WESTERN SYDNEY
UNIVERSITY

A study of the effects of chronic neuroinflammation on cognition and behaviour in the GFAP-IL6 transgenic mouse and investigation of the flavonoid apigenin as a neuroprotective agent

Sandra Sonego

School of Medicine, Western Sydney University

This thesis is submitted for the degree of Master in Science (Medicine)

March 2018

Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

Signature: 

Name: Sandra Sonego

Date: 21/3/18

Acknowledgements

This work is dedicated to my family and the tiny lives that were sacrificed.

Bushido, choose wisely, Waheguru.

Abstract

Neuroinflammation is a prominent feature of most neurodegenerative and affective disorders and has been increasingly implicated as a contributing factor in the disease development. The inflammatory process is closely linked with multiple neurodegenerative pathways and represents an important therapeutic target in halting or reversing disease progression.

This study explored the cognitive and behavioural effects of chronic neuroinflammation in the glial fibrillary acidic protein promoter-interleukin 6 (GFAP-IL6) transgenic mouse in which the pro-inflammatory cytokine interleukin 6 (IL-6) is overexpressed causing low level, chronic inflammation localised to the brain. Additionally the anti-inflammatory, anti-oxidant and neuroprotective potential of the dietary bioflavanoid apigenin was investigated to determine whether any deficits in behaviour and cognition could be rescued.

Male and female heterozygous GFAP-IL6 mice (n=32) and their non-transgenic littermates (C57/BL6J) (n=36) were introduced to either an apigenin enriched pellet diet (40mg/kg daily dose) or control pellet diet at the age of 3 months. After 3 months of feeding (6 months of age) the mice were subjected to a behavioural test battery including the elevated plus maze (EPM), open field test (OF), Barnes maze (BM) and functional observational tests.

The results showed that at 6 months of age, GFAP-IL6 mice exhibited alterations in anxiety-related behaviour in the EPM and OF, presenting a tendency toward an anxiolytic-like phenotype and demonstrated impairments in memory and spatial learning in the BM. GFAP-IL6 mice also displayed an ataxic phenotype and exhibited reduced locomotor activity compared to wild-type (WT) controls.

The effect of apigenin on anxiety-related behaviours were mixed, being sedative-like in reducing locomotion and explorative behaviour in the EPM and OF, and anxiolytic-like in reducing risk assessment behaviour. Cognition improving properties are indicated for apigenin in the BM probe trial, enhancing recall of the target hole location, particularly among GFAP-IL6 mice. Interestingly, apigenin was additionally associated with increased food consumption and weight gain, suggesting potential as an appetite stimulant.

List of publications

Journals:

1. Venigalla M, Sonego S, Gyengesi E, Sharman MJ, Münch G. (2016). Novel promising therapeutics against chronic neuroinflammation and neurodegeneration in Alzheimer's disease. *Neurochemistry International*, May; 95:63-74. doi: 10.1016/j.neuint.2015.10.011.
2. Venigalla M, Sonego S, Gyengesi E, Münch G. (2015). Curcumin and Apigenin - novel and promising therapeutics against chronic neuroinflammation in Alzheimer's disease. *Neural Regeneration Research*, 2015 Aug;10(8):1181-5. doi: 10.4103/1673-5374.162686.
3. Millington C, Sonego S, Karunaweera N, Rangel A, Aldrich-Wright JR, Campbell IL, Gyengesi E, Münch G. (2014). Chronic neuroinflammation in Alzheimer's disease: new perspectives on animal models and promising candidate drugs. *Biomed Research International*, 2014:309129. doi: 10.1155/2014/309129.

Conference papers:

4. H Liang, S Sonego, E Gyengesi, A Rangel, G Niedermayer, T Karl, G Münch.(2017) OP-25 - Anti-Inflammatory and Neuroprotective Effect of Apigenin: Studies in the GFAP-IL6 Mouse Model of Chronic Neuroinflammation (abstracts of the OCC World Congress and Annual SFRR-E Conference 2017 Metabolic Stress and Redox Regulation Berlin, Germany 21-23 June 2017) *Free Radical Biology and Medicine*, Volume 108, Supplement 1, July 2017, Page S10. https://doi.org/10.1016/j.freeradbiomed.2017.04.064

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1 Background

Neuroinflammation is now understood to be a fundamental process underlying the progression and perhaps even the initiation of a diverse range of neurological diseases and disorders, including neurodegenerative diseases such as Alzhemier's disease (AD) and neuropsychiatric disorders including major depressive disorder (MDD). Neurological diseases and disorders constitute a significant burden on healthcare systems globally. The World Health Organization (WHO) has predicted that by 2040 neurodegenerative diseases alone will exceed cancer as the second leading cause of death worldwide[1]. While neurodegenerative diseases and affective disorders exhibit vastly different clinical symptoms, they all share neuroinflammation as a common link, characterised by microglial activation, reactive astrogliosis, expression of pro-inflammatory cytokines, release of reactive oxygen species (ROS) and nitrogen species. Although immune activation in the central nervous system (CNS) occurs as a protective reaction against microbial infection, acute injury or disease, uncontrolled and sustained neuroinflammation can create a neurotoxic environment that leads to neuronal injury, synaptic loss and neuronal death.

In view of the evidence suggesting neuroinflammatory dysregulation underpins neurological diseases and disorders, it becomes critical to understand this association and its mechanisms in order to derive future intervention strategies that address these potential pathological targets. In particular, the mitigation of activated microglia toward reparative and neuroprotective processes rather than neurodestructive may be a key therapeutic approach.

A proposed animal model in which to study the effects of sustained neuroinflammation and neurological disease is the glial fibrillary acidic protein-interleukin 6 (GFAP-IL6) transgenic mouse[2,3]. In this model, inflammation is localised in the brain by triggering the chronic expression of the cytokine interleukin-6 (IL-6) from astrocytes, a cytokine frequently observed to be upregulated in neuroinflammation and pathophysiology of various neurological disorders[4]. This animal model adequately reproduces fundamental components of progressive neurodegeneration including neuronal loss and atrophy,

activated glia, increased expression of inflammatory mediators, breached blood brain barrier (BBB), and both motor and cognitive impairments[2,5,6]. As such, the GFAP-IL6 transgenic mouse could serve as a suitable model for the study of chronic neuroinflammation and a novel tool for drug discovery and validation *in vivo*.

Despite decades of intensive research into neurological disorders, current pharmacological treatment is focused on the management of symptoms, with few relevant medicines that actually address the progression or prevention of neurological diseases. In consideration of the global burden CNS disorders represents, particularly in an aging population, there is a pressing need for the investigation and development of novel therapies that have the capacity to halt or reverse the underlying neuroinflammatory and neurodegenerative processes.

The use of plant-derived polyphenolic compounds in the context of neurological diseases and disorders has increasingly gained interest, particularly in their capacity to address neuroinflammatory mechanisms. Dietary flavonoids such as apigenin show promise in being effective multipotent neuroprotective agents through their anti-inflammatory and anti-oxidant activity, in addition to acting as modulators in the signalling pathways that regulate inflammation in the brain[7]. There is both clinical and *in vivo* preclinical evidence demonstrating the neuroprotective properties of dietary flavonoids through their defence of neurons against oxidative stress, attenuation of neuroinflammation and improvements in cognition and learning[7,8]. Furthermore, epidemiological studies also show an association between dietary flavonoid intake and reduction in the risk of certain neurodegenerative disorders[9].

The specific neuroprotective potential of the dietary flavonoid apigenin is highlighted here, which is proposed to be evaluated in the GFAP-IL6 transgenic mouse model.

1.2 Neuroinflammation

Neuroinflammation refers to the self-defensive reaction by the CNS intended to counteract and eliminate harmful stimuli such as pathogens, acute injury or disease. Neuroinflammation is characterised by microglial and astroglial activation, release of inflammatory cytokines, increased permeability of the BBB and subsequent peripheral immune infiltration, oxidative and nitrosative stress[10-12]. The localized process of brain inflammation requires tight regulation due to the "two-edged sword" of inflammation[13], where both insufficient and excessive neuroinflammatory responses can be deleterious, resulting in pathological conditions. It is understood that a short-lived acute inflammatory response in the brain is favourable, in order to initiate tissue repair, minimise neuronal injury and clear cellular debris. However unregulated and chronic inflammation in the CNS, can contribute to events that conclude in the progressive neuronal damage seen in neurological disorders. The presence of excessive levels of pro-inflammatory cytokines in the brain can not only promote neurotoxicity but can also produce acute behavioural deficits[14]. The CNS is particularly vulnerable to uncontrolled inflammatory processes and resultant oxidative stress due to the restricted cell renewal and regenerative capacity of neural tissue[15].

1.2.1 Cytokines: immune-to-brain communicators

In early research, owing to the lack of lymphatic system and presence of the BBB, the brain was considered to be an "immune privileged" site[13]. Recent studies in neuroimmunology have challenged this concept and clearly demonstrate that there exists a significant and continual cross-talk between the nervous and immune systems, and that peripheral leukocytes indeed have the capacity to infiltrate the BBB[16,17]. Cytokines are small proteins that act as signalling molecules that are especially important in the regulation of inflammation, as well as regulating cell cycle processes. For instance, during peripheral infection, information is exchanged between the immune system and the brain via the release of inflammatory cytokines by peripheral innate immune cells. These cytokines convey information to the brain by binding receptors on the BBB that induce the activation of microglia within the CNS[16-18].

These interactions most often occur at brain sites without an intact BBB, such as the circumventricular organs and the choroid plexus[14,16,17]. Cytokines can also relay peripheral inflammatory messages via the vagus nerve, causing the activation of microglia, and the neural pathways involved with the hypothalamic-pituitary-adrenal (HPA) axis that leads to sickness behaviour[14].

The communication network between the nervous and immune systems is comprised of a vast array of immune, neuronal and hormonal signalling molecules. Key players in the CNS immune activation include neurons, microglia, astrocytes, endothelial cells and adaptive immune cells[12,13].

1.2.2 Threat detection and triggers of the CNS immune response

Innate and adaptive immune responses in the CNS are triggered by cellular interactions between pattern-recognition receptors (PRRs) expressed by glia, epyndymal and endothelial cells and neurons, and pathogenic or 'danger' molecules termed pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) respectively[19]. Among the best studied PRRs are toll-like receptors (TLRs), which bind the highly conserved PAMP or DAMP ligands to trigger innate and adaptive immune responses. In neurological disorders TLRs are frequently observed to be upregulated in glia. Examples include TLR2 and TLR4 expression in AD[19]. An increasing role has also been suggested for the receptor for advanced glycation end products (RAGE)[19].

1.2.3 Microglia: major players in neuroinflammation

Microglia are the principal cells involved in brain innate immune response. Microglia are similar in function to peripheral macrophages[7,20], and constitute between 10-15% of all brain cells[21]. Inflammation in the CNS is primarily mediated by microglia, interacting and influencing surrounding astrocytes and neurons[7]. Microglia possess a dualistic role in the ability to be both neuroprotective and neurotoxic, dependent upon the state and stimulus[22]. Under normal conditions, microglia classically termed as 'resting', continually survey the microenvironment of the brain, and are typified by a ramified

morphology with dynamic processes[10,20]. In this inactivated state microglia express various surface receptors that help to maintain brain homeostasis by the promoting clearance of cellular debris and aggregated or misfolded proteins[12].

Upon exposure to immunological stimuli including beta-amyloid (β-amyloid) peptide, lipopolysaccharide (LPS), ROS, or interferon-alpha (IFN- α), microglia become activated, leading to modification and up-regulation of microglial cell-surface receptors expression, increased proliferation, morphological change to amoeboid shape, increased phagocytic activity and secretion of pro-inflammatory cytokines[18,20]. In this activated state microglia have the capacity to clear cellular debris and amyloid fibrils, and release neurotrophic factors to promote the survival of neurons[23]. At the same time however, activated microglia can release cytotoxic factors and pro-inflammatory molecules such as tumor necrosis factor-alpha (TNF-α), prostaglandin E2 (PGE_2), activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and inducible nitric oxide synthase (iNOS), which can cause collateral damage to surrounding tissues[18]. It is proposed that there is a spectrum of activated microglial phenotypes rather than a singular activated phenotype[24], with the degree and type of microglial activation dependent upon their cytokine and cell-surface receptor profiles. However, two main activated microglia phenotypes have been described, M1-like and M2-like, based on their similitude to the classically and alternatively activated macrophage phenotypes in peripheral immune responses. M1-like activated microglia exhibit a neurotoxic phenotype by expression of pro-inflammatory cytokines, while the M2-like phenotype is neuroprotective by secretion of anti-inflammatory mediators. It is the over-activation of the inflammatory M1-like microglial phenotype that has been implicated in the pathogenesis of diverse neurodegenerative diseases[12].

Sustained and unregulated microglial activation can cause a perpetual cycle of inflammation by becoming a source of excess nitric oxide (NO), ROS, pro-inflammatory cytokines including TNF-α, IL-6, interleukin 1-alpha and interleukin 1-beta (IL-1α/β), chemokines, and glutamate. Excess NO can disturb mitochondrial function, leading to neuronal energy disruption and further augments ROS production. Surplus pro-inflammatory cytokines further enhance iNOS induction and stimulate the release more inflammatory cytokines, while excessive glutamate concentrations can exert neuronal

damage via excitotoxicity. Moreover, the release of TNF-α by microglia can directly promote neuronal apoptosis, feeding the inflammatory signalling cascade further. Regulation of microglial activation hinges upon the activities and interplay between neurons, BBB, astrocytes, cell surface receptors, cytokines, ROS, and neurotransmitters. The regulatory signalling pathways involved in microglial activation are understood to be the mitogen activated protein kinase (MAPK) and nuclear-factor-KB (NF-KB) cascades, including transcription factors such as activator protein 1 (AP1) and signal transducer and activator of transcription-1 (STAT-1). Irrespective of the triggers leading to neuroinflammation, microglia constitute a central point of convergence and regulation[10] and as such represent an important therapeutic target in neurological disorders.

1.2.4 Astrocytes: synergistic actions with microglia

In conjunction with microglia, astrocytes are involved in the induction of neuroinflammation. Astrocytes are suggested to play a significant role in the maintenance of neuroinflammatory states being the most abundant glial cell in the CNS and due to their longer lasting reactivation[25]. These star-shaped cells serve many functions in the regulation and optimization of the neuronal environment, including ion and pH homeostasis, glutamate control, metabolic support, induction and maintenance of the BBB[12,25].

In response to pathogenic factors, several studies have shown that astrocytes over-express pro-inflammatory mediators, indicating astrocytes could contribute to the neurodegenerative process through the release of various growth factors, chemokines and cytokines such as IL-1β and α, IL-6 TNF-α, and interferon–gamma (IFN-γ)[25]. Moreover, astrocytes are also influenced by activated microglia and are reported to adopt a neurotoxic phenotype similar to microglia during chronic and uncontrolled inflammation[12,25]. For example, secretion of TNF-α by microglia induces an increased release of glutamate by astrocytes, contributing to neuronal excitotoxicity[12]. Conversely, astrocytes are also neuroprotective, being actively involved in ROS clearance and removal of β-amyloid plaques[12].

Importantly, as integral components of the BBB, astrocytes may also play a role in the events leading to neurological diseases. For example, by releasing chemoattractant chemokines and leukocyte adhesion molecules, astrocytes are thought to facilitate leukocyte recruitment across the BBB into the CNS, initiating neuroinflammation[25,26].

1.3 The link between neurodegenerative diseases, neuropsychiatric disorders and neuroinflammation: A unifying hypothesis

Mental and neurological disorders are a major global public health burden, particularly among developed countries where the proportion of neurological deaths is steadily rising[27]. Despite several years of extensive research, the aetiology and pathogenesis of most neurological diseases and disorders are yet to be elucidated. Consequently there are currently no treatments that cure or reverse the progression of neurodegenerative diseases such as AD, and similarly, treatments for mood disorders like MDD fail to target the causal factors. Risk factors for disorders of the CNS appear to be heterogeneous in nature involving a diverse and complex number of environmental, lifestyle and genetic factors, often with compounding effects. Aging, peripheral inflammation and environmental stressors have been suggested to be factors rendering the CNS more vulnerable to damage[14].

It is now well documented that chronic neuroinflammation is a shared characteristic of neurodegenerative diseases and neuropsychiatric disorders. The neuroimmune hypothesis of neurological disease stems from evidence that reveals common features among different neurological conditions including: activated microglia, astrogliosis, protein aggregation, compromised BBB, infiltration and accumulation of leukocytes, changes in glucose metabolism, oxidative stress, elevated levels of various cytokines, in addition to alterations in several other inflammatory mediators[10,13,19,28]. As previously outlined, neuroinflammation is a necessary and beneficial process that confers the CNS protection from infection and injury, however it appears that the clearance of harmful agents by microglia frequently fails, and/or an exaggerated response is mounted which results in unresolved inflammation[16]. The exact mechanisms and events that cause the conversion from beneficial

neuroinflammation to chronic and detrimental neuroinflammation are not yet fully understood. However, it is postulated that secondary damage mediated by the initial inflammatory response continually disturbs and prevents the return to homeostasis[16]. It has also been suggested that in response to injury or threat, activated microglia may have trouble converting to a 'resolving-phenotype' or take too long to do so, which exacerbates and prolongs inflammation into a perpetual state[16]. Furthermore, chronic microglial activation alters the permeability of the BBB, resulting in an influx of peripheral immune cells that further "prime" microglia toward an aggressive M1-phenotype response[14].

Recent research increasingly suggests a significant role for low-grade systemic inflammation as an underlying mechanism in chronic neuroinflammation and resulting neurodegeneration[24]. It is hypothesised that during the course of a person's lifetime, the body accumulates pathology from repeated inflammatory insults via infections or chronic inflammatory diseases, and this renders the brain susceptible to inflammatory over-reactions, particularly in cases where microglia are already 'primed' (Fig. 1)[29].

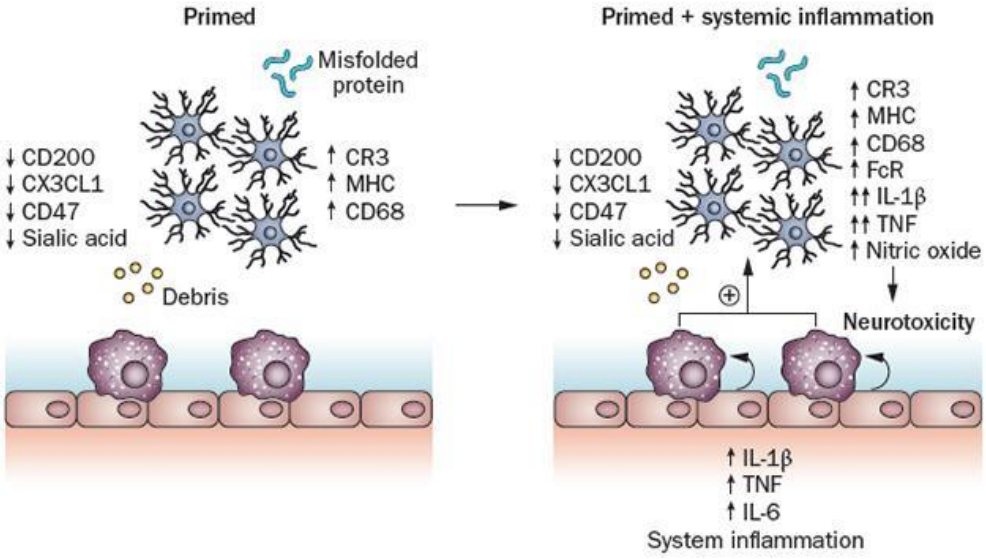


Figure 1.1 The effects of systemic inflammation on primed microglia

Source: Systemic Inflammation: A Driver of Neurodegenerative Disease? 2015. (Accessed 5th May, 2015, at http://www.alzforum.org/news/conference-coverage/systemic-inflammation-driver-neurodegenerative-disease.)

For example, it has been shown that neuroprotective M2-like microglia in the brains of prion-diseased mice rapidly change to more aggressive M1-like pro-inflammatory microglia after being administered an intraperitoneal dose of LPS[14,24]. Moreover, epidemiological studies show systemic infection is

associated as a risk factor for dementia in patients with AD, and a risk factor for relapse in multiple sclerosis (MS) patients[14].

Aside from infectious and inflammatory diseases, other sources of low-grade chronic systemic inflammation have been suggested. One example is intestinal permeability affecting the gut-brain axis as a possible internal stressor. It is thought that increased gut permeability results in increased translocation of LPS from enteric bacteria into the blood, thus contributing to chronic systemic inflammation[30]. Other sources such as heightened stress responses have also been demonstrated to increase the release of pro-inflammatory cytokines such as IFN-γ and TNF-α in humans, further contributing to chronic systemic inflammation[30].

Additionally it is well known that obesity induces low-grade chronic systemic inflammation. Recently, links have been made between neuroinflammation, neurodegeneration and overnutrition-induced diseases such as obesity, type 2 diabetes and metabolic syndrome through IKKβ/NF-κB-directed inflammation in the brain[31]. Aging is also associated with a chronic inflammatory state and is therefore a contributing factor in the predisposition to neuroinflammation and neurodegeneration.

Jointly, these findings support the rationale for the use of anti-inflammatory drugs to dampen systemic inflammation, thereby mitigating chronic neuroinflammation and potentially slowing neurological disease progression.

1.3.1 Neuroinflammation in AD

AD is the most common cause of dementia, and is associated with aging. Key pathological hallmarks linked to AD include extensive neuronal loss, extracellular β-amyloid plaques composed of aggregated, cleaved products of the amyloid precursor protein (APP), and intracellular neurofibrillary tangles. Clinical symptoms comprise impaired cognitive function, memory loss, disorientation and most often psychiatric manifestations. Whether neuroinflammation is the causative factor behind β-amyloid deposition in AD is still to be determined, however it is understood that the β-amyloid aggregation itself is a potent trigger of chronic inflammation in the CNS[13]. Neuroimmune involvement in AD is evidenced

by the following findings in the brains of AD patients: activation and proliferation of microglia and astrocytes in areas surrounding senile plaques, presence of acute phase proteins, components of the complement system, and elevated levels of pro-inflammatory mediators such as major histocompatibility complex (MHC) class II, cyclooxygenase-1 and 2 (Cox-1 and Cox-2), monocyte chemoattractant protein-1 (MCP-1), TNF-α, IL-1β, and IL-6, as well as upregulation of iNOS, and NADPH [2,32-35]. Similarly, experimental and meta-analytical studies confirm the inflammatory profile of AD patients, showing elevated peripheral concentrations of IL-6, TNF-α, IL-1β, interleukin-12 (IL-12) and interleukin-18 (IL-18) compared to healthy controls[36]. It has been suggested that in the presence of β-amyloid plaques, neuronal damage in AD is driven by a pro-inflammatory microenvironment in the CNS, and that neuroinflammation is an early hallmark in AD. For example, patients with mild cognitive impairment (MCI) who have elevated levels of pro-inflammatory cytokine TNFα and decreased concentrations of anti-inflammatory transforming growth factor-beta (TGF-β) in the CSF show an increased risk of progression to AD[37]. Other evidence pointing toward neuroinflammation as a contributing factor in AD comes from epidemiological studies that report a reduced risk of AD associated with chronic use of NSAIDs[38,39]. Moreover, findings that associate genes for immune receptors such as TREM2[40] and CD33[41] with AD further support the central role of neuroinflammation in disease development.

Animal models recapulate the links between chronic neuroinflammation and AD. For example, mice with LPS-induced neuroinflammation showed an increase in β-amyloid deposition and accompanying cognitive impairment[42], while in transgenic mice models of AD such as the APPV717F and APPswe, LPS aggravated the severity of AD pathology[10].

1.3.2 Neuroinflammation in MDD

Affective disorders such as MDD are severe and distressing conditions, causing significant disability and pose a major health concern. MDD is characterised by depressed mood, anhedonia, sleep and appetite disturbances, fatigue, feelings of worthlessness and cognitive impairments[43]. There is an

increasing body of evidence suggesting neuroinflammation may play a pathogenic role in psychiatric illnesses. Neuroimmune abnormalities including elevated pro-inflammatory cytokine expression, are consistently found in MDD and other neuropsychiatric disorders. Moreover, current research has postulated the "inflammatory and neurodegenerative hypothesis of depression",[28,30] linking both neuroinflammation and neurodegeneration to depression. It is important to note that depression is a common comorbidity and a risk factor for neurodegenerative diseases such as AD and Parkinson's disease (PD)[28,30]. It is suggested that chronic neuroinflammation may incite alterations in brain structure and induce impairments in synaptic plasticity, which lead to neurodegeneration. These degenerative changes coupled with reduced neuroprotection and increased glucocorticoid levels are proposed to set the pathoglogical state behind MDD[28,30].

A number of studies consistently report increased expression of pro-inflammatory mediators in persons with MDD. For instance, case-control studies showed MDD patients had increased PGE_2 and c-reactive protein (CRP) levels in saliva, plasma or CSF, with PGE_2 and CRP levels being positively correlated with the severity and risk of depressive symptoms[43-46]. Other studies have shown significantly elevated serum concentrations of pro-inflammatory cytokines IL-6, TNF-α and soluble IL-2 receptor in persons with MDD[15,43,47] compared to healthy controls. Also supporting the role of inflammation in mood disorders is the finding that patients receiving long-term treatment with IFN- α as therapy for infectious diseases resulted in approximately 30-45% of patients developing depression[15,44].

Preclinical studies in rodents are consistent with human clinical findings, demonstrating a clear association between elevated cytokine production and higher rates of and depressive-like behaviours[28,44]. For instance, healthy animals injected with peripheral immune mediators such as TNF-α and IL-1β induces 'sickness behaviour'[15]. Animal sickness behaviour symptoms such as anhedonia, low locomotor activity and cognitive disturbances strongly parallel depression symptoms in humans (Fig. 2)[48]. Moreover, evidence supports a bi-directional relationship between markers of inflammation and depression, exemplified by the enhancement of neuroinflammation in mice subjected to external mild chronic stress paradigms[28].

Although data is inconsistent, there is evidence indicating the involvement of an underlying inflammatory process in MDD found in anti-inflammatory mechanisms attributed to anti-depressant medication, that is perhaps more relevant than its other activities[30]. For example, anti-depressants have been demonstrated to suppress LPS-induced production of pro-inflammatory cytokines and decreased cytokine-induced depressive-like behaviour in mice[44].

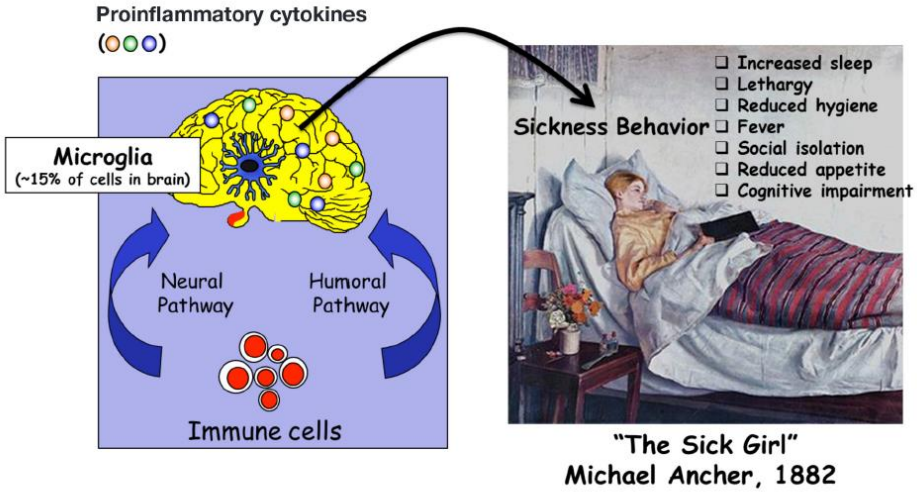


Figure 1.2. Immune-to-brain signalling activate microglia, leading to neuroinflammation, and cognitive and behavioural disturbances ('sickness behaviour').

Source: From Inflammation to Sickness and Cognitive Dysfunction: When the Immune System Subjugates the Brain 114th Abbott Nutrition Research Conference Cognition and Nutrition 2013

The HPA axis has also been implicated in the pathology of depression and mood disorders, showing pro-inflammatory cytokines alter HPA axis activity and sensitivity. For example in rodents, prior exposure to LPS was associated with greater HPA axis reactivity when challenged with TNF-α. Neuroinflammation has also been suggested to cause desensitization of glucocorticoid receptor-mediated negative feedback on the HPA axis[30,44].

Other compelling evidence shows similarities in structural brain features associated with MDD in humans and depressive-like behaviour in rodents. Structural changes in the brains of MDD patients include reduced hippocampal volume, reduction in neurogenesis, and enlargement of lateral

ventricles[44]. Strikingly, neuroinflammation induced by chronic central administration of LPS in rats show similar lateral ventrical enlargement and reduction in the size of hippocampus[44].

1.4 The role of the cytokine interleukin-6 in neurological disorders

Belonging to the neuropoietin family of cytokines, IL-6 is a pleiotropic cytokine involved in varied biological functions[4,49]. IL-6 activities include the mediation and regulation of immune and inflammatory responses, such as the growth and differentiation of T and B cells, as well as its function as a haemaopoietic factor. IL-6 is reported to act as both a pro-inflammatory and an anti-inflammatory cytokine[4,49], paradoxically having neurodegenerative and neuroprotective effects respectively. IL-6 has a prominent role in neuroinflammation, exerting neurotrophic effects directly and indirectly on neurons, activating glia and acute phase proteins, and influencing BBB integrity[4]. IL-6 and IL-6R expression in the CNS are observed in astrocytes, microglia, neurons and endothelial cells[49]. The two major signal transduction pathways connected to IL-6R are the JAK/STAT and Ras-MAPK/ERK pathways[4]. IL-6 also has an important role in neuronal development and function, and adult neurogenesis, which is typically affected in brain pathologies such as stroke, AD and PD[49]. Interestingly, connections between genetic variants of IL-6 and the volume of the hippocampus using voxel-based morphometry indicate the IL-6 allele has a significant role in the development of brain atrophy[50].

Under physiological conditions, concentrations of IL-6 in the CNS are very low. There is substantial evidence that along with other neuroimmune factors, IL-6 expression in the CNS is elevated in inflammatory states, including neurodegenerative and psychiatric diseases, and during CNS infection or injury[51]. Moreover, clinical, animal, and *in vitro* studies all suggest that IL-6 produced within the CNS has the capacity to modulate neuronal and synaptic function, behaviour, and is associated with altered cognitive function[51,52]. It has also been demonstrated that with advancing age, peripheral IL-6 levels are increased[53]. Consequently, dysregulation of IL-6 has been suggested to have a role in aging and age-associated diseases.

