THE IMPACTS OF A CANNABIS EXTRACT ON BRAIN PATHOLOGY AND MEMORY IMPAIRMENTS ASSOCIATED WITH ALZHEIMER'S DISEASE IN AN APP-NL-G-F TRANSGENIC MOUSE MODEL

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ABSTRACT

Alzheimer's Disease (AD) is a devastating neurodegenerative disorder that is categorized by progressive memory loss and cognitive deterioration. Amyloid-beta (Aβ) proteins accumulate within AD brains and trigger harmful chronic inflammation. By targeting both Aβ and inflammation, disease progression may be delayed. *Cannabis* is a plant containing THC and CBD. Previous work in our lab has shown that these compounds are ineffective at reducing AD pathology independently; however, research suggests they are more effective when combined. This project utilizes a THC/CBD combination extract to evaluate AD pathology in APP mice by assessing performance on memory-based behavioural tasks and by using protein markers to determine disease progression and severity. There were no treatment-related effects of the extract found via behavioural testing. However, the extract did decrease PFC 4G8-stained Aβ plaques. There were no other significant effects on Aβ, inflammation, or HPC cell volume found. Sex differences were found on various behavioural tasks.

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LIST OF ABBREVIATIONS

Aβ	Amyloid Beta
ACh	Acetylcholine
AD	Alzheimer's Disease
AMG	Amygdala
ANOVA	Analysis of Variance
ApoE	Apolipoprotein E
APP	Amyloid Precursor Protein
BBB	Blood-Brain Barrier
CA1	Cornu Ammonis 1
CA3	Cornu Ammonis 2
CB	Cannabinoid
CB1	Cannabinoid Receptor Type 1
CB2	Cannabinoid Receptor Type 2
CBD	Cannabidiol
ChAT	Choline Acetyltransferase
CNS	Central Nervous System
EC	Entorhinal Cortex
ECS	Endocannabinoid System
ERT	Estrogen Replacement Therapy
FAD	Familial Alzheimer's Disease
FC	Fear Conditioning
GFAP	Glial Fibrillary Acidic Protein
GWAS	Genome-wide Association Studies
HPC	Hippocampus
I.P	Intraperitoneal
Iba1	Ionized Calcium-binding Adapter Molecule 1
IHC	Immunohistochemistry
IR	Investigation Ratio
lPFC	Lateral Prefrontal Cortex
LTP	Long-term Potentiation
mPFC	Medial Prefrontal Cortex
MRI	Magnetic Resonance Imaging
MTL	Medial Temporal Lobe
MWT	Morris Water Task
NFT	Neurofibrillary Tangles
NOR	Novel Object Recognition
OFT	Open Field Task
PBS	Phosphate-Buffered Saline
PFA	Paraformaldehyde

PFC	Prefrontal Cortex
PRC	Perirhinal Cortex
PS1	Presenilin-1
PS2	Presenilin-2
SAD	Sporadic Alzheimer's Disease
SEM	Standard Error of the Mean
THC	Δ9-tetrahydrocannabinol

Introduction

Alzheimer's Disease (AD) was first described in 1907 by Alois Alzheimer in the autopsy results of his 55-year-old patient, Auguste Deter (Alzheimer, 1907; O'Brien & Wong, 2011). Deter experienced gradual cognitive decline due to a previously unknown behavioural and cognitive disorder. The very first symptom that Alzheimer reported in his seminal 1907 paper was the development of a 'rapid loss of memory' (Stelzmann et al., 1995). Later behaviours that arose as a result included confusion, helplessness, disorientation, auditory hallucinations, and paranoid thinking. Post-mortem analysis showed significant brain atrophy and tangled materials where cells should be (Stelzmann et al., 1995).

Alzheimer's Disease (AD) is now known to be the most common age-related neurodegenerative disorder. Neurodegeneration is the gradual loss of function and structure in both neuronal and glial cells, which results in mass cell death in the central nervous system (CNS) (Toro et al., 2019). AD is categorized by progressive memory loss and sweeping deterioration of one's cognitive ability (which is defined as a decline in executive function, attention, and working memory) (Calina et al., 2020; Guo et al., 2020; Hooli & Tanzi, 2009). This also translates to impairments in the ability to conduct daily routines and tasks. Preclinical symptoms can also include changes in one's thought patterns and/or unconscious behaviour as well as impairments in language and speech (Guo et al., 2020). These include more subtle, routine-related behaviours that a stranger may not notice, but a spouse would have caught. This may include confusion during tasks like dealing with money at the checkout till or locating a parked car in the lot. These are seemingly simple cognitive tasks, but as thinking becomes more disorganized it becomes more difficult to perform these actions. Communication also begins to break down as words are often misplaced in one's mind and used incorrectly in conversation, leading to a decreased ability to communicate effectively. Overall, AD slowly begins to disrupt every aspect of one's life from cognitive functions to interpersonal relationships.

Present findings from the *Journal of the Alzheimer's Association* estimates that over 50 million people are currently living with dementia globally, costing over a trillion US dollars annually (Gustavsson et al., 2023). Typically, those caring for dementia patients are unpaid family members, placing an even further burden on the loved ones of those suffering. Furthermore, individuals with preclinical AD are estimated to be 315 million worldwide; combined with dementia this roughly accounts for 22% of all people above the age of 50 (Gustavsson et al., 2023). The preclinical stage of AD develops slowly over the course of 20-30 years and shows virtually no symptoms. This suggests that treatment methods targeting brain pathology must be initiated during the early stages of disease progression to be most effective at preventing the more advanced, behavioural stages.

Disease Pathology

Amyloid Beta Plaques and Neurofibrillary Tangles

There are two main pathological hallmarks of AD: (1) extracellular amyloid beta (Aβ) plaques (also called senile plaques) and (2) intracellular neurofibrillary tangles (NFTs) produced by aggregated hyperphosphorylated tau (Aso et al., 2016; Aso & Ferrer, 2014; Bloom, 2014; Cooray et al., 2020; Mooko et al., 2021; Sasaguri et al., 2017; Zhao et al., 2020). Individuals with AD also experience both neuronal and synaptic loss, as well as neuroinflammation (Hooli & Tanzi, 2009).

The Aβ protein is one of the main constituents of senile plaques, thus making it a protein of interest (Awasthi et al., 2016). Aβ aggregates are composed of a 40-42 amino acid chain that develops from amyloid precursor protein (APP), through a successive cleavage process by β-secretase and γ-secretase (Aho et al., 2010; Farkhondeh et al., 2020; Russo, 2018; Zhao et al., 2020). Normally, APP functions to help form and maintain synapses, as well as promote neuronal survival and expansion (Guo et al., 2020; Penke et al., 2017). The mutation of the APP gene is one of the four most common genetic mutations responsible for the development of familial AD (FAD) (Cooray et al., 2020). FAD is brought about by a predetermined genetic code. Specifically, APP genetic mutations lead to the assembly of longer Aβ peptides that accumulate much more readily in the brain, producing neurotoxic effects (Rosenberg et al., 2020). Aβ first presents itself in the initial stages of all forms of AD, thus making it a 'preclinical' model of the disease (Sasaguri et al., 2017). Aβ plaques develop early in the progression of AD, whereas NFTs are indicative that the disease has reached more advanced stages.

NFTs first develop from aggregated tau proteins found within the brain. In healthy individuals, tau acts a microtubule binding component that helps polymerize and stabilize microtubules (Guo et al., 2020). Essentially, tau plays a crucial role in supporting the internal skeleton of neurons. However, in AD, tau becomes hyperphosphorylated which is a process where multiple phosphorylation sites on tau become fully saturated. This leads to decreased biological activity as NFTs develop from aggregates of hyperphosphorylated tau protein (Guo et al., 2020). NFTs cluster in the brain and produce neurotoxic effects as brain processes become disrupted (Guo et al., 2020).

Essentially, Aβ accumulation occurs first in the progression of AD pathology; this accumulation then triggers the transformation of tau from a normal state to a harmful one (Bloom, 2014). Moreover, there is supporting evidence that toxic tau, in turn, exacerbates the toxicity of Aβ through negative feedback (Bloom, 2014). Aβ and NFTs create toxic conditions in the brain that lead to compounding effects that trigger chronic neuroinflammation (which is heavily related to the pathology and cognitive deficits associated with AD) (Gowran et al., 2011). Thus, one key therapeutic target in the treatment of AD is glial cells which sustain prolonged activation and produce chronic inflammation in the presence of Aβ proteins; combined, these factors significantly contribute to AD pathology.

