracted and preserved in 10% formalin for the immunohistochemistry of glial cells. From the remaining half, the cervical lymph nodes were extracted for conducting fluorescent activated cell sorting to analyze changes in T cell and subsets count.

Three- and eight-months-old mice that were provided with 6 months and 4 weeks of EE respectively were tested for various behaviors using a standardized behavioral battery, the results of which are reported in chapter 5. Following this, half of the mice were sacrificed, their brains were extracted and preserved in 10% formalin for the immunohistochemistry of glial cells. From the remaining half, the cervical lymph nodes were extracted for conducting fluorescent activated cell sorting to analyze changes in T cell and phenotype count.

Our results indicate that both short- and long-term EE enhance the microglia number to the same extent, but EE of a longer duration may be required to modify the immunoreactive astrocytes count in the dentate gyrus and peripheral $CD8^+$ T cells and subsets count in middle-age mice. Hence, a longer duration of EE may be more beneficial in enhancing basal immunity at middle age than short-term EE, however, this may also correspond to the development of anxiety-like behavior that we reported in chapter 5. Since our research is the only one to elucidate the differential effects of the duration of the EE on neuroimmune functions, we opine that similar research in the future using bigger sample size and in old C57BL/6 mice may be required to develop a better understanding.

Chapter 7: Thesis Discussion

Preamble to the thesis discussion

During this research work, we conducted three sets of experiments with five sets of analyses. In total, we tested eight hypotheses and the results are reported and discussed systematically in individual results chapters 2 to 6. The aim of this chapter is to provide an overall summary of the results from this PhD research. The individual chapters discussions have been articulated to develop a comprehensive understanding of the overall research work.

This chapter, in turn, will help to reach the overall conclusions in chapter 8.

Discussion

During this research work, we investigated the effects of short-term PE, EE and the combination of PE and EE on brain functions, neuroimmune changes and hippocampal genes expression in healthy aging C57BL/6 mice at 4 (early age), 9 (middle age) and 14 (late-middle age) months. Prior to this, we investigated the effects of normal aging in C57BL/6 mice that provided us with results that we used as a reference for further study. Subsequent to our investigation on the effects of short-term PE, EE, and PE+EE, and based on the results obtained, a follow-up study was conducted to investigate the differential effects of short- and long-term EE on brain functions, neuroimmune changes and hippocampal genes expression in healthy middle-aged C57BL/6 mice. Overall, there were eight hypotheses that we developed and tested for validity. The overall results from this research are discussed pointwise below.

Bodyweight

Bodyweight increases significantly until middle age under the effect of normal aging, and short-term PE and PE+EE, and short- and long-term EE interventions have no effects on it.

The results from chapters 2, 3 and 5 suggest that there is an increase in the mean body weights of both male and female C57BL/6 mice until middle age but remain steady afterward during normal aging. The aging effects on the mean body weights of C57BL/6 mice are partially in compliance with the previously reported findings, which reported an increase in the mean body weights of C57BL/6 mice until 18-20 months of age [226, 234]. Mice during our experiments were fed with ad libitum standard laboratory food pellets and water, which is in contrast to some of the past studies where mice were fed with a high-fat diet [473, 474]. This led us to hypothesize that exposure to environmental interventions may influence body weight in C57BL/6 mice. However, short-term PE, EE, and PE+EE did not affect the mean body weights of male and female C57BL/6 mice at any age. Likewise, the longer duration of EE, which was investigated during follow-up research, also showed no effects on the mean body weights of male and female C57BL/6 mice at middle age.

Studies in the past have shown a decrease in the mean body weights, both in rodents [542-544] and humans [545, 546] after long-term continuous PE. Our study differs in providing PE only for the short-term, which may not have been enough to mobilize body fats and thereby explains the abovementioned findings. The effects of EE and PE+EE, however, are in compliance with previously reported results, whereby 2.5 months of EE [14] and 6 weeks of PE+EE [28] did not influence the mean body weights of young mice. Hence, while aging is directly related to body weights, short-term interventions PE, EE and the combination of PE+EE has no or minimal influence on body weights. Also, unlike long-term EE, long-term PE may cause a reduction in body weights, however, this is a topic for another research. It must be noted that we did not investigate the body composition of mice or food intake in the study, which could have influenced our findings on body weights.

Locomotion

Short-term EE, and not PE and PE+EE, reverses the aging-induced reduction in baseline locomotion from middle age onwards. Long-term EE affects baseline locomotion in a threatening environment.