1.4.1 Evidence of IL-6 involvement in AD

In AD, IL-6 expression has been repeatedly found altered in the brain, CSF and blood of AD patients in research[4,49,52]. Although discrepancies between studies exist, the overall finding is that IL-6 likely plays a role in the histopathological events of neurodegeneration in AD. For example, hippocampal neurons treated with physiological doses of IL-6, resulted in an increase in Alzheimer-type tau phosphorylation[54]. IL-6 also enhanced the expression of AD APP in human glial and neuronal cells[55,56]. In a study utilising the APPsw transgenic AD mouse model, IL-6 mRNA levels were observed to be significantly higher in the cortex and hippocampus, and occurred at ages preceding the detection of amyloid plaques[57]. Other evidence linking IL-6 with AD is the IL-6 over-expressing GFAP-IL6 transgenic mouse (discussed further in section 1.5), exhibiting neurodegenerative pathology and cognitive decline[2]. In AD patients, levels of IL-6 are increased in the CSF and surrounding β-amyloid plaques compared to healthy controls[49,58-61]. Moreover, IL-6 immunoreactivity studies found IL-6 in a significantly higher ratio in diffuse plaques in AD patients compared to controls[58,59]. The appearance of this cytokine at the early stages of plaque formation, suggests IL-6 could precede and be involved in the transformation of plaques from diffuse to neuritic[58,59]. Additionally the severity of dementia in AD has been connected to high levels of IL-6 in plaques[58]. Similar alterations are found in studies of peripheral IL-6 concentrations. A recent meta-analysis of cytokines in AD showed that along with several other cytokines, peripheral blood concentrations of IL-6 were significantly higher in AD patients compared to controls[36], with another study demonstrating IL-6 levels were significantly higher in late-onset AD compared to early onset AD[62]. Broader correlations related to AD have been established between peripheral IL-6 levels and cognitive decline in a 10-year longitudinal and cross-sectional study. This study provides evidence that peripheral IL-6 levels are elevated years before the onset of clinical symptoms of dementia, and suggests elevated IL-6 levels in midlife are a predictor of cognitive decline[63]. Although there are no conclusive human studies relating allele polymorphisms to AD, the -572C/G polymorphism of IL-6 gene promoter region and IL-6-174 G/C promoter allele are suggested to have an association with the AD[64,65].

1.4.2 Evidence of IL-6 involvement in MDD

Although several mediators of CNS immunity have been implicated in the neuroimmune hypothesis of depression, the elevation of cytokine IL-6 is the most consistent finding across clinical literature[43,45,66], suggesting IL-6 is a component of the molecular mechanisms in the pathogenesis of depression. For example, levels of IL-6 are significantly higher in CSF and plasma of suicidal MDD patients compared to healthy controls[67,68]. Moreover, elevated plasma IL-6 levels in MDD patients are reported to decrease following treatment with anti-depressant therapy, and most interestingly, plasma IL-6 levels remain elevated in MDD patients who fail to respond to anti-depressant therapy[69,70]. Rodent studies also confirm the association between IL-6 and depression, examples include the induction of depressive-like behaviour following central administration of IL-6[66], increased IL-6 levels in stress-induced rodent models of depressive behaviour[66,71], and resistance to stress-induced depressive-like behaviour exhibited by IL-6 knockout mice[72].

1.5 The GFAP-IL6 transgenic mouse as a model of neurological diseases and disorders linked to neuroinflammation

Animal models are essential to the development of our understanding of cellular and molecular mechanisms underpinning neurological diseases. The GFAP-IL6 transgenic mouse strain was originally developed to study the actions of IL-6 within the CNS, by triggering the constitutive expression of IL-6 in astrocytes under control of the GFAP promoter. As a result, inflammation in this transgenic model is confined to the CNS, allowing the opportunity to investigate a localised, progressive neuroinflammatory and neurodegenerative state. This model is relevant to the neuroinflammation and elevated IL-6 expression reported in various human disorders of the CNS[73]. Severity of neurologic disease in this model is both age- and transgene dose- related, with homozygous mice exhibiting more severe disease states earlier in their lifespan than the lower-level transgene heterozygous mice[74]. While IL-6 is undetectable in the brains of wild type (WT) mice, IL-6 expression in the GFAP-IL6 transgenic mouse is found elevated in the regions of the cerebellum, the striatum, the hippocampus, the

hypothalamus, the neocortex, and the pons, resulting in accelerated age-related structural changes seen within 3–6 months[75]. Levels of transgene-encoded IL-6 expression are comparable to the pathophysiological range of experimental autoimmune encephalomyelitis (EAE)[74], with the highest expression of IL-6 mRNA being found in the cerebellar region[2].

In many aspects, the GFAP-IL6 mouse shows pathological features commonly associated with human neurological diseases and disorders linked to neuroinflammation. For example, the GFAP-IL6 mouse displays progressive physical and motor traits consistent with neurologic disease such as hunched posture, tremor, cerebellar ataxia, spontaneous seizures, pilorerection and hindlimb weakness[2]. Moreover, just as neuroinflammation and elevated IL-6 levels are associated with cognitive decline in human neurological conditions, the GFAP-IL6 transgenic mice exhibits cognitive decline in avoidance learning performance[5], correlating with age and extent of neuroinflammation and neurodegenerative damage. Neuropathology of the GFAP-IL6 also reveals neurodegenerative changes paralleling those in human neurological diseases and disorders. These include: alterations in the hippocampal region such as reduced neurogenesis, dendritic vacuolization and stripping, loss of parvalbumin and calbindin immunoreactive neurons, as well degeneration in the cerebellum including spongiosis, Purkinje cell atrophy, granular cell layer disruption and axonal dystrophy[2,5,76]. Further replicating aspects of neuroinflammation in human neurological conditions, the GFAP-IL6 mice exhibits microglial and astroglial activation, proliferative angiopathy, loss of BBB integrity, and activation of acute-phase response genes such as α1-antichymotrypsin, complement C3, and metallothionein[2,3,74,77]. Pathophysiological investigations of GFAP-IL6 mice show increased hippocampal excitatory activity and suppressed theta rhythm in electroencephalographic (EEG) recordings[78]. The characteristic susceptibility to seizures in GFAP-IL mice is attributed to the loss of inhibitory control[78]. Additionally, analysis of hippocampal slices show reduced long-term potentiation in the dendate in GFAP-IL6, compared to WTs[79]. It is suggested that these functional alterations in the hippocampus lead to impaired hippocampal synaptic plasticity that may underpin the cognitive deficits in this transgenic mouse model[76]. Interestingly, the HPA axis function is also found altered in the GFAP-IL6, matching the disturbances in HPA axis implicated in human patients with AD and MDD[80], in which IL-6 levels in

the CNS are elevated[73]. Transgenic GFAP-IL mice presented normal basal plasma levels of the stress hormone corticosterone compared to WTs, however following restraint stress, the GFAP-IL6 mice showed elevated corticosterone plasma levels that correlated with adrenal hyperplasia, increased plasma arginine vasopressin (AVP), without disturbing plasma adrenocorticotrophic hormone (ACTH) or pituitary ACTH content[81].

While it is important to consider that neuroinflammation involves several pro-inflammatory cytokines, the IL-6 cytokine-induced neurological disease described in the GFAP-IL6 transgenic mouse suggests its suitability in closely replicating the neuroinflammatory and neurodegenerative processes underlying most neurological diseases and disorders. The GFAP-IL6 represents a relevant phenotype to further our understanding on the effects of chronic neuroinflammation and offers a valuable opportunity to investigate potential anti-inflammatory and neuroprotective compounds *in viv*o.

1.6 Apigenin as a potential neuroprotective agent in neurological diseases and disorders

In view of the increasing evidence that neuroinflammation may contribute to the pathophysiology of neurological diseases and disorders, the prevention and treatment of inflammatory disturbances could be a valid therapeutic target in these conditions. There has been a growing interest in the use of natural polyphenols as multipotent agents to combat neurological diseases. Epidemiological evidence shows that dietary flavonoid intake is associated with a decreased risk of neurodegenerative diseases[9]. Furthermore, there are both animal and clinical studies showing dietary flavonoids possess neuroprotective properties, defending neurons against oxidative stress, attenuating neuroinflammation and improving cognition and learning[7,8]. It is thought that flavonoids may exert anti-inflammatory effects on the brain by acting centrally and indirectly by soothing peripheral inflammation[14]. (Fig 3). Apigenin, a natural flavone is one such compound with neuroprotective potential.

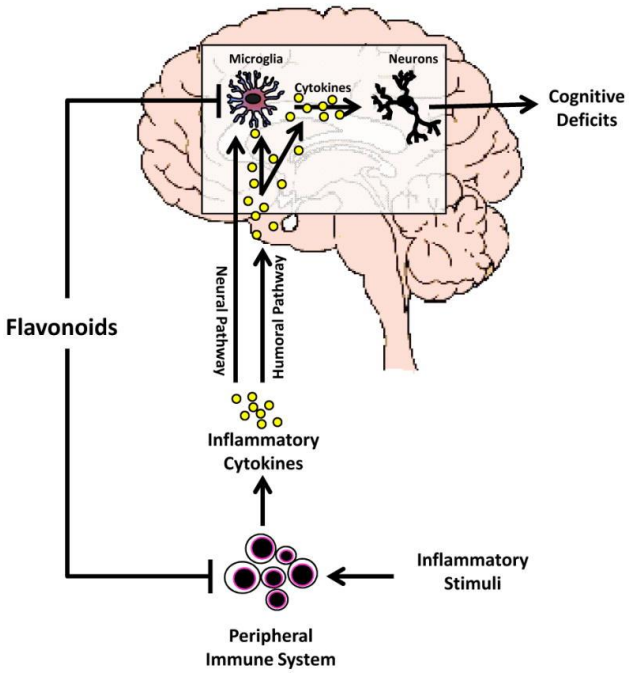


Figure 1.3. Direct and indirect anti-inflammatory effects of flavonoids

Source: Jang S, Johnson RW. Can consuming flavonoids restore old microglia to their youthful state? Nutrition reviews 2010;68:719-28

1.6.1 Pharmacological profile of apigenin

Apigenin (4',5,7-trihydroxyflavone) (Fig.4), is a flavonoid of low molecular weight (MW 270.24), found in a wide variety of plants, fruits, and vegetables, particularly abundant in the ligulate flowers of the chamomile plant (68% apigenin) and found in lesser concentrations in other sources such as celery, parsley, grapefruit[82]. Investigations of the biological activity of apigenin reveal potent antimicrobial, anti-inflammatory, antioxidant, antidepressive-like and antitumorigenic properties[83]. Apigenin is reported to exert many of its effects through interactions with the signaling molecules in the 3 major MAPK pathways (ERK, JNK, and p38) in both murine and human cell culture models[82,83]. Apigenin is considered very safe and even at high doses no toxicity was observed, however apigenin may induce muscle relaxation and sedation at high doses[84,85]. Apigenin is practically insoluble in water, moderately soluble in hot alcohol, and soluble in dilute KOH, and is incompatible with strong oxidizing agents[83]. Apigenin appeared to be absorbable by humans after intake of parsley, with the half-life for apigenin calculated to be in the order of 12 hr[86]. Apigenin crosses the brain-blood-barrier, and concentrations in rats reached 1.2 µM after daily i.p. administration of 20 mg/kg of apigenin for

one week[87]. A recent study utilising an in vitro model of the BBB has also shown measurable permeation of apigenin across the model membrane[88].

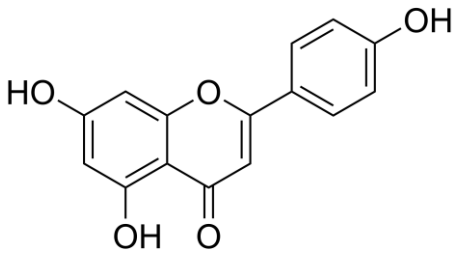


Figure 1.4. Structure of apigenin

Source: Retrieved 5th May 2015 from http://www.selleckchem.com/products/Apigenin.html

1.6.2 Neurological mechanisms and effects of apigenin

In vitro studies

Evidence of apigenin's anti-inflammatory properties is exemplified in studies that show dose-dependent suppression of the inflammatory mediators Nitric Oxide (NO) and prostaglandin, through inhibition of iNOS and Cox-2 in BV-2 murine microglial cell[89]. Apigenin also strongly suppressed levels of CD40, TFN-α and IL-6 production via inhibition of IFN-γ-induced phosphorylation of STAT1 in murine microglia[90]. Anti-inflammatory and anti-proliferative properties were additionally found for apigenin in cell cycle progression of activated BV-2 murine microglial cells[91]. Other important beneficial activities include apigenin's strong anti-oxidant and anti-apoptotic properties, shown in the protection of rat neuronal cells subjected to deprivation/reperfusion induced-injury[92], and neuroprotection of copper mediated β-amyloid-toxicity in a human neuroblastoma AD cell model[93]. Furthermore, apigenin conferred protection against β-amyloid25–35-induced toxicity in rat cerebral micro vascular endothelial cells[94], with an β-amyloid peptide and liposome assay showing apigenin as a potent inhibitor of liposome permeabilization by β-amyloid42 oligomers[95]. Apigenin exerted neuroprotective properties against endoplasmic reticulum stress-induced apoptosis in the HT22 murine hippocampal neuronal cells through the reduction of ROS, mitochondrial damage and endoplasmic reticulum-stress associated proteins[96]. In human neuronal cells apigenin protected against quinoloinic acid-induced excitotoxicity

via anti-oxidant mechanisms[97] and was neuroprotective against glutamate-induced neurotoxicity in murine cerebellar and cortical cell cultures[98].

***In vivo* studies**

Although few, studies in animal models are consistent with the anti-inflammatory, anti-oxidant and neuroprotective actions found in vitro. One recent study by Zhao et al. tested the neuroprotective effects of apigenin in the APP/PS1 double transgenic AD mouse model. Four month-old mice were orally treated with apigenin (40 mg/kg) for 3 months. Their results showed that apigenin-treated mice displayed improvements in memory and learning deficits, and a reduction of fibrillar amyloid deposits with lowered insoluble β-amyloid concentrations, mediated by a decrease in β-CTF and BACE1. Additionally, the apigenin-treated mice showed restoration of the cortical ERK/CREB/BDNF pathway involved in learning and memory typically affected in AD pathology. Enhanced activities of superoxide dismutase and glutathione peroxidase were also observed and increased superoxide anion scavenging[99]. Similarly, in another study β-amyloid-25-35-induced amnesia mouse models were treated with apigenin (20 mg/kg), resulting in improvements in spatial learning and memory, in addition to neurovascular protective effects[100]. Another study indicated neuroprotective effects in apigenin pre-treated mice (10 – 20 mg/kg) subjected to contusive spinal cord injury, including reduction in IL-1β, TFN-α, ICAM-1 and caspase-3, with an increase in Bcl-2:Bax ratio[101]. Cognitive enhancing effects have also been reported for apigenin in a recent study involving young male Wistar rats, showing apigenin improved memory in the passive avoidance task[87]. Similarly, another pre-clinical study utilising 7-week old mice found that compared to controls, administration of apigenin (25mg/kg) for 10 days resulted in improved performance in the Morris water maze and stimulated neurogenesis in the hippocampal region of the brain[102]. Additional animal study findings of apigenin that may be therapeutically relevant to neurological disorders include antidepressant-like activity[103-105], anti-convulsant effects[106], and improvement in motor skills and enhancement of neurotrophic potential seen in MPTP induced parkinsonism in mice[107].

1.7. Hypothesis and aims

Hypothesis:

Chronic neuroinflammation leads to progressive neurodegeneration, contributing to memory loss, cognitive decline and mood disturbances. This study proposes that the GFAP-IL6 transgenic mouse in which brain inflammation is triggered by the chronic production of the cytokine IL-6 in astrocytes, is a valid model to study the effects of sustained neuroinflammation on behaviour and cognition, with behavioural disturbances and cognitive dysfunction anticipated for this mouse line. Additionally this study proposes that administration of the anti-inflammatory dietary flavonoid apigenin, may result in the attenuation of brain inflammation in this mouse model, thereby having the potential to rescue cognitive deficits and ameliorate behavioural alterations.

Aims:

Aim 1. To improve our understanding of the effects of sustained neuroinflammation and neurodegeneration in the GFAP-IL6 mouse compared to WT mice, including any sex differences that may be present, in relation to cognitive and behavioural changes.

Aim 2. To investigate whether the natural anti-inflammatory compound apigenin can ameliorate brain inflammation, neurodegeneration, and any cognitive decline and behavioural alterations that exist in the transgenic model.

CHAPTER 2

METHODS

2.1 Animals

A total of sixty-eight heterozygous GFAP-IL6 mice (N=32; 16 males and 16 females), and their non-transgenic wild-type-like (WT) littermates (N=36; 14 males and 22 females) with a C57/BL6J background served as experimental subjects. The GFAP-IL6 mouse line, previously generated and characterised by Prof Ian Campbell was generously donated to the WSU animal house and bred at the facility. Mice were maintained in standard laboratory conditions, temperature 22°C (± 2°C) with a 12-h light:12-h dark cycle, same-sex group housed. Food and water were provided ad libitum with basic environmental enrichment. Individual mice were identified by ear punches made at 3 weeks of age, utilising the extracted tissue to determine genotype by quantitative polymerase chain reaction (qPCR). All testing was conducted in accordance and approved by WSU Animal Care and Ethics Committee (A10885).

2.2 Feeding

At 12 (±1) weeks the animals were switched over from the regular pellets provided at the animal facility to the experimental diet pellets. Animals were randomly assigned to either a control diet or apigenin diet, thus constituting four experimental groups (sex-matched):

Group I: WT mice (*N=20*), oral intake of control pellets

Group II: GFAP-IL6 mice (*N=16*), oral intake of control pellets

Group III: WT mice (*N=16*), oral intake of food pellets containing apigenin

Group IV: GFAP-IL6 mice (*N=16*), oral intake of food pellets containing apigenin

The experimental diet pellets were formulated by Specialty Feeds Pty Ltd, Western Australia. The pellets consisted of a standard irradiated rat and mouse fixed formulation diet for laboratory rodents

fortified with vitamins and minerals to meet the requirements of breeding animals (protein 20.00% total fat, 4.80% crude fibre 4.80%, digestible energy 14.0 MJ/Kg), milled with the compound apigenin K to the concentration of 400ppm (40mg/kg daily dose). Apigenin K was obtained from Nutrafur S.A., Murcia, Spain (min. purity 90% HPLC on dry basis). Mice were housed in groups of no more than 3 per cage in order to adequately monitor their food consumption. Mice were weighed every 2-3 weeks. The mice were closely monitored every 4-6 hours in the first 24-48 hours of commencing the experimental diet to check for any adverse effects.

2.3 General health monitoring

The general health of the animals was frequently monitored, daily by the animal facility staff and regularly during behavioural testing by investigators. Animals were monitored for any signs of stress, pain (Mouse Grimace Scale), discomfort, immobility, aggression, injuries or illness. Posture, gait, weight and body tone were observed, as well as behavioural abnormalities in grooming, nesting, or socialisation.

2.4 Behavioural test battery

After 12 weeks of experimental diet feeding, mice were tested in a number of behavioural tests at 24 (±2) weeks of age, over a 3-4 week period. Experimental diets were continued throughout the behavioural testing. The test battery included: 3 day escalated handling, elevated plus maze, open field, Barnes maze and a functional observational battery. All tests were conducted early in the light phase between 8:30 – 14:00 hours. A minimum 48 hour inter-test interval was maintained. Laboratory equipment, surfaces and apparatus were thoroughly cleaned between trials using 70% ethanol. To address the logistical requirements of behavioural testing and ensure the safety and monitoring required by ethics, all behavioural experiments were conducted as a research team with another investigator Chris Millington. The animal test order allocation for each experiment was always kept blind to one investigator and alternated equally between investigators.

Table 2.1 Behavioural test order of GFAP-IL6 and WT mice

Test Battery Day	Test administered
1-3	Escalated handling
7	Elevated Plus Maze
10	Open Field
15-17	Barnes Maze (Acquisition phase)
20	Barnes Maze (Probe trial)
23	FOB

2.4.1 Escalated Handling

Mice were handled for 3 consecutive days, utilising a modified escalated handling protocol by Fridgeirsdottir et.al.[108] (Appendix A). Mice were handled under the same conditions, quasi-randomised between one of two investigators (Sandra Sonego and Chris Millington) in equal numbers.

2.4.2 Elevated Plus Maze

Anxiety-like behaviour was assessed on the elevated plus maze (EPM), widely used to phenotype transgenic strains and detect anxiolytic effects of pharmacological agents (Appendix B). The test is based on the natural aversion of mice for open and elevated spaces and their drive to explore novel environments, the number of entries and time spent in the open arms is used as an indicator of open space-induced anxiety in mice. The apparatus consisted of two open arms (35 cm x 5 cm; without side walls) and two closed arms (35 cm x 5 cm; height of enclosing walls 15 cm) intersecting at the centre (5 cm x 5 cm) in the shape of a plus sign and elevated 40cm from the ground. Lighting was set at 900 lux. Animals were placed at the intersection of the four arms facing an open arm, and allowed to freely explore the maze. Each mouse received one five-minute trial in which data was captured by digital overhead camera and the ANY-Maze™ (Stoelting, Wood Dale, USA) video-tracking system. An observer was always present to check whether any tracking errors occurred during the trial. EPM

parameters included total distance travelled, percentage of time in the open arms, time in the closed arms, distance travelled in the closed arms, percentage of distance in the open arms, mean speed, head dips frequency, rearing frequency and stretched attend posture frequency. Mice that fell off the maze during the trial were excluded.

2.4.3 Open Field

The open field test was used to assess basic locomotor activity, anxiety and exploratory drive (Appendix C). The open field test provides a broad assessment of both behavioural and motor activities of mice, important in helping to define phenotypes. It is particularly useful in being able to non-invasively detect locomotor and neurobehavioural impairments in neurodegenerative disease models and evaluate the effects of therapeutic interventions. The test utilises an enclosed arena to track and assess the animal's amount of movement and quality of movement and is based on the natural tendency of mice to both explore, and avoid open spaces as a protective reaction. Normal animals are expected to spend more time in the periphery along the walls (thigmotaxis) than in the open central area of the arena (anxiogenic area). Mice were placed in an open box arena with covered walls and allowed to explore freely for a period of 10 minutes. Measures of interest were recorded using a digital overhead camera and ANY-Maze™ (Stoelting, Wood Dale, USA) video-tracking system including: total distance travelled, distance and time spent in central vs peripheral area, mean speed and latency to first exit of the periphery.

2.4.4 Barnes Maze

Cognitive performance was evaluated using the Barnes maze (Appendix D). The Barnes maze is a type of navigational maze used to assess spatial learning and memory in rodents. Although similar to Morris water maze and radial-arm maze task, this maze does not require strong aversive stimuli or food deprivation as motivation and is considered a more ecological, dryland alternative to the Morris water maze. The maze consists of an elevated circular platform with 20 evenly spaced holes around

the perimeter. Beneath one of the holes is an escape box which mice can get into to by moving through the hole on the surface. Animals were given reinforcement (bright lighting ~900lux and aversive stimuli – tone at 85dB) and visual intra-maze and extra-maze cues to escape from the exposed brightly lit platform to a dark recessed target hole. Improvement in their ability over trials is thought to signify the animal's learning and memory of the escape target hole. The maze uses the rodents' instinct to seek out dark enclosed spaces and avoid open bright spaces. It has been used to test both reference and working memory. The maze's capacity to measure spatial ability has been confirmed by rodents with hippocampal damage showing impaired performance, and was particularly relevant to testing the damage to hippocampal areas in the GFAP-IL-6 mouse brain. Performance was recorded by a digital overhead camera and ANY-Maze™ (Stoelting, Wood Dale, USA) video-tracking system, measuring such parameters as latency, path length and number of errors (entries to wrong hole) the mice make in finding the hole containing the escape box. Mice were provided 1 habituation trial on the first day, followed by 2 days of 180s acquisition trials (3 trials per day). Following a 2 day break the probe trial was administered (90s trial) in which the escape hole is no longer accessible to assess long-term memory retention of the escape hole location. A detailed description of procedures can be found in Appendix D.

2.4.5 Functional observational battery

The FOB is a neurobehavioural assessment tool consisting of various non-invasive procedures to detect gross functional deficits and characterise mice strains (Appendix E). Mice were observed in items of general health and gross neurological function including assessment of ataxia, fur state and grooming behaviours, reflexes, and auditory, visual and olfactory abilities. Animals were screened for any unusual or bizarre behaviours.

2.5 Statistical analysis

Data are shown as means ± standard error of the mean (SEM). Analysis of variance (ANOVA) were performed for each of the behavioural parameters. Three-way univariate ANOVAs were conducted for EPM, OF, BM probe trial and FOB items to detect the effects of 'genotype', 'diet' and 'sex' (between subject factors). For BM acquisition phase trials and food and weight data four-way repeated measures (RM) ANOVAs were performed to investigate the same between subject effects and the within subject factors of 'trials' (BM), 'holes' (BM) and 'month' (food and weight). Subsequent analyses were run to examine significant interaction effects. In cases of significant main effects ($p < 0.05$), pairwise post-hoc comparisons were performed using Fisher's LSD tests with significant differences indicated by $p < 0.05$. One sample t-test against chance 25% were performed for percentage of time in the target quadrant in the BM probe trial and Dunnett's multiple comparison test was used for hole entry distribution analysis. Analyses were conducted using IBM SPSS Statistics software 22.0 (IBM Corp, USA) for Windows and Microsoft Excel.

.

CHAPTER 3

RESULTS

3.1 Feeding, body weight and general health

3.1.1 Food consumption, starting body weight and weight gain

In order to ensure the mice consumed the drug-enriched diet at the assumed dose and that there were no effects of the drug on food palatability, the estimated average daily food consumption, starting body weight and weight gained was recorded for the first 2 months of experimental diet feeding (Table 3.1.1).

An effect of 'month' [$F(1,49) = 18.65$, $p < 0.0001$] found for the estimated average daily food consumption indicated that mice in all groups consumed ~9.6% more food in the first month (3.54g ± 0.06) compared to the second month (3.23g ± .06). An effect of 'diet' was also revealed for the estimated average daily food consumption [$F(1,49) = 35.35$, $p < 0.0001$] showing that mice on the apigenin-enriched diet consumed approximately 17% more food than those on the control diet (apigenin 3.65g ± 0.09, control 3.13g ± 0.07). Additionally a 'genotype' by 'diet' interaction was seen [$F(1,49) = 9.00$, $p < 0.01$], WT apigenin diet mice had an increased average daily consumption compared to GFAP-IL6 apigenin diet animals and WT control diet. GFAP-IL6 apigenin mice also had an increased food consumption compared to GFAP-IL6 control diet mice. No effect of sex was found for food consumption.

Analysis of the starting weight (3 months of age) showed there were sex differences [$F(1,61) = 169.07$, $p < 0.0001$], males weighing more than females (26.11g ± 0.39, 20.04g ± 0.32) with no genotype or diet effects present.

The 4-way RM ANOVA showed an effect of 'sex' in the amount of weight gained over the 2 months [$F(1,44) = 22.43$, $p < 0.0001$] males gaining more weight than females (4.27g ± 0.32, 2.38g ± 0.24), as well as an effect of 'diet' [$F(1,44) = 20.35$, $p < 0.0001$] with apigenin fed animals gaining ~74%

more weight (4.2g ± 0.28) than control fed animals (2.43g ± 0.28). A significant 'sex' by 'genotype' interaction was also found [$F(1,44) = 6.99$, $p < 0.05$], male WT mice gained ~42% more weight than GFAP-IL6 male mice, regardless of diet. A within-subject effect of 'month' was additionally found [$F(1,44) = 28.43$, $p < 0.0001$], showing all mice gained more weight in the second month (3.91g ± 0.19) compared to the first month (2.74g ± 0.26).

It is important to note that there were missing data for several animals in the weight and food consumption data presented here. The differences between group numbers were in part due to the staggering of the timing of animal breeding, however primarily due to logistical issues encountered with maintaining animal and food consumption weights and managing the demands of lengthy time-critical behavioural testing that often conflicted with scheduled weighing.

3.1.2 General health and gross neurological function

Differences were found among GFAP-IL6 mice for some general health and gross neurological function items on the functional observational test (Appendix E). These were gait observation, kyphosis observation and hindlimb clasping scores (Fig 3.1.2). A three-way ANOVA yielded a strong effect of 'genotype' for these 3 parameters: gait [$F(1,60) = 14.04$, $p < 0.001$], kyphosis [$F(1,60) = 35.51$, $p < 0.0001$], hindlimb clasping [$F(1,60) = 7.29$, $p < 0.01$], showing an increased score of abnormalities in these measures for the GFAP-IL6 mouse model. Furthermore there were GFAP-IL6 mice that experienced spontaneous seizures and tremoring either during behavioural tests, handling, being transferred, weighing or feeding (Table 3.1.2). If seizures occurred during behavioural testing they were excluded from the test. If seizures occurred during feeding, weighing, or transfer they were placed in a quiet area and monitored until seizures ceased (maximum seizure episode length ~10secs), animal housing staff were advised, and the event was reported in the monitoring sheets and animal log. These incidents were reported as adverse events to animal ethics in accordance with standard procedures. No seizures were reported among the WT mice. The ledge test item in the functional observational battery was identified as a common trigger for the GFAP-IL6 tremoring and seizures,

subsequently it was removed from the battery to minimise any possible harm to the animals and due to ethical concerns. There were no other differences between groups for any other FOB items assessed.

Table 3.1.1 Starting body weight, average weight gain and estimated average daily food consumption

	Control Diet				Apigenin Diet			
	WT		GFAP-IL6		WT		GFAP-IL6	
	Male	Female	Male	Female	Male	Female	Male	Female
Starting body weight (g)	24.30 ± 1.86	20.82 ± 0.75	25.29 ± 0.71	19.81 ± 0.28	27.88 ± 0.70	19.71 ± 0.45	25.86 ± 0.77	19.87 ± 0.19
Weight gain after 1 month (g)	2.60 ± 0.98	0.97 ± 0.13	2.63 ± 1.02	1.71 ± 0.20	4.70 ± 0.77	2.40 ± 0.32	3.61 ± 0.90	2.86 ± 0.23
Weight gain after 2 months (g)	4.10 ± 1.33	1.66 ± 0.22	3.52 ± 0.30	2.23 ± 0.20	8.20 ± 0.92	3.29 ± 0.42	4.31 ± 0.86	3.96 ± 0.28
Average daily food consumption 1st month (g)	3.28 ± 0.17	2.91 ± 0.05	3.41 ± 0.12	3.18 ± 0.14	3.82 ± 0.22	4.01 ± 0.08	3.84 ± 0.28	3.65 ± 0.12
Average daily food consumption 2nd month (g)	3.00 ± 0.16	2.85 ± 0.09	3.28 ± 0.05	3.08 ± 0.11	3.79 ± 0.00	3.36 ± 0.11	3.05 ± 0.35	3.43 ± 0.12

Starting body weight: WT control male n = 3, WT control female n = 9, GFAP-IL6 control male n = 9, GFAP-IL6 control female n = 7, WT apigenin male n = 6, WT apigenin female n= 10, GFAP-IL6 apigenin male n = 7 , GFAP-IL6 apigenin female n = 9.
Weight gain 1 month: WT control male n = 3, WT control female n = 9, GFAP-IL6 control male n = 9, GFAP-IL6 control female n = 7, WT apigenin male n = 4, WT apigenin female n= 7, GFAP-IL6 apigenin male n = 7 , GFAP-IL6 apigenin female n = 9.
Weight gain 2 months: WT control male n = 3, WT control female n = 7, GFAP-IL6 control male n = 9, GFAP-IL6 control female n = 7, WT apigenin male n = 3, WT apigenin female n= 7, GFAP-IL6 apigenin male n = 7 , GFAP-IL6 apigenin female n = 9.
Average daily food consumption 1st month: WT control male n = 5, WT control female n = 12, GFAP-IL6 control male n = 9, GFAP-IL6 control female n = 7, WT apigenin male n = 4, WT apigenin female n= 7, GFAP-IL6 apigenin male n = 7 , GFAP-IL6 apigenin female n = 9.
Average daily food consumption 2nd month: WT control male n = 5, WT control female n = 10, GFAP-IL6 control male n = 9, GFAP-IL6 control female n = 7, WT apigenin male n = 3, WT apigenin female n= 7, GFAP-IL6 apigenin male n = 7 , GFAP-IL6 apigenin female n = 9.
Food intake was measured per cage- then divided by number of animals in cage to provide an estimate average intake per animal
Abbreviations: WT = Wild-type, GFAP-IL6 = Glial fibrillary acidic protein interleukin 6

Table 3.1.2 Log of adverse events among GFAP-IL6 mice

Event No	Sex	Diet	Age	Type of adverse event
1	Female	Control	6 months	Seizures and tremors during functional observational test- ledge test
2	Male	Apigenin	6 months	Severe seizures, freezing and strong tremoring unable to walk during functional observational
3	Female	Apigenin	6 months	Tremoring, fell off elevated plus maze during test
4	Male	Control	6 months	Seizures and tremoring during functional observational test – ledge test
5	Male	Control	6 months	Tremoring, fell off elevated plus maze during test
6	Male	Control	6 months	Tremoring, fell off elevated plus maze during test
7	Female	Control	5 months	Strong tremoring during weighing/feeding
8	Male	Control	5 months	Severe seizures during cage transfer
9	Male	Apigenin	6 months	Tremoring during handling
10	Female	Control	6 months	Seizures and tremors during functional observational test – ledge test
11	Male	Apigenin	6 months	Strong tremoring during functional observational test – ledge test

.