Glial Cells

Glial cells (including microglia and astrocytes) consist of more than 70% of the population of cells in the brain and spinal cord. Glial cells play a critical role in regulating the immune system, a necessary process that induces inflammatory responses to maintain overall body health and function (Bie et al., 2018; Romero-Sandoval et al., 2008). Acute inflammation is an essential component of the body's immune response against a wide variety of insults such as trauma, infection, and disease, however disrupted and prolonged inflammatory responses (in the form of chronic inflammation) can become extremely harmful over time (Ativie et al., 2018; Newcombe et al., 2018). Microglia are essential macrophages (or immune cells) in the CNS that play a fundamental role in brain development and aging by helping the brain maintain homeostasis (Rossum, 2004; Van Rossum & Hanisch, 2004) and mediate synaptic communication (Garland et al., 2022). Essentially, they help ensure that cellular processes, interactions, and turnover is always running smoothly. Microglia have been shown to exist in two distinct states known as 'activated' or 'resting'; which are mainly categorized by their distinct morphology in the two states (Rossum & Hanisch, 2004). In an 'activated' state, microglia possess visibly enlarged cell bodies compared to 'resting' state size (Garland et al., 2022). Microglia are the 'first responders' when the immune system becomes activated due to pathogens or other unfamiliar molecules. In AD, microglia are thought to be a critical part of the early stages of the disease and thus are key therapeutics targets (Hemonnot et al., 2019; Martín-Moreno et al., 2011). This is likely due to the important role they play in initial inflammatory responses. Microglia become mobile in response to Aβ deposition and become highly activated to mitigate immune responses within the brain; however, they end up chronically producing a large variety of pro-inflammatory molecules that lead to AD-related neuronal damage (Benito et al., 2007). This chronic activation essentially wears down processes over time. Astrocytes, on the other hand, remain relatively fixed in space and function to supply nutrients and structural support. Astrocytes help form one key factor in the immune system, the blood-brain barrier (BBB), which prevents pathogens and dangerous substances from entering the brain (Newcombe et al., 2018). In the progression of AD, astrocytes have been found to lose their protective ability (Siracusa et al., 2019). Initially, astrocytes function to remove Aβ, however, as the disease progresses there is a reduction in removal (Garwood et al., 2017). Similarly, to microglia, the Aβ accumulation stimulates astrocytes to produce more pro-inflammatory molecules that induce a dangerous positive feedback cycle of continuous activation (Siracusa et al., 2019). This chronic inflammation will dimmish the structural integrity of astrocytes over time. Overall, microglia and astrocytes play a fundamental role in both acute and chronic inflammation, making them important therapeutic targets in the treatment of AD.

Microglia

As a key regulator of inflammation in the brain, microglia play a vital role in neuroinflammation-causing neurodegeneration (Cooray et al., 2020). Unfortunately, microglia are unable to clear Aβ effectively, which leads to prolonged inflammatory responses and a buildup of hazardous peptides (Cooray et al., 2020). Acute inflammation is an effective immune system response that oftentimes quickly resolves insults; however, in AD, Aβ plaques prolong the immune response resulting in chronic inflammation and ultimately neuronal degeneration (Spangenberg & Green, 2017). Microglia employ phagocytosis, a process that clears neurons of debris and targets damaged neurons; however, when this process is disrupted, cells become increasingly toxic due to accumulated waste (Spangenberg & Green, 2017). Interestingly, the size and number of microglia are directly proportionate to the size of Aβ plaques in Aβ-containing transgenic mice brains (Wegiel et al., 2001). This shows that microglia are closely linked with AD pathology. Overall, these studies demonstrate that downregulating microglial activation early in disease progression may be essential to reducing pathology and improving behavioural outcomes in AD.

Similarly, aging plays a significant role in microglial function, even on a phenotypic level (Newcombe et al., 2018). Naturally, as time progresses cells and their constituents lose their ability to function properly as they slow and degenerate over time; this includes a decrease in housekeeping abilities such as synaptic maintenance, neurogenesis, cognitive organization, and immunological care (Newcombe et al., 2018). Aging combined with disease progression is likely the reason age is the predominant risk factor for AD.

Astrocytes

Astrocytes vastly outnumber microglia in the brain (Wyss-Coray et al., 2003); this is likely because astrocytes remain fixed in place whereas microglia are recruited in inflammatory responses. Like microglia, astrocytes play a key role in organizing and maintaining brain structure and function, however they also help control information processing, neural and synaptic plasticity, signal transmission, and BBB homeostasis (Newcombe et al., 2018). In AD, astrocytes, like microglia, are shown to form clusters around Aβ plaques (Gomez-Arboledas et al., 2018). Although, Wyss-Coray and colleagues found that astrocytes could reuptake and degrade Aβ using *in vitro* mouse astrocytes (Wyss-Coray et al., 2003). This shows that astrocytes are key therapeutic targets in AD treatments. However, over time astrocytes lose their functional ability as they sustain prolonged activation, resulting in secondary plaques accumulating as they die (Garwood et al., 2017). This means that neurodegeneration is occurring faster than the rate of astrocyte mediated Aβ clearance. Astrocytes, along with microglia, respond to the accumulation of Aβ plaques, making their activation a crucial aspect of AD pathology. The combined findings of these studies suggest that while astrocytes may help facilitate Aβ clearance, the inflammatory mechanisms preceding them are too harmful to be left unmediated. Overall, reducing inflammation early in disease onset remains a critical target for reducing resulting AD pathology.

Overall, research shows that low chronic inflammatory levels correlate with better brain health (Newcombe et al., 2018). Chronic inflammation is a main contributor to AD pathology, thus controlling prolonged, chronic inflammation via microglia and astrocyte activation is a crucial component of AD treatment and prevention.

Other systems besides the ones described above become dysregulated in the progression of AD, especially, the cholinergic system. The cholinergic system is a key ascending neurotransmitter system for cognitive functioning and becomes a main area of alteration in the brain of AD patients.

Cholinergic System

Along with Aβ and NFTs, another main hallmark of AD is the degradation of cholinergic neurons (Fisher, 2008; Mehla et al., 2022; Thathiah & De Strooper, 2009). Cholinergic neurons are shown to play a key role in cognition and attention, and they also provide acetylcholine (ACh) to various brain regions including, the cerebral cortex, hippocampus, and amygdala

(Fisher, 2008; Mehla et al., 2022), which are major areas affected by AD. ACh is an essential neurotransmitter that plays a crucial neuromodulatory role in cognition and memory. AD pathology seems to closely correlate with cholinergic dysfunction and decreased ACh in the brain (Mehla et al., 2022). Cholinergic dysfunction is described as a decrease in ACh synthesis due to low levels of choline acetyltransferase (ChAT), ACh reuptake, cholinergic neuronal and axonal abnormalities, and degeneration of cholinergic neurons (Fisher, 2008). Specifically, research has found that ACh levels are significantly reduced in various brain regions, including the frontal cortex and hippocampus of AD patients compared to healthy controls (Quirion, 1993). Furthermore, decreased levels of ACh has been reported in Aβ-containing transgenic mice, as well as post-mortem analysis of AD brains (Mehla et al., 2019). Quirion (1993) first proposed that the cholinergic system produces far spanning effects on brain function, including the degradation of Aβ. In a follow up study, it was found that Aβ-related proteins, including APP, act as physiological modulators of cholinergic function (Kar & Quirion, 2004). Pittel and colleagues found that cholinergic muscarinic receptor agonists enhanced soluble APP secretion in rats while inhibiting insoluble Aβ production (Pittel et al., 1996). This suggests that treatments targeting the cholinergic system may help regulate Aβ formation. Along with significant effects on Aβ pathology, cholinergic agonists have been found to attenuate the hyperphosphorylation of tau (Kar et al., 2004). Ultimately, changes in the cholinergic system seem to produce changes in APP processing and promotion of tau hyperphosphorylation, although the cellular mechanisms remain elusive (Kar et al., 2004). Alterations in the cholinergic system and overall accumulation of Aβ and hyperphosphorylated tau likely create a feedback loop that furth exacerbates AD pathology. Overall, Aβ (1) decreases ACh synthesis and release, (2) downregulates cholinergic receptor signaling, (3) increases tau phosphorylation and (4) increases overall neuronal toxicity.

Combined these findings stress the significant role of cholinergic system modulation in AD pathology.

In summary, key therapeutic targets in AD treatment involves addressing Aβ plaques and the ensuing pathologies they contribute to, such as the functioning of glial cells and the regulation of various brain systems, including the cholinergic system. By controlling the number of Aβ plaques present in the brain, the progression and development of AD may be slowed. Aβ plaques are one of the earliest physical signs, along with neuroinflammation, of AD; thus, by targeting the early pathology there is hope that the later developments will not occur.

Amyloid Beta Cascade Hypothesis

Research has shown that Aβ pathology presents itself two decades before tau pathology and the onset of behavioural symptoms (Sasaguri et al., 2017). This suggests that Aβ plays a critical role in the early development of the disease and ultimately, in prevention. One proposed theory behind how AD develops is the 'Aβ cascade hypothesis (ACH)', which argues that the principal event in AD pathology is the excess production of Aβ (Awasthi et al., 2016; Zhao et al., 2020). Awasthi and colleagues (2016) attribute this accumulation of Aβ as being the main pathological event of AD, which results in numerous 'secondary neuropathological changes' including the latter presentation of the disease in the form of NFTs, neuronal and synaptic loss, and dementia. The ACH suggests that deposits of Aβ in the brain are a pathological instigator for the development of AD and that NFTs are likely due to an imbalance in the assembly of Aβ and removal (Awasthi et al., 2016). However, it should be noted that the ACH is largely contended as Aβ accumulation does not appear to closely correlate with cognitive deficits (Simón et al., 2010). This suggests that the presence of Aβ does not necessarily give insights into how developed the disease is. Furthermore, it has been shown that healthy, elderly patients do display Aβ plaques without showing any behavioural symptoms of AD (Simón et al., 2010). Although, as Aβ plaques are the early signs of the disease it can be expected that cognitive deficits would not yet have the chance to develop. The early stages of AD progression are an important area of study as it offers invaluable insights into initial dysregulation, potentially eliminating the presentation of more advanced and severe disease stages. Ultimately, once individuals begin to display more advanced behavioural symptoms of AD it is often too late to remedy. It is important to keep in mind that roughly 80% of those age 65 and above demonstrate Aβ plaques and/or NFTs, however only 5% suffer from dementia (Aso & Ferrer, 2016). This shows that the presence of both Aβ plaques and NFTs is typical in normal aging and not always a major cause for concern.