In chapters 2, 3 and 5, we hypothesized that normal aging will result in a decrease in locomotion which will be reversed by short-term PE, EE, and PE+EE and that the effects of PE+EE and long-term EE on locomotion would be significantly greater than PE or EE alone and short-term EE, respectively. However, in chapter 2, we observed a significant decrease in locomotor activity at late-middle age during normal aging only under the basal conditions of the Home Cage, but not under the challenging conditions of the Open Field which was illuminated with artificial light. Past studies have reported no loss of locomotion in the Open Field until 7-8 months of age, but unlike our findings, reduced it significantly in mice 8 months onwards [226, 227]. This decrease in baseline locomotion during normal aging was, however, reversed by both short- and long-term EE treatments at middle age (chapters 3 and 5), with short-term EE also improving baseline locomotion at late-middle age. Interestingly, the effects of short-term EE on baseline locomotion is primarily driven by a significant increase in the baseline locomotion of female mice and not male mice. Conversely, long-term EE showed no sexually dimorphic effects on the baseline locomotion of C57BL/6 mice. Under more threatening environmental conditions in the open field, no significant differences in locomotor behavior relative to controls were noted. Interestingly, long-term EE mice traveled significantly shorter distances than short-term EE mice in the open field, thus suggesting that locomotor activity in a threatening environment is dependent on the duration of EE, with short-term EE leading to an increase in locomotion relative to the long-term EE group. The reported effects of EE on locomotion comply with previously published findings. For example, EE without a running wheel for 39 days has been shown to enhance locomotion in young male Sprague-Dawley rats in the Open Field test [384]. Similarly, EE without running wheel for 11 weeks enhanced play behavior in home conditions in newly weaned male mice [547]. In chapter 3, we also reported a significant reduction in the baseline locomotion of female mice at 4 months after short-term PE, which was, however, compensated by the baseline locomotion of the 4-month male PE mice, thereby resulting in an overall non-significant difference between 4-month PE and age-matched control PE mice. It must be noted that we observed no sex differences in the open field locomotor activity for any of the treatment groups. Overall, our result suggests that EE primarily enhances the locomotor activity of female C57BL/6 mice. Further research into differentiating the effects of PE, EE, and PE+EE on locomotion after a longer duration of treatment in C57BL/6 mice is required.

Anxiety-like behavior

EE, PE, and PE+EE do not reverse aging-induced anxiety-like behavior but show effect across the lifespan. Long-term EE is anxiogenic at middle age.

In chapters 2, 3 and 5, we hypothesized that normal aging will result in an increase in anxiety-like behavior which will be reversed by short-term PE, EE, and PE+EE and that the anxiolytic effects of PE+EE and long-term EE would be significantly greater than PE or EE alone and short-term EE, respectively. In chapter 2, results indicated that our hypothesis was true at $p < .05$. 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 arms and the number of head dips by 14 months mice in the EZM. However, in the Open Field, 4-months old mice spent significantly less time in the inner zone compared to 9 months old mice, suggesting that 4-month 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-months-old and 28-month-old [225-228], but not in 5 and 11 months old [225, 229] 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 not male mice [225], we observed no significant effect of sex on anxiety-like behavior. Our findings from EZM, therefore, suggest that normal aging is directly associated with the onset of anxiety-like behavior after middle age.

During the subsequent analysis, results from chapter 3 indicate that while none of the treatments were different from controls at all ages for anxiety-like behavior, we found age effects within treatments. Short-term EE and PE mice appeared to exhibit less anxiety-like behavior at 9 months compared to their 4- and 14-months cohorts as evident by significantly more time spent in the open quadrants of EZM and an inner zone of Open Field respectively. However, sex influenced anxiety-like behavior. We found that EE mice were different due to the significantly less anxiety-like behavior of 9-month male EE mice compared to 4- and 14-months male EE mice. Similarly, PE was found to be anxiogenic for females compared to age-matched controls at 4 months. Indeed, PE, when added to EE at 4 months, increased anxiety, as evident by significantly fewer head dips by 4-month PE+EE mice compared to 4-month EE mice in the EZM. Furthermore, the observed significant higher anxiety-like behavior of 14-month PE+EE mice compared to 9-month counterparts, as evident by differences between the treatment matched age cohorts for a time in open arms of EZM, was primarily due to higher anxiety-like behavior in male PE+EE mice. The 14-month controls and EE mice showed significantly fewer head dips in the EZM than their cohorts at 4 months, suggesting significantly higher anxiety-like behavior at late-middle age compared to early age cohorts in untreated and EE mice during our experiments. In chapter 5, while short-term EE treatment showed improvement in anxiety-like behavior, with mice showing increased interest in the open arm of EZM and a significantly higher number of head dips than short-term controls, long-term EE of 6 months showed anxiogenic effects, with mice spending significantly less time in the inner zone than long-term controls. Together, our findings point towards short-term EE being anxiolytic at young and middle age, long-term EE being anxiogenic at middle age, PE being anxiogenic for females at young age and EE when combined with PE being anxiogenic for males at late-middle age.

Depressive-like behavior

Aging is not a controlling factor for depressive-like behavior. Short-term PE induces depressive-like behavior at an early age and EE, irrespective of duration, reduces depressive-like behavior at middle age.

In chapters 2, 3 and 5, we hypothesized that normal aging will result in an increase in depressive-like behavior which will be reversed by the short-term PE, EE, and PE+EE and that the anti-depressive effects of PE+EE and long-term EE would be significantly greater than PE or EE alone and short-term EE, respectively. However, in chapter 3, no significant differences between any of the age groups for depressive-like behavior suggested 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 [229, 230] while others are suggesting that aging has a significant effect on depressive-like behavior [227, 231]. C57BL/6 mice, 11 and 18 months old, showed no differences in the parameters of behavioral despair when

evaluated against young mice (2-3 months old) in the FST [229, 230]. 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 [227]. 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 [231]. 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.

In chapter 3, PE acted as an inducer of depressive-like behavior at 4 months of age, as evidenced by significantly higher immobility time of 4-month PE mice compared to both 4-month-old controls and EE mice in the FST. However, research has shown that moderate PE alleviates depressive-like behavior in rodents [402, 525, 553]. The discrepancy in our findings for PE in FST from past studies could be due to differences in cages, running wheel dimensions, or handling time during the experiment and may require further validation. Conversely, EE mice showed the least depressive-like behavior among all groups at 4, 9, and 14 months of age, although results were not significant when compared to controls. However, EE has been shown to attenuate depressive-like behavior associated with prenatal chronic stress [377] but not with early life stress [376]. We housed mice in controlled environmental conditions where chances of stress were minimal. Perhaps the non-significant results for EE and PE+EE in FST were due to the short duration of the treatment. Hence, we hypothesized that longer treatment with EE could be more effective in the treatment of depressive-like behavior. The follow up research, as reported in chapter 5, revealed that while both short-term and long-term EE significantly reduced depressive-like behavior compared to respective controls, there were no significant differences between the short- and long-term EE mice. The results are, however, consistent with all previously reported findings [15, 63, 64]. Interestingly, the difference was statistically significant only for male mice, suggestive of EE, irrespective of duration, affects male mice more than female mice while ameliorating depressive-like behavior.