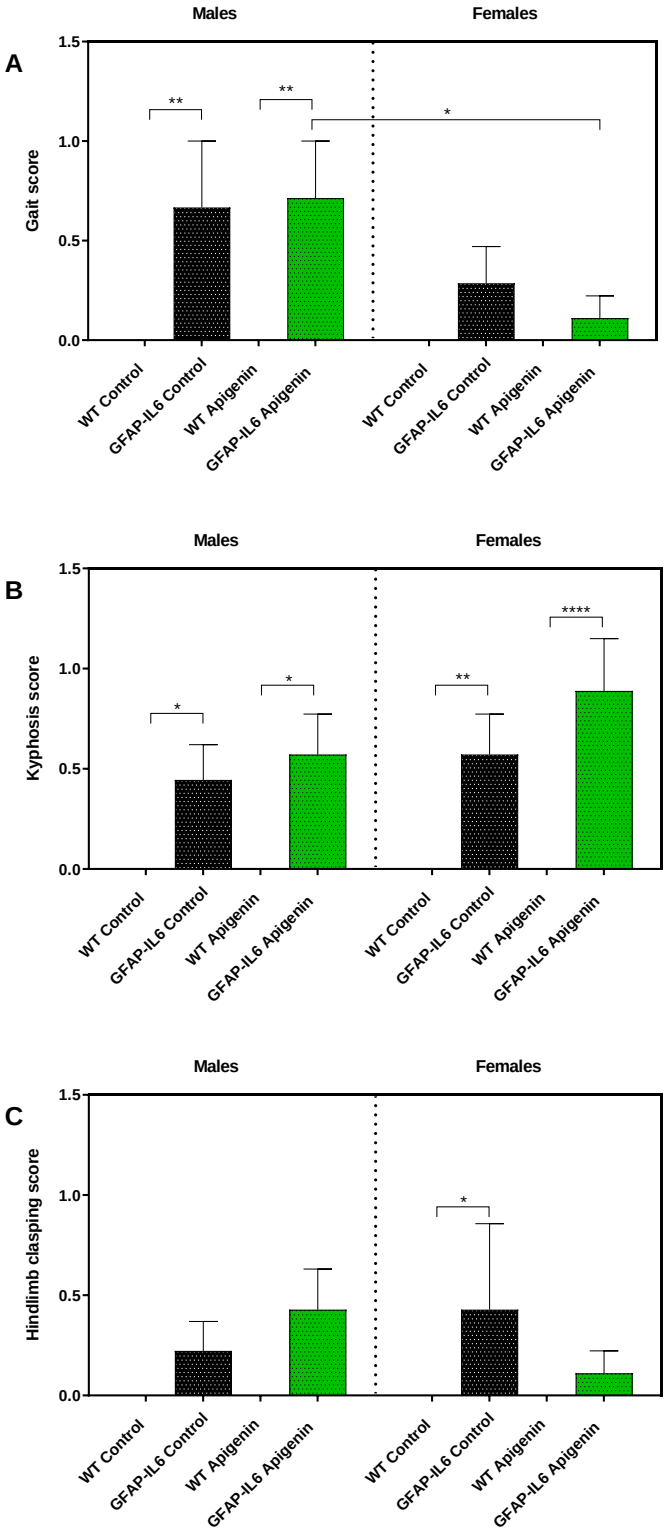


Figure 3.1.2 Gait, kyphosis and hindlimb clasping scores in the functional observational battery. *Mean (± SEM)* ***A)*** *gait observation score,* ***B)*** *kyphosis observation score,* ***C)*** *hindlimb clasping score. A significant effect of 'genotype' is reported for gait ($p < 0.001$), kyphosis ($p < 0.0001$) and hindlimb clasping ($p < 0.01$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.*

Scores of 0 = normal function, scores 1-3 = abnormalities increasing in severity, refer to Appendix E for exact definitions. WT control male n = 8, WT control female n = 12, GFAP-IL6 control male n = 9, GFAP-IL6 control female n = 7, WT apigenin male n = 6, WT apigenin female n= 10, GFAP-IL6 apigenin male n = 7, GFAP-IL6 apigenin female n = 9. Abbreviations: WT = Wild-type, GFAP-IL6 = Glial fibrillary acidic protein interleukin 6.

3.2 Anxiety-like behaviour, locomotion and exploration

3.2.1 Elevated Plus Maze (EPM)

Anxiety-like and locomotive behaviours were assessed in the EPM. A three-way ANOVA revealed a significant effect of 'genotype' in the total distance travelled [$F(1,58) = 27.87$, $p < 0.0001$], distance travelled in the closed arms [$F(1,58) = 16.32$, $p < 0.0001$] and mean speed [$F(1,58) = 28.32$, $p < 0.0001$] showing transgenic GFAP-IL-6 mice exhibited reduced locomotor activity and velocity compared to WT mice (Figure 3.2.1a). No significant effects of 'sex' or 'diet' were obtained in these measures.

While significant differences were not found in the percentage of time spent in the open arms, there was a trend toward an effect of 'diet' [$F(1,58) = 3.32$, $p <$ **0.074**], apigenin treatment being associated with a decrease in the percentage of time spent in the open arms. A main effect of 'diet' was seen for the percentage of distance travelled in the open arms [$F(1,58) = 4.56$, $p < 0.05$] (Figure 3.2.1b) such that apigenin-fed mice exhibited a lower percentage of distance travelled in the open arms compared to control fed. A trend tending toward a significant interaction between 'diet' and 'sex'[$F(1,58) = 3.08$, $p <$ **0.084**], also suggested this particular effect of apigenin was pronounced among male mice, apigenin appearing to reduce the percentage of distance travelled in the open arms compared to control fed males [$F(1,58) = 6.79$, $p < 0.05$] . In the frequency of stretched attend postures there was a significant effect of 'sex' [$F(1,57) = 9.11$, $p < 0.01$] showing females had an increased frequency of stretched attend posture compared to males. An increasing trend for a main effect of 'diet' was also observed [$F(1,57) = 3.10$, $p <$ **0.084**] with animals on the apigenin diet displaying a decreased frequency of stretched attend posture compared to control fed animals. Additionally a trend toward a 'genotype' by 'diet' by 'sex' interaction [$F(1,57) = 3.21$, $p <$ **0.078**] indicated that for GFAP-IL6 females the apigenin diet tended to lower the frequency of stretched attend posture in comparison to control fed [$F(1,57) = 4.03$, $p < 0.05$] (Figure 3.2.1c). This interaction also suggested sex differences among GFAP-IL6 control fed mice where females tended toward a higher frequency of stretched attend posture compared to males [$F(1,57) = 8.14$, $p < 0.01$].

There was a main effect of ‘diet’ found in the head dip frequency [$F(1,57) = 7.23$, $p < 0.01$], animals treated with the apigenin diet exhibited a reduction in the number of head dips compared to animals on the control diet (Figure 3.2.1c). There were no other effects or interactions found for any another exploration-relevant behaviour such as total rearing frequency (Table 3.2.1).

No further interactions between ‘genotype’ ‘diet’ and ‘sex’ for any other measures investigated in the EPM were reported (Table 3.2.1).

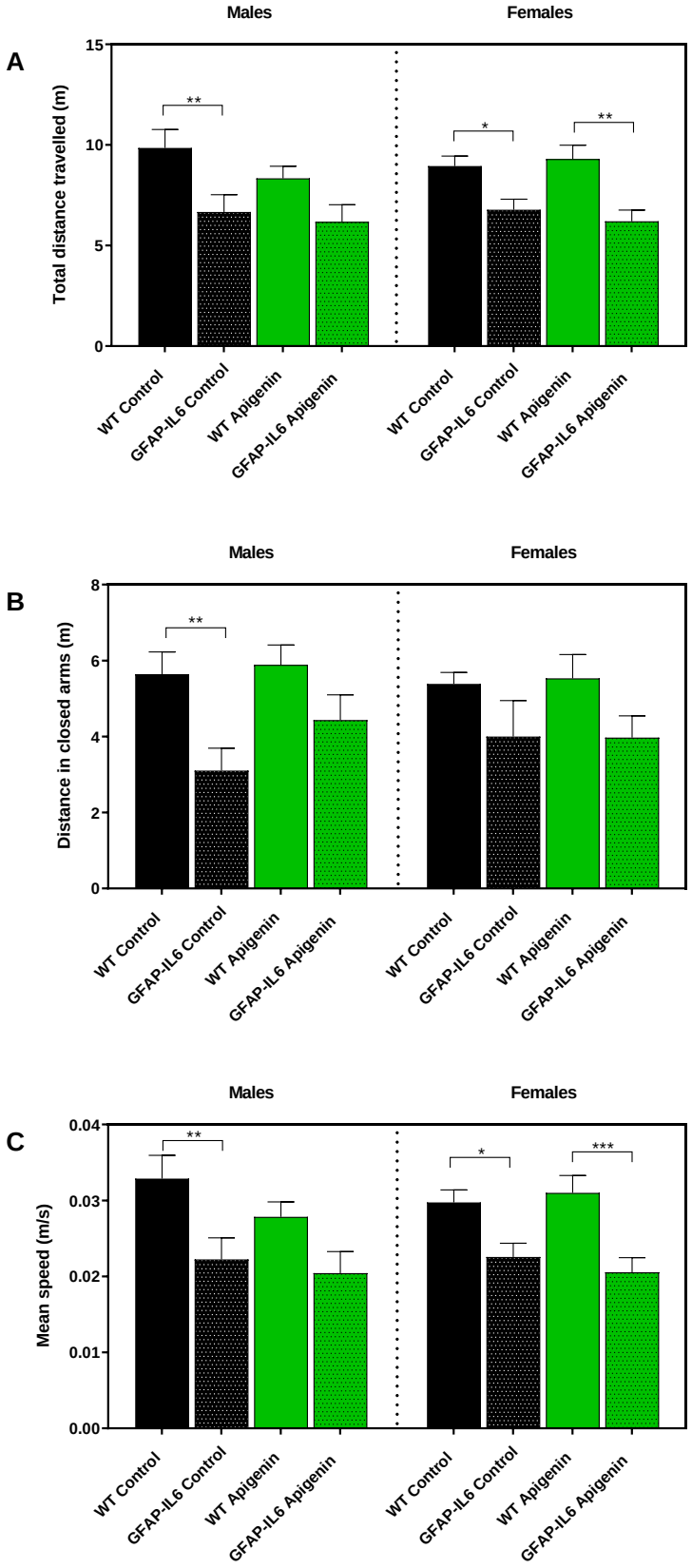


Figure 3.2.1a. Locomotor activity and velocity in the elevated plus maze. *Mean (± SEM) **A)** total distance travelled, **B)** distance in the closed arms and **C)** mean speed in the EPM. A significant effect of 'genotype' in the total distance travelled ($p < 0.0001$), distance travelled in the closed arms ($p < 0.0001$) and mean speed ($p < 0.0001$) was found. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.*

WT control male n = 8, WT control female n = 12, GFAP-IL6 control male n = 8, GFAP-IL6 control female n = 7, WT apigenin male n = 6, WT apigenin female n= 9, GFAP-IL6 apigenin male n = 7, GFAP-IL6 apigenin female n = 8. Abbreviations: WT = Wild-type, GFAP-IL6 = Glial fibrillary acidic protein interleukin 6.

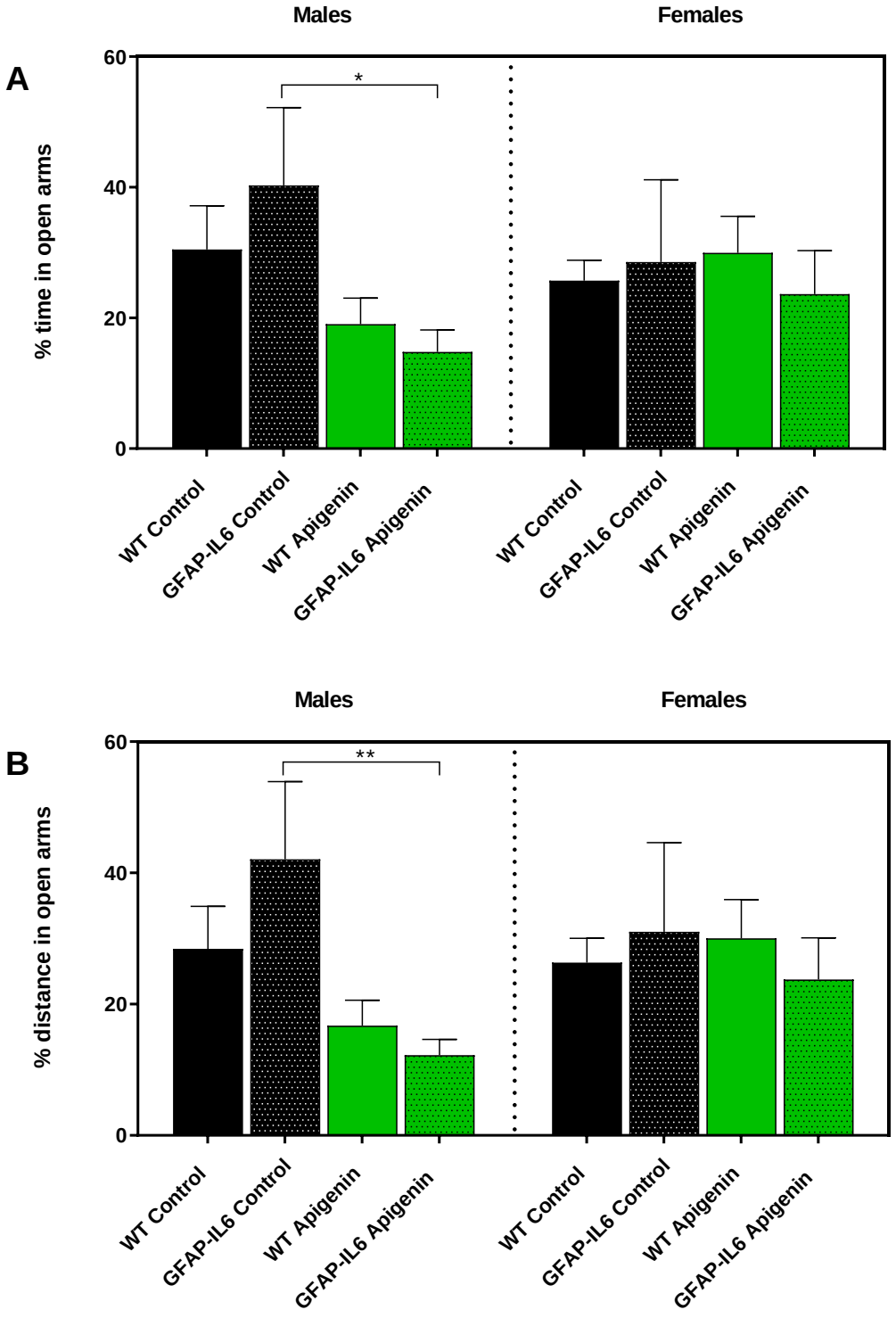


Figure 3.2.1b. Time and locomotion in the open arms of the elevated plus maze. *Mean (± SEM) **A)** percentage of time spent in the open arms, **B)** percentage of distance travelled in the open arms in the EPM. A main effect of 'diet' is seen for the percentage of distance travelled in the open arms ($p < 0.05$). *$p < 0.05$, **$p < 0.01$.*

WT control male n = 8, WT control female n = 12, GFAP-IL6 control male n = 8, GFAP-IL6 control female n = 7, WT apigenin male n = 6, WT apigenin female n= 9, GFAP-IL6 apigenin male n = 7, GFAP-IL6 apigenin female n = 8. Abbreviations: WT = Wild-type, GFAP-IL6 = Glial fibrillary acidic protein interleukin 6.

.

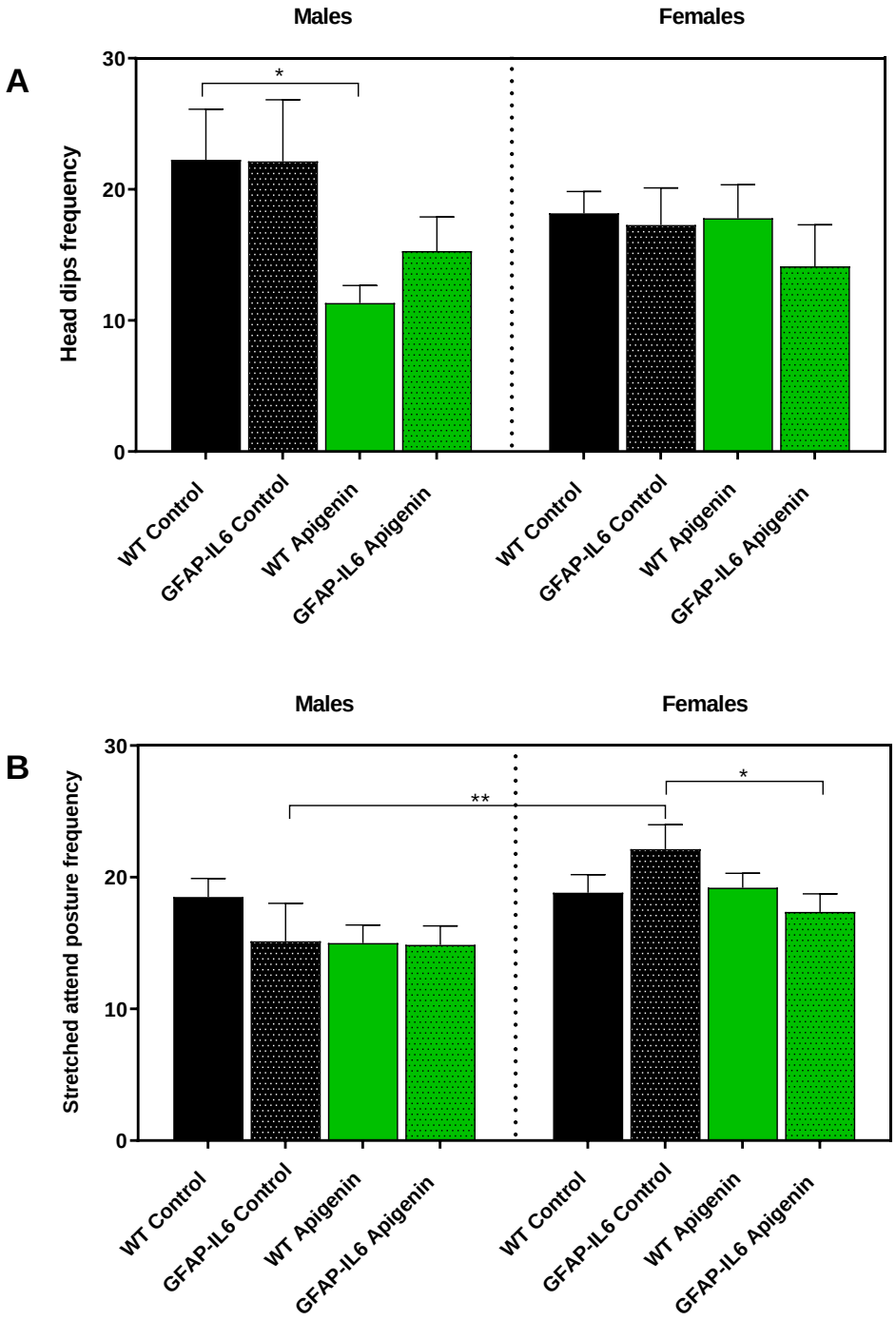


Figure 3.2.1c. Head dips and stretched attend posture frequency in the elevated plus maze. *Mean (± SEM)* ***A)*** *head dips frequency,* ***B)*** *stretched attend posture frequency in the EPM. A main effect of 'diet' is seen in the head dips frequency ($p < 0.01$). A main effect of 'sex' ($p < 0.01$) is reported for the stretched attend posture frequency. *$p < 0.05$, **$p < 0.01$.*

WT control male n = 8, WT control female n = 12, GFAP-IL6 control male n = 7, GFAP-IL6 control female n = 7, WT apigenin male n = 6, WT apigenin female n= 9, GFAP-IL6 apigenin male n = 7, GFAP-IL6 apigenin female n = 8. Abbreviations: WT = Wild-type, GFAP-IL6 = Glial fibrillary acidic protein interleukin 6.

Table 3.2.1 Summary of elevated plus maze parameters: 3-way univariate ANOVA p values

Parameters:	Factors:							
	Genotype	**Diet**	**Sex**	**Genotype * Diet**	**Genotype * Sex**	**Diet * Sex**	**Genotype * Diet * Sex**	**Description of significant effects or interactions**
Total distance travelled	**0.000**	0.262	0.961	0.997	0.995	0.414	0.323	**Effect of genotype:** GFAP-IL-6 mice displayed reduced locomotor activity in the total distance travelled compared to WT mice
Percentage of time in the open arms	0.951	**0.074**	0.907	0.258	0.641	0.097	0.844	
Time in the closed arms	0.800	0.103	0.889	0.551	0.758	0.229	0.779	
Distance travelled in the closed arms	**0.000**	0.341	0.910	0.610	0.560	0.400	0.470	**Effect of genotype:** GFAP-IL-6 mice showed decreased locomotor activity in the closed arms compared to WT mice
Percentage of distance in the open arms	0.740	**0.038**	0.600	0.175	0.607	**0.084**	0.754	**Effect of diet:** Mice consuming the apigenin diet had a reduced percentage of distance travelled in the open arms compared to mice on the control diet
Mean speed	**0.000**	0.259	0.942	0.995	0.952	0.367	0.337	**Effect of genotype:** GFAP-IL-6 mice exhibited reduced velocity compared to WT mice
Head dips frequency	0.933	**0.015**	0.669	0.882	0.325	0.097	0.421	**Effect of diet:** Apigenin treated mice had less frequency of head dips compared to control fed mice
Rearing frequency	0.284	0.279	0.117	0.671	0.319	0.164	0.503	
Stretched attend posture frequency	0.667	**0.084**	**0.004**	0.682	0.289	0.895	**0.078**	**Effect of sex:** Females had an increased frequency of stretched attend posture compared to males

3.2.2 Open Field (OF)

Locomotor activity and anxiety-related behaviour was analysed in the OF. Total distance travelled and mean speed were not affected by 'diet', 'genotype' or 'sex' (all $p > 0.05$) (Table 3.2.2).

In the percentage of time spent in the centre there was a significant main effect of 'sex' [$F(1,60) = 5.37$, $p < 0.05$], with female mice spending a greater percentage of time in the centre compared to male mice. A very strong trend for an anxiolytic-like effect of the GFAP-IL6 genotype was also observed in the percentage of time spent in the centre [$F(1,60) = 5.55$, $p <$ **0.055**], mice of the GFAP-IL6 strain had an increased percentage of time spent in the centre in comparison to WT mice (Figure 3.2.2). Furthermore an increasing trend toward a significant 'genotype' by 'diet' interaction [$F(1,60) = 3.35$, $p <$ **0.072**] showed that for control diet mice, the GFAP-IL6 genotype was associated with an increase in a percentage of time in the centre [$F(1,60) = 7.63$, $p < 0.01$], and among GFAP-IL6 mice the apigenin diet led to a decrease in the percentage of time in the centre [$F(1,60) = 4.77$, $p < 0.05$].

Similar to the percentage of time spent in the centre, an effect of 'sex' was also seen for the percentage of distance travelled in the centre [$F(1,60) = 8.63$, $p < 0.01$] with female mice displaying increased ambulatory activity in the centre zone compared to that of male mice. A main effect of 'diet' was additionally found for the percentage of distance travelled in the centre [$F(1,60) = 4.09$, $p < 0.05$], where consumption of the apigenin diet was associated with a significant reduction in the percentage of distance travelled in the centre zone compared to the control diet (Figure 3.2.2).

As for the other behavioural measures analysed in the open field including distance travelled in the peripheral zone and latency to exit the peripheral zone, there were no significant effects or interactions found (Table 3.2.2).

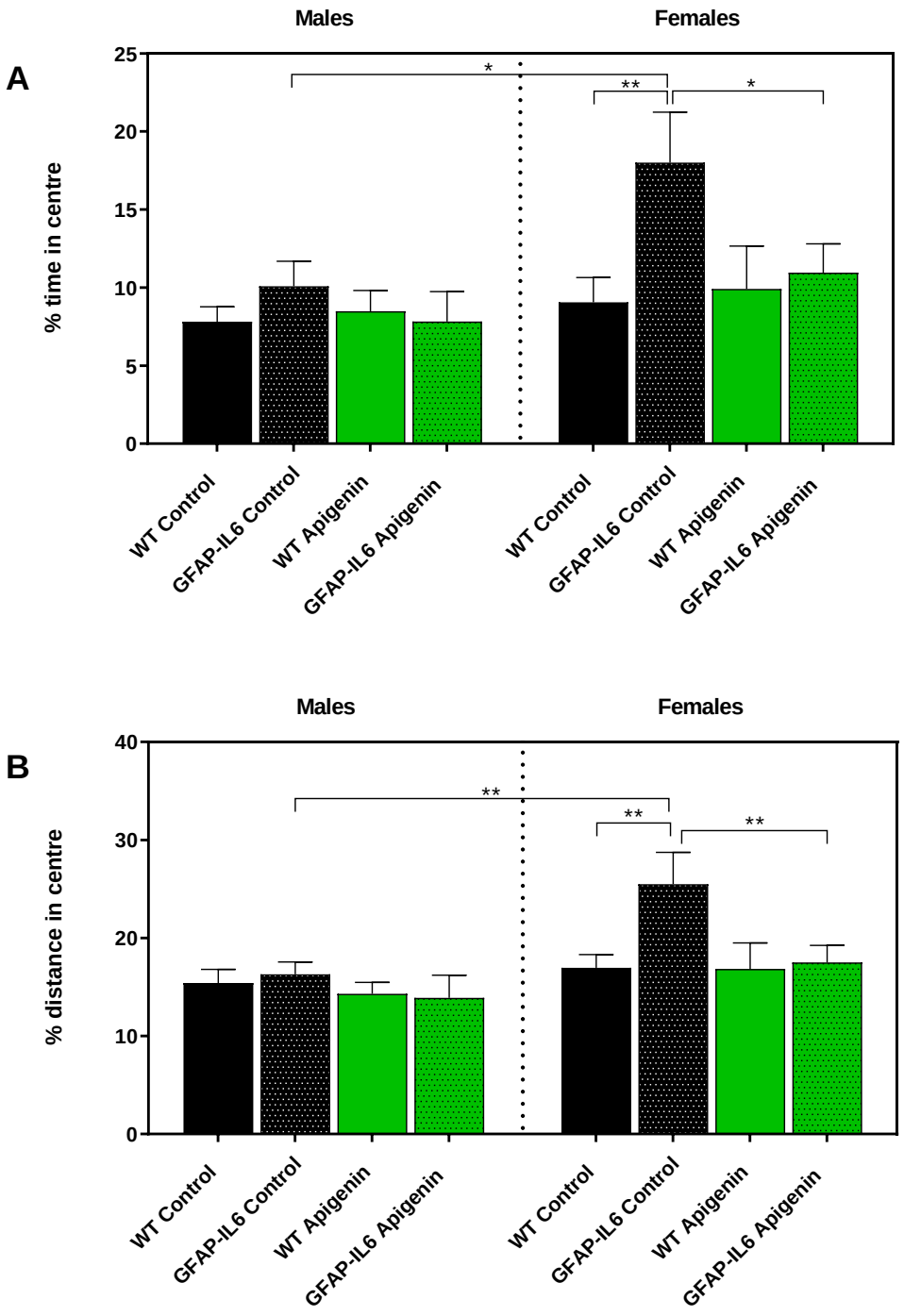


Figure 3.2.2. Time and locomotion in the centre in the open field test. *Mean (± SEM)* ***A)*** *percentage of time spent in the centre,* ***B)*** *percentage of distance travelled in the centre in the OF (10 minute trial). A significant effect of 'sex' (p <0 .05) and a very strong trend for an effect of 'genotype' (p < **0.055**) is reported for the percentage of time spent in the centre. A main effect of 'sex' (p < 0.01) and 'diet' (p < 0.01) was found for the percentage of distance travelled in the centre. *p < 0.05, **p < 0.01.*

WT control male n = 8, WT control female n = 12, GFAP-IL6 control male n = 9, GFAP-IL6 control female n = 7, WT apigenin male n = 6, WT apigenin female n= 10, GFAP-IL6 apigenin male n = 7, GFAP-IL6 apigenin female n = 9. Abbreviations: WT = Wild-type, GFAP-IL6 = Glial fibrillary acidic protein interleukin 6.

Table 3.2.2 Summary of open field test parameters: 3-way univariate ANOVA p values

Parameter:	Factors:							
	Genotype	**Diet**	**Sex**	**Genotype * Diet**	**Genotype * Sex**	**Diet * Sex**	**Genotype * Diet * Sex**	**Description of significant effects or interactions**
Total distance travelled	0.964	0.246	0.873	0.764	0.476	0.442	0.752	
Percentage of time in the centre	**0.055***	0.195	**0.024**	**0.072**	0.162	0.441	0.403	**Effect of sex:** Female mice spent a greater percentage of time in the centre compared to male mice **Effect of genotype*:** GFAP-IL6 mice spent a higher percentage of time spent in the centre compared to WT mice
Percentage of distance in the centre	0.098	**0.048**	**0.005**	0.115	0.135	0.426	0.260	**Effect of diet:** Apigenin treated mice had a lower percentage of distance travelled in the centre zone compared to control fed mice **Effect of sex:** Female mice displayed increased ambulatory activity in the centre zone compared to male mice
Distance travelled in periphery	0.726	0.117	0.561	0.967	0.746	0.363	0.578	
Mean speed	0.962	0.229	0.895	0.759	0.476	0.447	0.756	
Latency to first exit of the periphery	0.262	0.273	0.377	0.517	0.751	0.115	0.114	

*almost reached statistical significance

3.3 Cognition: Spatial Learning and Memory

3.3.1 Barnes Maze (BM): Acquisition Phase

Spatial learning and memory were evaluated using the BM. The acquisition phase took place on days 1-3 of the test. In general mice learned to locate the escape hole and showed improved performance over trials in the acquisition phase evidenced by a RM ANOVA within subject effect of 'trials' in total latency [$F(5,300) = 24.25$, $p < 0.0001$], primary latency [$F(5,300) = 6.13$, $p < 0.0001$], total path length [$F(5,120) = 4.55$, $p < 0.001$], primary path length [$F(5,150) = 4.55$, $p < 0.01$], primary errors [$F(5,150) = 3.25$, $p < 0.01$], percent success rate [$F(5,300) = 10.95$, $p < 0.0001$], and mean speed [$F(5,300) = 6.67$, $p < 0.0001$] (Figures 3.3.1a-b). However task acquisition performance was influenced by the factors of genotype, diet and sex, varying for each parameter.