Many brain areas become affected by the accumulation of Aβ plaques throughout the development of AD as processes breakdown and memory networks cease to function properly. Some brain areas implicated in AD-pathology are the various regions found within the medial temporal lobe.

Medial Temporal Lobe

The previously unknown function of the medial temporal lobe (MTL) was discovered following bilateral removal from patient H.M in 1953. Following surgery, H.M presented an inability to form new memories and a temporally-graded retrograde amnesia (Scoville & Milner, 1957). The MTL consists of the hippocampus (HPC), amygdala (AMG), and parahippocampal formations (including the perirhinal and entorhinal cortices); this led to difficulty determining which structures were responsible for which aspects of memory formations (Rudy, 2021). Later, researchers were able to implicate the HPC in a particular form of memory formation called episodic memory (Preston & Eichenbaum, 2013). Episodic memory arises from a memory system within the MTL that is responsible for extracting and storing information from our personal experiences (Tulving & Markowitsch, 1998). Episodic memory allows us to recall who we met up with at the movies last week or in, a rodent's case, what distinct, new stimuli they encountered yesterday.

As AD primarily affects one's memory (including episodic memory), the HPC is a key region of degradation in disease progression. Interestingly, in human studies, researchers have found that the HPC and associated cortical regions is where the most profound neuronal loss occurs during the progression of AD (Kril et al., 2002). This is likely why episodic memory is one of the first processes affected by the disease.

Parahippocampal formation

The parahippocampal formation consists of the entorhinal cortex (EC), perirhinal cortex (PRC), postrhinal, presubiculum, and parasubiculum and helps support memory formation in the MTL (Burwell & Amaral, 1998).

The EC transmits information from sensory cortices to the HPC and further on to the neocortex (including the prefrontal cortex) (Sipos et al., 2007). Due to its significant role in HPC processes, it is thought to be a key component in memory processing. Furthermore, studies have shown that EC lesions lead to various impairments in memory, including recognition and spatial memory (Sipos et al., 2007). This further demonstrates that the EC plays a crucial role in relaying memory information within and outside of the MTL. Furthermore, Sipos and colleagues also found that Aβ-plaque formation in the EC of rodents inhibited performance on memory-related tasks compared to controls (Sipos et al., 2007). This study shows the damaging affects of Aβ-plaque accumulation in memory processing regions, such as the EC. The EC also sends and receives information from the PRC. The PRC sits next to the EC in the parahippocampal formation.

The PRC has been attributed to recognition memory in mammals and has been extensively studied in rats, primates, and humans (Antunes & Biala, 2012; Mehla et al., 2019; Suzuki & Naya, 2014). Recognition memory consists of recollection (specific contextual details of a memory) and familiarity (awareness of previous exposure to a memory without any contextual cues or information) (Suzuki & Naya, 2014). PRC lesions in rodents have also been found to consistently impair recognition memory (Ennaceur et al., 1996; Mumby & Pinel, 1994; Warburton & Brown, 2015); which demonstrates that the PRC is a key area in recognition memory. The PRC has been shown to degrade in the progression of AD, thus making it an area of interest. Specifically, Juottonen and colleagues investigated the differences in volume of various memory-associated brain regions including, the PRC and EC using magnetic resonance imaging (MRI) and found a significant reduction in patients with AD compared to healthy controls (Juottonen et al., 1998). This demonstrates the importance of investigating changes in these regions throughout the progression of AD.

Hippocampus

Memory is a highly evolutionary process that has been the difference between the survival or extinction of our species (Brown & Aggleton, 2001). A key component of memory is brain plasticity, or the brain's ability to adapt via rigorous and constant restructuring (Rudy, 2021). The brain mechanisms responsible for synaptic plasticity have been studied more than any other region in the brain; namely, the mechanism of long-term potentiation (LTP) which is crucial for memory formation in the HPC (Bliss & Lømo, 1973). LTP is responsible for mediating brain plasticity by being able to adapt and change in the presence of varying situations and stimuli

(Rudy, 2021). Essentially, LTP is what makes the brain moldable and able to survive and thrive in an ever-changing environment. The HPC is a main brain region known to undergo LTP (LeDoux, 1993) as well as neurogenesis (Jin et al., 2004). Neurogenesis is a crucial way to combat AD-related neurodegeneration as it facilitates the creation of new neurons (Jin et al., 2004). Furthermore, the HPC is responsible for unifying memories of specific events in the context in which they took place (Preston & Eichenbaum, 2013) through episodic memory formation.

As AD primarily affects one's episodic memory, it is no surprise that the HPC is a key region of degradation in disease progression (Götz et al., 2018). It has been long established that the HPC and associated cortical regions is where the most profound neuronal loss occurs during the progression of AD (Hyman et al., 1984); this is likely why episodic memory is one of the first processes affected by the disease. In an MRI study conducted by Ardekani and colleagues (2016) they found that not only did AD patients show significant hippocampal atrophy, but females demonstrated a faster rate of atrophy than males (Ardekani et al., 2016). The reason for this profound sex difference remains unclear in present-day research; however, HPC atrophy measures using MRI may offer a more accessible diagnostic tool in the initial stages of AD. Furthermore, a study conducted by Kril and colleagues found that neurodegeneration in the HPC of AD patients exceeded NFT formations, wherein a ~60% neuronal loss only showed roughly ~8% NFT accumulation (Kril et al., 2002). This suggests that NFT formation is not strongly associated with neurodegeneration and the resultant behavioural effects that arise. This means that targeting NFTs in AD treatments may not be the most effective way of treating the disease. Ultimately, early pathological signs of AD remain necessary therapeutic targets to interrupt further progression and development.

Amygdala

Another key element of the MTL is the AMG, which plays a key role in ‘emotional memory’ and essentially links memories to an emotional component (LeDoux, 1993). Findings suggest that memories with stronger emotional significance are far more adhesive in the brain (i.e., salient emotional memories are the most difficult to forget) (Morena & Campolongo, 2014). The AMG is also a key player in memory formation, especially when related to fear (LeDoux, 1993). This is likely due to the evolutionary advantage of remembering negative events over positive ones (i.e., remembering where you saw that bear when you were gathering food is more important for your survival than remembering how delicious the berries that you ate from that area were). Studies have shown that the AMG becomes severely atrophied in the progression of AD (Poulin et al., 2011; Vogt et al., 1990), making it a region of interest.

In summary, various regions of the MTL combine to facilitate memory. The PRC is responsible for encoding an item in memory, the HPC is responsible for associating those items within memory, and the AMG is responsible for linking memories with an emotional factor (LeDoux, 1993; Squire & Zola-Morgan, 1991; Suzuki & Naya, 2014). All together, these brain regions play significant roles in the limbic system. The limbic system is a large, over-arching brain system consisting of the aforementioned structures and is responsible for learning and memory, as well as a myriad of other functions that are crucial to survival (LeDoux, 1993). These areas will be the primary focus of the present research project as they are responsible for various components of memory and become disrupted in the progression of AD.

Another key symptom that arises in AD are changes to one’s thoughts and perceptions of the world and of themselves. These cognitive processes arise in an important brain region known as the prefrontal cortex (PFC). The PFC also plays a crucial role in memory processes that become severely dysregulated in the progression of AD.

Prefrontal cortex

The PFC, amongst other things, is responsible for the top-down process that is executive function, which includes: reasoning, planning, language, and social interactions (Jobson et al., 2021). Essentially, the PFC functions as the command centre for the brain. Initially, rodents were believed not to have a PFC; it was first postulated by Kolb in 1984 that this is simply not the case as PFC lesion studies in rodents and primates revealed considerable similarities (Kolb, 1984). Although the rodent brain is roughly 1000 times smaller than that of the human brain (Kolb & Cioe, 2004), it is evident that there are still analogous structures that allow for applicable research to be conducted using rodent models.