Spatial learning and memory

Normal aging causes a significant reduction in spatial learning and memory. Short-term PE adversely affects the spatial learning of female mice at early and late-middle age. Short-term EE improves retention of spatial learning at late-middle age.

In chapters 2, 3 and 5, we hypothesized that normal aging will result in an impairment in spatial learning and memory which will be reversed by short-term PE, EE, and PE+EE, and that the effects of PE+EE and long-term EE on spatial learning and memory would be significantly greater than PE or EE alone and short-term EE, respectively. True to our hypothesis, we observed that 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 in Barnes maze when compared to both 4 and 9-month-old mice. 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 during our experiments indicates impaired spatial learning, memory, and cognitive flexibility. Indeed, 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) [225]. 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 [233]. Evidence suggests that while the spatial learning and memory functions significantly decline from 12 months of age [235], this process can start as early as 8 months of age in C57BL/6 mice [227].

Interestingly, treatment with PE also showed impairment in spatial learning at 4- and 14-months of age, which was primarily driven by 4- and 14-month female PE showing significantly higher latency to escape than age-matched female controls. PE has widely been accepted as a modulator of brain functions and shown to improve spatial memory in young, middle age and old age male rodents [31, 517, 519-521, 557-559]. Perhaps, the species and strain of rodents could have a significant influence on the rate of spatial memory acquisition. Moreover, PE in the short-term may have served as a stressor at an early age for female C57BL/6 mice. Other factors, such as the design of our study, cage dimensions, the presence of one wheel per cage, handling frequency, and mouse-mouse interactions, may have played a role as well.

Conversely, EE reversed the age-related decline in spatial memory at 14 months, but without any significant sex differences. Short-term EE with or without running wheels has been shown to improve the spatial memory in young rats by promoting hippocampal neurogenesis, dendritic arborization, and spine density in the brain [374, 375, 381, 383, 555]. We found no change in cognitive functionality after both short-term and long-term EE when 9-month-old mice were tested on Barnes Maze over four days of training. This may be because naïve early-middle-age mice were exposed to EE, as opposed to aged mice. Previous research has shown improved cognition and spatial memory in aged mice after EE. For example, exposure of aged (21-23 months) C57BL/6 mice to 24-hour EE for ten weeks improved spatial learning and cognitive abilities [28]. Likewise, EE mitigated cognitive deficits in aged mouse models of Alzheimer's disease [6, 65].

A short note on the sex-specific differences of the effects of PE and EE on various behaviors

From the above discussion on the effects of PE, EE and PE+EE on baseline locomotion, affective-like and cognitive behaviors, we observed sexually dimorphic effects of PE and EE on baseline locomotion, anxiety-like and cognitive behaviors but not on depressive-like behavior. It is important to note that sexually dimorphic effects on behaviors were noted after the short-term PE and EE treatments and not after short-term PE+EE and long-term EE treatments.

Short-term EE female mice showed a significant increase in the baseline locomotion compared to short-term EE male mice at 9 months of age. Contrary to this, we observed a significant reduction in the baseline locomotion of female mice at 4 months after short-term PE. Similarly, PE was found to be anxiogenic for females compared to age-matched controls at 4 months in the elevated zero maze. Short-term PE also adversely affected the spatial learning of female mice at early and late-middle age. We speculate that these sexually dimorphic effects of behaviors could be due to significantly more interaction of female mice with EE objects and running wheels, and hence, greater effects of PE and EE treatments on female mice compared to male

mice. Other factors, such as genetics may also play a role. Since the effects of PE and EE that we observed were opposing, this could be the reason for no sexually dimorphic effects seen in PE+EE treated mice. Yet, it is interesting to note that that short-term PE reduced baseline locomotion, enhanced anxiety-like behavior and reduced cognition in young mice. We recommend further study to develop a full understanding of the sexually dimorphic effects of aging, and short- and long-term PE, EE and PE+EE on various behaviors.

Expression of genes of interest in the hippocampus

Normal aging significantly affects the expression of hippocampal genes. PE and PE+EE elicit differential hippocampal gene expression at middle age, but EE, irrespective of duration, has no effect on gene expression.

In chapters 2, 3 and 5, we hypothesized that normal aging will induce a change in the expression of hippocampal genes of interest and that the effects of aging on gene expression would be reversed by short-term PE, EE, and PE+EE, as well as long-term EE. Indeed, we found significant differences in the expression of 29 genes out of 43 GOI between the three age groups at adjusted $p < 0.05$. Several hippocampal genes differentially expressed at middle age during the comparisons between the three treatments. However, the absence of treatment effects on the expression of hippocampal genes during early and late-middle age was unexpected. This could be attributed to genes being more responsive to the external environment during middle age than young and late-middle age. In middle age mice, we observed 11, 3, 6, and 7 differentially expressed genes during PE vs. controls, PE+EE vs. controls, PE vs. EE and PE+EE vs. EE comparisons, respectively. None of the genes differentially expressed during EE vs. C and PE vs. PE+EE comparisons. We found no significant differences in expression of the 43 genes of interest after both short-term and long-term EE at adjusted $p<0.05$.