The RM ANOVA yielded a significant between subject interaction of 'genotype', 'diet' and 'sex' for total latency [$F(1,60) = 11.56$, $p < 0.01$] (Figure 3.3.1a). This interaction showed that for control diet male mice, the GFAP-IL6 genotype was associated with an increased total latency [$F(1,60) = 6.01$, $p < 0.05$], while among apigenin diet male mice, the GFAP-IL6 genotype had the effect of reducing in total latency [$F(1,60) = 6.89$, $p < 0.05$]. For WT male mice, the apigenin diet increased the total latency compared to control diet [$F(1,60) = 14.32$, $p < 0.001$]. Among WT mice on the apigenin diet, females appeared to perform better at the acquisition task exhibiting lower overall total latencies than males [$F(1,60) = 9.68$, $p < 0.01$], similarly for GFAP-IL6 mice on the control diet, the female sex displayed a lower total latency compared to males [$F(1,60) = 4.14$, $p < 0.05$]. Additionally there was a strong trend for a 'genotype' by 'diet' interaction in total latency [$F(1,60) = 3.81$, $p <$ **0.056**], indicating that for WT mice, the apigenin diet had the effect of increasing the total latency compared to the control diet [$F(1,60) = 6.56$, $p < 0.05$]. Data for total latency are shown in Figure 3.3.1a.

A significant between subject main effect of 'genotype' was obtained for the primary path length [$F(1,30) = 5.31$, $p < 0.05$] as shown in Figure 3.3.1b, interestingly GFAP-IL6 mice had a reduced primary path length compared to WT mice. An effect of 'diet' was also found for the primary path length [$F(1,30) = 4.28$, $p < 0.05$], mice that consumed the apigenin diet had a greater primary path length than those on the control diet. A 'genotype' by 'diet' interaction [$F(1,30) = 6.54$, $p < 0.05$]

showed that among mice consuming the apigenin diet, the GFAP-IL6 genotype displayed lower primary path lengths compared to WT [$F(1,30) = 8.69$, $p < 0.01$]. For WT mice, consumption of the apigenin diet led to an increased primary path length compared to control diet [$F(1,30) = 8.43$, $p < 0.01$]. Moreover an interaction between 'genotype', 'diet' and 'sex' was obtained [$F(1,30) = 8.94$, $p < 0.01$], among male mice treated with the apigenin diet, the GFAP-IL6 mice presented with reduced primary path length compared to WT mice [$F(1,30) = 10.75$, $p < 0.01$], for WT male mice, the apigenin diet had the effect of increasing the primary path length compared to the control diet [$F(1,30) = 9.71$, $p < 0.01$], and in WT mice that were fed the apigenin diet, females showed reduced primary path lengths compared to males [$F(1,30) = 6.16$, $p < 0.05$].

A significant between subject main effect of 'diet' was obtained for percent success rate [$F(1,60) = 4.22$, $p < 0.05$], such that mice consuming the apigenin diet had a lower percent success rate than control fed animals (Figure 3.3.1c). Additionally a significant main effect of 'sex' was found for percent success rate [$F(1,60) = 5.29$, $p < 0.05$], with female mice performing better at completing the escape task than male mice. An interaction was also obtained for percent success rate between 'genotype' and 'diet' [$F(1,60) = 4.10$, $p < 0.05$], indicating that for WT mice, the apigenin diet was associated with decreased percent success rate compared to control diet [$F(1,60) = 8.61$, $p < 0.01$]. Lastly, another interaction of 'trials' by 'diet' was found for percent success rate [$F(5,300) = 3.33$, $p < 0.01$]. The apigenin diet impacted on task acquisition performance effecting a decrease in percent success rate during trials 2 [$F(1,60) = 8.15$, $p < 0.01$] and 4 [$F(1,60) = 7.20$, $p < 0.01$] among mice treated with apigenin, regardless of genotype or sex.

A univariate 3-way ANOVA was performed to investigate initial mean speed in the acquisition phase for trial 1 in order to determine whether there were any starting differences among the groups. This analysis yielded a main effect of 'genotype' [$F(1,60) = 4.39$, $p < 0.05$] and 'sex'[$F(1,60) = 9.49$, $p < 0.01$], the GFAP-IL6 genotype exhibiting a lower velocity than WT and females presenting with a higher mean speed than males (Figure 3.3.1d).

No further main effects or interactions were obtained for any other acquisition phase parameters investigated including primary latency, primary and total errors and total path length (Table 3.3.1).

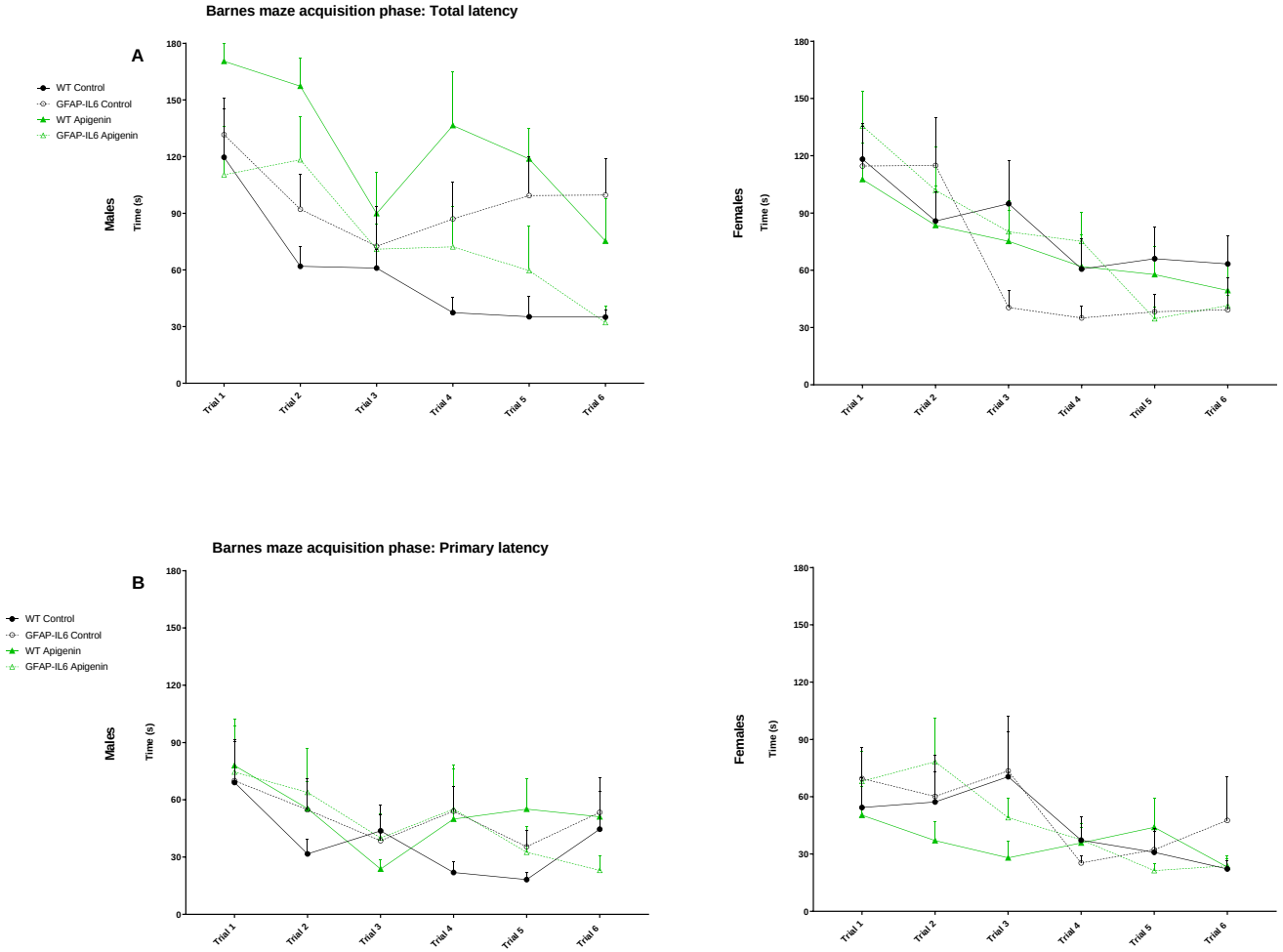


Figure 3.3.1a. Total and primary latency in the Barnes maze. *Mean (± SEM) A) total latency of the 6 trials in the acquisition phase of BM, out of 180s. An interaction of 'genotype', 'diet' and 'sex'($p < 0.01$) and an almost significant 'genotype' by 'diet' interaction ($p <$ **0.056**] was found. A within subject effect of 'trials' is also observed ($p < 0.0001$). B) primary latency of the 6 trial in the acquisition phase. Primary latency defined as the time to the first encounter with the escape hole. A within subject effect of 'trials' was reported ($p < 0.0001$).*

Trials 1-6: WT control male n= 8, WT control female n= 12, GFAP-IL6 control male n= 9, GFAP-IL6 control female n=7, WT apigenin male n= 6, WT apigenin female n= 10, GFAP-IL6 apigenin male n= 7, GFAP-IL6 apigenin female n= 9. Abbreviations: WT = Wild-type, GFAP-IL6 = Glial fibrillary acidic protein interleukin 6.

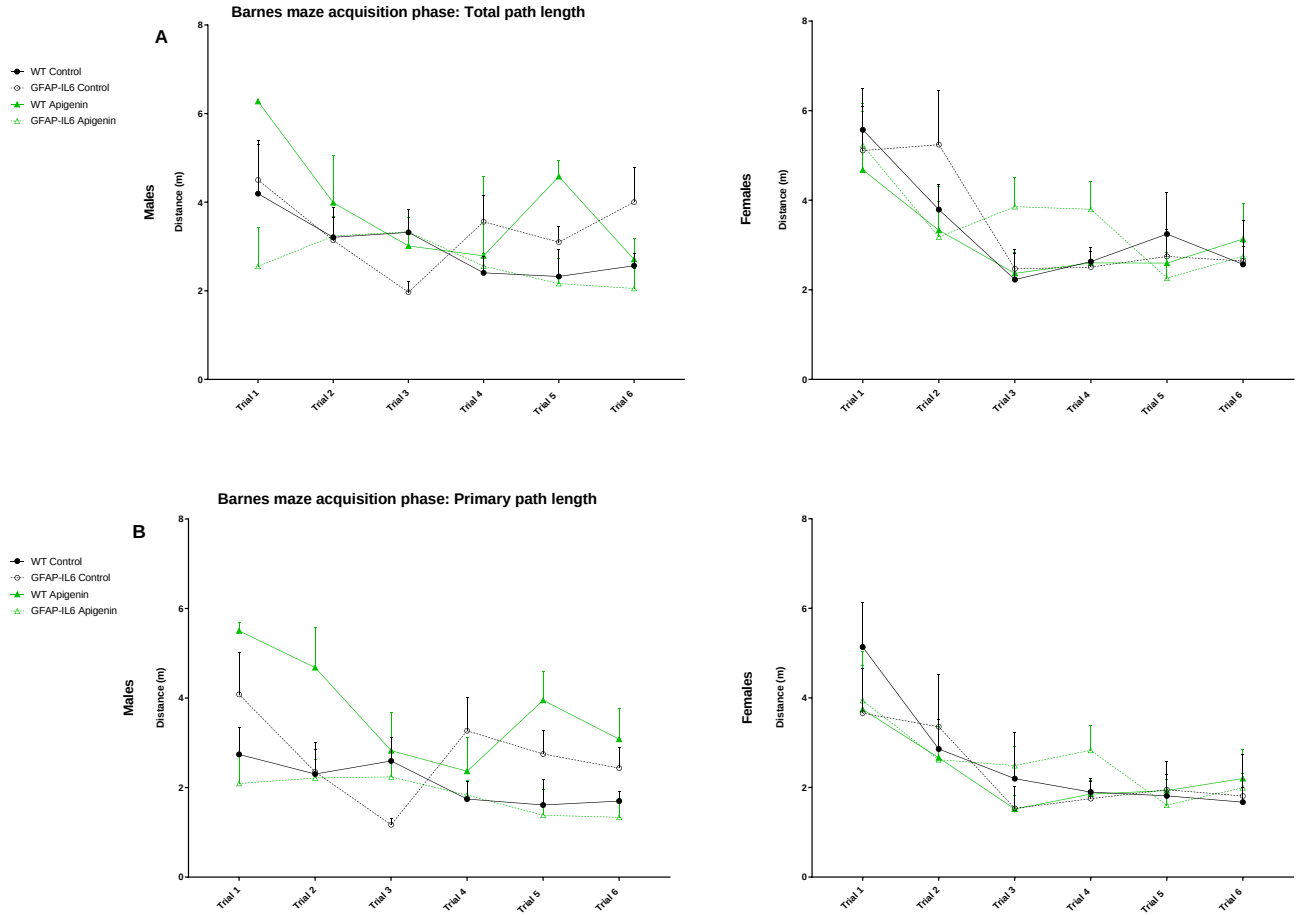


Figure 3.3.1b. Total and primary path length in the Barnes maze. *Mean (± SEM) A) total path length of the 6 trials in the acquisition phase of the BM, a within subject effect of 'trials' ($p < 0.001$) is reported. B) primary path length of the 6 trials in the acquisition phase of BM. A main effect of 'genotype' ($p < 0.05$) and 'diet'($p < 0.05$) was found. Significant interactions included 'genotype' by 'diet' ($p < 0.05$), 'genotype', diet' and 'sex' ($p < 0.01$). A within subject effect of 'trials' ($p < 0.01$) was also reported.*

Trial 1: WT control male n= 6, WT control female n= 10, GFAP-IL6 control male n= 7, GFAP-IL6 control female n=5, WT apigenin male n= 4, WT apigenin female n= 7, GFAP-IL6 apigenin male n= 6, GFAP-IL6 apigenin female n= 7. Trial 2: WT control male n= 8, WT control female n= 11, GFAP-IL6 control male n= 8, GFAP-IL6 control female n=5, WT apigenin male n= 5, WT apigenin female n= 9, GFAP-IL6 apigenin male n= 5, GFAP-IL6 apigenin female n= 6. Trial 3: WT control male n= 8, WT control female n= 8, GFAP-IL6 control male n= 8, GFAP-IL6 control female n=7, WT apigenin male n= 6, WT apigenin female n= 10, GFAP-IL6 apigenin male n= 7, GFAP-IL6 apigenin female n= 9. Trial 4: WT control male n= 8, WT control female n= 11, GFAP-IL6 control male n= 9, GFAP-IL6 control female n=7, WT apigenin male n= 3, WT apigenin female n= 10, GFAP-IL6 apigenin male n= 6, GFAP-IL6 apigenin female n= 9. Trial 5: WT control male n= 8, WT control female n= 12, GFAP-IL6 control male n= 7, GFAP-IL6 control female n=7, WT apigenin male n= 6, WT apigenin female n= 9, GFAP-IL6 apigenin male n= 6, GFAP-IL6 apigenin female n= 9. Trial 6: WT control male n= 8, WT control female n= 11, GFAP-IL6 control male n= 7, GFAP-IL6 control female n=7, WT apigenin male n= 6, WT apigenin female n= 10, GFAP-IL6 apigenin male n= 7, GFAP-IL6 apigenin female n= 9. Abbreviations: WT = Wild-type, GFAP-IL6 = Glial fibrillary acidic protein interleukin 6.

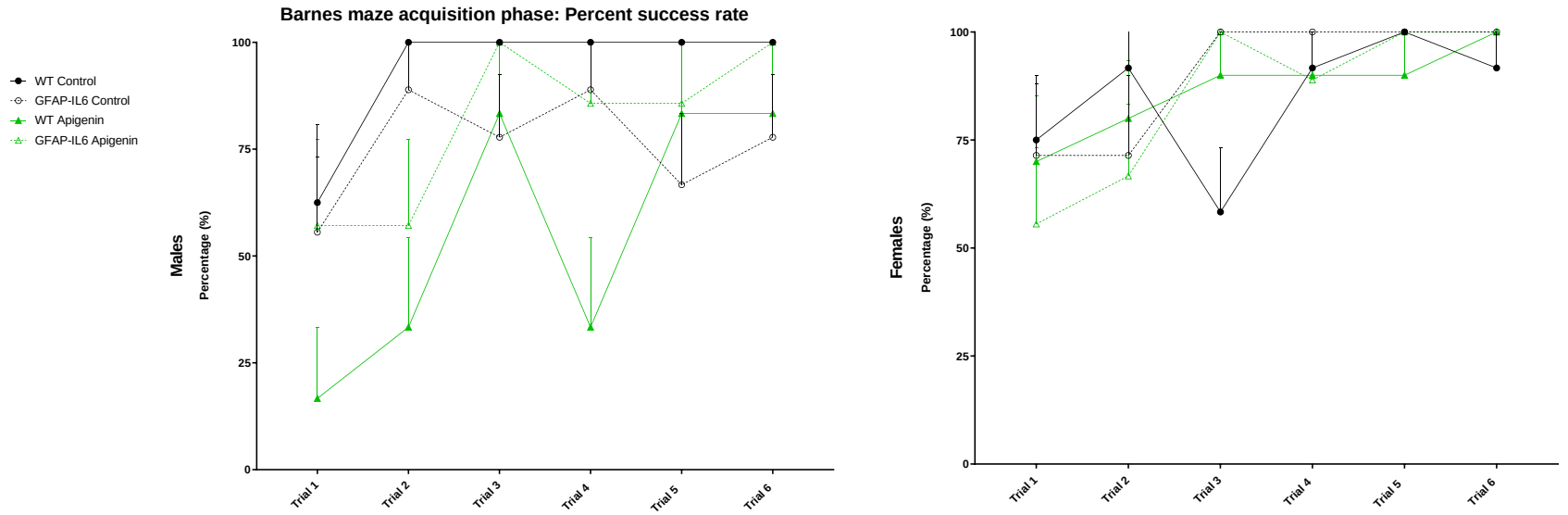


Figure 3.3.1c. Percent success rate in the Barnes maze. *Mean (± SEM) percent success rate of the 6 trials in the acquisition phase of BM. Success is defined as escaping through the target hole in under 180s. A significant main effect of 'diet' ($p < 0.05$) and 'sex' ($p < 0.05$) was observed. Interactions included 'genotype' by 'diet' ($p < 0.05$) and 'trials' by 'diet' ($p < .01$). A within subject effect of 'trials' is also reported ($p < 0.0001$).*

Trials 1-6: WT control male n= 8, WT control female n= 12, GFAP-IL6 control male n= 9, GFAP-IL6 control female n=7, WT apigenin male n= 6, WT apigenin female n= 10, GFAP-IL6 apigenin male n= 7, GFAP-IL6 apigenin female n= 9. Abbreviations: WT = Wild-type, GFAP-IL6 = Glial fibrillary acidic protein interleukin 6.

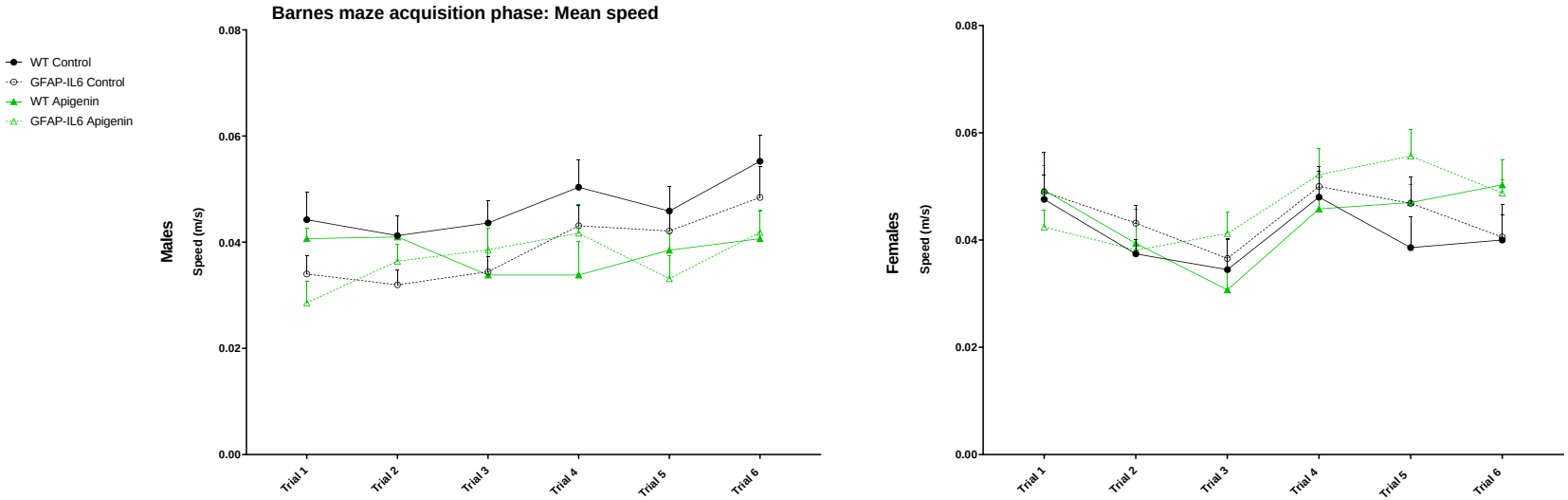


Figure 3.3.1d. Mean speed in the Barnes maze. *Mean (± SEM) Mean speed of the 6 trials in the acquisition phase of BM. For trial 1, a main effect of 'genotype' ($p < 0.05$) and 'sex'($p < 0.01$) was obtained. A within subject effect of 'trials' is also reported ($p < 0.0001$).*

Trials 1-6: WT control male n= 8, WT control female n= 12, GFAP-IL6 control male n= 9, GFAP-IL6 control female n=7, WT apigenin male n= 6, WT apigenin female n= 10, GFAP-IL6 apigenin male n= 7, GFAP-IL6 apigenin female n= 9. Abbreviations: WT = Wild-type, GFAP-IL6 = Glial fibrillary acidic protein interleukin 6.

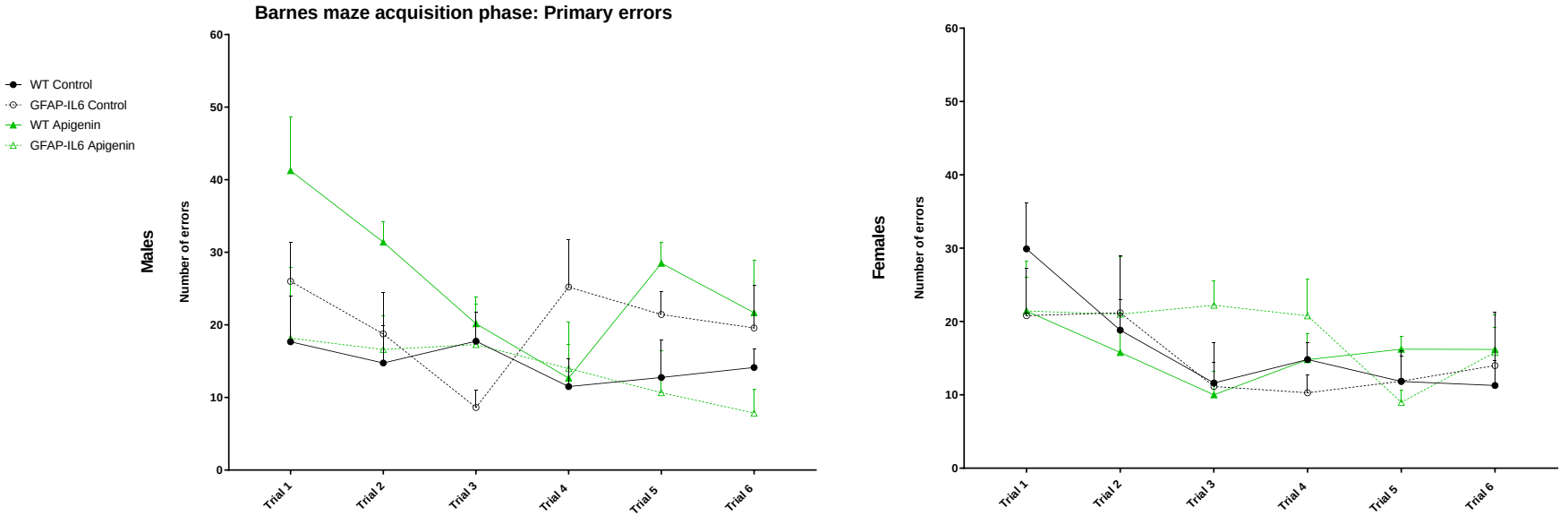


Figure 3.3.1e. Primary errors in the Barnes maze. *Mean (± SEM) primary errors of the 6 trials in the acquisition phase of BM. Primary error is defined as entries to holes other than the escape hole made before the first encounter of the escape hole. A within subject effect of 'trials' is reported ($p < 0.01$).*

Trial 1: WT control male n= 6, WT control female n= 10, GFAP-IL6 control male n= 7, GFAP-IL6 control female n=5, WT apigenin male n= 4, WT apigenin female n= 7, GFAP-IL6 apigenin male n= 6, GFAP-IL6 apigenin female n= 7. Trial 2: WT control male n= 8, WT control female n= 11, GFAP-IL6 control male n= 8, GFAP-IL6 control female n=5, WT apigenin male n= 5, WT apigenin female n= 9, GFAP-IL6 apigenin male n= 5, GFAP-IL6 apigenin female n= 6. Trial 3: WT control male n= 8, WT control female n= 8, GFAP-IL6 control male n= 8, GFAP-IL6 control female n=7, WT apigenin male n= 6, WT apigenin female n= 10, GFAP-IL6 apigenin male n= 7, GFAP-IL6 apigenin female n= 9. Trial 4: WT control male n= 8, WT control female n= 11, GFAP-IL6 control male n= 9, GFAP-IL6 control female n=7, WT apigenin male n= 3, WT apigenin female n= 10, GFAP-IL6 apigenin male n= 6, GFAP-IL6 apigenin female n= 9. Trial 5: WT control male n= 8, WT control female n= 12, GFAP-IL6 control male n= 7, GFAP-IL6 control female n=7, WT apigenin male n= 6, WT apigenin female n= 9, GFAP-IL6 apigenin male n= 6, GFAP-IL6 apigenin female n= 9. Trial 6: WT control male n= 8, WT control female n= 11, GFAP-IL6 control male n= 7, GFAP-IL6 control female n=7, WT apigenin male n= 6, WT apigenin female n= 10, GFAP-IL6 apigenin male n= 7, GFAP-IL6 apigenin female n= 9. Abbreviations: WT = Wild-type, GFAP-IL6 = Glial fibrillary acidic protein interleukin 6.

Table 3.3.1. Summary of Barnes Maze acquisition phase parameters

Parameter	Effect or interaction	Significance value	Description of the nature of significant effects or interactions
Total latency	Within-subject main effect of 'trials'	[F(5,300) = 24.25, p < 0.0001]	Total latency decreased over trials for all groups
	Between-subject interaction of 'genotype' and 'diet'*****	[*F*(1,60) = 3.81, *p* < **0.056**]	Strong trend toward WT apigenin mice ↑ total latency compared to WT control mice
	Between-subject interaction of 'genotype', 'diet' and 'sex'	[F(1,60) = 11.56, p < 0.01]	Apigenin GFAP-IL6 males ↓ total latency compared to control GFAP-IL6 males Apigenin WT males ↑ total latency compared to control WT males WT apigenin females ↓ total latency compared to WT apigenin males GFAP-IL6 control females ↓ total latency compared to GFAP-IL6 control males
Primary latency	Within-subject main effect of 'trials'	[F(5,300) = 6.13, p < 0.0001]	Primary latency decreased over trials for all groups
Total path length	Within-subject main effect of 'trials'	[F(5,120) = 4.55, p < 0.001]	Total path length decreased over trials for all groups

Primary path length	Within-subject main effect of ‘trials’	[F(5,150) = 4.55, p < 0.01]	Primary path length decreased over trials for all groups
	Between-subject main effect of ‘genotype’	[F(1,30) = 5.31, p < 0.05]	GFAP-IL6 mice ↓ primary path length compared to WT mice
	Between-subject main effect of ‘diet’	[F(1,30) = 4.28, p < 0.05]	Apigenin diet mice ↑ primary path length compared to control diet mice
	Between-subject interaction of ‘genotype’ and ‘diet’	[F(1,30) = 6.54, p < 0.05]	GFAP-IL6 apigenin mice ↓ primary path length compared to WT apigenin mice WT apigenin mice ↑ primary path length compared to WT control mice
	Between-subject interaction of 'genotype', 'diet' and 'sex'	[F(1,30) = 8.94, p < 0.01]	GFAP-IL6 apigenin males ↓ primary path length compared to WT apigenin males WT apigenin males ↑ primary path length compared to WT control males WT apigenin females ↓ primary path length compared to WT apigenin males
Total errors	**No significant effects or interactions**	-	-
Primary errors	Within-subject main effect of ‘trials’	[F(5,150) = 3.25, p < 0.01]	Primary errors decreased over trials for all groups

Percent success rate	Within-subject main effect of 'trials'	[$F(5,300) = 10.95$, $p < 0.0001$]	Percent success rate increased over trials for all groups
	Between-subject main effect of 'diet'	[$F(1,60) = 4.22$, $p < 0.05$]	Apigenin diet mice ↓ percent success rate than control fed animals
	Between-subject main effect of 'sex'	[$F(1,60) = 5.29$, $p < 0.05$]	Female mice ↑ percent success rate than male mice
	Between-subject interaction of 'genotype' and 'diet'	[$F(1,60) = 4.10$, $p < 0.05$]	WT apigenin mice ↓ percent success rate compared to WT control mice
	Between-subject and within-subject interaction of 'trials' and 'diet'	[$F(5,300) = 3.33$, $p < 0.01$]	Apigenin diet ↓ percent success rate during trials 2 and 4
Mean speed	Within-subject main effect of 'trials'	[$F(5,300) = 6.67$, $p < 0.0001$]	Mean speed increased over trials for all groups
	Between-subject main effect of 'genotype' (3-way univariate ANOVA trial 1 only)	[$F(1,60) = 4.39$, $p < 0.05$]	GFAP-IL6 mice ↓ mean speed than WT in trial 1
	Between-subject main effect of 'sex' (3-way univariate ANOVA trial 1 only)	[$F(1,60) = 9.49$, $p < 0.01$]	Female mice ↑ mean speed than male mice in trial 1

All parameters analysed by 4-way RM ANOVA including between-subject factors of 'genotype', 'diet', 'sex' and within-subject factor of 'trials' unless stated otherwise. Abbreviations WT = Wild-type, GFAP-IL6 = Glial fibrillary acidic protein interleukin 6.