The PFC is located in the most anterior part of the cerebral cortex and can be divided into two regions based on neuroanatomical connections: 1) the medial prefrontal cortex (mPFC) and 2) the lateral prefrontal cortex (lPFC) (Kolb & Tees, 1990; Jobson et al., 2021). The mPFC shares connections with the AMG, HPC, and other temporal areas, which unifies information from environmental stimuli; whereas the lPFC shares connections with the basal ganglia, cingulate cortex, and parietal cortex areas in order to regulate feedback from the environment (Jobson et al., 2020). The mPFC will be the primary focus in the present research as its connections and role in memory is an important factor in AD progression and severity. The mPFC plays a role in working memory, decision-making, cognitive flexibility, and attention (Jobson et al., 2021). In 1984, Kolb found that mPFC lesions resulted in impaired memory processes; specifically, regarding working memory, but not spatial memory acquisition (Kolb, 1984). Furthermore, mPFC lesions produced impairments in temporally linked working memory (memory that involves temporarily remembering stimuli in the present moment), whereas HPC lesions produce impairments in episodic memory (memory that involves recalling previously encountered stimuli). Impairments in the mPFC translate as an inability to keep track of sensory events in real time. However, there is also an important pathway present between the mPFC and the HPC that aids in the consolidation of memory (Preston & Eichenbaum, 2013); thus, it makes sense that a breakdown of these systems and their resultant connections would produce global impairments in memory.

Aging studies have shown a decline in spatial working memory over the lifespan, a primary function of the mPFC. Bizon and colleagues found that aged male Fischer rats performed worse at tasks involving spatial working memory compared to their young counterparts (Bizon et al., 2009). This suggests that working memory deteriorates with age, however neurodegenerative-related disorders may greatly exacerbate these impairments.

Over time, the PFC becomes affected by AD as it is one of the first brain regions vulnerable to Aβ accumulation (Latif-Hernandez et al., 2020). The pattern of effects in AD is of interest because the PFC and HPC appear to work together to encode, consolidate, and recall memories (Euston et al., 2012). As the mPFC is the 'conductor' in the metaphorical orchestra that is the brain this means that its removal does not necessarily correlate with an inability to perform any one specific function, but rather the connectivity of various parts. This demonstrates how the mPFC and MTL work in tandem to produce various components of memory.

MTL-mPFC Connections

Various regions within the MTL are strongly connected with one another; however, they also send/receive projections from the mPFC. Namely, the HPC (specifically, the ventral portion in rodents and the anterior portion in humans) sends projections to the mPFC; studies suggest that the mPFC is responsible for gathering information about the context of linked memories (Preston & Eichenbaum, 2013). The HPC and parahippocampal formation (including PRC and EC) form a feedback loop that allow the HPC to retrieve information about the events of a memory based on context, and vice versa (Preston & Eichenbaum, 2013). This means that the PRC and EC send and receive information on items within memory that allow the HPC to place them within memory. These areas working together is crucial to generating complete, coherent memories. Thus, dysregulation within any single aspect of this system will impair memory function, which is seen in individuals suffering from AD. Overall, the aforementioned brain regions must work together in order to facilitate memory; it is through the degradation of these systems in AD progression that memory becomes disrupted. Thus, by improving the functioning and longevity of these brain systems memory may be saved.

A relatively new idea in AD research is the different effects pathology seemingly has on females and males. Exploring sex differences is vital in AD research, as there are distinct manifestations across the sexes.

Sex Differences

Historically, scientific research has failed to consider the sex differences that naturally occur in the brain due to various factors such as hormones, maturation rate, and environmental influences. AD research is no exception. A recent review found that only one-third of recent preclinical AD-related articles published between 2013 and 2018 included female subjects (Waters et al., 2021). A quick search through the method section of any date of Alzheimer's research will show that almost exclusively male subjects are used. It should be noted that 'sex' in this context refers to both biological and physiological differences between males and females, specifically regarding hormones. The present thesis will not touch on the nuances of gender. In this research project, I will be using an equal number of both female and male subjects, as AD presents itself differently in female and male brains.

Overall, AD disproportionately affects women, by nearly two-fold, even when natural female longevity is accounted for (Hemonnot et al., 2019; Nebel et al., 2018; Oveisgharan et al., 2018; Toro et al., 2019). Specifically, female brains tend to experience faster HPC degeneration compared to male brains (Ardekani et al., 2016). This means that once disease progression has started, deterioration is accelerated in females. One main proposed contributor to sex dimorphisms in AD are sex hormones due to their fundamental role in sex-related differences in cognition and brain function (Toro et al., 2019). AD risk is elevated in postmenopausal women (which is when a reduction in estrogen is experienced) (Toro et al., 2019), suggesting that altered hormone levels may be to blame. Estrogen is a neuroprotective, sex hormone that acts as a neurotransmitter and hormone mediator, synaptogenesis and LTP modulator, and an anti-inflammatory (Cholerton et al., 2002). A study conducted by Shaywitz and colleagues found that estrogen levels affected working memory in post menopausal women (1999); this suggests that estrogen has widespread affects on cognitive function.

Estrogen replacement therapy (ERT) was first proposed to counteract the memory-related risk factors of depleted protective estrogen levels related to post menopause in women. A recent meta-analysis looking at 16 different studies found that ERT improved AD-related outcomes in post menopausal women (Song et al., 2020). This suggests that hormone therapy may be crucial in delaying the progression of AD in female subjects.

Perhaps most importantly, sex differences have been revealed in treatment efficacy in clinical trials (Kim et al., 2015); stressing the importance of investigating these sex differences at the preclinical level.

Current Treatment Methods

Presently, there are no effective treatments for many neurodegenerative disorders (including AD) (Sasaguri et al., 2017). Current drugs approved for the AD treatment include: 1) acetylcholinesterase inhibitors (Aricept, Exelon, Razadyne) and 2) NMDA-antagonist memantine (Namenda) (Fisher, 2008; Karl et al., 2012; Zhao et al., 2020). However, these drugs do not modify disease pathology or progression, they simply treat symptoms of AD (Cooray et al., 2020). Evidently, previous drug treatment methods have failed.

What follows, is a need to establish alternative treatment modalities and methods in order to generate multifaceted treatments for AD and other complex diseases.

Endocannabinoid System

The endocannabinoid system (ECS) is an immense and complex network that is not completely understood. The ECS, a lipid-signaling system, encompasses the cannabinoid (CB) receptors, endogenous ligands, numerous enzymes for the synthesis and degradation of the endocannabinoids, as well as the reuptake transport system (Kruk-Slomka et al., 2017). Studies have shown that the ECS is operational early in brain development and continues to remain active throughout the lifespan (Kruk-Slomka et al., 2017), indicating that the ECS is a critical brain system. The ECS has been thought to be neuromodulatory in nature as it directly engages in many important biological functions (Benito, 2007). Recently, the ECS has been a promising new target for the treatment of various neurodegenerative diseases, including AD (Calina et al., 2020).

The two main targets in the ECS are G-coupled protein receptors: (1) cannabinoid receptor type 1 (CB1) and (2) cannabinoid receptor type 2 (CB2) (Bahr et al., 2006; Gowran et al., 2011; Morena et al., 2014).

CB1 receptors are one of the most abundant G-coupled receptors in the brain and are most concentrated in the HPC, basal ganglia, cortex, striatum, and cerebellum (Bahr et al., 2006; Cassano et al., 2020; Howlett et al., 2004; Karl et al., 2017; Jiang et al., 2005). As the HPC contains one of highest densities of CB1 receptors (Howlett et al., 2004; Mechoulam & Parker, 2013) and is a key brain area for memory, it will be a main area of enquiry in this research project. The concentration of CB1 receptors in the brain is comparable to that of GABA- and glutamate-gated ion channels (Howlett et al., 2004) as well as striatal dopamine receptors (Fowler, 2021). CB1 receptors are mainly located on presynaptic neurons that inhibit neurotransmitter release (one of the main functions of the ECS) (Mechoulam & Parker, 2013). Due to the myriad of CB1 receptors in the CNS, it can be inferred that they play a critical role in overall brain function. Particularly, CB1 receptors play a crucial role in aging-related processes (Nidadavolu et al., 2021; Sarne et al., 2018). A study conducted by Zimmer (2009) showed that a reduction in CB1 receptors was associated with an increased rate of mortality (Zimmer et al., 1999). CB1 receptors primarily engage in functions that help achieve cell maintenance and they play a vital role in memory consolidation (Bahr et al., 2006; Howlett et al., 2004). A study conducted by De Oliveira Alvares and colleagues (2005) showed that intra-hippocampal administration of a CB1 antagonist in male rats impaired memory during an aversive hippocampal-dependent task. Furthermore, intra-hippocampal administration of CB1 agonists, showed an impairment of long-term memory consolidation, however spared acquisition, and short-term memory (Yim et al., 2008). These studies show how short-term and long-term memories are differently mediated via CB1 receptors.

CB2 receptors are mainly located on immune cells (namely, activated microglia) within the CNS and play a crucial role in immune function (Brown, 2007; Cassano et al., 2020; Koppel & Davies, 2008; Maresz et al., 2005). Interestingly, CB2 receptors primarily only appear when there is active inflammation present (Bie et al., 2018). CB2 activation produces a myriad of immune effects, including the reduction of cytokine production, cell migration regulation, cell proliferation inhibition, and downregulation of surface marker expression and cell function impairment (Romero-Sandoval et al., 2008). There is robust evidence suggesting that neuroinflammation may be a key component in the pathology of AD via inflammation-causing cytokines, chemokines, and neurotoxins that are triggered by microglia (Benito et al., 2007; Masliah & Selman, 2008; Perry et al., 2010). Cannabinoids have been shown to be neuroprotective against Aβ-induced activation of microglia (Martín-Moreno et al., 2011). Furthermore, Ehrhart and Colleagues (2005) found that stimulation of CB2 receptors suppressed microglia activation in mice. Supressing microglia activation downregulates harmful, chronic inflammatory processes. This demonstrates the vital role that cannabinoids play in inflammatory control.