Aging has been shown to influence gene expression and gene-gene interactions in the hippocampus regulating neuronal growth, structure, and functions [229, 468, 469]. We observed a non-significant increase in the expression of the *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. It has been reported that the Bdnf gene plays a role in the biology of mood disorders, and its expression is reduced during aging-related neurodegenerative disorders [481-483]. Conversely, the expression of the *Bdnf* gene within the hippocampus has been shown to increase after external interventions, such as physical activity [454] and environmental enrichment [388]. Hence, 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. However, it must be noted that mice during our experiments were aged only until late-middle age and not old age, hence, this could be a reason for no significant decrease in the expression of *Bdnf* gene during our work. Nonetheless, the findings are still interesting as we are able to determine that the beneficial effects of EE on cognitive and affective-like behaviors at late-middle age is independent of BDNF expression in the brain.

An 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 [484, 485]. 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 [486-489], at 14 months suggests that oxidative and inflammatory stress increased with age. This explains the increase in anxiety-like behavior and spatial learning and memory impairment 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 the *Aif1* gene, which promotes macrophage activation and phagocytosis, growth of T-lymphocytes, and peripheral inflammation [490-492], 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 the *Aif1* gene, the expression of the *Nlrp3* gene decreased significantly between 4 and 9 months and non-significantly between 9 and 14 months. The decreased expression of the *Nlrp3* gene indicates a lowered expression of the proteins of cellular senescence at nine months, although the levels of senescence proteins have been reported to increase during normal aging [290]. We believe the controlled external environment for aging mice is responsible for the lowered expression of *Nlrp3* gene during our experiments. However, we observed no change in the expression of any of the abovementioned genes after the three short-term treatments and long-term EE.

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 afterward. 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 [446, 493-501]. 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 [498, 499, 502-507], 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. In contrast, 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 afterward 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 afterward until 14 months of age. 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 [447, 448, 508-515]. Together, the results from chapter 2 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.

When we tested the effects of external interventions on the hippocampal gene expression, we observed that three of the genes, i.e., *Creb1*, *Smad2,* and *Il1r1* were upregulated in all groups with PE when compared to groups without PE, confirming the effects of PE and not EE on these genes. *Creb1* encodes for the transcription factor CREB (cyclic adenosine monophosphate response element-binding protein), which is involved in the regulation of genes important for neuronal regulation and survival of proteins such as brain-derived neurotrophic factor [493, 560, 561]. *Smad2* mediates TGF-β signals controlling a wide variety of cellular processes, including cell differentiation, proliferation, apoptosis, developmental fate specification, and differentiation of hippocampal granule neurons [505-507]. Similarly, IL-1R1 signaling mediates innate and acquired immune responses [502], associated with protection or aggravation of the disease states in the CNS, infections, and cancers [503, 562]. Knocking out these genes have shown to result in apoptotic cell death and affects brain functions and neurobiology adversely [505, 563, 564]. The upregulation of these three genes in all groups after PE at nine months suggests the neuroprotective role of PE, which has also been reported earlier [565].

Similarly, two of the genes, i.e., *Sirt1* and *Il12a,* were upregulated in PE mice compared to control and EE mice. These genes were not differentially expressed in PE+EE mice. This suggests that the addition of EE to PE reduces the expression of these two genes. *Sirt1* gene is a mitochondrial gene that encodes for SIRT1 protein, which influences energy metabolism and is important for neuronal plasticity, cognitive functions, as well as protection against aging-associated neuronal degeneration and cognitive decline [500, 501]. PE has been shown to enhance *Sirt1* activity [451]. *Il12a* gene encodes cytokine IL 12a, which act as a growth factor for activated T and NK cells, enhance the lytic activity of NK cells, and stimulate the production of IFN-γ by resting peripheral blood mononuclear cells. The upregulation of these two genes after PE is again suggestive of the neuroprotective role of PE at nine months of age.

Other genes that were upregulated in response to PE or PE+EE when compared to control or EE mice are *Foxo3*, Cs, *Actb*, *Prkaa1*, *Prkaa2*, *Htr1a,* and *Htr2a*. *Foxo3* activates genes that preserve cellular quiescence, prevents premature differentiation, and controls oxygen metabolism during aging [566]. A study has shown that *Foxo3* deficient mice are significantly less immobile in FST [568], suggesting that *Foxo3* regulates the behavioral manifestation of depression. We, however, observed no significant change in depressive-like behavior after PE at middle age. This is because none of the groups, including controls, showed the development of depressive-like behavior at middle age during our experiments. Mitochondrial genes Cs, *Actb*, *Prkaa1,* and *Prkaa2* were also upregulated in the hippocampus of 9-month-old PE and PE+EE mice when compared to control and EE mice. A study has indeed shown that PE stimulates the expression of mitochondrial genes in the hippocampus [327], thus suggesting that there can be a possible link between mitochondrial function and behavioral changes. Moderate exercise has been reported to reverse mitochondrial dysfunction and lower content of oxidation products and improve related neurological performances [570]. The upregulated *Htr1a* and *Htr2a* genes encode serotonin 1A and 1B receptors which mediate inhibitory neurotransmission. Inactivation of *Htr1a* and *Htr2a* genes in mice results in behavior consistent with an increased anxiety and stress response [499]. The upregulation of *Htr1a* and *Htr2a* genes, therefore, suggests

improved serotonin neurotransmission in the hippocampus after short-term PE at middle age.