3.3.2 Barnes Maze (BM): Probe Trial

On day 6 of the BM the mice underwent the probe trial to observe differences in memory retrieval among the experimental groups.

The percentage of time in the target quadrant data is depicted in Figure 3.3.2a. While ANOVA did not reveal any main effects or interactions, the one sample t-test analysis against chance level (25%) demonstrated that the WT female control diet group [$t(11)= 2.35$, $p < 0.05$], WT male apigenin diet group[$t(5)= 4.01$, $p < 0.05$], WT female apigenin diet group [$t(9)= 4.62$, $p < 0.01$] and the GFAP-IL6 male apigenin diet group [$t(6)= 2.91$, $p < 0.05$] all displayed a preference for the target quadrant, suggesting successful recall of the location of the escape hole. In this case the apigenin diet appeared to enhance the preference for the target quadrant.

Figure 3.3.2b shows data for the number of entries made to the escape hole. A main effect of 'genotype' was found for the number of entries made to the escape hole [$F(1,60) = 11.40$, $p < 0.01$] such that mice of GFAP-IL6 genotype made significantly fewer entries to the escape hole than WT mice and indicating a poorer memory retrieval of the escape hole location. An effect of 'diet' was also obtained for entries to the escape hole in the probe trial [$F(1,60) = 5.77$, $p < 0.05$], with mice consuming the apigenin diet having a significantly higher number of entries to the escape hole.

The hole entry distribution was also evaluated by ANOVA, applying Dunnetts's multiple comparison tests to compare the average escape hole entries against all other hole entry averages (Figure 3.3.2c). All groups searched in the right location in and around the target area, with non-escape hole entries being statistically different to the escape hole entries, indicating preference for the target hole. The degree of preference however is shown to vary depending on diet, genotype and sex. For example, comparing the GFAP-IL6 apigenin diet and GFAP-IL6 control diet groups, apigenin appears to improve the hole entry distribution profile toward greater preference of the escape hole.

Total distance travelled and mean speed in the probe trial was analysed as additional locomotor measures to compare to the results from the EPM and OF (Figure 3.3.2e). The three way ANOVA showed a main effect of 'genotype' for both the total distance travelled [$F(1,60) = 4.75$, $p < 0.05$] and

mean speed [$F(1,60) = 4.79$, $p < 0.05$], GFAP-IL6 mice travelling less and moving slower than WT mice.

There were no significant effects or interactions found for other probe trial parameters investigated including time in escape hole, latency to escape hole, and distance to escape hole (Table 3.3.2).

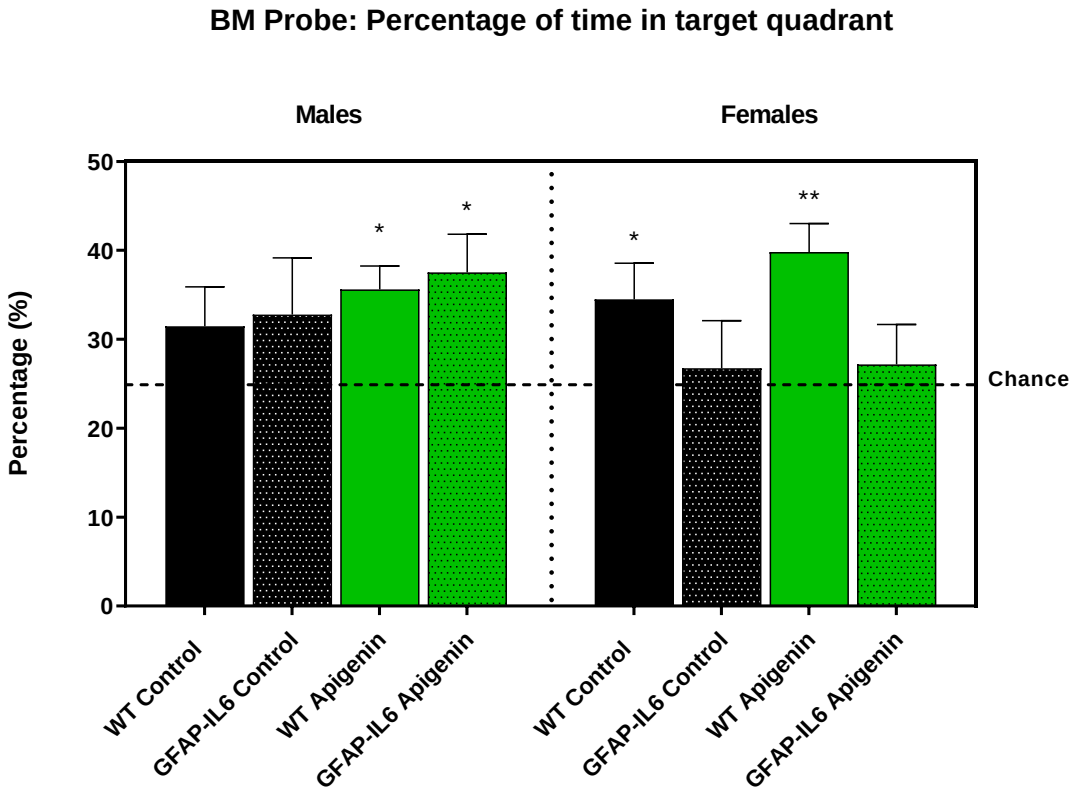


Figure 3.3.2a. Percentage of time in the target quadrant during probe trial in the Barnes maze.
Mean (± SEM) percentage of time in the EHQ vs chance (25%) in the probe trial of the BM.
**p < 0.05, **p < 0.01, ***p < 0.00.*

WT control male n = 8, WT control female n = 12, GFAP-IL6 control male n = 9, GFAP-IL6 control female n = 7, WT apigenin male n = 6, WT apigenin female n= 10, GFAP-IL6 apigenin male n = 7, GFAP-IL6 apigenin female n = 9. Abbreviations: WT = Wild-type, GFAP-IL6 = Glial fibrillary acidic protein interleukin 6.

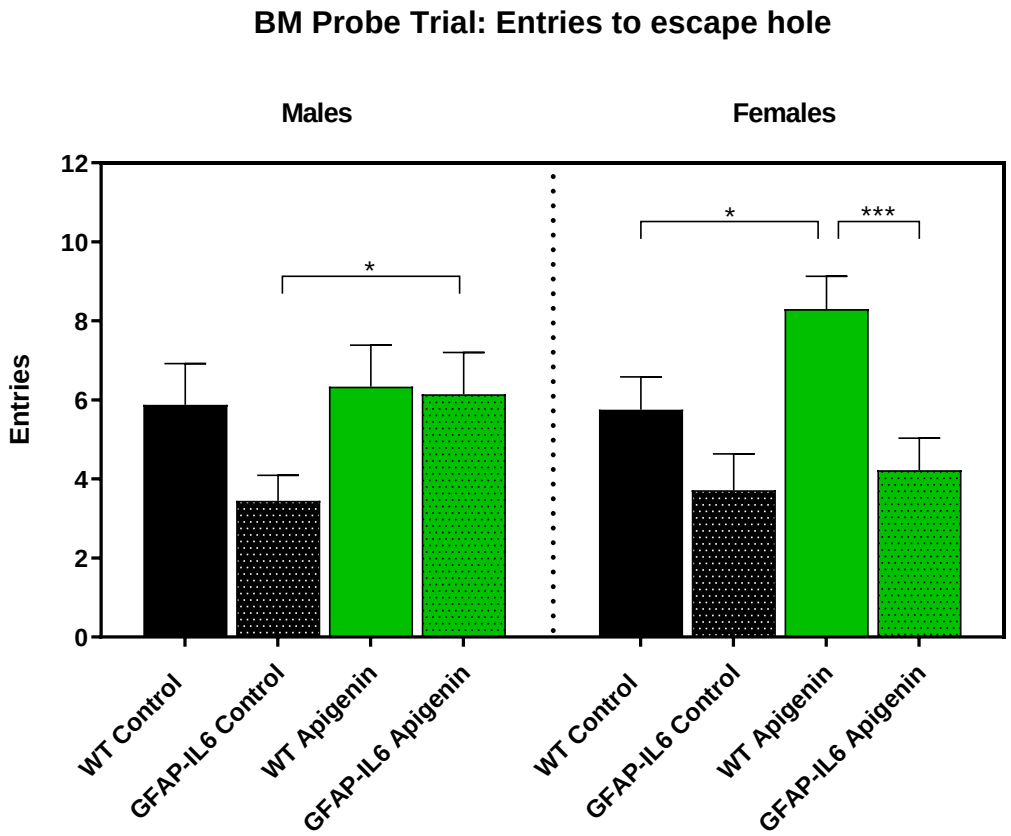


Figure 3.3.2b. Number of entries to the escape hole during probe trial in the Barnes maze. *Mean (± SEM) number of entries to the escape hole in the probe trial of the BM. A main effect of 'genotype' ($p < 0.01$) and 'diet' ($p < 0.05$) was reported. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.*

WT control male n = 8, WT control female n = 12, GFAP-IL6 control male n = 9, GFAP-IL6 control female n = 7, WT apigenin male n = 6, WT apigenin female n= 10, GFAP-IL6 apigenin male n = 7, GFAP-IL6 apigenin female n = 9. Abbreviations: WT = Wild-type, GFAP-IL6 = Glial fibrillary acidic protein interleukin 6.

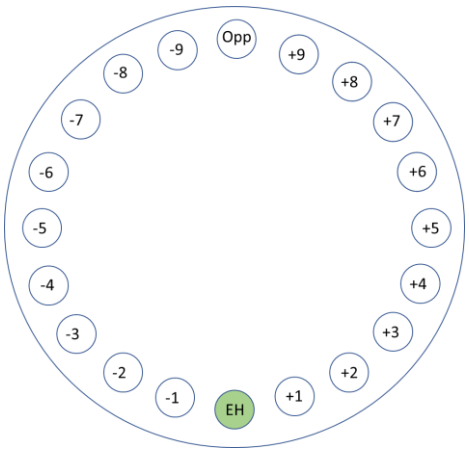
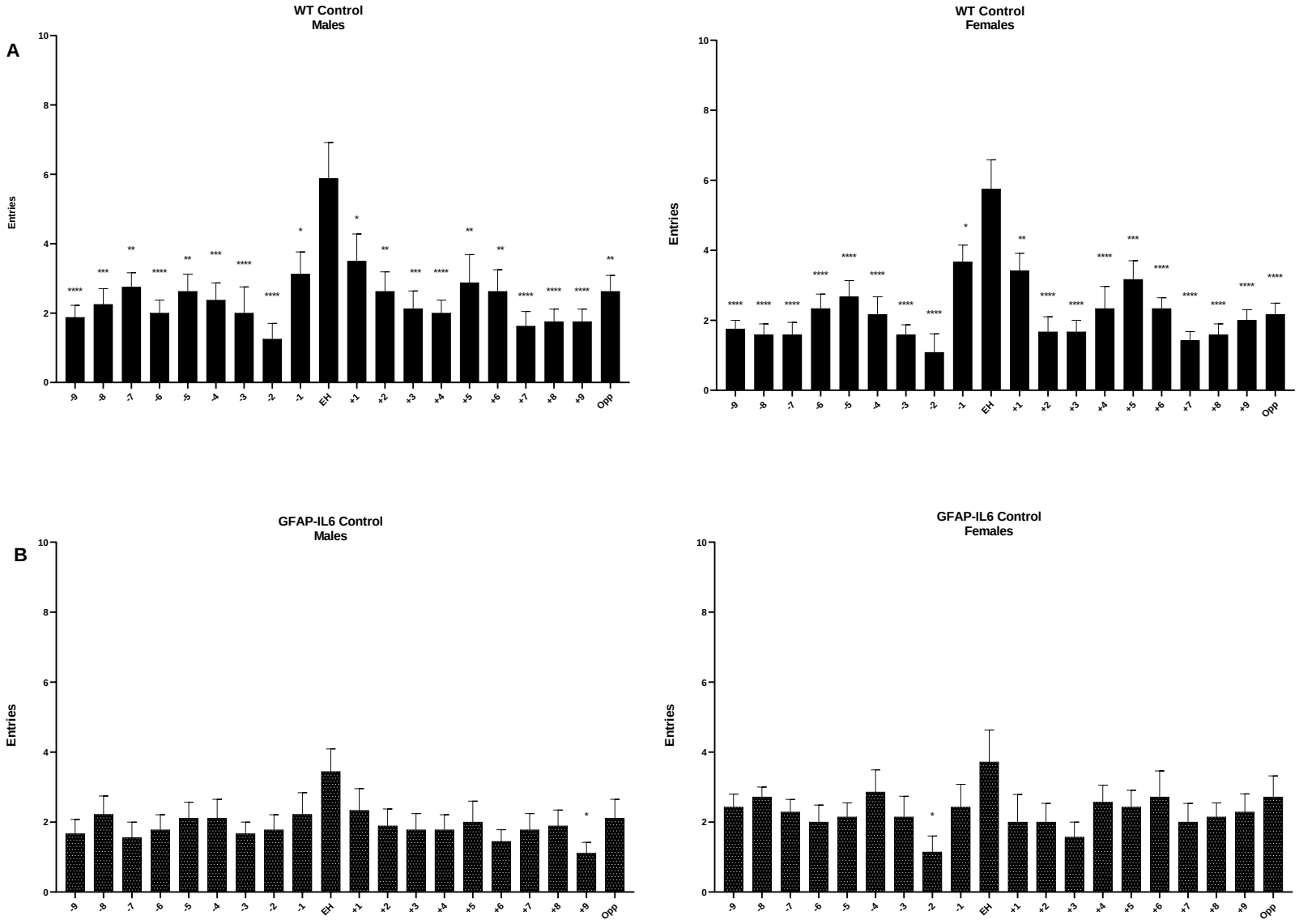


Figure 3.3.2c Scheme of the Barnes Maze hole locations


A
WT Control
Males
Entries
WT Control
Females
Entries
B
GFAP-IL6 Control
Males
Entries
GFAP-IL6 Control
Females
Entries
-9
-8
-7
-6
-5
-4
-3
-2
-1
EH
+1
+2
+3
+4
+5
+6
+7
+8
+9
Opp

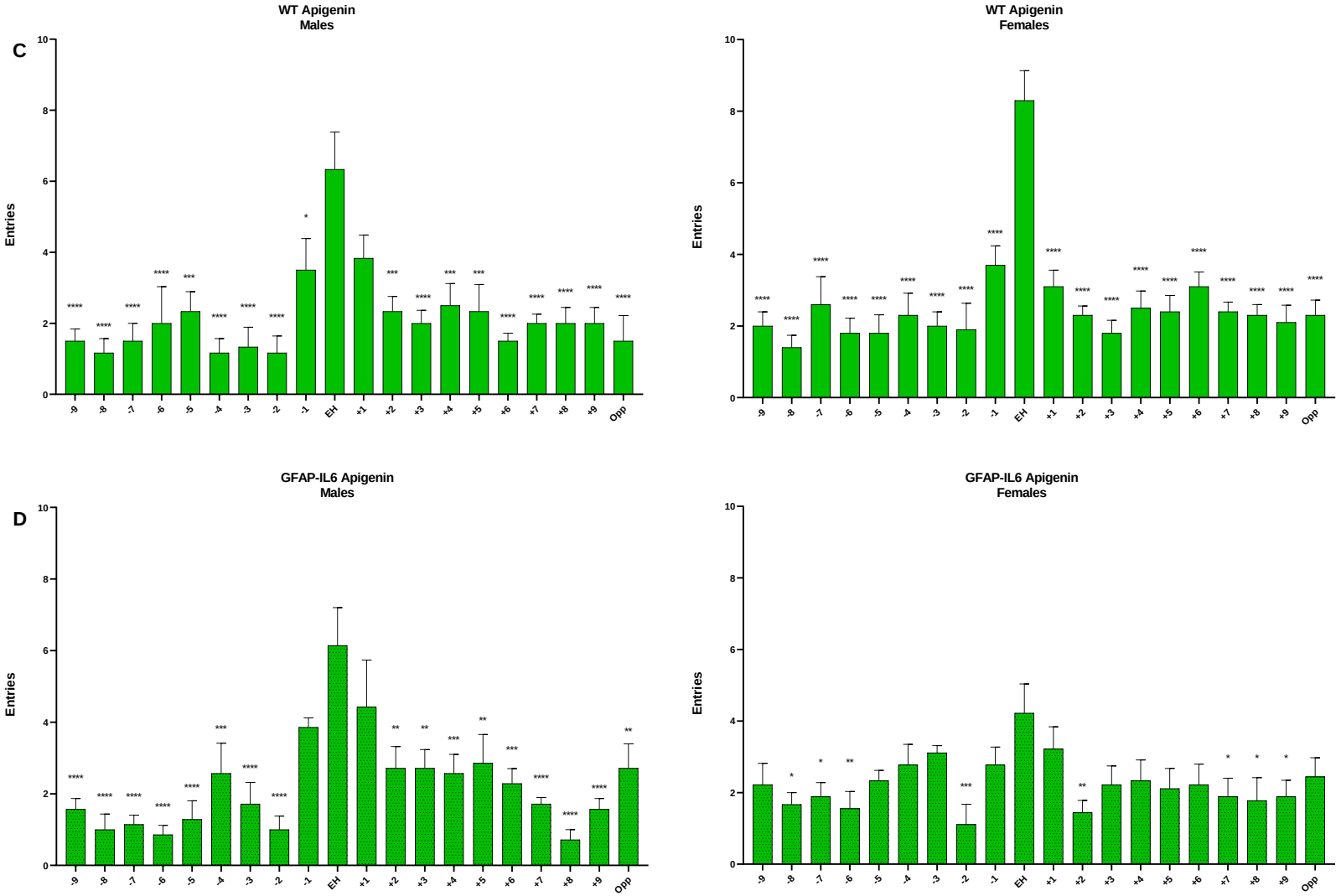


Figure 3.3.2d. Hole entry distribution in the probe trial of the Barnes maze. Mean *(± SEM) average number of entries made in each hole during the probe trial (90s). A) WT Control, B) GFAP-IL6 Control, C) WT Apigenin, D) GFAP-IL6 Apigenin. Asterisks indicate level of significance in Dunnett's multiple comparison test comparing all hole entries to the escape hole entry. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.* WT control male n = 8, WT control female n = 12, GFAP-IL6 control male n = 9, GFAP-IL6 control female n = 7, WT apigenin male n = 6, WT apigenin female n= 10, GFAP-IL6 apigenin male n = 7, GFAP-IL6 apigenin female n = 9. Abbreviations: WT = Wild-type, GFAP-IL6 = Glial fibrillary acidic protein interleukin 6, EH =Escape hole, Opp = Opposite hole. (Refer to Figure 3.3.2c for locations of holes +1 to +9 and -1 to -9).

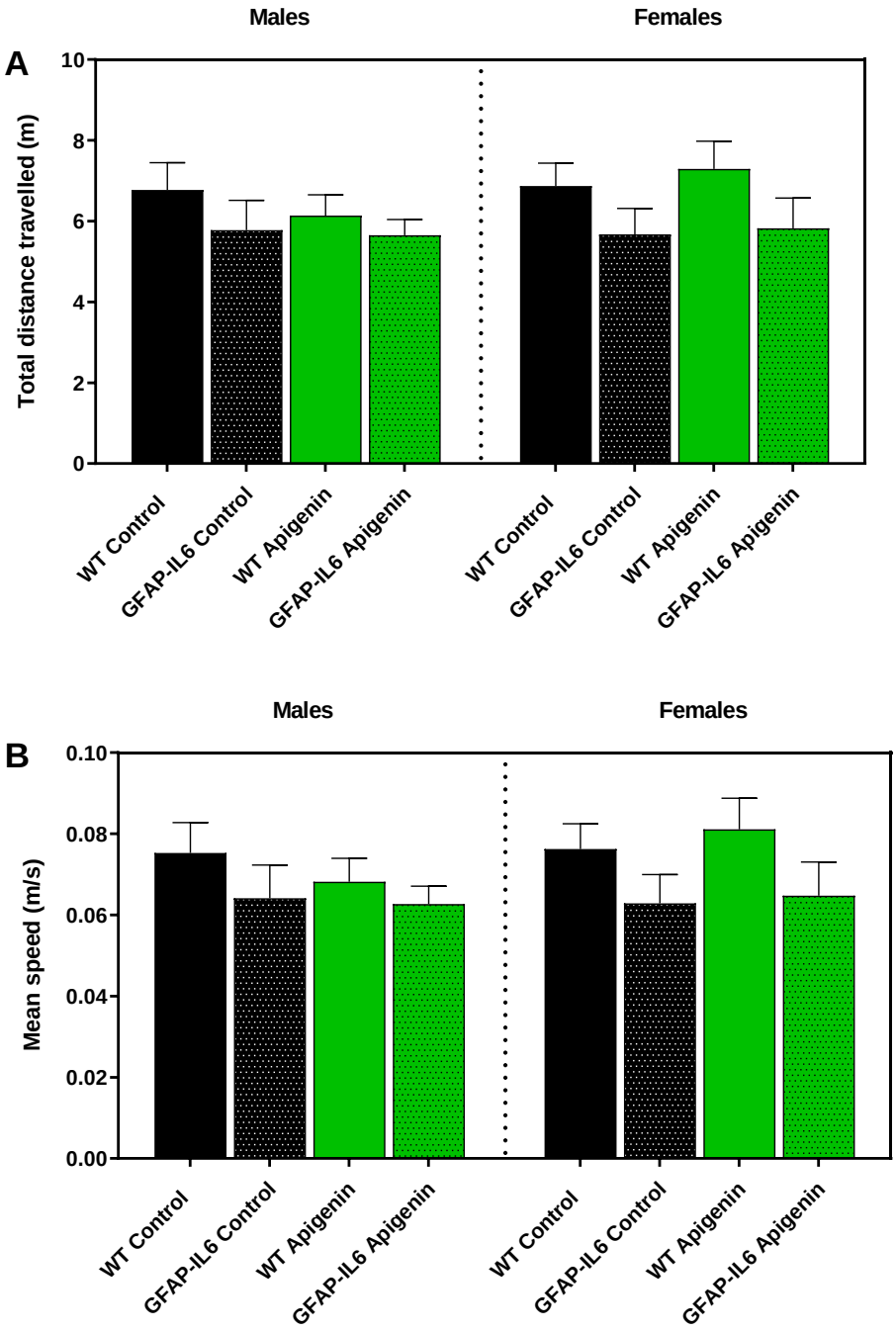


Figure 3.3.2e. Locomotion during the probe trial of the Barnes maze. *Mean (± SEM) (A) total distance travelled (B) mean speed in the probe trial of the BM. A main effect of 'genotype' was obtained for total distance travelled ($p < 0.05$) and mean speed ($p < 0.05$).*

WT control male n = 8, WT control female n = 12, GFAP-IL6 control male n = 9, GFAP-IL6 control female n = 7, WT apigenin male n = 6, WT apigenin female n= 10, GFAP-IL6 apigenin male n = 7, GFAP-IL6 apigenin female n = 9. Abbreviations: WT = Wild-type, GFAP-IL6 = Glial fibrillary acidic protein interleukin 6.

Table 3.3.2 Summary Barnes Maze probe trial parameters

Parameter	Effect or interaction	Significance value	Description of the nature of significant effects or interactions
Percentage of time in target quadrant	No significant effects or interactions by 3-way ANOVA	-	-
	4 groups showed preference for the target quadrant in the one sample t-test analysis against chance level (25%)	WT control females [$t(11)= 2.35$, $p < 0.05$] WT apigenin males [$t(5)= 4.01$, $p < 0.05$] WT apigenin females [$t(9)= 4.62$, $p < 0.01$] GFAP-IL6 apigenin males [$t(6)= 2.91$, $p < 0.05$]	All 4 listed groups displayed a preference for the target quadrant, suggesting successful recall of the location of the escape hole. The apigenin diet appeared to be associated with increased preference for the target quadrant
Number of entries made to escape hole	Between-subject main effect of 'genotype'	[$F(1,60) = 11.40$, $p < 0.01$]	GFAP-IL6 mice ↓ number of entries to escape hole compared to WT
	Between-subject main effect of 'diet'	[$F(1,60) = 5.77$, $p < 0.05$]	Mice treated with apigenin ↑ number of entries to escape hole compared to control diet
Hole entry distribution	No significant effects or interactions by 3-way ANOVA or 4-way RM ANOVA	-	-

	Number of non-escape hole entries were significantly different to the number escape hole entries (Dunnett's multiple comparison test comparing all hole entries to the escape hole entry)	Numerous and varied – refer to Figure 3.3.2d (A-D)	Preference for the target hole is indicated for most groups. GFAP-IL6 control mice showed almost no preference for the target hole. Apigenin appears to increase the preference for the escape hole and improve the hole entry distribution profile.
Total distance travelled	Between-subject main effect of 'genotype'	[$F(1,60) = 4.75$, $p < 0.05$]	GFAP-IL6 mice ↓ locomotion compared to WT
Mean speed	Between-subject main effect of 'genotype'	[$F(1,60) = 4.79$, $p < 0.05$]	GFAP-IL6 mice ↓ velocity compared to WT
Time in escape hole	No significant effects or interactions by 3-way ANOVA	-	-
Latency to escape hole	No significant effects or interactions by 3-way ANOVA	-	-
Distance to escape hole	No significant effects or interactions by 3-way ANOVA	-	-

Abbreviations WT = Wild-type, GFAP-IL6 = Glial fibrillary acidic protein interleukin 6.

CHAPTER 4

DISCUSSION

The alarming increase in neurodegenerative diseases and affective disorders has surfaced as a global health problem with a clear need for therapies that can halt or reverse disease progression. The present study proposed the use of the GFAP-IL6 transgenic mouse as a tool to investigate the effects of chronic neuroinflammation, a common underlying feature in the development of human neurological diseases. At the same time, this mouse line was presented as a suitable model to screen potential therapeutic agents against neuroinflammation, in this case testing the neuroprotective potential of the anti-inflammatory dietary flavonoid apigenin. Specifically, the focus of this research was to investigate these effects in relation to cognition and behaviour, anticipating that the chronic neuroinflammation in the GFAP-IL6 mouse would manifest cognitive impairments and behavioural alterations, and that apigenin may ameliorate any abnormalities found. While there have been earlier behavioural studies conducted on the GFAP-IL6 mouse [2,5], based on the current literature this study is the first to tests this transgenic model in the arenas of the BM, OF and EPM.

The principal findings of this study will be discussed in relation to the two main aims of this project, these being: 1) evaluating the physiological and behavioural (i.e. anxiety response and spatial memory) effects of IL-6 mediated chronic neuroinflammation, including any sex differences that may exist, and 2) evaluating the effects of the natural anti-inflammatory agent apigenin, with both aims in respect to cognition and behaviour.

4.1 Feeding, body weight and general health

Apigenin fed animals consumed significantly more food and gained more weight in the first 2 months of the diet. It is unclear whether apigenin caused an increase in appetite or whether the addition of apigenin to the feed mix improved its palatability. These findings are not consistent with the reported effects of apigenin on body weight and appetite, which has been shown to reduce food intake and

produce weight loss in mice [109]. Apigenin's reported sedative-like effects may have also contributed to the increased weight gain by reduction in ambulation.

The abnormalities in gait, kyphosis and hindlimb clasping found in this study describe an ataxic phenotype for the GFAP-IL6 mouse, which is consistent with previously reported findings[2] and aligns with the cerebellar neurodegeneration found in this mouse model.

4.2 Anxiety-like behaviour, locomotion and exploration (EPM and OF)

In the EPM, the GFAP-IL6 genotype significantly impacted on locomotor activity including a decrease in the total distance travelled, distance travelled in the closed arms and a reduced mean speed. This reduced locomotor activity in the EPM is in agreement with previously reported findings of locomotor impairments in the GFAP-IL6 mouse including tremor, ataxia and gait abnormalities that again correlate with neuropathology found in the cerebellum[2], which were also found in the functional observational battery in this present study. As there were no genotype differences found in the percentage of distance travelled in the open arms, the reduced total distance travelled was driven by decreased ambulatory activity within the closed arms. This finding may indicate behavioural alterations in this mouse line related to interactions with specific environmental conditions, in this case perhaps triggered by the dark and enclosed space of the closed arms. In the open field, chronic IL-6 driven neuroinflammation in the GFAP-IL6 mouse produced an anxiolytic-like effect evidenced by a greater percentage of time in the centre zone compared to WT mice, while the locomotor measures of total distance travelled and mean speed remained unaffected. Studies have linked IL-6 to emotionality and anxiety-like behaviours in mice[110-113], with IL-6 said to likely affect the stress response and other behaviours through modulation of the hypothalamus [114,115]. It is known that the HPA axis function is perturbed in the GFAP-IL6 mouse [2,81]. In total and astrocytic IL-6 knockout mice behavioural characterization studies revealed an anxiogenic-like effect for IL-6 deficiency as well as alterations in exploratory and social behaviours [110-112]. However these studies have returned some mixed results suggesting that the role of IL-6 is highly dependent on age, sex, background strain

and apparatus[112]. The fact that the locomotor abnormalities captured in the EPM did not translate across to the OF is of interest. By additionally analysing the total distance travelled and mean speed during the Barnes maze probe trial it was possible to determine that motor impairments in the GFAP-IL6 strain appear to be test/apparatus-dependant, revealing a genotype effect for both of these locomotor measures. Similarities in the more challenging and fear-provoking elevated position of the EPM and the BM make a case toward this conclusion. Furthermore, the finding of increased gait abnormalities and the propensity to spontaneous seizures during environmental changes in the GFAP-IL6 animals (eg. handling, changing cages, during testing) proposes a possible combined effect of : impaired fine motor ability within a challenging environment (BM and EPM both are elevated and induce anxiety, mice may not move as freely as in the OF), an inefficiency or altered processing in the perception of their environment due to their neuropathology; and additional anxiolytic-like genotype effects related specifically to the role of IL-6 on behaviour. The GFAP-IL6 anxiolytic-like phenotype is contrary to the commonly seen effects of neuroinflammation on OF and EPM behaviours. Previous studies have demonstrated neuroinflammation induces anxiety-like behaviour in the EPM and OF resulting in a reduction in the time and entries in the open arms and centre zone respectively [116-119]. However, many of these studies have been conducted in lipopolysaccharide peripherally induced models of acute neuroinflammation [117-119], or traumatic brain injury neuroinflammation [116]. The CNS-targeted chronic overexpression of IL-6 in this transgenic mouse represents an atypical form of neuroinflammation and concomitant neurodegeneration[3], and as such may explain the absence of the typical sickness behaviour and increases in anxiety-related behaviours that are normally expected. The sustained over-expression of IL-6 is likely to be the driving factor contributing toward the anxiolytic-like phenotype, rather than the general neuroinflammatory load or neurodegenerative processes.