The ECS is a promising therapeutic target for AD due to the broad range of functions that are conducted within this system, including memory-related processes and inflammatory mechanisms. The ECS is activated through various cannabinoids found both externally, in nature, and internally, within the body.

Cannabinoids

Cannabinoids are a group of compounds that modulate and act on the cannabinoid receptors (CB1 and CB2) of the ECS and are divided into 3 categories: (1) endogenous (endocannabinoids), (2) synthetic cannabinoids, and (3) phytocannabinoids (Gonçalves et al., 2020; Rom & Persidsky, 2013). *Cannabis* consists of naturally occurring cannabinoids (or phytocannabinoids); the two most well-known and commonly researched are cannabidiol (CBD) and Δ9-tetrahydrocannabinol (THC) (Anderson et al., 2019; Calina et al., 2020). *Cannabis* has been used for thousands of years as it is a naturally occurring plant that encompasses both THC, CBD, and over 100 other cannabinoids (Booth & Bohlmann, 2019; Pellati et al., 2018; Schubert et al., 2019). As a result of the vast number of cannabinoids, the therapeutic potential of *cannabis* is largely unexplored.

THC is the main psychotropic component of *cannabis* and acts as a partial agonist on both CB1 and CB2 receptors (Wang et al., 2022). THC targets the ECS via CB1 receptors and produces psychoactive effects at high doses (typically above 3 mg/kg) (Puighermanal et al., 2009). Interestingly, THC has a biphasic effect, meaning that the effects at a low dose are opposite of those that occur at a high dose (Calabrese & Rubio-Casillas, 2018). Studies have shown that THC, at low doses, encourages HPC neurogenesis (Jiang et al., 2005), inhibits neurodegeneration (Gowran et al., 2011), protects the brain from neuroinflammatory-driven cognitive deterioration (Martín-Moreno et al., 2012), and strengthens both memory and cognition (Bilkei-Gorzo et al., 2017). Neurogenesis is a process where HPC cells are able to generate new neurons (Jiang et al., 2005); this offers a crucial treatment modality to combat neurodegeneration (the opposite of neurogenesis) in AD. As there are large quantities of CB1 receptors in the HPC (Calabrese & Rubio-Casillas, 2018), an area of the brain that is important for episodic memory, CB1 receptors may be crucial targets for diseases affecting memory, such as AD. Interestingly, Jin and colleagues (2004) found that CB1 receptor knockout mice showed significantly less HPC neurogenesis compared to controls. This was further supported by Jiang and colleagues (2005) that found a cannabinoid-induced increase in neurogenesis as a direct result of CB1 receptors. Furthermore, Zimmer and colleagues (1999) found that CB1 knockout mice displayed a higher mortality rate than controls, showing that these receptors seem to play a key role in long-term brain health. These studies demonstrate the importance of CB1 receptors for neurogenesis and cognitive processes in the brain, especially in the HPC. Evidence has shown that low doses of THC encourage HPC neurogenesis, prevents age-related neurodegeneration, and protects against inflammatory-related cognitive deterioration (Calabrese & Rubio-Casillas, 2018). These findings illustrate the exciting potential of low doses of THC against AD pathology.

CBD is a non-competitive negative allosteric modulator of CB1 receptors (Hughes & Herron, 2019), this means that CBD diminishes the effects of CB1 agonists. Interestingly, CBD attenuates the metabolism of THC by preventing its conversion to the more psychoactive form, 11-hydroxy-THC (Costa et al., 2007; Klein et al., 2011; Russo & Guy, 2006; Varvel et al., 2006). CBD is a non-psychotropic cannabinoid that interacts with various receptors and proteins (Wang et al., 2022); specifically, CBD acts as an inverse agonist at CB2 receptors (Watt & Karl, 2018). CB2 receptors play a role in the clearance of Aβ (Aso et al., 2016; Wu et al., 2013), making them an important target for AD treatments. Furthermore, CBD has been shown to significantly reduce neuroinflammation and significantly increase cognitive function (Sánchez-Sarasúa et al., 2020). CBD reduces neuroinflammation via CB2 receptors and results in the downregulation of immune cells, including microglia and astrocytes (Martín-Moreno et al., 2011). CBD treatments have been shown to decrease Aβ-induced inflammation (Esposito et al., 2007) and improve deficits in spatial memory (Martín-Moreno et al., 2011). This demonstrates the importance of decreasing chronic inflammation and the resultant role that inflammatory-reducing cannabinoids may play when treating AD pathology.

THC, CBD, and Terpenoids

Interestingly, THC presents itself as a paradox in terms of cognitive functioning as research has shown that THC, in low concentrations, improves both memory and cognition (Calabrese & Rubio-Casillas, 2018). This means that a low concentration of THC may help save memory and cognitive functions impaired by AD pathology. This is why the dosage of THC, CBD, and terpenoids, as well as the ratio among them must be carefully considered. Using cannabinoids in combination is not a novel concept; Carlini and colleagues (1974) found that using CBD inhibited the negative effects of THC in rodents. Additionally, Watt and colleagues (2020) found that high doses of CBD (50 mg/kg) reduced Aβ accumulation and improved cognition in APP/PS1 mice (Watt et al., 2020). Furthermore, a recent study also found that low doses (0.02 and 0.2 mg/kg) of THC lowered Aβ aggregation and increased spatial learning ability in Aβ-containing mice in a dose-dependent manner (Wang et al., 2022). These studies display the combined beneficial effects of CBD and THC.

CBD has been extensively researched using various dosages and treatment models (Johnson et al., 2010); however, THC is still relatively novel to AD research due to more restrictive drug control. Early research has shown that the presence of CBD inhibits the psychoactive effects of THC (Karniol et al., 1973). This is incredibly useful as one of the main roadblocks of *cannabis* treatments is that it produces undesired psychoactive effects; by incorporating both CBD and THC this can be avoided. Aso and colleagues (2015) also found that combination extracts incorporating terpenoids improved long-term cognitive deficits (Aso et al., 2015).

Terpenoids are the cannabinoids responsible for *cannabis'* unique and characteristic aroma (Gallily et al., 2018; Hanuš and Hol, 2020). Terpenoids are used to treat a wide variety of illnesses and diseases including fungal infections, viruses, cancer, inflammation, parasites, and bacteria (Russo et al, 2011; Hanuš and Hol, 2020; Vieira et al., 2018; Yoo & Park, 2012). Interestingly, terpenoids are the most diverse category of organic compounds found in nature (Yoo & Park, 2012). Terpenoids, when combined with THC and CBD, activate various 'entourage effects' that produce positive changes in the brain. The idea of an 'entourage effect' was first described by Mechoulam and Ben-Shabat in 1998, as being the way in which the ECS responds differently in the presence of 'active components' combined with 'entourage compounds' which result in more naturally occurring effects (Ben-Shabat et al., 1998). This is a classic example of the sum being greater than any isolated, individual component, which is a common theme in scientific findings. Furthermore, Ben-Shabat and colleagues (1998) found that using both 'inactive' metabolites and active primary endogenous cannabinoids increased overall activity. This suggests that increasing the variety of cannabinoids used in treatment composition may boost therapeutic potential. However, the mechanisms underlying the entourage effect remain elusive as they do not occur directly via CB1 or CB2 receptors.

Ultimately, medicinal therapeutic theories argue that all the various compounds within the *cannabis* plant produce enhanced therapeutic effects when used in combination, more so than any isolated, single compound (Berenbaum, 1989; Hanuš and Hol, 2020; Russo, 2009; Russo et al., 2011). As mentioned earlier, the *cannabis* plant consists of hundreds of different compounds (Gallily et al., 2018); this is far too great a number to synthesize all the different varieties of possible extracts from, however incorporating more rather than fewer components may be the key to therapeutic success. Combination *cannabis* extracts seem to exert a more therapeutic effect than using any individual compound solely (Kim et al., 2019; Watt & Karl, 2017). One study found that combination THC + CBD extracts were significantly better at reducing Aβ compared to THC or CBD alone (Aso et al., 2015). Overall, combination *cannabis* extracts that incorporate THC, CBD, and terpenoids may prove to be the most effective treatment option for targeting the various pathologies implicated in AD.

Various methodologies have been used to explore AD treatment efficacy, however transgenic mouse models are one of the leading approaches as they produce insights into memory pathways and molecular changes that would not be possible in human subjects.

APP-NL-G-F/APP-NL Mouse Model

There are over 100 different mouse models presenting various aspects of AD-like pathology, including Aβ and NFT accumulation, inflammation, and/or neurodegeneration (Jankowsky & Zheng, 2017). One roadblock in these models is that these pathologies are generally not all seen in one single mouse model alone (Jankowsky & Zheng, 2017; Webster et al., 2014). However, as these pathologies emerge at different time points in the progression of AD, this allows for investigation of various stages in AD development; this means that later progressions of the disease may be prevented. Aβ pathology was of particular interest in this research project as it represents the early manifestation of the disease, thus allowing for optimal intervention and prevention. Whereas NFT-related pathology presents itself later in the progression of AD, often after it is too late to remedy. It remains crucial to slow down the progression and development of AD as this could be the difference between losing memories sooner rather than later.