The downregulated immune genes Ifng and *Tnfrsf1a* encode IFN-γ and members of the TNF receptor family of membrane-bound and soluble proteins that interact with the ligand TNF-α. Both IFN-γ and TNF-α regulate complex injury promoting proinflammatory and apoptotic and protective cell proliferation and survival roles in the CNS depending on dose, disease phase, and cell development stage [447, 514]. Moreover, evidence suggests that both TNF-α and IFN-γ are involved in the modulation of CNS plasticity, regulates memory function and anxiety and depressive states [448, 515, 575, 576]. The downregulation of Ifng and *Tnfrsf1a* again suggests that PE at nine months is neuroprotective.

EE has been shown to modulate the expression of genes in the hippocampus that regulate neuronal growth, structure, and functions [67]. Results on EE during our study, however, contrasts with previously published literature. For example, a study reported that EE upregulates the *Htr5B* gene expression and reverses the depression-like state caused by early life seizure experiences [355]. Likewise, EE in the absence of exercise showed anxiolytic effects in 5-HT1AR KO mice [356]. EE has also been shown to modulate the expression of 5-HT receptors in the hippocampus of R6/1 transgenic mice enriched for four weeks, from 8 to 12 weeks of age [577]. However, the authors also noted gender effects, and the *Htr2a* gene differentially expressed only in females. Hence, the observed change in the behavior despite no GOI being expressed differentially after EE could be attributed to gender effects that we could not measure due to a low sample size for gene expression analysis. While our behavioral results suggest that EE elicits age-dependent effects on anxiety-like behavior and could be anti-depressive in long-term, we hypothesize that more research with long-term EE using a wide variety of objects may be required to fully understand the effects of EE on hippocampal gene expression.

Microglia and astrocyte number in the dentate gyrus

Aging, as well as short-term PE and PE+EE, and short- and long-term term EE affect microglial number in the dentate gyrus.

Astrocytes and microglia are the primary immune effector cells in the CNS and regulate the extensive bi-directional communication between the nervous and the immune systems in response to different immunological, physiological and psychological stressors, as well as external laboratory treatments. Indeed, in constitutive states, they are essential for the maintenance of neurobiological homeostasis and play a vital role in neuroprotection and neuroplasticity [17, 18]. Glial cells are not only altered in density and function during normal aging and psychiatric disorders but also under improved environmental conditions, particularly in the hippocampus. In chapters 2, 4 and 6, we hypothesized that the counts of immunopositive microglia and astroglial cells, and T cells subsets in the cervical lymph nodes will increase during normal aging, in particular at late-middle age, which will be reversed by short-term PE, EE, and PE+EE, and that the effects of PE+EE and long-term EE on brain glial cells and T cells subsets in the cervical lymph nodes would be significantly greater than PE or EE alone and short-term EE, respectively. We observed a significant reduction in the expression of microglia in the dentate gyrus from 4 to 9 months but a significant increase from 9 to 14 months. This suggests that aging is a controlling factor for microglial numbers and may have significant implications in the microglia-mediated neuroimmune pathways.

However, contrary to the effects of aging, we observed a significant increase in the number of immunopositive IBA1$^+$ microglia after short-term EE at both 4 and 9 months and after short-term PE and PE+EE at 9 months. None of the treatments significantly differed from controls at 14 months. Furthermore, we observed an increase in microglial numbers after both short- and long-term EE during middle age and no significant differences between the two EE groups were noted.

During aging, there is an increase in the expression of cellular senescence proteins. This results in an increased expression of reactive microglia with age [101], 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 [289, 479], depression [480], and neurodegenerative diseases such as AD [44]. 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.

The abovementioned increased expression of reactive microglia during old age leads to enhanced glial pro-inflammatory cytokine signaling, impaired neurobiology, and an increase in apoptotic cell death [101]. Hence, it is hypothesized that any beneficial effects of the treatment on brain functions during normal aging would be accompanied by a decrease in the number of IBA1$^+$ microglia in the dentate gyrus. However, the significant increase observed after EE, PE, and PE+EE at middle age could, in turn, be the result of an unusual reduction in the number of IBA1$^+$ microglia in controls during middle age. Also, short-term PE+EE mice had significantly more IBA1$^+$ microglial cells in the dentate gyrus at 14 months compared to 4 and 9 months. The results for EE are in line with previously published findings where short-term EE significantly increased microglia antigen (IBA1$^+$) expression within the dentate gyrus of adult male Sprague–Dawley rats [371]. This, however, fails to explain the beneficial effects of the short-term EE on locomotor activity, anxiety-like behavior and spatial memory at middle and late-middle age that we have reported in chapter 3 [592]. Furthermore, the IBA1$^+$ microglia result does not explain the reported spatial learning impairment and depressive-like behavior at an early age after short-term PE. These results are interesting given the microglia are considered the primary immune effector cells in the brain and any dysfunction in microglial functioning is often associated with impaired brain functions and vice versa. However, we investigated mice that aged from early to late middle age, and not old age, in a relatively stress-free environment, thereby reducing the likelihood of pathogen- and damage-associated molecular patterns accelerating the microglia-related process. As such, an increase in microglial number during our work is indicative of the effects of normal aging with other external confounders significantly filtered out, and could primarily be due to an increase in the number of quiescent and not reactive

microglia. Future research investigating the change in the proportion of two phenotypes after the three treatments is required to confirm this hypothesis.