In the EPM, agipenin produced a reduction in ambulatory activity in the open arms, decreased head dips frequency and exhibited a trend toward decreasing stretched attend posture frequency. In the OF apigenin decreased the percentage of locomotion in the centre zone. These findings do not describe clear-cut effects for apigenin, but rather suggest it may be acting in both a sedative-like and anxiolytic-like manner. Previous studies have shown apigenin to have anxiolytic-like and sedative-

like effects in rodent models in the EPM[84], and sedative-like effects in the OF[120,121]. In rats, apigenin reduced ambulation in the OF at doses of 25 and 50 mg/kg[120,121]. The findings of this study appear to align with these demonstrated effects. Although a reduction in locomotion in the open arms (EPM) and centre zone (OF) can also signify an anxiogenic-like effect, these prior findings suggest that the depressive effect on locomotion is more likely due to apigenin's sedative properties. The same significant reduction in head dips were also found by Kumar and colleague in the EPM, however their study utilised the glycosyloxyflavone, apigenin-7-glucoside[122], which is considered to have increased solubility and stability compared to apigenin. While the exact interpretation of ethological measures can be arguable, the tendency of apigenin to reduce stretched attend postures seems to also support an anxiolytic-like activity, as a lower frequency of stretched attend postures have been associated with administration of benzodiazepine receptor agonists[123]. However, apigenin did not increase the time spent in the open arms of the EPM or the centre zone in the OF, which are typical effects of anxiolytic agents. It may indicate apigenin is exerting a more sedative-like effect than an anxiolytic-like effect and may be related to the dose. Apigenin is proposed to exert anxiolytic effects by acting as a benzodiazepine ligand, with no myorelaxant or sedative effects at normal dosages (3-10mg/kg bodyweight) however sedation is reported at 3 and 10-fold of this dose (30-100mg/kg bodyweight)[84]. The dose of 40mg/kg bodyweight used in this study lies within the starting range of sedative effects. Sedative effects of apigenin have also been attributed to enhancement of GABAergic systems[124].

Apigenin's known anti-inflammatory, anti-oxidant, neuroprotective and anti-depressant-like activities *in vitro* and *in vivo* include suppression of oxidative stress, migroglial activation, NLRP3 activation and inflammatory cytokines, restoration of HPA axis function, and upregulation of BDNF[99,100,104,105,125-129]. Moreover, histological investigations in our own laboratory of apigenin treated GFAP-IL6 mice from this present study confirmed apigenin's anti-inflammatory effects, showing a ~25-30% decrease in the number of activated microglia in the cerebellum and hippocampus[130]. It could be anticipated that apigenin's variety of activities in the CNS would potentially normalise any disturbances in anxiety-related behaviours found in the GFAP-IL6 mouse mediated by neuroinflammatory and neurodegenerative processes. However there were no reported

effects that shifted the anxiolytic-like phenotype of the GFAP-IL6 closer toward WT anxiety-like behaviour, and it would likely be masked by the effects of apigenin's anxiolytic-like activity, matching the anxiolytic-like phenotype of the GFAP-IL6.

An important consideration is the potential impact of the increased bodyweight among apigenin treated mice on the EPM and OF parameters. Anticipated effects of increased bodyweights would likely manifest as decreased total distance travelled and velocity, as previously reported[131]. However, while apigenin was shown to reduce the distance travelled in the open arms of the EPM and lower the percentage of distance travelled in the centre zone of the OF, there were no main effects found for the apigenin diet in the total distance travelled or mean speed in either of these tests as would be expected. This seems to suggest that although possible, the increased bodyweight was unlikely to have impacted significantly on their behaviour and the reduced ambulation in the open arms and centre zone of the EPM and OF respectively relates to apigenin's sedative-like effects. Similarly, when considering the possible contribution of increased bodyweight on the reduction in head dips associated with apigenin treatment, this effect seems more likely to be mediated by apigenin's known anxiolytic and sedative properties as reported in similar studies[122] and not by any mobility issues caused by increased body mass.

In the OF and EPM females displayed greater activity in stretched attend posture frequency and percentage of time and distance in the centre zone of the OF. These sex differences are comparable to previous studies of activity and emotionality[132].

4.3 Cognition: Spatial Learning and Memory (BM)

The effects of chronic neuroinflammation in the GFAP-IL6 model on hippocampal-dependent learning was evaluated in the BM. In the acquisition phase, evidence of cognitive dysfunction was present only in the total latency and only among male GFAP-IL6 control mice where their performance was significantly impaired in comparison to WT control diet males. Interestingly, the primary latency during the acquisition phase for GFAP-IL6 mice was found to be significantly lower

than that of WT mice. Shorter primary path lengths usually indicate improved learning and memory of the escape hole location [133], however this result was likely attributed to the altered locomotor profile of the GFAP-IL6 model rather than being evidence of better cognitive performance than WT mice.

The most compelling finding demonstrating cognitive impairment in this model however was found in the number of escape hole entries made during the probe trial, where the GFAP-IL6 genotype was associated with a significantly reduced number of entries, suggesting impaired memory retrieval. The percentage of time in the target quadrant also indicate cognitive impairment in that the GFAP-IL6 control fed animals did not show a preference for the target quadrant in these parameters, further implying deficits in learning and recall of the location of the escape hole. These results compare to the learning impairments described in earlier studies of the GFAP-IL6 mouse where cognitive decline in avoidance learning correlated with progressive neuropathological changes [3,5]. It is well understood that hippocampal lesions and inflammatory processes contribute to spatial memory deficits[134], and the functional alterations in hippocampal synaptic plasticity in this model are consistent with the associated cognitive decline, including dendridic vacuolization, loss of parvalbumin and calbunin immunoreactive neurons, decreased neurogenesis, reduced long-term potentiation in the dentate gyrus, increased hippocampal excitatory activity and suppressed theta rhythm [5,78,79].

The IL-6 cytokine has also been shown to be involved in memory processes [135]. In like manner to the inconsistencies found in the role of IL-6 in anxiety-related and emotional behaviours in mice, the role of IL-6 in hippocampal-dependent learning has also returned conflicting results with some studies reporting cognitive improvements in IL-6 knockout mice while others suggesting the absence of IL-6 impaired cognition [135-137]. An additional consideration that cannot be ruled out is the possibility of any non-detectable affectations in vision in the GFAP-IL6 mouse that may hamper their ability to learn using distal cues in the BM.

While the indications of cognitive dysfunction in this transgenic mouse are in line with previously published studies, and the links between cognitive impairment, neuroinflammation and IL-6 have been reported in both animal models and in humans [138,139], these results must be evaluated considering

the alterations in anxiety-like behaviours and locomotor activity of the GFAP-IL6 found in the EPM and OF. For example, if the locomotor function in this mouse is impaired, assessing cognition with activities that rely on the ability to move, such as the BM can be confounded. Similarly, the baseline differences in anxiety-related behaviours in this model will undoubtedly influence performance in cognitive tests that use anxiety as a motivation for learning, also a feature of the BM[140]. These factors may shadow whether true alterations in spatial navigation are present in this model and need to be considered for any future testing to minimise confounding.

Unexpectedly, apigenin treatment was associated with a poorer performance in learning and memory during the acquisition phase of the BM. Apigenin fed animals exhibited higher latencies in the total time taken to escape and an increased primary path length, with WT apigenin diet males exhibiting the most pronounced effects. Examination of the percent success rate revealed a decrease in this parameter for apigenin fed animals, again suggesting decreased cognitive ability. A genotype by diet interaction for these three parameters showed the cognitive-impairing effect of apigenin was most prominent among WT apigenin diet animals. The effects found for apigenin in the acquisition phase are not consistent with the improving effects of apigenin on learning and memory reported in other studies [99,100,102,129,141,142]. Specific sex differences were observed in the BM acquisition phase showing females obtained a higher success rate and increased mean speed compared to males. The higher mean speed among females is in keeping with other studies reporting similar findings[143].

Surprisingly, there was a complete reversal of the acquisition phase findings for apigenin, displaying cognition enhancing properties during the probe trial. Apigenin treated animals exhibited higher perseveration behaviour evidenced by an increased number of escape hole entries compared to control diet mice and indicating improved memory of the escape hole location. Additionally apigenin appeared to increase the preference for the target quadrant in the percentage of time spent in the target zone, apigenin being associated with significantly increased exploration of the target quadrant against chance. Apigenin's cognition improving properties appear most evident in the comparison of the hole entry distribution during the probe trial between apigenin and control fed GFAP-IL6 mice. In this

opportunity apigenin clearly improved the hole entry distribution profile toward greater preference of the escape hole.

In contrast to the acquisition phase, apigenin's effects during the probe trial are in line with the previously mentioned studies demonstrating cognitive improvement. These effects are attributed to apigenin's multiple modes of action some of which include suppressing oxidative stress and nitric oxide synthase pathway, suppressing amyloidogenic process, restoration of ERK/CREB/BDNF pathway and modification of BNDF, TrkB, and phospho-CREB levels, neurovascular coupling protection, improvement of the cholinergic system, and stimulation of adult neurogenesis[99,100,102,129,141,142].

The poorer performance of the apigenin fed mice in the acquisition phase may be explained by the anxiolytic-like and sedative-like effects of apigenin. Anxiety is a core motivator to learn the location of the escape hole in the BM [140]. It stands to reason that anti-anxiety agents would weaken this learning drive. Similarly, sedative drugs would also affect learning. However, it may be that the possible anti-anxiety effect of apigenin produced a stronger consolidated memory of what they learned in the acquisition phase, and despite the poorer results attained in the acquisition phase, an improved memory recall was demonstrated in the probe trial. These findings also indicate apigenin may be acting differentially in short-term vs long-term memory. In considering apigenin as a potential cognitive enhancer it becomes essential to factor apigenin's anxiolytic-like and sedative-like activities, which are dependent on dose. As previously mentioned, the dose utilised in this study may be in the range sufficient to produce sedative-like effects that may impact on cognitive performance.

One potential criticism of the BM is the degree to which the persistence to re-visit the learned location of the escape hole during the probe trial is a measure of improved memory retrieval or a lack of cognitive flexibility. Reversal learning of the escape hole in the BM would perhaps further elucidate apigenin's effects on cognition.

.

There does not appear to be any evidence of the increased body weight among apigenin fed animals affecting performance in the BM task in the way of mobility issues, such as decreasing the total distance travelled or mean speed. In fact the opposite effect is found, with apigenin treatment being associated with a greater distance travelled in the primary path length parameter of the acquisition phase.

These results add to the body of current research surrounding apigenin's anxiolytic-like, sedative-like and cognition enhancing effects and suggests appropriate doses for humans may help to achieve similar outcomes.

4.4 Study limitations and future work

There are inherent limitations in working with experimental animal models in that they re-create one particular aspect of a disease rather than the complexity of the whole disease. Although the GFAP-IL6 transgenic mouse has been valuable to study the biological activities of the IL-6 cytokine in the CNS, its neuropathology is not representative of a natural disease state found in either mice or humans. These findings must be then considered in the context of these limitations, viewing apigenin's neuroprotective potential within the low translational capacity of murine models to human conditions and disease[144].

Another significant limitation was the impact that the locomotor deficits and altered anxiety-like behaviour found in the GFAP-IL6 mice had on the BM cognitive task, that relies on anxiogenic stimulus as motivation to locate the escape hole and mobility to navigate the maze. Considering that the locomotor impairments in the GFAP-IL6 mice were not detected in the OF test setting, a task such as the novel object recognition test[145] that is conducted within a similar environment may be better suited to assess aspects of learning and memory for this model and may reduce confounding. Another consideration is the radial arm maze[146], that utilises food as the task acquisition motivation rather than anxiety and consists of enclosed, non-elevated corridors as its environment to navigate. However,

further profiling of the GFAP-IL6 anxiety-related behaviours is recommended in order to confirm and establish the anxiety-like phenotype and accordingly factor this in into any future cognitive testing.

Although histological investigations were undertaken as part of the same research[130], these were not officially part of this study and can only be referred to as a supplementary information. Experimentation with different doses would also strengthen this study, as would investigation of the concentrations of apigenin in the CNS for confirmation and comparison of the flavanoid's ability and level of BBB crossing.

Given apigenin is found ubiquitously in various fruits, herbs and vegetables, and the low toxicity of apigenin at the doses found in food naturally, the future of this work lies in human studies.

4.5 Summary and conclusion

This thesis set out to investigate the effects of chronic, IL-6-driven, low-level neuroinflammation on cognition and behaviour in the GFAP-IL6 transgenic mouse. Alongside the behavioural characterisation of the GFAP-IL6 transgenic strain (including any sex differences), the potential of the dietary bioflavanoid apigenin as a neuroprotective agent was also evaluated, to determine whether any alterations in behaviour and cognition in the GFAP-IL6 mouse could be ameliorated. To achieve this, GFAP-IL6 and WT male and female mice were fed either control or apigenin-enriched pellets for 3 months and were assessed at 6 months of age in the commonly used behavioural phenotyping assays of the EPM, OF and BM, in addition to the FOB. To the researcher's best knowledge, this is the first study that profiled the behavioural parameters of the GFAP-IL6 mouse utilising the EPM, OF and BM.

This study has found that at the age of 6 months, chronic neuroinflammation mediated by the over-expression of the pro-inflammatory cytokine IL-6 in the GFAP-IL6 transgenic mouse manifests cognitive, behavioural and motor alterations. Functional observational assessments detected abnormalities in gait, kyphosis and hindlimb clasping, as well as spontaneous seizures that confirmed the ataxic phenotype previously identified in the GFAP-IL6 line and appears to be linked to the

cerebellar neurodegeneration found in this mouse model[2]. Genotype effects were also uncovered in measures of locomotion in the EPM and BM, further establishing the presence of motor deficits in this mouse line. The absence of any evidence of motor impairments in the OF test seem to suggest that motor impairments in the GFAP-IL6 mice may be test-specific and more likely to be detected in arenas with challenging environments (such as EPM and BM), requiring higher level motor function. Analysis of the OF parameters revealed that the GFAP-IL6 exhibited a strong tendency toward an anxiolytic-like phenotype, the GFAP-IL6 genotype being associated with an increased percentage of time in the centre zone compared to WT. These findings are contrary to the expected sickness behaviour and increases in anxiety-like behaviours found in other models of neuroinflammation[116-119], with these unexpected results being attributed to specific actions of IL-6 overexpression that produce an atypical form of neuroinflammation[3].

With the exception of increased total latencies among male GFAP-IL6 control mice, there were no detectable deficits in cognition for the GFAP-IL6 strain in the acquisition phase of the BM. However, in agreement with previous findings reporting cognitive impairments in this mouse line[5], the probe trial of the BM showed clear evidence of cognitive dysfunction in their learning and recall of the location of the escape hole, making significantly fewer visits to the escape hole and failing to show preference for the target quadrant.

Investigation of the effects of apigenin on cognition and behaviour in this study suggest several properties for this anti-inflammatory flavonoid, including anxiolytic-like and sedative-like effects, cognitive enhancing effects, as well as increasing food consumption and associated weight gain. Inconsistent with previous studies of apigenin's effects on weight[109], mice on the apigenin diet consumed significantly more food and put on more weight in the first 2 months of the experiment, appearing to act as an appetite stimulant. In the EPM and OF agipenin showed sedative-like properties in its depressive effect on locomotion, reducing the distance travelled in the open arms, decreasing the percentage of distance travelled in the centre zone and decreasing the frequency of head dips. At the same time however, apigenin produced a trend toward lowering the frequency of stretched attend posture, indicating an anxiolytic-like activity. This study suggests that apigenin may be acting in both

a sedative-like and anxiolytic-like manner and appears to be related to the dose used in these experiments that borders between anxiolytic and sedative activity[84]. In the BM task apigenin was associated with poorer cognitive performance during the acquisition phase, evidenced by main effects for the apigenin diet producing higher primary path lengths (more pronounced among WT mice and among WT males) and lower percent success rates (especially among WT mice). However, apigenin was found to have the opposite effect in the probe trial as a cognitive enhancer, in keeping with apigenin's previously reported effects[99,100,102,129,141,142]. Apigenin significantly improved the memory recall of the escape hole location in the probe trial, showing a main effect of the apigenin diet in increasing the number of visits to the escape hole location, was associated with higher preference for the target quadrant and appeared to comparatively improve the hole entry distribution profile among GFAP-IL6 mice.

Sex differences were additionally uncovered in the OF and EPM with females exhibiting higher stretched attend posture frequency and displaying increased anxiolytic-like behaviour in their greater percentage of time and distance in the centre zone. In the BM acquisition phase females attained higher percent success rates and reached a greater mean speed compared than males. These sex-based differences align with other studies reporting sex differences in behavioural measures[132,143].

In conclusion, this thesis validated the hypothesis that inflammatory and degenerative neuropathology in the GFAP-IL6 mouse line produces disturbances on parameters of behavioural and cognitive assessments. However, while the GFAP-IL6 mouse model may have potential in elucidating neuroinflammatory and neurodegenerative processes and testing drug effects at a histological level, the current study found this model is less amenable to evaluate drug effects at the behavioural level. The evaluation of memory and learning is particularly problematic due to the model's impairments in motor function and altered anxiety-related behaviour that can confound findings.

Three-month treatment with apigenin appeared to improve memory retention in the BM probe trial and demonstrated a potential role for apigenin as a neuroprotective agent warranting further research, particularly at lower doses that are not approaching the sedative range. The findings of this research confirm apigenin's known activity as a sedative-like and anxiolytic-like agent in the OF and EPM,

which is dose-dependent. Apigenin may additionally have properties as a natural appetite stimulant and should similarly be investigated. These findings may offer new therapeutic avenues for the treatment of neuroinflammation in neurological and affective disorders.

References

1. World Health Organization. Atlas: Country Resources for Neurological Disorders. Geneva2004.
2. Campbell IL, Abraham CR, Masliah E, et al. Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin 6. Proc Natl Acad Sci U S A 1993;90:10061-5.
3. Campbell IL, Hofer MJ, Pagenstecher A. Transgenic models for cytokine-induced neurological disease. Biochim Biophys Acta 2010;1802:903-17.
4. Spooren A, Kolmus K, Laureys G, et al. Interleukin-6, a mental cytokine. Brain Res Rev 2011;67:157-83.
5. Heyser CJ, Masliah E, Samimi A, Campbell IL, Gold LH. Progressive decline in avoidance learning paralleled by inflammatory neurodegeneration in transgenic mice expressing interleukin 6 in the brain. Proc Natl Acad Sci U S A 1997;94:1500-5.
6. Powell HC CI. Ultrastructural abnormalities of glia and the blood brain barrier in a transgenic mouse overexpressing interleukin-6. Brain Pathol 1994;4:278.
7. Spencer JP, Vafeiadou K, Williams RJ, Vauzour D. Neuroinflammation: modulation by flavonoids and mechanisms of action. Mol Aspects Med 2012;33:83-97.
8. Vauzour D, Vafeiadou K, Rodriguez-Mateos A, Rendeiro C, Spencer JP. The neuroprotective potential of flavonoids: a multiplicity of effects. Genes Nutr 2008;3:115-26.
9. Bhullar KS, Rupasinghe HPV. Polyphenols: Multipotent Therapeutic Agents in Neurodegenerative Diseases. Oxidative Medicine and Cellular Longevity 2013;2013:891748.
10. Frank-Cannon TC, Alto LT, McAlpine FE, Tansey MG. Does neuroinflammation fan the flame in neurodegenerative diseases? Mol Neurodegener 2009;4:47.
11. Tansey MG, McCoy MK, Frank-Cannon TC. Neuroinflammatory mechanisms in Parkinson's disease: potential environmental triggers, pathways, and targets for early therapeutic intervention. Exp Neurol 2007;208:1-25.
12. Gonzalez H, Elgueta D, Montoya A, Pacheco R. Neuroimmune regulation of microglial activity involved in neuroinflammation and neurodegenerative diseases. Journal of neuroimmunology 2014;274:1-13.
13. Wyss-Coray T, Mucke L. Inflammation in neurodegenerative disease--a double-edged sword. Neuron 2002;35:419-32.
14. Jang S, Johnson RW. Can consuming flavonoids restore old microglia to their youthful state? Nutrition reviews 2010;68:719-28.
15. Najjar S, Pearlman DM, Alper K, Najjar A, Devinsky O. Neuroinflammation and psychiatric illness. J Neuroinflammation 2013;10:43.
16. Schwartz M, Baruch K. The resolution of neuroinflammation in neurodegeneration: leukocyte recruitment via the choroid plexus. The EMBO journal 2014;33:7-22.
17. Rivest S. Regulation of innate immune responses in the brain. Nat Rev Immunol 2009;9:429-39.
18. Block ML, Hong JS. Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism. Prog Neurobiol 2005;76:77-98.
19. Amor S, Puentes F, Baker D, van der Valk P. Inflammation in neurodegenerative diseases. Immunology 2010;129:154-69.
20. Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. Trends Neurosci 1996;19:312-8.
21. Lawson LJ, Perry VH, Gordon S. Turnover of resident microglia in the normal adult mouse brain. Neuroscience 1992;48:405-15.
22. Choi DK, Koppula S, Suk K. Inhibitors of microglial neurotoxicity: focus on natural products. Molecules (Basel, Switzerland) 2011;16:1021-43.
23. Garden GA, La Spada AR. Intercellular (mis)communication in neurodegenerative disease. Neuron 2012;73:886-901.

24. Cunningham C. Microglia and neurodegeneration: the role of systemic inflammation. Glia 2013;61:71-90.
25. Li C, Zhao R, Gao K, et al. Astrocytes: implications for neuroinflammatory pathogenesis of Alzheimer's disease. Curr Alzheimer Res 2011;8:67-80.
26. Moynagh PN. The interleukin-1 signalling pathway in astrocytes: a key contributor to inflammation in the brain. J Anat 2005;207:265-9.
27. Pritchard C, Mayers A, Baldwin D. Changing patterns of neurological mortality in the 10 major developed countries--1979-2010. Public health 2013;127:357-68.
28. Hurley LL, Tizabi Y. Neuroinflammation, neurodegeneration, and depression. Neurotox Res 2013;23:131-44.
29. Systemic Inflammation: A Driver of Neurodegenerative Disease? 2015. (Accessed 5th May, 2015, at http://www.alzforum.org/news/conference-coverage/systemic-inflammation-driver-neurodegenerative-disease.)
30. Maes M, Yirmyia R, Noraberg J, et al. The inflammatory & neurodegenerative (I&ND) hypothesis of depression: leads for future research and new drug developments in depression. Metab Brain Dis 2009;24:27-53.
31. Cai D. Neuroinflammation and neurodegeneration in overnutrition-induced diseases. Trends Endocrinol Metab 2013;24:40-7.
32. McGeer EG, McGeer PL. Inflammatory processes in Alzheimer's disease. Progress in neuro-psychopharmacology & biological psychiatry 2003;27:741-9.
33. McGeer PL, Itagaki S, Tago H, McGeer EG. Reactive microglia in patients with senile dementia of the Alzheimer type are positive for the histocompatibility glycoprotein HLA-DR. Neuroscience letters 1987;79:195-200.
34. Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH. Mechanisms Underlying Inflammation in Neurodegeneration. Cell 2010;140:918-34.
35. Heneka MT, Carson MJ, El Khoury J, et al. Neuroinflammation in Alzheimer's disease. The Lancet Neurology 2015;14:388-405.
36. Swardfager W, Lanctot K, Rothenburg L, Wong A, Cappell J, Herrmann N. A meta-analysis of cytokines in Alzheimer's disease. Biological psychiatry 2010;68:930-41.
37. Tarkowski E, Andreasen N, Tarkowski A, Blennow K. Intrathecal inflammation precedes development of Alzheimer's disease. Journal of neurology, neurosurgery, and psychiatry 2003;74:1200-5.
38. Delanty N, Vaughan C. Risk of Alzheimer's disease and duration of NSAID use. Neurology 1998;51:652.
39. Vlad SC, Miller DR, Kowall NW, Felson DT. Protective effects of NSAIDs on the development of Alzheimer disease. Neurology 2008;70:1672-7.
40. Guerreiro R, Wojtas A, Bras J, et al. TREM2 variants in Alzheimer's disease. The New England journal of medicine 2013;368:117-27.
41. Griciuc A, Serrano-Pozo A, Parrado AR, et al. Alzheimer's disease risk gene CD33 inhibits microglial uptake of amyloid beta. Neuron 2013;78:631-43.
42. Lee JW, Lee YK, Yuk DY, et al. Neuro-inflammation induced by lipopolysaccharide causes cognitive impairment through enhancement of beta-amyloid generation. Journal of neuroinflammation 2008;5:37.
43. Dowlati Y, Herrmann N, Swardfager W, et al. A meta-analysis of cytokines in major depression. Biol Psychiatry 2010;67:446-57.
44. McNamara RK, Lotrich FE. Elevated immune-inflammatory signaling in mood disorders: a new therapeutic target? Expert Rev Neurother 2012;12:1143-61.
45. Howren MB, Lamkin DM, Suls J. Associations of depression with C-reactive protein, IL-1, and IL-6: a meta-analysis. Psychosom Med 2009;71:171-86.
46. Ohishi K, Ueno R, Nishino S, Sakai T, Hayaishi O. Increased level of salivary prostaglandins in patients with major depression. Biol Psychiatry 1988;23:326-34.

47. Liu Y, Ho RC, Mak A. Interleukin (IL)-6, tumour necrosis factor alpha (TNF-alpha) and soluble interleukin-2 receptors (sIL-2R) are elevated in patients with major depressive disorder: a meta-analysis and meta-regression. J Affect Disord 2012;139:230-9.
48. Johnson RW. From Inflammation to Sickness and Cognitive Dysfunction: When the Immune System Subjugates the Brain 114th Abbott Nutrition Research Conference Cognition and Nutrition2013.
49. Erta M, Quintana A, Hidalgo J. Interleukin-6, a Major Cytokine in the Central Nervous System. International Journal of Biological Sciences 2012;8:1254-66.
50. Baune BT, Konrad C, Grotegerd D, et al. Interleukin-6 gene (IL-6): a possible role in brain morphology in the healthy adult brain. Journal of neuroinflammation 2012;9:125.
51. Gruol DL. IL-6 Regulation of Synaptic Function in the CNS. Neuropharmacology 2014.
52. Trapero I, Cauli O. Interleukin 6 and cognitive dysfunction. Metabolic brain disease 2014;29:593-608.
53. Ershler WB, Keller ET. Age-associated increased interleukin-6 gene expression, late-life diseases, and frailty. Annual review of medicine 2000;51:245-70.
54. Quintanilla RA, Orellana DI, Gonzalez-Billault C, Maccioni RB. Interleukin-6 induces Alzheimer-type phosphorylation of tau protein by deregulating the cdk5/p35 pathway. Exp Cell Res 2004;295:245-57.
55. Altstiel LD, Sperber K. Cytokines in Alzheimer's disease. Progress in neuro-psychopharmacology & biological psychiatry 1991;15:481-95.
56. Ringheim GE, Szczepanik AM, Petko W, Burgher KL, Zhu SZ, Chao CC. Enhancement of beta-amyloid precursor protein transcription and expression by the soluble interleukin-6 receptor/interleukin-6 complex. Brain research Molecular brain research 1998;55:35-44.
57. Tehranian R, Hasanvan H, Iverfeldt K, Post C, Schultzberg M. Early induction of interleukin-6 mRNA in the hippocampus and cortex of APPsw transgenic mice Tg2576. Neuroscience letters 2001;301:54-8.
58. Huell M, Strauss S, Volk B, Berger M, Bauer J. Interleukin-6 is present in early stages of plaque formation and is restricted to the brains of Alzheimer's disease patients. Acta neuropathologica 1995;89:544-51.
59. Hull M, Berger M, Volk B, Bauer J. Occurrence of interleukin-6 in cortical plaques of Alzheimer's disease patients may precede transformation of diffuse into neuritic plaques. Annals of the New York Academy of Sciences 1996;777:205-12.
60. Blum-Degen D, Muller T, Kuhn W, Gerlach M, Przuntek H, Riederer P. Interleukin-1 beta and interleukin-6 are elevated in the cerebrospinal fluid of Alzheimer's and de novo Parkinson's disease patients. Neuroscience letters 1995;202:17-20.
61. Martinez M, Fernandez-Vivancos E, Frank A, De la Fuente M, Hernanz A. Increased cerebrospinal fluid fas (Apo-1) levels in Alzheimer's disease. Relationship with IL-6 concentrations. Brain research 2000;869:216-9.
62. Jellinger KA. Intrathecal levels of IL-6 in Alzheimer's disease. Journal of neurology 2010;257:142.
63. Singh-Manoux A, Dugravot A, Brunner E, et al. Interleukin-6 and C-reactive protein as predictors of cognitive decline in late midlife. Neurology 2014;83:486-93.
64. He MX, Yang WL, Zhang MM, et al. Association between interleukin-6 gene promoter -572C/G polymorphism and the risk of sporadic Alzheimer's disease. Neurological sciences : official journal of the Italian Neurological Society and of the Italian Society of Clinical Neurophysiology 2010;31:165-8.
65. Capurso C, Solfrizzi V, D'Introno A, et al. Interleukin 6-174 G/C promoter gene polymorphism and sporadic Alzheimer's disease: geographic allele and genotype variations in Europe. Experimental gerontology 2004;39:1567-73.
66. Sukoff Rizzo SJ, Neal SJ, Hughes ZA, et al. Evidence for sustained elevation of IL-6 in the CNS as a key contributor of depressive-like phenotypes. Transl Psychiatry 2012;2:e199.

67. Lindqvist D, Janelidze S, Hagell P, et al. Interleukin-6 is elevated in the cerebrospinal fluid of suicide attempters and related to symptom severity. Biological psychiatry 2009;66:287-92.
68. Janelidze S, Mattei D, Westrin A, Traskman-Bendz L, Brundin L. Cytokine levels in the blood may distinguish suicide attempters from depressed patients. Brain, behavior, and immunity 2011;25:335-9.
69. O'Brien SM, Scully P, Fitzgerald P, Scott LV, Dinan TG. Plasma cytokine profiles in depressed patients who fail to respond to selective serotonin reuptake inhibitor therapy. Journal of psychiatric research 2007;41:326-31.
70. Yoshimura R, Hori H, Ikenouchi-Sugita A, Umene-Nakano W, Ueda N, Nakamura J. Higher plasma interleukin-6 (IL-6) level is associated with SSRI- or SNRI-refractory depression. Progress in neuro-psychopharmacology & biological psychiatry 2009;33:722-6.
71. Voorhees JL, Tarr AJ, Wohleb ES, et al. Prolonged Restraint Stress Increases IL-6, Reduces IL-10, and Causes Persistent Depressive-Like Behavior That Is Reversed by Recombinant IL-10. PLoS ONE 2013;8:e58488.
72. Chourbaji S, Urani A, Inta I, et al. IL-6 knockout mice exhibit resistance to stress-induced development of depression-like behaviors. Neurobiology of disease 2006;23:587-94.
73. Gruol DL, Nelson TE. Physiological and pathological roles of interleukin-6 in the central nervous system. Mol Neurobiol 1997;15:307-39.
74. Campbell IL. Structural and functional impact of the transgenic expression of cytokines in the CNS. Ann N Y Acad Sci 1998;840:83-96.
75. Vallieres L, Campbell IL, Gage FH, Sawchenko PE. Reduced hippocampal neurogenesis in adult transgenic mice with chronic astrocytic production of interleukin-6. The Journal of neuroscience : the official journal of the Society for Neuroscience 2002;22:486-92.
76. Campbell IL, Stalder AK, Chiang CS, et al. Transgenic models to assess the pathogenic actions of cytokines in the central nervous system. Mol Psychiatry 1997;2:125-9.
77. Chiang CS, Stalder A, Samimi A, Campbell IL. Reactive gliosis as a consequence of interleukin-6 expression in the brain: studies in transgenic mice. Developmental neuroscience 1994;16:212-21.
78. Steffensen SC, Campbell IL, Henriksen SJ. Site-specific hippocampal pathophysiology due to cerebral overexpression of interleukin-6 in transgenic mice. Brain research 1994;652:149-53.
79. Bellinger FP, Madamba SG, Campbell IL, Siggins GR. Reduced long-term potentiation in the dentate gyrus of transgenic mice with cerebral overexpression of interleukin-6. Neurosci Lett 1995;198:95-8.
80. Du X, Pang TY. Is Dysregulation of the HPA-Axis a Core Pathophysiology Mediating Co-Morbid Depression in Neurodegenerative Diseases? Front Psychiatry 2015;6:32.
81. Raber J, O'Shea RD, Bloom FE, Campbell IL. Modulation of hypothalamic-pituitary-adrenal function by transgenic expression of interleukin-6 in the CNS of mice. The Journal of neuroscience : the official journal of the Society for Neuroscience 1997;17:9473-80.
82. Shukla S, Gupta S. Apigenin: a promising molecule for cancer prevention. Pharm Res 2010;27:962-78.
83. Patel D, Shukla S, Gupta S. Apigenin and cancer chemoprevention: progress, potential and promise (review). Int J Oncol 2007;30:233-45.
84. Viola H, Wasowski C, Levi de Stein M, et al. Apigenin, a component of Matricaria recutita flowers, is a central benzodiazepine receptors-ligand with anxiolytic effects. Planta medica 1995;61:213-6.
85. Ross JA, Kasum CM. Dietary flavonoids: bioavailability, metabolic effects, and safety. Annual review of nutrition 2002;22:19-34.
86. Nielsen SE, Young JF, Daneshvar B, et al. Effect of parsley (Petroselinum crispum) intake on urinary apigenin excretion, blood antioxidant enzymes and biomarkers for oxidative stress in human subjects. Br J Nutr 1999;81:447-55.