APP models, displaying Aβ-related pathology solely, are crucial to employ when analyzing AD as the pathology is less aggressive, allowing for longer and more varied behavioural testing paradigms. APP models are also considered to be ‘preclinical’ models of AD as they do not display more advanced AD pathology, such as tau (Sakakibara et al., 2019; Sasaguri et al., 2017). This is beneficial as it allows for intervention early in the progression and development of AD.

First-generation APP mouse models were known to overexpress APP, leading to a misrepresentation of AD-pathology (Mehla et al., 2019; Sasaguri et al., 2017; Whyte et al., 2018). Second-generation models, which use knock-in methods, were able to circumvent this issue. Another benefit of knock-in models is the ability to produce relevant controls that were unable to be produced with first generation models (Sasaguri et al., 2017). This means that more accurate comparisons can be drawn between the experimental and control groups.

The APP-NL-G-F and APP-NL knock-in mouse models are commonly used in AD research to overcome the previous issue of overexpressed APP (Mehla et al., 2019; Sasaguri et al., 2017; Whyte et al., 2018). APP-NL-G-F mice display AD pathology including, Aβ plaques and neuroinflammation throughout the brain, whereas APP-NL mice do not (Sakakibara et al., 2019). In a study conducted by Whyte and colleagues, they showed no evidence of memory deficits in APP-NL-G-F or APP-NL mice on memory-based tasks at 6 months of age (Whyte et al., 2018); suggesting that a testing time point after 6 months is more appropriate. Furthermore, a study conducted by Mehla and colleagues found that impairments begin to develop at 12 months of age in APP-NL-G-F mice (Mehla et al., 2019). This reinforces the idea that behavioural testing should be initiated between 6-12 months of age to see memory-related deficits related to AD pathology. APP-NL control mice have been shown to display no pathology or cognitive deficits related to AD (Sasaguri et al., 2017). In summary, the knock-in APP-NL-G-F mouse model displays key pathologies related to the initial stages of AD.

APP-NL-G-F and APP-NL subjects will help demonstrate how *cannabis* extracts may reduce memory impairments, Aβ accumulation, and neuroinflammation in AD by investigating early progression and development.

Purpose of the Present Study

The purpose of the present study is to investigate if combination cannabinoid extracts can help mitigate disease pathology and memory impairments related to AD.

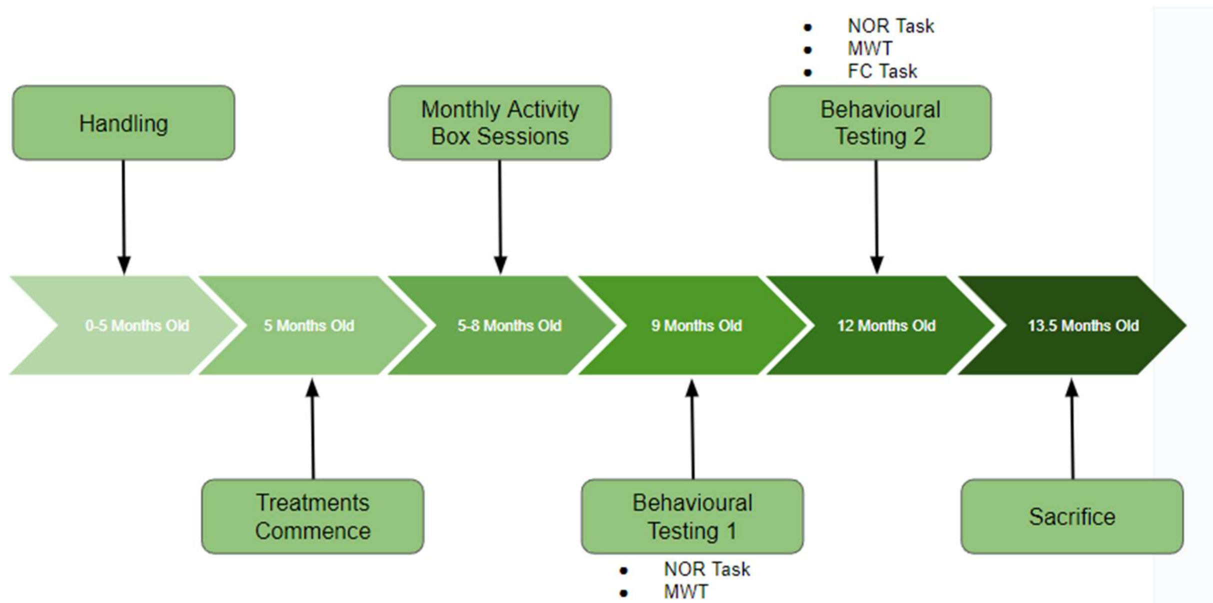
Hypothesis: A combination *cannabis* extract (20:1 mg/kg CBD:THC) should reduce pathology and memory impairments related to AD in APP-NL-G-F transgenic mice.

Our lab has recently investigated treatments of THC (0.2, 0.5, or 1 mg/kg) or CBD (5, 10, or 20 mg/kg) alone on APP-NL-G-F mice and found no treatment-specific effect on measures assessing memory-related behavioural tasks (namely, the NOR task, FC task, and MWT), Aβ-load (4G8/82E1-stained), or inflammation (GFAP/Iba1) (Nixon, 2022).

We are now combining these two cannabinoid compounds and incorporating various terpenoids to potentially boost the therapeutic potential of these cannabinoids. The present study will also incorporate longer treatment times to ensure that early pathological development of the disease is treated. In a recent study assessing aged mice (18 months of age), we showed improved performance on memory-based tests compared to vehicle-treated counterparts (unpublished).

This shows that *cannabis* extracts may be effective in ameliorating age-related cognitive impairments as well as those found in AD.

The purpose of the present study is to investigate the effectiveness of combination *cannabis* extracts at treating memory-related impairments and pathological symptoms of AD. Past studies in our lab have shown that CBD (20mg/kg) and THC (1mg/kg) have shown little or no effects when used separately. The entourage effect offers promise in the therapeutic potential of combining cannabinoids to maximize therapeutic effect. Overall, this study seeks to investigate if a later, longer dose of *cannabis* will enhance therapeutic effects.



Methodology

Subjects

Eighty homozygous amyloid-β precursor protein (APP) knock-in positive (APP-NL-G-F, n= 40) and negative (APP-NL, n= 40) mice weighing 18-45g were obtained from the University of Lethbridge and housed in a temperature-controlled (20–22°C) vivarium, with a 12h/12h light/dark cycle. Food and water were available *ad libitum* in their housing cages. The mice were housed singly or in groups of 2-5 per cage. The eight experimental groups consisted of positive extract-treated females (n=10), positive extract-treated males (n=10), positive vehicle-treated females (n=10), positive vehicle-treated males (n=10), negative extract-treated females (n=10), negative extract-treated males (n=10), negative vehicle-treated females (n=10), and negative vehicle-treated males (n=10). It should be noted that 2 subjects from each experimental group were used for alternative analysis for the pathology portion of the data, reducing each experimental group to n=8 for pathological analysis.

Apparatus

A *cannabis* extract of THC (1 mg/kg), CBD (20 mg/kg), and terpenoids (1.6 mg/kg) were administered orally daily starting at 5 months of age until sacrifice at 13.5 months of age, for a total of 8 months. Treatments were administered at the same time daily. All treatments were administered by a female experimenter.

Oral administration via an oil extract allows for accurate and reliable dosing, as it is difficult to pinpoint an accurate dosage using inhalation. However, oral administration of cannabinoids does prove to be slower and more erratic due to their lipid soluble nature (Russo et al., 2008). Oral ingestion of cannabinoids typically results in a slower absorption time and lower, more-delayed THC concentration peaks. However, this also means that the effects of orally administered cannabinoids linger longer in the body. For this reason, we can provide daily doses that will remain in the system for up to 20 hours (Bruni et al., 2020). Furthermore, human trials would likely support an oral administration or oromucosal administration of treatment over intraperitoneal injections or inhalation.

Dosing Paradigm

The mice were weighed every other day to ensure that corresponding dosages remained consistent and accurate. The *cannabis* extract was prepared by combining 10 g of extract #81 in ethanol with 3 g of #81 in ethyl-acetate in grapeseed oil. This was thoroughly vortexed several times daily over 5 days and left in a shake and bake oven overnight. Once dissolved, the final volume of grapeseed oil was added and left to mix.

One week prior to treatments being initiated at 5 months of age, mice were exposed to Nutella in a weigh boat within their housing cages to become accustomed to the taste. They were given 0.25 g/mouse. One day prior to treatments being initiated, the mice were placed in the treatment cages for 1 hour with Nutella (void of any extract or vehicle). The mice that more readily ate the Nutella were the mice selected to receive the *cannabis* extract treatment. For the first 21 days, the mice were placed on food restriction for 4 hours prior to treatments to increase their motivation to eat the treatments. For the first 21 days, the mice were also left in their treatment cages for 90 minutes (this slowly decreased to 15 minutes as they became faster at eating the treatments). Due to the relatively long duration spent in the treatment cages, the mice also had water bottles in the cages until the time spent in the treatment cages decreased to less than 40 minutes. For the first 28 days, the mice also received 0.4 g of Nutella (to better mask the strong taste/odour of the *cannabis* extract); this slowly decreased to 0.25 g as the mice more readily ate the treatments. All mice received the same amount of Nutella to maintain consistency.