We also = observed that the behavioral changes in response to either short or long-term EE, i.e., enhanced baseline locomotion and reduced depressive-like behavior after both short and long-term EE, and reduced locomotion in a threatening environment and increased anxiety-like behavior after only long-term EE, as reported in chapter 5 [613], were accompanied by an increase in microglial number within the dentate gyrus of the hippocampus of middle age C57BL/6 mice. The increase in the number of IBA^+ microglia in the dentate gyrus after EE is consistent with the previously published findings in adult rats [371]. This also indicates enhanced neuroprotection at middle age after EE, irrespective of the duration, which in turn, improves brain functions such as baseline locomotion and depressive-like behavior via the microglia-mediated molecular mechanism.

Short-term PE, EE, and their combination has no effect, but long-term EE enhances astroglia number in the dentate gyrus

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. During aging the quiescent astrocytes reduce in number and turn into reactive phenotype characteristic of neuroinflammatory reactive astrocytes [434]. 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.

None of the short-term treatments differed significantly from controls for $GFAP^+$ astroglia cells at any of the ages. However, 15-month PE mice showed significantly lower expression of $GFAP^+$ astroglia cells than 5-month PE mice, suggesting that the short-term PE might influence astrocyte-related processes during old age. We hypothesize that the longer duration of PE, EE, and PE+EE treatments may be required to observe a significant change in the $GFAP^+$ astroglia cell number in the dentate gyrus. No effects of short-term EE on $GFAP^+$ astrocyte expression in the brain is contrary to the previously reported findings of a significant increase in the number of astrocytes in 2-month-old C57BL/6 mouse brains after 7 weeks of EE, albeit the increase was shown in layer 1 of the motor cortex and not in the dentate gyrus [587]. Nonetheless, a significant increase in astrocyte ($GFAP^+$) number within the dentate gyrus has also been reported in adult male rats that were housed for 12 hours in an enriched environment daily for seven weeks [371]. Hence, we hypothesized that no effects of EE on immunopositive astroglia cells during our work could be due to the shorter duration of EE. When we investigated the effects of long-term EE on $GFAP^+$ astrocyte number in the dentate gyrus, we indeed observed an increase in the number of $GFAP^+$ astrocyte in the dentate gyrus. A study has shown that long-term EE of 3 months, from

5 to 8 months of age, increased the number of astrocytes in a mouse model of Alzheimer's disease, albeit in the presence of a running wheel [620]. It must be noted that long-term physical exercise alone can enhance the number of hippocampal GFAP$^+$ astrocytes [621]. Together, the abovementioned evidence suggests that the age of the rodents, duration of EE, as well as the presence or absence of running wheels in EE protocol, may alter astrocytes number within the dentate gyrus.

T cell immunophenotyping of cervical lymph node cells

Short-term EE and PE+EE increase the proportion of peripheral CD8+ T_N cells in young and late middle age mice, respectively, reversing the effects of normal aging. Long-term EE alters peripheral T cell numbers at middle age.

In chapters 2, 4 and 6, we hypothesized that normal aging will result in decrease in T helper and increase in T cytotoxic cell subsets in the cervical lymph nodes, which will be reversed by PE, EE, and PE+EE, and that the beneficial effects of PE+EE and long-term EE would be significantly greater than PE or EE alone and short-term EE, respectively.

No significant differences were observed in the proportion of total CD4$^+$ and CD8$^+$ T cells, CD4 T_N, T_{CM} and T_{CM} and CD8 T_{EM} cells, and CD4 CD25 cells between the three age groups. There was a significant decrease in the proportion of the CD8$^+$ T_N cells from 9 to 14 months. Conversely, a significant increase was observed in the proportion of CD8$^+$ T_{CM} cells between 4 and 9, and 4- and 14-months groups. Our results indicate that aging alone does not induce proliferation of T cells or change in T cells phenotype and 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 T_N cells [297, 298], which, in turn, reduces the ability to establish immunological memory in response to new antigens. 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 [307]. Similarly, the adverse effects of aging on the CD8$^+$ T cells receptor repertoire diversity has also been reported in another study [309]. Overall, it is clear that normal aging has limited effects on the T cell number and diversity until the late-middle age.

Short-term PE and EE did not affect the cervical lymph nodes CD4$^+$ T cell subsets until late-middle age. When further subset analyses of the proportion of CD4$^+$ T cells were carried out, we observed a significantly higher proportion of T_N cells in the 5-month PE+EE mice compared to 5-month controls and PE+EE mice at 10 and 15 months. Moreover, the proportion of T_{CM} cells was significantly higher in 5-month EE and PE mice when compared to 5-month control mice, and 10- and 15-month mice that received the same treatments. Interestingly, the proportions of T_{EM} in 5-month PE and EE groups were significantly lower than the same age controls as well as older mice receiving the same treatments. Overall, this suggests that while the combination of short-term PE+EE increased the proportion of CD4$^+$ T_N cells, PE or EE alone did not. On the other hand, short-term PE and EE in isolation not only induced an increase in

the proportion of $CD4^+$ T_{CM} cells but also reduced the proportion of $CD4^+$ T_{EM} cells at an early age. Hence, in our study, exposure to either physical or environmental enrichment appears to induce an earlier expansion of CD4+ T_N to $CD4^+$ T_{CM} cells. Also, the lower proportions of CD4+ T_{EM} cells in 5-month PE and EE can be explained by the fact that mice during our experiments were raised in a relatively stress-free environment also devoid of PAMPs. However, aging can also result in the production of DAMPs, which can act as cognate antigens. This might explain the higher proportions of CD4+ T_{EM} cells at 10 and 15 months. The controlled environment could also be the reason for no differences noted in the proportion of $CD4^+$ $CD25^+$ cells except for a 5-month PE group showing a significantly higher proportion of $CD4^+$ $CD25^+$ cells than the 5-month controls and the older mice receiving the same treatment. Since $CD4^+$ $CD25^+$ cells have been reported to suppress the proliferation of effector T cells [603, 604], this suggests that short-term PE might help to develop immunological self-tolerance at an early age.