87. Popovic M, Caballero-Bleda M, Benavente-Garcia O, Castillo J. The flavonoid apigenin delays forgetting of passive avoidance conditioning in rats. Journal of psychopharmacology (Oxford, England) 2014;28:498-501.
88. Yang Y, Bai L, Li X, et al. Transport of active flavonoids, based on cytotoxicity and lipophilicity: an evaluation using the blood-brain barrier cell and Caco-2 cell models. Toxicology in vitro : an international journal published in association with BIBRA 2014;28:388-96.
89. Ha SK, Lee P, Park JA, et al. Apigenin inhibits the production of NO and PGE2 in microglia and inhibits neuronal cell death in a middle cerebral artery occlusion-induced focal ischemia mice model. Neurochemistry international 2008;52:878-86.
90. Rezai-Zadeh K, Ehrhart J, Bai Y, et al. Apigenin and luteolin modulate microglial activation via inhibition of STAT1-induced CD40 expression. Journal of Neuroinflammation 2008;5.
91. Elsisi NS, Darling-Reed S, Lee EY, Oriaku ET, Soliman KF. Ibuprofen and apigenin induce apoptosis and cell cycle arrest in activated microglia. Neuroscience letters 2005;375:91-6.
92. Guo H, Kong S, Chen W, et al. Apigenin mediated protection of OGD-evoked neuron-like injury in differentiated PC12 cells. Neurochemical research 2014;39:2197-210.
93. Zhao L, Wang JL, Wang YR, Fa XZ. Apigenin attenuates copper-mediated beta-amyloid neurotoxicity through antioxidation, mitochondrion protection and MAPK signal inactivation in an AD cell model. Brain research 2013;1492:33-45.
94. Zhao L, Hou L, Sun H, et al. Apigenin isolated from the medicinal plant Elsholtzia rugulosa prevents β-amyloid 25-35-induces toxicity in rat cerebral microvascular endothelial cells. Molecules (Basel, Switzerland) 2011;16:4005-19.
95. Gauci AJ, Caruana M, Giese A, Scerri C, Vassallo N. Identification of polyphenolic compounds and black tea extract as potent inhibitors of lipid membrane destabilization by Abeta(4)(2) aggregates. Journal of Alzheimer's disease : JAD 2011;27:767-79.
96. Choi AY, Choi JH, Lee JY, et al. Apigenin protects HT22 murine hippocampal neuronal cells against endoplasmic reticulum stress-induced apoptosis. Neurochemistry international 2010;57:143-52.
97. Braidy N, Grant R, Adams S, Guillemin GJ. Neuroprotective effects of naturally occurring polyphenols on quinolinic acid-induced excitotoxicity in human neurons. The FEBS journal 2010;277:368-82.
98. Losi G, Puia G, Garzon G, de Vuono MC, Baraldi M. Apigenin modulates GABAergic and glutamatergic transmission in cultured cortical neurons. European journal of pharmacology 2004;502:41-6.
99. Zhao L, Wang JL, Liu R, Li XX, Li JF, Zhang L. Neuroprotective, anti-amyloidogenic and neurotrophic effects of apigenin in an Alzheimer's disease mouse model. Molecules (Basel, Switzerland) 2013;18:9949-65.
100. Liu R, Zhang T, Yang H, Lan X, Ying J, Du G. The flavonoid apigenin protects brain neurovascular coupling against amyloid-beta(2)(5)(-)(3)(5)-induced toxicity in mice. Journal of Alzheimer's disease : JAD 2011;24:85-100.
101. Zhang F, Li F, Chen G. Neuroprotective effect of apigenin in rats after contusive spinal cord injury. Neurol Sci 2013.
102. Taupin P. Apigenin and related compounds stimulate adult neurogenesis. Mars, Inc., the Salk Institute for Biological Studies: WO2008147483. Expert opinion on therapeutic patents 2009;19:523-7.
103. Nakazawa T, Yasuda T, Ueda J, Ohsawa K. Antidepressant-like effects of apigenin and 2,4,5-trimethoxycinnamic acid from Perilla frutescens in the forced swimming test. Biological & pharmaceutical bulletin 2003;26:474-80.
104. Yi LT, Li JM, Li YC, Pan Y, Xu Q, Kong LD. Antidepressant-like behavioral and neurochemical effects of the citrus-associated chemical apigenin. Life sciences 2008;82:741-51.
105. Li R, Zhao D, Qu R, Fu Q, Ma S. The effects of apigenin on lipopolysaccharide-induced depressive-like behavior in mice. Neuroscience letters 2015;594:17-22.

106. Han JY, Ahn SY, Kim CS, et al. Protection of apigenin against kainate-induced excitotoxicity by anti-oxidative effects. Biological & pharmaceutical bulletin 2012;35:1440-6.
107. Patil SP, Jain PD, Sancheti JS, Ghumatkar PJ, Tambe R, Sathaye S. Neuroprotective and neurotrophic effects of Apigenin and Luteolin in MPTP induced parkinsonism in mice. Neuropharmacology 2014;86:192-202.
108. Fridgeirsdottir GA, Hillered L, Clausen F. Escalated handling of young C57BL/6 mice results in altered Morris water maze performance. Ups J Med Sci 2014;119:1-9.
109. Myoung H-J, Kim G, Nam K-W. Apigenin isolated from the seeds of Perilla frutescens britton var crispa (Benth.) inhibits food intake in C57BL/6J mice. Archives of Pharmacal Research 2010;33:1741-6.
110. Butterweck V, Prinz S, Schwaninger M. The role of interleukin-6 in stress-induced hyperthermia and emotional behaviour in mice. Behavioural brain research 2003;144:49-56.
111. Armario A, Hernandez J, Bluethmann H, Hidalgo J. IL-6 deficiency leads to increased emotionality in mice: evidence in transgenic mice carrying a null mutation for IL-6. Journal of neuroimmunology 1998;92:160-9.
112. Erta M, Giralt M, Esposito FL, Fernandez-Gayol O, Hidalgo J. Astrocytic IL-6 mediates locomotor activity, exploration, anxiety, learning and social behavior. Hormones and behavior 2015;73:64-74.
113. Quintana A, Erta M, Ferrer B, Comes G, Giralt M, Hidalgo J. Astrocyte-specific deficiency of interleukin-6 and its receptor reveal specific roles in survival, body weight and behavior. Brain, behavior, and immunity 2013;27:162-73.
114. Vallieres L, Rivest S. Interleukin-6 is a needed proinflammatory cytokine in the prolonged neural activity and transcriptional activation of corticotropin-releasing factor during endotoxemia. Endocrinology 1999;140:3890-903.
115. Wang J, Dunn AJ. Mouse interleukin-6 stimulates the HPA axis and increases brain tryptophan and serotonin metabolism. Neurochemistry international 1998;33:143-54.
116. Broussard JI, Acion L, De Jesus-Cortes H, et al. Repeated mild traumatic brain injury produces neuroinflammation, anxiety-like behaviour and impaired spatial memory in mice. Brain injury 2018;32:113-22.
117. Sulakhiya K, Keshavlal GP, Bezbaruah BB, et al. Lipopolysaccharide induced anxiety- and depressive-like behaviour in mice are prevented by chronic pre-treatment of esculetin. Neuroscience letters 2016;611:106-11.
118. Jangra A, Lukhi MM, Sulakhiya K, Baruah CC, Lahkar M. Protective effect of mangiferin against lipopolysaccharide-induced depressive and anxiety-like behaviour in mice. European journal of pharmacology 2014;740:337-45.
119. Swiergiel AH, Dunn AJ. Effects of interleukin-1beta and lipopolysaccharide on behavior of mice in the elevated plus-maze and open field tests. Pharmacology, biochemistry, and behavior 2007;86:651-9.
120. Zanoli P, Avallone R, Baraldi M. Behavioral characterisation of the flavonoids apigenin and chrysin. Fitoterapia 2000;71 Suppl 1:S117-23.
121. Avallone R, Zanoli P, Puia G, Kleinschnitz M, Schreier P, Baraldi M. Pharmacological profile of apigenin, a flavonoid isolated from Matricaria chamomilla. Biochemical pharmacology 2000;59:1387-94.
122. Kumar D, Bhat ZA. Apigenin 7-glucoside from Stachys tibetica Vatke and its anxiolytic effect in rats. Phytomedicine : international journal of phytotherapy and phytopharmacology 2014;21:1010-4.
123. Grewal SS, Shepherd JK, Bill DJ, Fletcher A, Dourish CT. Behavioural and pharmacological characterisation of the canopy stretched attend posture test as a model of anxiety in mice and rats. Psychopharmacology 1997;133:29-38.

124. Gazola AC, Costa GM, Castellanos L, et al. Involvement of GABAergic pathway in the sedative activity of apigenin, the main flavonoid from Passiflora quadrangularis pericarp. Revista Brasileira de Farmacognosia 2015;25:158-63.
125. Ha SK, Lee P, Park JA, et al. Apigenin inhibits the production of NO and PGE2 in microglia and inhibits neuronal cell death in a middle cerebral artery occlusion-induced focal ischemia mice model. Neurochemistry international 2008;52:878-86.
126. Rezai-Zadeh K, Ehrhart J, Bai Y, et al. Apigenin and luteolin modulate microglial activation via inhibition of STAT1-induced CD40 expression. Journal of Neuroinflammation 2008;5:41-.
127. Zhang X, Wang G, Gurley EC, Zhou H. Flavonoid apigenin inhibits lipopolysaccharide-induced inflammatory response through multiple mechanisms in macrophages. PloS one 2014;9:e107072.
128. Weng L, Guo X, Li Y, Yang X, Han Y. Apigenin reverses depression-like behavior induced by chronic corticosterone treatment in mice. European journal of pharmacology 2016;774:50-4.
129. Chen L, Xie W, Xie W, Zhuang W, Jiang C, Liu N. Apigenin attenuates isoflurane-induced cognitive dysfunction via epigenetic regulation and neuroinflammation in aged rats. Archives of gerontology and geriatrics 2017;73:29-36.
130. Liang H, Sonego S, Gyengesi E, et al. OP-25 - Anti-Inflammatory and Neuroprotective Effect of Apigenin: Studies in the GFAP-IL6 Mouse Model of Chronic Neuroinflammation. Free Radical Biology and Medicine 2017;108:S10.
131. Gelineau RR, Arruda NL, Hicks JA, Monteiro De Pina I, Hatzidis A, Seggio JA. The behavioral and physiological effects of high-fat diet and alcohol consumption: Sex differences in C57BL6/J mice. Brain and behavior 2017;7:e00708-e.
132. Podhorna J, Brown RE. Strain differences in activity and emotionality do not account for differences in learning and memory performance between C57BL/6 and DBA/2 mice. Genes, brain, and behavior 2002;1:96-110.
133. Sunyer B, Patil S, Höger H, Lubec G. Barnes maze, a useful task to assess spatial reference memory in the mice. 2007.
134. Deacon RM, Rawlins JN. Hippocampal lesions, species-typical behaviours and anxiety in mice. Behavioural brain research 2005;156:241-9.
135. Braida D, Sacerdote P, Panerai AE, et al. Cognitive function in young and adult IL (interleukin)-6 deficient mice. Behavioural brain research 2004;153:423-9.
136. Baier PC, May U, Scheller J, Rose-John S, Schiffelholz T. Impaired hippocampus-dependent and -independent learning in IL-6 deficient mice. Behavioural brain research 2009;200:192-6.
137. Sparkman NL, Buchanan JB, Heyen JR, Chen J, Beverly JL, Johnson RW. Interleukin-6 facilitates lipopolysaccharide-induced disruption in working memory and expression of other proinflammatory cytokines in hippocampal neuronal cell layers. The Journal of neuroscience : the official journal of the Society for Neuroscience 2006;26:10709-16.
138. Trapero I, Cauli O. Interleukin 6 and cognitive dysfunction. Metabolic Brain Disease 2014;29:593-608.
139. Simen AA, Bordner KA, Martin MP, Moy LA, Barry LC. Cognitive Dysfunction with Aging and the Role of Inflammation. Therapeutic Advances in Chronic Disease 2011;2:175-95.
140. Rosenfeld CS, Ferguson SA. Barnes Maze Testing Strategies with Small and Large Rodent Models. Journal of Visualized Experiments : JoVE 2014:51194.
141. Miroljub P, María C-B, Obdulio B-G, Julián C. The flavonoid apigenin delays forgetting of passive avoidance conditioning in rats. Journal of Psychopharmacology 2013;28:498-501.
142. Mao X-Y, Yu J, Liu Z-Q, Zhou H-H. Apigenin attenuates diabetes-associated cognitive decline in rats via suppressing oxidative stress and nitric oxide synthase pathway. International Journal of Clinical and Experimental Medicine 2015;8:15506-13.
143. Fritz A-K, Amrein I, Wolfer DP. Similar reliability and equivalent performance of female and male mice in the open field and water-maze place navigation task. American journal of medical genetics Part C, Seminars in medical genetics 2017;175:380-91.

144. Martić-Kehl MI, Schibli R, Schubiger PA. Can animal data predict human outcome? Problems and pitfalls of translational animal research. European Journal of Nuclear Medicine and Molecular Imaging 2012;39:1492-6.
145. Lueptow LM. Novel Object Recognition Test for the Investigation of Learning and Memory in Mice. Journal of visualized experiments : JoVE 2017:55718.
146. Wenk GL. Assessment of spatial memory using the radial arm maze and Morris water maze. Current protocols in neuroscience 2004;Chapter 8:Unit 8.5A.

WESTERN SYDNEY UNIVERSITY

Subject:	3-Day Escalated Handling
Protocol No:	WSU-ABL_3EH.2015.01
Version:	1.0
Issued:	December 1st, 2015
Last Reviewed:	March 21st, 2016
Authors:	Chris Millington Sandra Sonego
Working group members:	Prof. Gerald Muench (Principal Supervisor) Dr. Erika Gyengesi (Supervisor) Chris Millington (Masters Candidate) Sandra Sonego (Masters Candidate)

1. Objective:

Basic handling of mice is conducted prior to any behavioural or functional testing to help animals acclimatize to being lifted/transferred , the investigator, testing room, or in some cases to the testing equipment itself. This protocol is adapted from the protocol by Fridgeirdottir, Hillered & Clausen, 2014 and Deacon, 2006. It describes a structured method for handling mice that gradually increases the contact with the investigator over 3 days, with the aim of reducing the stress and anxiety caused by handling.

2. Scope and Applicability:

This handling protocol can be applied prior to behavioural or functional testing of mice. In view that the basic handling technique used before an experiment can affect the final outcome of the test, this protocol provides a controlled handling procedure that can be clearly reported and replicated.

3. Safety Requirements:

Individuals must be trained and competent with the procedures, equipment and handling of animals described in this protocol.

General PC2 laboratory safety procedures must befollowed.

4. Materials/Apparatus:

- Quiet space in animal facility or animal laboratory
- Empty homecages
- Stopwatch
- Paper towels
- 70% ethanol solution
- Personal protective equipment – eg. gloves and lab coat

5. Procedure:

Choose a quiet place either in the animal housing facility handling area, animal procedure room or behavioural lab to conduct handling of the mice. Once a place is selected as the handling space this must remain consistent for all animal experimental groups that are to be tested as part of a project.

At all times mice are handled with care and respect, with the aim to minimise stress and anxiety. Ensure that there is appropriate sterilisation of surfaces and gloves in between different cages/animals.

Day 1

1. Remove lid and food grill from homecage and leave open undisturbed for 60 seconds.
2. Place hand in a corner of the cage and leave it there for 60 seconds allowing them to freely sniff the hand. Do this without disturbing the enrichment material.
3. Gently move around the enrichment material.
4. Taking turns one by one pick up each mouse by the base of tail in the homecage for no longer than 3-4 seconds, then place them immediately back down in the homecage. Repeat 3 times for each animal.

Day 2

1. Remove lid and food grill from homecage and leave open undisturbed for 30 seconds.
2. Place hand in a corner of the cage and leave it there for 30 seconds allowing them to freely sniff the hand. Do this without disturbing the enrichment material.
3. Gently move around the enrichment material.
4. Taking turns one by one pick up each mouse by the base of the tail and transfer them from the homecage to an adjacent empty cage allowing them to stay in there for 10 seconds before picking them up again and transferring back to the homecage. Ensure that the mice are held by the tail no longer than 3-4 seconds each time they are picked up. Conduct 5 of these 'homecage-empty tub-homecage' transfers for each mouse.

Day 3

1. Remove lid and food grill from homecage and leave open undisturbed for 15 seconds.
2. Place hand in a corner of the cage and leave it there for 15 seconds allowing them to freely sniff the hand. Do this without disturbing the enrichment material.
3. Remove the enrichment material from the cage and place aside.
4. Taking turns one by one pick up each mouse by the base tail and transfer them from the homecage to an adjacent empty cage allowing them to stay in there for 5 seconds before picking them up, placing the mouse on the arm for 5 seconds before transferring back to the homecage. Ensure that the mice are held by the tail no longer than 3-4 seconds each

time they are picked up. Conduct 7 of these 'homecage-empty tub-arm-homecage' transfers for each mouse.

5. Following handling, transport animals to the testing room and leave to habituate for 1 hour. Ensure to cover animals on the trolley when transporting them to and from the animal facility.

Fig 1. Example of mouse being picked up by base of the tail. Do not grasp the tip of the tail. This method is only used for brief restraint-eg. transferring animals from cage to cage. Never suspend the mouse for prolonged periods of time by its tail.
(Source: http://www.theodora.com/rodent_laboratory/restraint.html)

Supporting Sources of Information:

Fridgeirsdottir, G. A., Hillered, L., & Clausen, F. (2014). Escalated handling of young C57BL/6 mice results in altered Morris water maze performance. Upsala Journal of Medical Sciences, 119(1), 1–9. http://doi.org/10.3109/03009734.2013.847511.

Deacon RM. Housing, husbandry and handling of rodents for behavioral experiments . Nat Protoc. 2006;1:936–46.

WESTERN SYDNEY UNIVERSITY

Subject:	Elevated Plus Maze Test
Protocol No:	WSU-ABL_EPM.2015.01
Version:	1.0
Issued:	July 10th, 2015
Last Reviewed:	March 21st, 2016
Authors:	Sandra Sonego Chris Millington
Working group members:	Prof. Gerald Muench (Principal Supervisor) Dr. Erika Gyengesi (Supervisor) Chris Millington (Masters Candidate Sandra Sonego (Masters Candidate)

1. Objective:

The elevated plus maze (EPM) is one of the most popular tests used to measure anxiety-like behaviour in rodents. The test is based on the natural aversion of mice for open and elevated spaces and their drive to explore novel environments. The maze consists of two open arms and two closed arms intersecting at the centre in the shape of a plus sign. Animals are placed at the intersection of the four arms, and allowed to freely explore the maze. The number of entries and time spent in the open arms is used as an indicator of open space-induced anxiety in mice.

2. Scope and Applicability:

The EPM is widely used to phenotype transgenic strains and detect anxiolytic effects of pharmacological agents. Anxiolytic drugs increase the number of entries into the open arms and the amount of time spent there. The test is also useful as a behavioural assay to study specific brain regions underlying anxiety-like behaviour.

3. Safety Requirements:

Individuals must be trained and competent with the procedures, equipment and handling of animals described in this protocol.

General PC2 laboratory safety procedures must befollowed.

4. Materials/Apparatus:

The Maze:

The EPM is in the configuration of a plus sign, consisting of two open arms across from each other and perpendicular to two closed arms with a center platform. The arms are 5cm wide, and 35cm long, with 15cm walls for the enclosed arms. The arms of the maze are attached to steel legs so that the maze is elevated 40cm from the ground.

Figure 1. The Stoelting elevated plus maze used at WSU Animal Behaviour Laboratory

Room configuration:

The maze is centrally placed in the laboratory floor in a curtained-off section of the room. Typical illumination is 850-950 lux – even lighting. Soft padding material is placed beneath the maze to ensure the animals are not injured if they fall or attempt to escape.

Video-tracking Software:

A digital camera and the ANY-maze video-tracking system is utilised to capture all data. An observer is always present to check whether any tracking errors occur during the trial.

Other:

- Paper towels
- 70% ethanol solution
- Clean empty cages for animal transfer
- Personal protective equipment – eg. gloves and lab coat

5. Procedure:

1. Tests are performed following an initial 3 consecutive days of escalated handling (protocol WSU-ABL_3EH.2015.01) with a 1 hour transportation habituation session of the experimental room conducted on the third day.

2. Animals are transported in their home cages from the animal facility to the behaviour laboratory on one of the animal facility's corridor trolleys, with a sheet covering the cages during the transport to the laboratory.

3. Animals are left on the trolley uncovered in a designated holding area in the laboratory, for 30 minutes to habituate to the laboratory environment.

4. Excess noise, human activity, odours, and visual distractions are also avoided during habituation and testing.

5. The Any-Maze software protocol is programed to capture mouse activity in the EPM for a 5 minute period. Five zones are delineated using the software: 2 open and 2 closed arm zones, and 1 center zone. An entry into an arm of the maze is considered to be when the centre of the mouse has crossed onto an arm. The following are examples of some of the parameters set up in Any-maze for data capture:

    - total distance travelled and distance in each zone
    - number of entries into each arm
    - % number of entries into each arm
    - time spent in each arm
    - time spent in central zone
    - number of nose dips
    - immobilization/freezing time

6. A record sheet with all test animal IDs, cage number, and ear markings is kept with the randomised order of the animal trials pre-determined for efficiency.

7. The open field arena, and all benches where animals are to be handled are wiped down with 70% ethanol.

8. Once the habituation period is over the testing can commence. Each mouse receives one 5 minute trial. Animals are carefully identified by matching their ear marks with their cage number and animal ID. To minimise the cross-over of odours between animals and sex, mice are carried over individually in their clean transfer cage from the holding area to the experimental bench area.

9. The animal is picked up by the base of the tail, and placed at the centre junction of the EPM with its head pointed toward one of the open arms.

10. The Any-maze program is the prompted to commence recording and tracking of the animal's movements for a trial period of 5 minutes. Once the trial is finished, the animal is removed from the maze, placed back in its transfer cage and carried over back to its home cage.

11. After each trial, the apparatus is cleaned with 70% ethanol solution spray to remove any urine or faeces, as well as any benches in use and allowed time to dry. The experimenter's gloves are also sprayed with ethanol and allowed to evaporate before picking up the next test animal.

12. The process is repeated for each test animal.

13. Once all testing is completed for the day, they are returned to the animal facility as per step 2.

Supporting Sources of Information:

Bailey KR, Crawley JN. Anxiety-Related Behaviors in Mice. In: Buccafusco JJ, editor. Methods of Behavior Analysis in Neuroscience. 2nd edition. Boca Raton (FL): CRC Press; 2009. Chapter 5.

Crawley JN, Paylor R (1997) A proposed test battery and constellations of specific behavioral paradigms to investigate the behavioral phenotypes of transgenic and knockout mice. Horm Behav. 31(3):197-211.

Crawley JN. (2007) What's Wrong With My Mouse? Behavioral Phenotyping of Transgenic and Knockout Mice. New York: John Wiley and Sons, Inc.

Gould T.D.(ed.), Mood and Anxiety Related Phenotypes in Mice, Neuromethods 42, DOI 10.1007/978-1- 60761-303-9_1, ª Humana Press, a part of Springer Science+Business Media, LLC 2009

Karl T, Pabst R, von Hörsten (2003) Behavioral phenotyping of mice in pharmacological and toxicological research. Exp Toxicol Pathol. 55(1):69-83.

Komada, M., Takao, K., & Miyakawa, T. (2008). Elevated Plus Maze for Mice. Journal of Visualized Experiments : JoVE, (22), 1088. doi:10.3791/1088.

Walf AA & Frye CA (2007) The use of the elevated plus maze as an assay of anxiety-related behavior in rodents, Nature Protocols 2, - 322 – 328, doi:10.1038/nprot.2007.44.

Nagaraju, K. (2008) Behavioural and locomotor measurements using open field animal activity monitoring system. TREAT-NMD. DMD_M.2.1.002.

WESTERN SYDNEY UNIVERSITY

Subject:	Open Field Test
Protocol No:	WSU-ABL_OF.2015.01
Version:	1.0
Issued:	July 10th, 2015
Last Reviewed:	March 21st, 2016
Authors:	Chris Millington Sandra Sonego
Working group members:	Prof. Gerald Muench (Principal Supervisor) Dr. Erika Gyengesi (Supervisor) Chris Millington (Masters Candidate) Sandra Sonego (Masters Candidate)

1. Objective:

The Open Field test is commonly used to measure anxiety, exploratory drive and locomotor activity. The test utilises an enclosed arena to track and assess the animal's amount of movement and quality of movement. The test is based on the natural tendency of mice to both explore, and avoid open spaces as a protective reaction. Normal animals are expected to spend more time in the periphery along the walls (thigmotaxis) than in the open central area of the arena (anxiogenic area). Common measures of interest include total distance travelled, distance and time spent in central vs peripheral area, freezing and rearing.

2. Scope and Applicability:

The Open Field test provides a broad assessment of both behavioural and motor activities of mice, important in helping to define phenotypes. It is particularly useful in being able to non-invasively detect locomotor and neurobehavioural impairments in neurodegenerative disease models and evaluate the effects of therapeutic interventions.

3. Safety Requirements:

Individuals must be trained and competent with the procedures, equipment and handling of animals described in this protocol.

General PC2 laboratory safety procedures must be followed.

4. Materials/Apparatus:

- The open field apparatus used in the WSU Animal Behaviour Laboratory is the open field activity test chamber MED Associates Inc., (43.2 x 43.2 cm). The walls are covered with black card on the outside to prevent animals from seeing out. The arena is evenly and indirectly illuminated (900 lux). Direct beams of light and shadowed corners are avoided.
- Videotracking system with dedicated tracking software Any-maze, for the automated recording of data.
- Paper towels
- 70% ethanol solution
- Clean empty cages for animal transfer
- Personal protective equipment – eg. gloves and lab coat

5. Procedure:

1. Tests are performed following an initial 3 consecutive days of escalated handling (protocol WSU-ABL_3EH.2015.01) with a 1 hour transportation habituation session of the experimental room conducted on the third day.

2. Animals are transported in their home cages from the animal facility to the behaviour laboratory on one of the animal facility's corridor trolleys, with a sheet covering the cages during the transport to the laboratory.

3. Animals are left on the trolley uncovered in a designated holding area in the laboratory, and left 30 minutes to habituate to the laboratory environment.

4. Excess noise, human activity, odours, and visual distractions are also avoided during habituation and testing.

5. The Any-Maze software protocol is set up to capture mouse activity in the arena for a 10 minute period. Two zones are delineated using the software, central and peripheral. The central region size is designated 22 x 22 cm.

6. A record sheet with all test animal IDs, cage number, and ear markings is kept with the randomised order of the animal trials pre-determined for efficiency.

7. The open field arena, and all benches where animals are to be handled are wiped down with 70% ethanol.

8. Once the habituation period is over the testing can commence. Animals are carefully identified by matching their ear marks with their cage number and animal ID. To minimise the cross-over of odours between animals and sex, mice are carried over individually in their clean transfer cage from the holding area to the experimental bench area. The animal is picked up by the tail, and placed in a same corner of the arena.

9. The Any-maze program is the prompted to commence recording and tracking of the animal's movements for a trial period of 10 minutes. Once the trial is finished, the animal is removed from the arena, placed back in its transfer cage and carried over back to its home cage.

10. After each trial, the apparatus is cleaned with 70% ethanol solution spray to remove any urine or faeces, as well as any benches in use and allowed time to dry. The experimenter's gloves are also sprayed with ethanol and allowed to evaporate before picking up the next test animal.

11. The process is repeated for each test animal.

Supporting Sources of Information:

Bailey KR, Crawley JN. Anxiety-Related Behaviors in Mice. In: Buccafusco JJ, editor. Methods of Behavior Analysis in Neuroscience. 2nd edition. Boca Raton (FL): CRC Press; 2009. Chapter 5.

Crawley JN, Paylor R (1997) A proposed test battery and constellations of specific behavioral paradigms to investigate the behavioral phenotypes of transgenic and knockout mice. Horm Behav. 31(3):197-211.

Crawley JN. (2007) What's Wrong With My Mouse? Behavioral Phenotyping of Transgenic and Knockout Mice. New York: John Wiley and Sons, Inc.

EMPReSS: European Mouse Phenotyping Resource for Standardised Screens (2007) Open Field: Standard Operating Procedure. ESLIM_007_001.

Gould T.D.(ed.), Mood and Anxiety Related Phenotypes in Mice, Neuromethods 42, DOI 10.1007/978-1- 60761-303-9_1, ª Humana Press, a part of Springer Science+Business Media, LLC 2009

Karl T, Pabst R, von Hörsten (2003) Behavioral phenotyping of mice in pharmacological and toxicological research. Exp Toxicol Pathol. 55(1):69-83.

Mandillo S1, Tucci V, Hölter SM, Meziane H, Banchaabouchi MA, Kallnik M, Lad HV, Nolan PM, Ouagazzal AM, Coghill EL, Gale K, Golini E, Jacquot S, Krezel W, Parker A, Riet F, Schneider I, Marazziti D, Auwerx J, Brown SD, Chambon P, Rosenthal N, Tocchini-Valentini G, Wurst W. Reliability, robustness, and reproducibility in mouse behavioral phenotyping: a cross-laboratory study. Physiol Genomics. 2008 Aug 15;34(3):243-55. doi: 10.1152/physiolgenomics.90207.2008

Nagaraju, K. (2008) Behavioural and locomotor measurements using open field animal activity monitoring system. TREAT-NMD. DMD_M.2.1.002.