Both the *cannabis* extract and grapeseed oil vehicle remained at refrigerator temperature (4 °C) prior to being administered, then it was kept on ice until it was pipetted. Mass stores of both the *cannabis* extract and grapeseed oil vehicle were kept at freezer temperature (-18 °C) prior to being moved to the refrigerator the day prior to use to thaw.

The *cannabis* extract and vehicle were prepared by adding their weight ratio (g: ul) to 0.25 g of Nutella. The Nutella was placed on a halved plastic weigh boat using a wooden tongue depressor and a wooden stir rod that was replaced daily. Then the wooden stir rod was used to mix the extract or vehicle in with the Nutella, until the oily texture was gone. To limit cross contamination, the vehicles were pipetted and stirred prior to the extracts. Each mouse had their own personal weigh boat that was marked clearly with their animal number and treatment type (vehicle or extract). These weigh boats were replaced weekly. In between treatments, the weigh boats were stored on a plastic tray (one for the vehicles and one for the extracts). Following preparation, the treatment weigh boats were placed in an empty housing cage. Each mouse was placed in its own corresponding treatment cage. The same cages were used each day for treatments. The mice were left in the treatment cages for 15 minutes to allow ample time to finish the treatments.

Following treatments, the mice were removed from the treatment cages and returned to their housing cages. The order in which they were removed was reversed every other day to account for the time spent cleaning each cage at the end of treatments. Each treatment cage was cleaned following the mouse being removed. Each treatment weigh boat was cleaned using a fresh paper towel then wiped with a warm, damp cloth before being placed back on the corresponding tray. A separate cloth was used for extract and vehicle weigh boats to limit cross contamination and the cloth was replaced daily.

The mice began receiving treatments at 5 months of age and continued until sacrifice. They were sacrificed once they began to show memory deficits on various memory-based behavioural tasks. Namely, the Morris Water Task, which assesses spatial memory through the employment of the HPC, a key target area for THC. Previous work in our lab showed no genotype-related differences at 9 months of age, which is why we began testing at this time point rather than earlier.

Cytology

To account for the rapidly changing estrous cycle of mice, vaginal lavage was completed for two weeks prior to behavioural testing. The estrous cycle in mice occurs in four successive stages (proestrus, estrus, metestrus, and diestrus) (figure 1) and repeats every 4-5 days (Byers et al., 2012; Caligioni, 2009). Interestingly, mice reach a period of post estrous around 12 months of age (Brinton, 2012). This may hint at correlations between menopause and the progression of AD. Furthermore, estrogen, which fluctuates throughout the estrous/menstrual cycle has been found to play a significant role in neuromodulation and neuroprotection, including anti-inflammatory actions, protection against cell apoptosis, and the regulation of neurotransmitters and hormones. Studies have found that estradiol, an estrogen steroid hormone, levels peak during the proestrus phase in mice (Lovick & Zangrossi, 2021). This suggests that proestrus is an essential estrus phase for receiving the protective effects of estrogen. The main purpose of observing the estrous cycle is to confirm that the drug treatments are not influencing the natural estrous cycle of the mice.

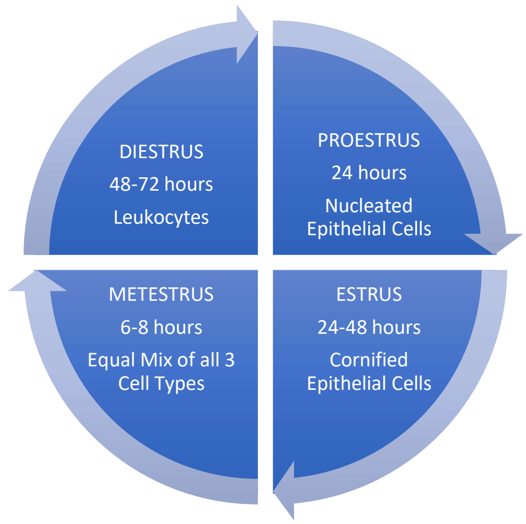


Figure 1. Estrous cycle in mice. Visual representation of estrous cycle stage, stage duration, and primary cell type present. The size of each quadrant does not correlate to the length of each stage.

Vaginal lavage was conducted on females 14 days prior to the start of behavioral testing to study the effects of *cannabis* treatments on estrous cycle. Females were scruffed and 50ul of distilled water was gently pipetted in and out of the vaginal canal to obtain a vaginal cell sample. Each sample was placed on a Superfrost plus microscope slide corresponding to the correct mouse number and allowed to dry for 24 hours before being placed in a microscope slide box. Males were scruffed and gently poked in the abdomen to simulate a similar experience.

Cytology was conducted using a Zeiss Axioskop-2 Mot Plus Upright Fluorescence Microscope with an objective of 20x. As previously mentioned, there are specific cells that show a majority representation based on the stage underwent while the samples were collected. A trained experimenter, who was blind to the experimental groups, manually observed which cell type was most present in each sample to deduce the estrus stage present in each sample.

Behavioural Tests

Activity Boxes

Exploratory behaviours were recorded using open field locomotor activity, a standard measurement of activity levels in mice (Gould et al., 2009). The mice were individually placed in AccuScan activity monitoring Plexiglas boxes (42 cm x 42 cm x 30 cm) and recorded using VersaDatTM software (AccuScan Instruments Inc., OH, USA). The mice were run on activity boxes monthly from 5 months of age (once they commenced treatments) until they began behavioural testing at 9 months of age. Each mouse underwent a total of 4 activity box sessions. The mice were placed in the activity boxes for 10 minutes and allowed to roam freely. The experimenter left the room during the trials to eliminate any distractions or additional sound. To ensure that the treatments were no longer in their system, the mice were run on the activity box 24 hours after being treated the previous day.

Novel Object Recognition (NOR) Task

Berlyne (1950) first observed that rodents naturally spend more time exploring novel objects compared to familiar ones (Berlyne, 1950). The novel object recognition (NOR) task utilizes spontaneous exploratory behaviour in rodents, which is their natural proclivity to assess objects unfamiliar to them. This task is particularly attractive as it requires no reward or punishment, as it is a purely observation-based task. This behavioural task evaluates recognition memory and is modulated by the PRC in the brain (Antunes & Biala, 2012). The PRC has been implicated in AD pathology as human MRI studies have shown a significant decrease in volume compared to healthy controls (Juottonen et al., 1998). Furthermore, PRC removal has been shown to impair recognition memory in rodents (Brown & Aggleton, 2001). This suggests that we should see impairments in PRC function with the onset of AD.

Object recognition, which utilizes recognition memory, can be determined by comparing the amount of time that the mice spend assessing each object. The parameters being that the mouse must be within 1 cm of the object, they cannot have their paws on the object, and they cannot be sitting on top of the object. Each object was placed an equal distance from the wall and the objects were across from one another diagonally (see figure 2). The NOR task has been used to evaluate memory in a variety of mouse models, including APP-NL-G-F (Grayson et al., 2015). A study conducted by Cheng et al., showed that a chronic dose of 20 mg/kg CBD improved object recognition impairments in APP/PS1 mice (2014). This suggests that the extract may improve memory recognition deficits compared to vehicle-treated subjects.

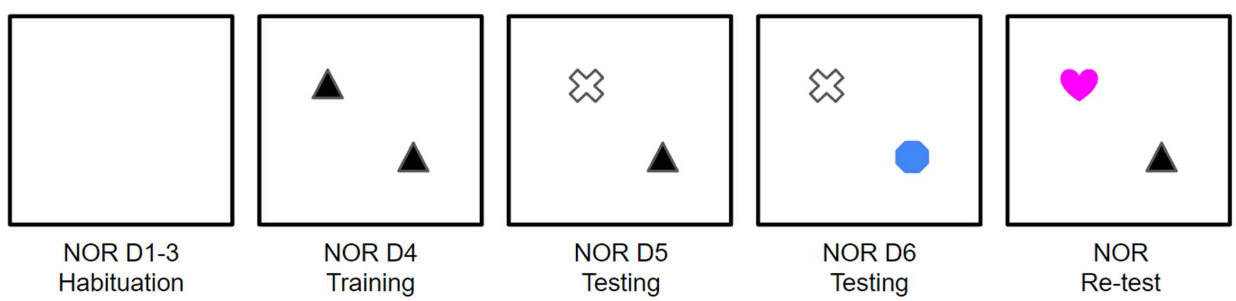


Figure 2. A diagram of each stage of the NOR task. Objects are not shown to scale.

Object exposure was counterbalanced to eliminate any potential bias or arena side/object preference. On the first training day, the mice were either exposed to A + A or B + B configurations, then on the second testing day they were either exposed to LA RB or LB RA (see Figure 3 below). Each configuration group was assigned randomly and had an equal number of subjects from each experimental group.