During the analysis of $CD8^+$ cells population, we observed that the proportion of peripheral $CD8^+$ T cells at late-middle age increased significantly after short-term EE. However, no change in the proportion of $CD8^+$ T cells was noted after both short-term PE and PE+EE at all ages. This was contrary to the hypothesis that short-term PE will improve neuroimmune functions during normal aging by reducing the proportion of $CD8^+$ T cells in C57BL/6 mice in a controlled environment. Further subset analyses of the proportion of $CD8^+$ T cells revealed an increase in the proportion of $CD8^+$ T_N but not T_{CM} and T_{EM} cells after short-term EE at late-middle age. This suggests that EE improves basal cytotoxic immunity during the late-middle-age reversing the age-related effects. This, possibly also explains the reported improvement in brain functions after short-term EE [592]. In contrast, we observed that the combination of short-term PE and EE also increased the proportion of $CD8^+$ T_N cells, but only at an early age and not at middle and late-middle age. These results are interesting and show the influence that the inclusion of a running wheel in EE paradigms can have on cell-mediated immunity. Furthermore, the proportion of $CD8^+$ T_{CM} cells in the cervical lymph nodes of control mice significantly increased from middle to late-middle age. This can again be explained by the fact that there is an increase in the production of DAMPs during aging, which may act as cognate antigens stimulating the conversion of the peripheral $CD8^+$ T_N cells to $CD8^+$ T_{CM} cells. The latter have little or no effector function but mediate reactive memory where they migrate to secondary lymphoid organs, and some of them proliferate and differentiate into effector cells in response to antigenic stimulation, thus helping the body to resolve inflammation [304]. The proportion of $CD8^+$ T_{EM} cells in the cervical lymph nodes of 5-month PE mice was significantly lower compared to older PE groups. Since the $CD8^+$ T_{EM} cells are important mediators of protective memory as mentioned before, the lower number of $CD8^+$ T_{EM} cells after PE at 5 months suggests that perhaps the cell-mediated immunity gets compromised after short-term PE at an early age.

Our results also suggest that the duration of EE has no differential effects on the expression of $CD4^+$ T cells and their subsets. However, a longer duration of EE may be beneficial in improving cytotoxic immunity at middle age, as shown by an increase in $CD8^+$ and $CD8^+$ T_N cell numbers in the long-term EE mice in comparison to short-term EE mice. The controlled environment, absence of pathogens and stressful stimuli, age and strain of the rodents, EE paradigm structure, size and traits of EE objects, and the frequency of changing the objects that we utilized during our study, may all have a

role to play in the observed findings. Further research into the differential effect of the various EE objects on neuroimmune functions in older mice may be required. Furthermore, the results need to be followed up in old-age and psychiatric disease rodent models to further validate and extend the findings. Additionally, since running wheels have been used as an EE tool in past studies, the differential effects of long-term EE, long-term PE, and their combination, need to be explored.

Chapter 8: Thesis Conclusion

Preamble to the thesis conclusion

In chapters 2 to 6, we provided the results of the research, which were then discussed in chapter 7. Together, the findings provide a broader understanding of the effects of PE, EE and the combination of PE and EE on brain functions during aging and the overall results could be translated to clinical trials in ways better than ever before.

The aim of the chapter is to conclude the research work that I conducted during my degree in Doctor of Philosophy.

Conclusion

There are only a few studies where the effects of aging on brain functions and underlying molecular biology are investigated in the absence of all external interventions. Our results of an increase in anxiety-like behavior, spatial learning and memory impairment, and microglial expression within the dentate gyrus at late-middle age, as well as alterations in hippocampal gene expression with age, complied to the previously published results. However, unlike previously reported findings, we observed that aging alone does not induce depressive-like behavior and change in astrocyte expression within the dentate gyrus until late-middle age in C57BL/6 mice. This finding is important as aging has often been associated with the onset of depression and pathological astroglia changes in the past.

When we investigated the differential effects of short-term PE, EE and PE+EE on brain functions and associated molecular changes, we observed that EE mice performed better than both PE and PE+EE mice during behavioral testing, which suggests that in the short-term, EE alone is more beneficial than PE or their combination to alleviate anxiety and depression symptoms. This was despite short-term PE modifying the expression of hippocampal genes of interest while short-term EE showing no effects on hippocampal genes. However, given a large number of studies on the beneficial effects of PE on brain functions, we cannot ignore the possibility that PE and PE+EE in the long-term may elicit equivalent or even greater effects than EE alone, the hypothesis that could be tested in future research. We found no previous evidence to suggest that EE in the short-term also affects hippocampal genes, hence this makes our study one of a kind. Furthermore, both short-term PE and EE modulated the brain glial cells and the peripheral $CD8^+$ T_N cells count. These observed greater effects of short-term EE on brain functions and immune factors in the absence of any change in hippocampal gene expression could be attributed to an increasing in the likelihood of survival of new cells in the hippocampus after EE as described before.