WESTERN SYDNEY UNIVERSITY

Subject:	Barnes Maze Test
Protocol No:	WSU-ABL_BM.2015.02
Version:	2.0
Issued:	December 1st, 2015
Last Reviewed:	March 21st, 2016
Authors:	Chris Millington Sandra Sonego
Working group members:	Prof. Gerald Muench (Principal Supervisor) Dr. Erika Gyengesi (Supervisor) Chris Millington (Masters Candidate) Sandra Sonego (Masters Candidate)

1. Objective:

The Barnes Maze (BM) is a type of navigational maze used to assess spatial learning and memory in rodents. The maze consists of an elevated circular platform with evenly spaced holes around the perimeter. Animals are given reinforcement and visual cues to escape from the exposed brightly lit platform to a dark recessed target hole. Improvement in their ability over trials is thought to signify the animal's learning and memory of the escape target hole. The maze uses the rodents' instinct to seek out dark enclosed spaces and avoid open bright spaces. It has been used to test both reference and working memory. The protocol described here is a shortened protocol adapted from the methods by Sunyer et al. 2007 and Attar et al. 2013.

2. Scope and Applicability:

The BM was invented by Carol Barnes in 1979 as an alternative to the stress caused by the Morris Water Maze (MWM), initially developed for rats. However the BM has now been adapted to and more frequently used in mice, as it utilises the natural tendencies and abilities of mice to search and escape through small holes. The maze's capacity to measure spatial ability has been confirmed by rodents with hippocampal damage showing impaired performance.

Although similar to MWM and radial-arm maze task, this maze does not require strong aversive stimuli or food deprivation as motivation.

3. Safety Requirements:

Individuals must be trained and competent with the procedures, equipment and handling of animals described in this protocol.

General PC2 laboratory safety procedures must be followed.

4. Materials/Apparatus:

The Maze:

The BM is made of a circular platform 91cm in diameter, with 20 equally spaced holes of 5cm diameter around the periphery, elevated 90cm above the floor. Only one of the holes is fitted with a small dark recessed chamber through which mice can escape, and the escape hole can be rotated with the maze to different positions. The mouse cannot visually discriminate between the escape hole and the closed holes from the maze surface. Soft padding material is laid down beneath the apparatus to prevent any injuries should the mice fall off during the test.

Figure 1. The Stoelting Barnes Maze apparatus used at WSU Animal Behaviour Laboratory.

Reinforcement:

Our laboratory uses aversive noise of 85dB as a motivator, in addition to bright lighting (950lux). The noise type is kept the same throughout testing.

Room configuration and visual cues:

Reference points for rodents to locate the target escape hole are provided. These include 4 different shapes as visual cues mounted around the maze, in addition to spatial cues from furniture and equipment located in the room. These cues are kept exactly the same throughout testing.

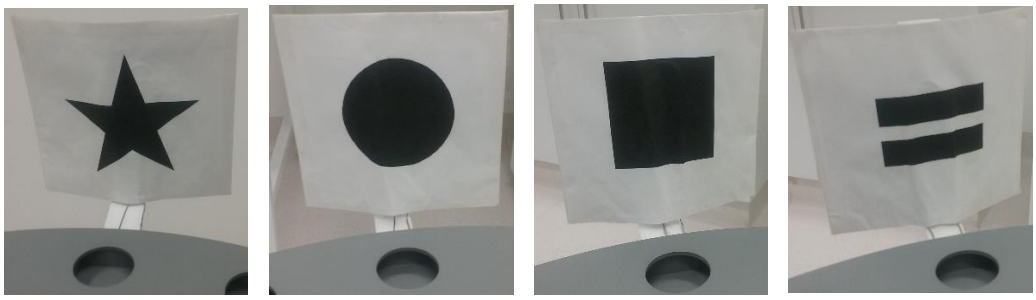


Figure 2. The 4 maze visual cues used.

Figure 3. The Barnes Maze set up with visual cues.

The position of the target hole is randomised for each mouse in order to minimise location bias. Each animal has 1 of 4 possible fixed positions allocated as shown in figure 5. Once an escape hole location has been assigned to a mouse it remains the same throughout testing.

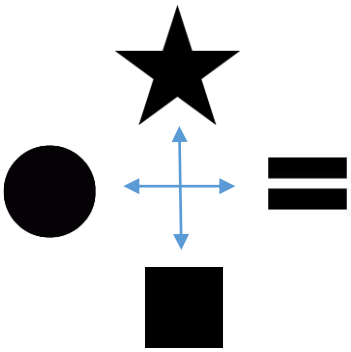


Figure 4. The cues move with the maze when the platform is rotated to the 4 fixed positions, ie, the star always remains above the target hole, followed by the circle to the right, square (opposite) and equals sign to the left.

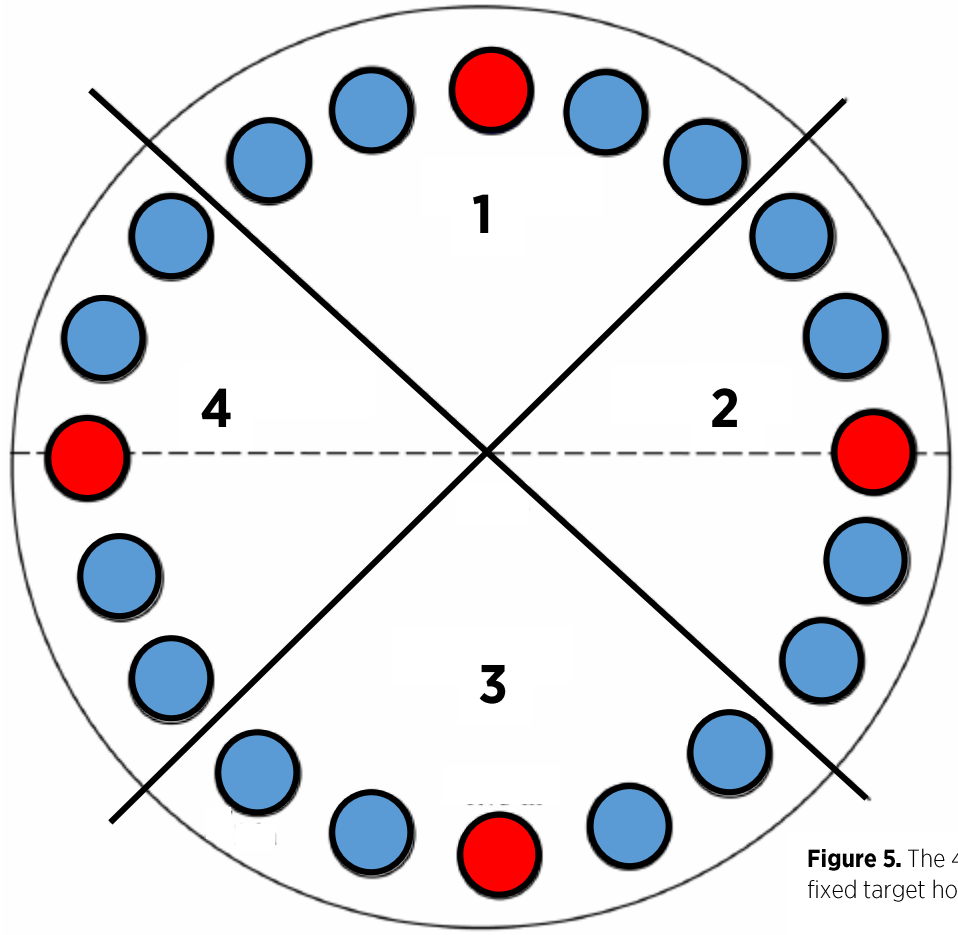


Figure 5. The 4 quadrants with 4 possible fixed target hole locations.

Video-tracking Software:

Digital camera and the ANY-maze tracking system is utilised to capture all data. An observer is always present to check whether any tracking errors occur during the trial.

Other:

- Isolation box
- Black starter chamber
- Stopwatch
- Paper towels
- 70% ethanol solution
- Clean empty cages for animal transfer
- Personal protective equipment – eg. gloves and lab coat

5. Procedure:

The BM test is typically performed as part of a test battery following an initial 3 consecutive days of escalated handling (protocol WSU-ABL_3EH.2015.01) with a 1 hour transportation habituation session of the experimental room conducted on the third day. Below is the experimental sequence, all work is conducted between 8:00am – 2:00pm:

Day 1:	1 habituation trial
Day 2:	3 acquisition trials
Day 3:	3 acquisition trials
	48 Hour break
Day 4:	1 probe trial

5.1 Preparation:

The following steps are followed in the morning of all experimental days:

1. Animals are transported in their home cages from the animal facility to the behaviour laboratory on one of the animal facility's corridor trolleys, with a sheet covering the cages during the transport to the laboratory.

2. The mice to be tested are placed in the low lighting isolation box (~10 lux) with the fan on to habituate in the setting for 30 minutes.

3. Excess noise, human activity, odours, and visual distractions are avoided during habituation and testing.

4. A record sheet with all test animal IDs, cage number, and ear markings is kept with the randomised order of the animal trials and escape hole location pre-determined for efficiency.

5. The BM, and all benches where animals are to be handled are wiped down with 70% ethanol.

5.2 Day 1: 1 Habituation Trial

Testing order: As there is only 1 habituation trial performed for the day, all animals in a cohort can be run one after the other.

1. Mice are carefully identified by matching their ear marks with their animal ID. With the laboratory lights dimmed (~30 lux), the mouse to be tested is carried over in a clean transfer cage to the experimental area. This aids to minimise the cross-over of odours between animals and sex.

2. The mouse is then carefully placed in the cylindrical black start chamber. The start chamber with the mouse is placed at the centre of the maze for 10s. After the 10s have passed, the chamber is lifted, and the aversive stimuli is turned on (sound-85dB and all laboratory lights turned on-~950 lux).

3. The mouse is gently guided to the escape hole. The mouse should not be forced to enter the escape hole, instead if it is resisting entering the hole, the animal is placed gently to the side of the hole and pulled gently by the base of the tail in the direction away from the hole, which should induce the mouse to move in the opposite direction to enter the hole. Should this manoeuvre not work, the mouse is placed directly into the hole.

4. When the mouse is in the escape hole, the aversive stimuli is turned off.

5. The mouse is kept in the escape hole for 2 min. Place a cover over the hole to prevent the mouse from exiting the hole during the 2 min.

6. After the 2 minutes have passed the mouse is placed back in its transfer cage, returned to its homecage and placed in a designated holding area just outside the lab so that they are not exposed to the aversive noise.

7. Between each trial, the apparatus and experimental surfaces are thoroughly cleaned with 70% ethanol solution spray to remove any urine or faeces, and allowed time to dry. The experimenter's gloves are also sprayed with ethanol and allowed to evaporate before picking up the next test animal.

8. Recording and tracking of the habituation trial is optional.

9. Once all testing is completed for the day, they are returned to the animal facility as per step 1, section 5.1.

5.3 Days 2-3: 3 Acquisition Trials

Testing order: Mice are tested in groups of 5, in order to maintain a 20-30 minute inter-trial-interval (ITI). Once the first group of 5 mice have completed their 3 trials for the day, the next group of 5 mice is then tested.

1. The Any-maze program is prompted to commence recording and tracking of the animal's movements in the maze for a fixed trial period of 3 minutes. Examples of parameters to be recorded include: primary and total latency, primary and total path length, as well as primary and total errors.

2. Mice are carefully identified by matching their ear marks with their animal ID. With the laboratory lights dimmed (~30 lux), the mouse to be tested is carried over to the experimental area in its transfer cage, carefully taken out and placed in the black cylindrical starting chamber. The starting chamber with the mouse inside is placed at the centre of the maze. After 10s the aversive stimuli is turned on (sound-85dB and all laboratory lights turned on-~950 lux) and the mouse is allowed to explore the maze for 3 min. The experimenter should move completely out of view behind the black curtain.

3. The trial ends when the mouse enters the escape hole or after 3 min have elapsed.

4. If the mouse has successfully entered the escape hole before the 3 minutes, the aversive stimuli is turned off and the mouse is made to stay in the escape hole for 1 min, closing the entrance so that it does not venture out.

5. If the mouse does not reach the escape hole within 3 minutes, the mouse is gently guided by the experimenter to the correct hole and the mouse is left inside for 1 min, turning aversive stimuli off once it is in the escape hole.

6. The mouse is placed back in its transfer cage and returned to its homecage in the isolation box.

7. After each trial, the apparatus is cleaned with 70% ethanol solution spray to remove any urine or faeces, as well as any benches in use and allowed time to dry. The experimenter's gloves are also sprayed with ethanol and allowed to evaporate before picking up the next test animal.

8. Steps 1-7 (Sec. 5.3) are repeated until the animal has performed the 3 trials for the day. Animals will receive a total number of 6 acquisition trials over the 2 days.

9. A 20-30 min ITI is maintained for all animals between trials.

10. Once one group of 5 animals have finished testing for the day, they are placed in a designated holding area just outside the lab so that they are not exposed to the aversive noise.

11. When all groups have completed testing they are returned to the animal facility as per step 1, section 5.1.

5.4 Day 4: 1 Probe Trial

Testing Order: All animals in a cohort can be run one after the other as there is only 1 probe trial conducted.

48 hours after the last day of acquisition trials, the probe trial is conducted. The experiment is run exactly as during the acquisition trials, except the escape hole is replaced with a closed hole, and the test duration is reduced to 90 seconds.

Fig. 6 Showing the closed hole and escape hole brackets. The escape hole bracket is replaced with a closed hole bracket during the probe trial.

The mouse is placed in the middle of the maze in the starter chamber for 10s, then aversive stimuli is turned on, and the mouse is free to explore the maze for a fixed interval of 90s. The probe trial is performed to determine if the mouse remembers where the target hole was located. Number of pokes (errors) in each hole, latency and path length to reach the virtual target hole are measured.

Supporting Sources of Information:

Attar A, Liu T, Chan WT, Hayes J, Nejad M, Lei K, Bitan G. (2013) A shortened Barnes maze protocol reveals memory deficits at 4-months of age in the triple-transgenic mouse model of Alzheimer's disease. PLoS One. Nov 13;8(11):e80355. doi: 10.1371/journal.pone.0080355.

Barnes, CA. (1979) Memory deficits associated with senescence: a neurophysiological and behavioral study in the rat. J Comp Physiol Psychol. 93:74-104.

Crawley JN. (2007) What's Wrong With My Mouse? Behavioral Phenotyping of Transgenic and Knockout Mice. New York: John Wiley and Sons, Inc.

Dawood MY, Lumley LA, Robison CL, Saviolakis GA, Meyerhoff JL.(2004) Accelerated Barnes maze test in mice for assessment of stress effects on memory. Ann N Y Acad Sci.Dec;1032:304-7.

Inman-Wood SL, Williams MT, Morford LL, Vorhees CV. (2000) Effects of prenatal cocaine on Morris and Barnes maze tests of spatial learning and memory in the offspring of C57BL/6J mice.Neurotoxicol Teratol. Jul-Aug;22(4):547-57.

O'Leary TP, Brown RE. (2009) Visuo-spatial learning and memory deficits on the Barnes maze in the 16-month-old APPswe/PS1dE9 mouse model of Alzheimer's disease. Behav Brain Res. Jul 19;201(1):120-7. doi: 10.1016/j.bbr.2009.01.039.

O'Leary TP, Brown RE. (2012) The effects of apparatus design and test procedure on learning and memory performance of C57BL/6J mice on the Barnes maze. J Neurosci Methods.Jan 30;203(2):315- 24. doi: 10.1016/j.jneumeth.2011.09.027.

O'Leary TP, Brown RE. (2013) Optimization of apparatus design and behavioral measures for the assessment of visuo-spatial learning and memory of mice on the Barnes maze. Learn Mem. Jan 15;20(2):85-96. doi: 10.1101/lm.028076.112.

Patil SS, Sunyer B, Höger H, Lubec G. (2009) Evaluation of spatial memory of C57BL/6J and CD1 mice in the Barnes maze, the Multiple T-maze and in the Morris water maze. Behav Brain Res. Mar 2;198(1):58-68. doi: 10.1016/j.bbr.2008.10.029.

Rosenfeld, C. S., Ferguson, S. A. (2014) Barnes Maze Testing Strategies with Small and Large Rodent Models. J. Vis. Exp. (84), e51194, doi:10.3791/51194.

Sunyer B, Patil S, Höger H, Lubec G. (2007) Barnes maze, a useful task to assess spatial reference memory in the mice. Protocol Exchange doi:10.1038/nprot.2007.390

Urbach A, Robakiewicz I, Baum E, Kaczmarek L, Witte OW, Filipkowski RK.(2013) Cyclin D2 knockout mice with depleted adult neurogenesis learn Barnes maze task. Behav Neurosci. Feb;127(1):1-8. doi: 10.1037/a003122

WESTERN SYDNEY UNIVERSITY

Subject:	Functional Observational Battery
Protocol No:	WSU-ABL_FOB.2015.01
Version:	1.0
Issued:	July 10th, 2015
Last Reviewed:	March 21st, 2016
Authors:	Chris Millington Sandra Sonego
Working group members:	Prof. Gerald Muench (Principal Supervisor) Dr. Erika Gyengesi (Supervisor) Chris Millington (Masters Candidate) Sandra Sonego (Masters Candidate)

Functional Observational Battery Items:

1. General Health

1.1. Body weight (g): _____

1.2. Rectal temperature (°C; rectal probe lubricated with peanut oil is inserted for 5-10 seconds to obtain a stable reading): _____

(to be completed last after all other tests are done)

While the animals are weighed, the animals are visually inspected and the following are recorded:

1.3. Presence of whiskers

0 = None

1 = A few

2 = Most, but not a full set

3 = A full set

1.4. Appearance of fur (not counting patches of missing fur)

0 = Ungroomed and disheveled

1 = Somewhat disheveled

2 = Well-groomed (normal)

1.4a Indicate the presence and location of staining or abnormal coloration of the hair.

1.4b Indicate alopecia (thinning of the hair).

1.5. Piloerection

To differentiate piloerection from a scruffy or ungroomed coat, simply stroke the back of the mouse in a rostral to caudal direction. Piloerection will still be apparent after stroking.

0 = None

1 = Most hairs standing on end

1.6. Patches of missing fur on face

0 = None

1 = Some

2 = Extensive

1.7. Patches of missing fur on body

0 = None

1 = Some

2 = Extensive

1.8. Wounds

0 = None

1 = Signs of previous wounding

2 = Slight wounds present

3 = Moderate wounds present

4 = Extensive wounds present

2. Gross Neurological function

Novel cage Test: The animal is placed in a clean tub cage for a period of 3 minutes and the following behaviours are recorded without disturbing the mouse, (can be video recorded):

2.1 Assess reactivity/excitability

Observe the level of excitability or resistance of a subject animal in response to handling and/or removal from the home cage.

Handling may precipitate the expression of certain spontaneous neurological abnormalities, and therefore some degree of assessment should be made before handling.

Rank the level of reactivity/excitability using scoring criteria such as:

1 = Low (no resistance, easy to hold or pick up)

2 = Moderately low (slight resistance)

3 = Moderately high (some squirming or moving around)

4 = High (excited, squirming, twisting)

5 = Very high (aggressive actions, e.g., biting, tail and throat rattling).

2.2 Assess Arousal

Observe whisking, rearing, exploration, or responses to sudden in order to make an overall ranking of arousal. Rank arousal over the entire observation period, using the scale:

1 = Very low (stupor, coma, or prostrate)

2 = Low (sluggish, only some movements)

3 = Somewhat low (slightly sluggish, some exploratory movements)

4 = Moderate (alert, exploratory behavior)

5 = Somewhat high (slight excitement, tenseness)

6 = Very high (very alert, very excited or tense, sudden running or movements).

2.3 Vocalization

No. of times during handling _____

2.4. Transfer behavior

0 = Coma

1 = Prolonged freeze (>10 sec.), then slight movement

2 = Extended freeze, then moderate movement

3 = Brief freeze (a few seconds), then active movement

4 = Momentary freeze, then swift movement

5 = No freeze, immediate movement

6 = Extremely excited ("manic")

2.5. Body position

0 = Completely flat (on stomach)

1 = Lying on side

2 = Lying on back

3 = Sitting or standing

4 = Rearing on hind legs

5 = Repeated vertical leaping

2.6. Spontaneous activity

0 = None, resting

1 = Casual scratch, groom, slow movement

2 = Vigorous scratch, groom, moderate movement

3 = Vigorous, rapid/dart movement

4 = Extremely vigorous, rapid/dart movement

2.7. Respiration rate

0 = Gasping, irregular

1 = Slow, shallow

2 = Normal

3 = Hyperventilation

2.8. Tremor

0 = None

1 = Mild

2 = Marked

2.9. Palpebral closure

0 = Eyes wide open

1 = Eyes 1/2 closed

2 = Eyes closed

2.10. Examination of eyes

1. Indicate corneal opacity or cloudiness for each eye.

Ocular abnormalities involve changes in the cornea or eyeball.

2. Indicate whether the eyeball appears to bulge (termed exophthalmia) for each eye.

2.11. Examination of lacrimation

1. Score lacrimation on a two- or three-point scale.

Lacrimation is evidenced by wetness around the eyes. The normal state is a low basal level of lacrimation, so it is difficult if not impossible to observe decreased lacrimation.

2. Indicate whether tears are clear or tinged

2.12. Examination of salivation

1. Score salivation on a two- or three-point scale.

Salivation is evidenced by wetness around the mouth and chin. As with lacrimation, it is difficult to observe decreased salivation.

2.13. Pelvic elevation

0 = Markedly flattened

1 = Barely touches

2 = Normal (3mm elevation)

3 = Elevated (more than 3mm elevation)

2.14. Tail elevation (during forward motion)

0 = Dragging

1 = Horizontally extended

2 = Elevated (Straub tail)

2.15. Urination

0 = None

1 = Little

2 = Moderate amount

3 = Extensive

2.16. Defecation (count the number of fecal boli emitted during the 3-min. period):

_____

2.17. Number of Rearings in 3 minutes: _____

2.18. Scoring of behaviours from video: Record behaviour every 10 seconds, taking note of the presence of: wild running, constant circling, excessive grooming, excessive stereotyped sniffing, excessive stereotyped head bobbing, hunched body posture, frozen immobility. No. of rearings counted in real-time can also be confirmed.

3. **<u>After the 3 minute observation, the animal is taken out of the tub cage and the following tests are performed on a flat surface or as indicated by each test.</u>**

3.1. Gait Observation Score:

The animal is removed from its cage and placed on a flat surface with its head facing away from the investigator. The mouse is observed from behind as it walks. The mouse is placed back into its cage and a gait score is recorded from the following:

0 = If the mouse moves normally, with its body weight supported on all limbs, with its abdomen not touching the ground, and with both hindlimbs participating evenly.

1= If it shows a tremor or appears to limp while walking.

2 = If it shows a severe tremor, severe limp, lowered pelvis, or the feet point away from the body during locomotion (“duck feet”)

3 = If the mouse has difficulty moving forward and drags its abdomen along the ground.

3.2. Kyphosis Observation Score:

The mouse is removed from its cage and and placed on a flat surface. The animal is observed as it walks. After observation the mouse is placed back in its cage and a kyphosis score is assigned from the following:

0 = Able to easily straighten its spine as it walks, and does not have persistent kyphosis.

1 = If the mouse exhibits mild kyphosis but is able to straighten its spine.

2 = If it is unable to straighten its spine completely and maintains persistent but mild kyphosis.

3 = If the mouse maintains pronounced kyphosis as it walks or while it sits.

3.3 Ledge Test

Lift the mouse from the cage and place it on the cage's ledge. The tendency will be for the animals to walk along the ledge and then attempt to descend back into the cage. Observe the mouse walk on the ledge and lower itself into the cage. Assign a score:

0 = (Normal) If the mouse walks along the ledge without losing its balance, and lowers itself back into the cage gracefully, using its paws

1 = If the mouse loses its footing while walking along the ledge, but otherwise appears coordinated

2 = If it does not effectively use its hind legs, or lands on its head rather than its paws when descending into the cage

3 = If it falls off the ledge, or nearly so, while walking or attempting to lower itself, or shakes and refuses to move at all despite encouragement

3.4. Hindlimb clasping

Grasp the tail near its base and lift the mouse clear of all surrounding objects. Observe the hindlimb position for 10 seconds. Place the mouse back into its cage and record its hindlimb clasping score from the following:

0 = If the hindlimbs are consistently splayed outward, away from abdomen

1 = If one hindlimb is retracted toward the abdomen for more than 50% of the time suspended

2 = If both hindlimbs are partially retracted toward the abdomen for more than 50% of the time suspended

3 = If its hindlimbs are entirely retracted and touching the abdomen for more than 50% of the time suspended

Reflexes:

3.5. Trunk curl (grip base of tail between thumb and forefinger and lift about 30 cm)

0 = Absent

1 = Present

3.7. Postural Reflex

The animal is placed in an empty cage and the cage is rapidly moved side to side then up and down. Normal postural reflex is extension of all four legs outward to maintain balance and upright posture.

0 = Normal

1 = Abnormal

3.8. Righting Reflex

(10 sec. maximum)

0 = No impairment

1-10 = Number of seconds required to right

3.9. Response to Approaching Object:

Animal response to an approaching cotton-tip swab is recorded. A normal response is for mice to sniff or approach the object, then turn away and avoid the object. An abnormal response is indicated by attacking or ignoring the object.

0 = Normal

1 = Abnormal

3.10. Palpebral reflex (eye blink)

1. Use a cotton-tip swab and touch the edge of the object to the inside (nasal) point of the eye.

The palpebral reflex tests the trigeminal and facial nerves in response to a light touch (somatomotor stimulus). The eyelids should quickly close.

Indicate the presence/absence of the reflex:

0 = Absent

1 = Present

3.11. Pinna reflex (while the mouse is gently restrained, touch each auditory meatus lightly with the tip of a 2mm stainless-steel wire probe; watch for either ear retraction or head movement).

The pinna reflex is a somatomotor test of the cranial nerves. The ear should either shake or flatten against the head

0 = None

1 = Active retraction, moderately brisk flick

2 = Hyperactive, repetitive flick

3.12. Whisker-orienting reflex

The whiskers of the animal are lightly touched with a swab while the animal is moving freely. A normal response is for the whiskers to stop moving, and the head of the animal usually turns to orient toward the stimulus.

0 = Absent

1 = Present

3.13. Test extensor thrust reflex

1. Hold the animal in one hand. Gently press the fingertips into the plantar surface of the animal's two hindfeet.

The extensor thrust reflex evaluates the motor/sensory components of the spinal response to pressure in the footpads. The mouse will extend the hindlimbs, pushing against the fingertips, and curl the toes around the fingertips. It may be necessary to press against the hindfeet several times to elicit this response.

2. Indicate the presence/absence as well as the strength of the extensor response. Note whether the response is equal in both hindlimbs. If desired, note the number of stimuli required to elicit the response.

0 = Absent

1 = Present

3.14. Visual Placing

1. Hold the mouse by the tail and position it above the edge of a table or cart.

2. Slowly move the mouse down toward the surface or edge.

As it approaches, the mouse should extend its head and neck and reach towards the edge with the forelimbs.

3. Indicate the presence/absence of the placing response

0 = Absent

1 = Present

3.15. Proprioceptive Positioning

1. Gently hold the mouse on a surface and flex a hindlimb so that the dorsal surface of the paw is down on the surface.

2. Relax the hold on the hindlimb.

The mouse should immediately return the paw to the normal position under the body.

3. Evaluate the presence/absence and the strength of the response. Note whether the response is equal in both hindlimbs.

0 = Absent

1 = Present

3.17. Auditory response test

1. Position a sound stimulus ~5 cm above the back of the rat (i.e., outside the field of view).

Various stimuli may be used, including a metal clicker, finger snap, or hand clap.

2. Make a sudden sound. Be sure that the stimulus is consistent from test to test.

The mouse should react immediately with pinna movement, flinch, or whole-body startle.

3. Score the magnitude of the response using the following scale:

1 = No reaction or response
2 = Slight or sluggish reaction (flinch or startle as evidence of perception)
3 = Obvious reaction (locomotor orientation as evidence of perception)
4 = Clear reaction or response (more intense startle or locomotion)
5 = Exaggerated reaction (may jump, bite, or attack).

3.18. Olfactory Response Test

1. Position the mouse away from the edge of an open field.
2. Dip a cotton swab in an odorant and approach the mouse at nose level with the swab. Be sure that the stimulus is consistent from test to test.
3. Hold the stimulus ~3 cm from the face for ~4 sec to give the mouse time to make a response.
4. Indicate the presence/absence of a response, or score the magnitude of the response using the following scale:

1 = No reaction or response
2 = Slight or sluggish reaction (flinch or startle as evidence of perception)
3 = Obvious reaction (locomotor orientation as evidence of perception)
4 = Clear reaction or response (more intense startle or locomotion)
5 = Exaggerated reaction (may jump, bite, or attack).

3.19 Tail-pinch response

1. Position the mouse away from the edge of an open field. Pinch the tail ~2 to 3 cm from the tip, or pinch the toes or foot pad. Pinch hard enough to produce a reliable response in control mice, and be consistent in the intensity of the pinch from test to test.
The very tip of the tail is relatively insensitive. The mouse should react immediately with a flinch, whole-body startle, or an escape response. Metal tweezers may be used, or any other device that will make a quick squeeze without damaging tissues.

2. Score the magnitude of the response with the following criteria:

1 = No reaction or response
2 = Slight or sluggish reaction (flinch or startle as evidence of perception)
3 = Obvious reaction (locomotor orientation as evidence of perception)
4 = Clear reaction or response (more intense startle or locomotion)
5 = Exaggerated reaction (may jump, bite, or attack).

3.20. Body tone (compress both sides of the mouse between thumb and index finger)

0 = Flaccid, no return of cavity to normal
1 = Slight resistance
2 = Extreme resistance, board like

4. Splash Test: Grooming Behaviour

200µl of a 10% sucrose solution is squirted on the mouse's snout. Subsequent behavior is videotaped for 5 min. The duration of grooming is later recorded from the videos. (David et al. 2009, Smolinsky et al. 2009)

Supporting Sources of Information:

Crawley JN, Paylor R (1997) A proposed test battery and constellations of specific behavioral paradigms to investigate the behavioral phenotypes of transgenic and knockout mice. Horm Behav. 31(3):197-211.

David, D. J., Samuels, B. A., Rainer, Q., Wang, J.-W., Marsteller, D., Mendez, I., … Hen, R. (2009). Behavioral effects of fluoxetine in an animal model of anxiety/derpression are mediated by both neurogenesis-dependent and independent mechanisms. Neuron, 62(4), 479–493. http://doi.org/10.1016/j.neuron.2009.04.017

Irwin S. Comprehensive observational assessment: A systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse. Psychopharmacologia 1968; 13:222-57.

Karl T, Pabst R, von Hörsten (2003) Behavioral phenotyping of mice in pharmacological and toxicological research. Exp Toxicol Pathol. 55(1):69-83.

Moser VC, (2001) Neurobehavioral screening in rodents. Curr Protoc Toxicol. Chapter 11:Unit11.2

Rogers DC, Fisher EM, Brown SD, Peters J, Hunter AJ, Martin JE. (1997) Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. Mamm Genome. 8(10):711-3.

Smolinsky AN, Bergner CL, LaPorte JL, Kalueff AV (2009) Analysis of Grooming Behavior and Its Utility in Studying Animal Stress, Anxiety, and Depression, Mood and Anxiety Related Phenotypes in Mice, Neuromethods Volume 42 , pp 21-36.