However, the duration of the environment may not play a significant role in the modulation of brain functions and underlying molecular changes. We observed that both short- and long-term EE alleviates depressive-like behaviors in the absence of any change in the expression of hippocampal genes of interest. Similarly, both short- and long-term increased the number of immunoreactive microglia within the dentate gyrus but only the long-term EE increased the number of immunoreactive astrocytes within the dentate gyrus and modulated peripheral T cell number and phenotype during middle age in C57BL/6 mice. Together, this suggests that the duration of EE has only subtle effects on behaviors and possibly on molecular profiles.

It is important to note that unlike many past studies, our EE paradigm has no running wheel included. This also makes our study the only one to investigate the effects of EE in the absence of PE on hippocampal gene expression. Overall, our results also suggest that PE can significantly affect the behavioral testing results in EE paradigms, as well as the expression of central and peripheral immune factors and hippocampal genes.

Chapter 9: Limitations of the study and future directions

Preamble to the limitations of the study and future directions

We conducted research on C57BL/6 mice and developed our own PE, EE, and PE+EE paradigms. The mice were aged from early to late-middle age. There were a few limitations of the study that are mentioned in this last chapter. Also, we aim to provide future research directions based on our results and discussion to help research works currently being planned.

Limitations of the study and future directions

At the start of this study, we investigated the effects of normal aging on brain functions and underlying changes in immune factors and hippocampal gene expression in C57BL/6 mice in the absence of all external interventions. During our extensive literature search, we found that only a few studies have investigated the effects of aging alone on the brain of mice. Most others have used one or the other form of external intervention on aging mice. These interventions include isolation, maternal separation, auditory or olfactory exposure, physical exercise, environmental enrichment, dietary supplements, and pharmacological drugs. Since it has been reported that the external environment can have a profound effect on brain functions and underlying molecular biology, findings from such studies may not be comparable with our reported results on the effects of aging alone.

We then provided the external treatments PE, EE and their combination to C57BL/6 mice and investigated their outcome on the brain functions and underlying molecular biology. Yet, other confounders such as age of mice, design of our study, cage dimensions, handling frequency, mouse-mouse interactions, number of wheels per cage, differences in the delivery of enrichment such as box dimensions, number/type of toys and how frequently they were changed may have accounted for those results during our study that were different from previously reported findings, for example unlike many past studies, we showed the aging alone may not be enough for the onset of depressive-like behavior until late-middle age in C57BL/6 mice. Hence, in order to understand the effects of external interventions on depressive-like behavior, it is advisable to first induce depressive-like behavior in mice or use transgenic mouse models of depression. Furthermore, our EE paradigm did not include running wheel as a tool of EE and running wheel activity alone could be a strong modulator of hippocampal gene expression as we have shown in this study. Also, from our first set of experiments, we know that aging alone can have a significant effect on the underlying molecular biology. Taken together, this suggests that findings from our second and third set of experiments may have also been affected by the age of mice we used during our study. These limitations must be considered in future studies investigating the effects of PE, EE or their combination on brain functions, neuroimmune mechanisms and hippocampal gene expression in aging mice. We also suggest that external environmental conditions and the strain and age of mice must be considered prior to using our findings as reference points in future research.

More research is required to further understand the effects of the presence and absence of running wheels, long-term PE, EE and their combination in older C57BL/6 mice, as well as in transgenic strains of mice relevant to the specific disease model. Also, the differential effects of the delivery of enrichment such as box dimensions, number/type/physical characteristics of toys and how frequently they were changed need to be explored. Other molecular factors, such as neurotrophins and neurotransmitters may also show variations in response to the treatments we provided and can be investigated in future research. Indeed, impaired neurotransmission [276-278] and growth hormones [276, 443] and glucose metabolism [277] in the aging brain have been reported to result in dysregulated synaptic plasticity and neurogenesis, and impaired cognitive and motor performances. Conversely, the levels of NGF and BDNF have been reported to increase in specific regions of the brain in an enriched environment [388, 627-629].

Furthermore, we conducted behavioral testing for 3 weeks on mice immediately before molecular analysis. Hence external factors, such as social or handling stress [29, 30, 630] and sensory stimulation [631, 632] during behavioral testing, could have modulated the brain glia number and cervical lymph node T cell subset numbers. However, it must be noted that all mice underwent the same behavioral testing protocol which should control for any of these effects. It is also possible that the immune response may not have been strong in response to treatments at middle age (9-month) in C57BL/6 mice. Furthermore, it is the primed phenotype of glial cells that drives functional changes. This encompasses much more than an increase in the number of immunopositive glial cells in the dentate gyrus. Hence, examining the morphology of glial cells and levels of other activation markers, for example, cytokines, chemokines, C-reactive protein, and protein kinases is required to develop a full understanding of neuroimmune response after the short- and long-term EE treatments. Our results, therefore, do not make a full profile of the neuroimmune response and may not fully explain the reported change in brain functions after the two treatments, however, we believe these provide a head start and would contribute significantly to the understanding of overall neuroimmune changes in response to EE together with future work. Nonetheless, it must also 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.

Another limitation of our study is that we could not analyze the effect of sex due to the low sample size (when the groups were divided into males and females). Sex could have significant effects on the reported results and as such we believe an independent study investigating a three-way interaction between treatment, duration of EE and sex would be very useful. Also, although the starting age of long-term EE mice is different from short-term EE mice, it was not possible to have the same starting age. In the latter case, we would have been comparing mice of different ages during behavioral testing (after 4 weeks and 6 months of EE). This would have led to even greater differences not related to the EE since aging is a known risk factor for change in brain functions and underlying molecular factors. Furthermore, during this study, no measures of neurogenesis were analyzed. The questions we have raised should be explored in future EE studies on rodents, including those in preclinical models of brain disorders.

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