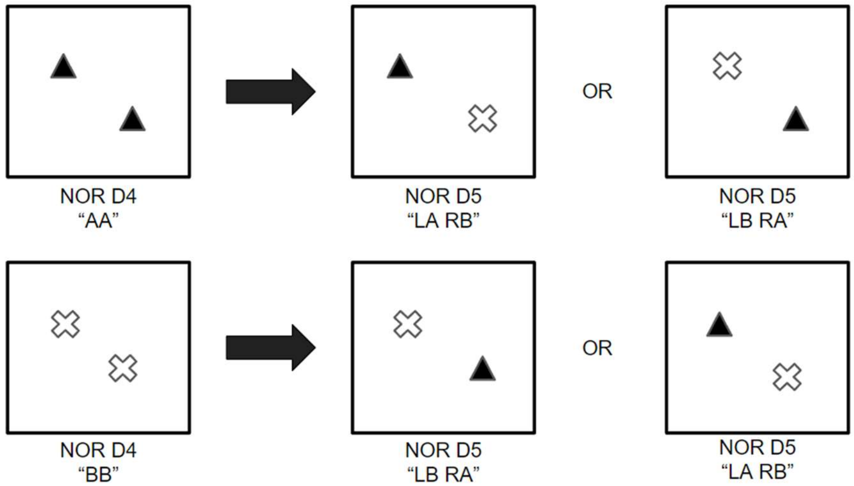


Figure 3. Object configurations in the NOR task.

Mice were placed facing the wall of the front right corner of an empty arena (58 cm x 58 cm x 38 cm) to initiate each trial. The arena consisted of a white tub with ~1 cm of bedding lining the entirety of the floor. The bedding was changed out between male and female trials; however, the same bedding was used for the entire initial 6 days of testing (to ensure that the environment was familiar to the mice). *Habituation:* The mice underwent habituation for a duration of 10 minutes, daily for 3 days. The first day of habituation was recorded and scored using an open field program to measure anxiety. *Training:* On day 4, the mice were exposed to 2 identical objects that were placed an equal distance from the walls on opposite sides of the arena for 10 minutes. *Testing:* On day 5, the mice experienced a 5-minute exposure to one object from the previous day (day 4) and one completely new object placed in the same orientation as the objects from the previous day. On day 6, the mice experienced a 5-minute exposure to the novel object from the previous day (day 5) and one completely new object that were also placed in the same orientation as the objects from the previous day. Please note, the objects that were used from the previous day were placed in the same location that they had previously been in. Objects were taped to the bottom of the tub using surgical tape to prevent them from falling over. The tape was rolled and out of the reach/view of the mice to avoid distractive picking. The males were run first on the task, then the bedding in the arena was switched out and the arena was cleaned using 2-3 sprays of isopropyl alcohol to limit scent transfer. This task was conducted at 9 and 12 months of age. Completely new and different objects were used for each round of testing. *One-month re-test:* Four weeks following the last habituation day during the 12-month testing period, the mice were re-tested on the novel object recognition task with one object from day 4 and one completely novel object. The mice were placed in the exact same arena with the exact same orientation of the objects for a single 5-minute trial. The re-test was used to investigate long-term recognition memory function.

Open Field Task

The open field task (OFT) is commonly used to examine exploratory behaviour (Gould et al., 2009) and anxiety-like behaviour (Latif-Hernandez et al., 2019) in rodents. In this project, exploratory behaviour and anxiety was measured by analyzing how much time the mice spent in the periphery of the arena, as more anxious mice tend to stick closer to the walls of a confined space.

The OFT was performed on day 1 of the NOR task (during the habituation period). The subjects were recorded, and the video files were processed using Noldus EthoVision XT version 16 software to track the movement of the mice over the 10-minute testing period. The software provided data on how long the mice spent in the 'outer rim' of the arena, this was defined as 1) when the nose of the subjects was in the outer periphery of the arena and 2) the subjects were not climbing on the walls of the arena.

Balance Beam

The balance beam task is used to assess motor ability in rodents, to ensure that any behavioural impairments found are due to memory deficits and not motor problems (Carter et al., 2001; Luong et al., 2011). The balance beam task utilizes an 80 cm pole that is suspended by two tripods at either end at a height of 50 cm. There are black tape rungs marked off in 10 cm intervals down the length of the pole. The mice are initially exposed to the tube that is at one end of the pole, to eliminate any fear associated with entering the escape tube. During training, the mice are then set on the pole at 10 cm and must make their way to the other end of the pole, the interval is subsequently increased to 50 cm, then lastly, to 80 cm. During the training day, the mice were caught by the experimenter to limit stress associated with falling. The mice received one day of training at the various intervals, then on the testing day, they were video recorded and ran for 3 trials from the 80 cm interval. To remove the mouse from the tube, they were gently prodded using a rolled-up bunch of paper towel.

Mice displaying a strong motor ability should be able to traverse the beam relatively easily. However, it should be noted that male mice exceeding 40 g did present a difficulty with balancing on the beam (due to their mass). The main purpose of this task is to ensure that any impairments found in behavioural tasks are due to memory deficits and not poor motor skills.

The mice were tested at both 9 and 12 months of age, 2 days following testing day 6 of the NOR task.

Morris Water Task (MWT)

The Morris Water Task (MWT) was first developed by Richard Morris in the early 1980s and, to this day, remains one of the most common tasks for assessing spatial learning and memory in rodents (Barnhart et al., 2015; Sakakibara et al., 2019; Sutherland et al., 1982). Spatial memory arises when animals (including humans) acquire map-like mental representations of their environment in order to navigate and learn where objects are in space (O'Keefe & Nadel, 1978). This is what allows us to orient ourselves in space and was likely first used to navigate toward a food source and/or away from harm. MWT-based lesion studies remain crucial ways to investigate and understand the role of the HPC (and other brain structures) in spatial memory. Morris and others demonstrated that rodents with HPC lesions could not perform this task as well as those with intact HPC (Morris et al., 1982; Morris, 1984; Sutherland et al., 1982; Sutherland et al., 1983). These studies were some of the first to demonstrate the crucial role of the HPC in spatial memory. Astur and colleagues went on to investigate this using a virtual MWT with human subjects that received unilateral HPC removals for epilepsy treatment and found significant impairments in spatial memory (Astur et al., 2002). This demonstrates the applicability of various animal models in the assessment of brain systems.

The HPC is one of the first areas of insult in the progression of AD (Götz et al., 2018). Therefore, it is extremely useful to investigate if this brain region can be saved using *cannabis* treatments. A previous study within our lab showed that APP-NL-G-F mice display significant impairments in acquisition during the training portion of the MWT (Mehla et al., 2019). This mouse model is expected to begin to show spatial memory deficits at roughly 12 months due to the accumulation of Aβ and resultant HPC deterioration (Mehla et al., 2019).

Previous studies have shown improved performance on the MWT in subjects treated with a low dose of THC. For example, in a study conducted by Sarne and colleagues they found that an ultra-low intraperitoneal (IP) injection of THC (0.002 mg/kg) significantly improved performance on the MWT in 24-month-old, aged mice (2018). Similarly, subcutaneous injections of THC (1 mg/kg) improved measures of spatial memory on the MWT in 18-month-old, aged mice (Nidadavolu et al., 2021). Furthermore, 3-month chronic THC (0.02 and 0.2 mg/kg) IP injections improved spatial memory performance in 14-month-old, aged APP/PS1 mice in a dose-dependent fashion (Wang et al., 2022). Additionally, as CBD is known to attenuate THC-mediated impairments in spatial memory (Calabrese & Rubio-Casillas, 2018); it can be expected that combination *cannabis* extracts (containing low THC and high CBD) will help improve spatial memory deficits. Ultimately, the ECS likely plays a key role in spatial memory due to the predominance of endocannabinoid receptors in the HPC.

In the MWT task, the mice are trained for an 8-day period on the location of a hidden platform located under the surface of opaque water. There are various visual cues located around the pool that the subjects use to learn the location of the platform. Over time, subjects with functioning spatial navigation abilities become faster at locating the platform. Ultimately, latency to reach the escape platform should decrease as a function of day as the mice learn where the platform is located. Therefore, mice with extensive damage to their HPC should show impairments in task acquisition.

The MWT consisted of a pool 154 cm in diameter divided into four quadrants (NW, SW, NE, and SE) and filled to a depth of 40 cm with water. The water was kept at a consistent temperature between 20-22°C. White paint was added to the pool to obscure the view of the platform, which had a surface 1 cm below the surface of the water. The Plexiglas, circular platform had a radius of 11 cm. Three distinctive visual cues (displaying a black square, triangle, and plus-sign on white, cardboard backgrounds) were placed around the edge of the pool, approximately 1 foot above the rim, and remained fixed in place throughout all testing days. The mice were individually housed in empty cages (with a single piece of paper towel) in-between testing. They were placed in their testing cages in the MWT testing room 30 minutes prior to testing to decrease any stress associated with a novel location.

Training: The mice were placed into the pool and had to navigate to the escape platform in order to be removed. If a mouse failed to locate the platform, then they are picked up and placed on the platform for 10 seconds prior to being removed. If the mouse did not stay on the platform, then they are held in place by the base of their tail. The mice were placed in the pool facing the wall of the pool and underwent four 60-second training trials per day from each cardinal direction (North, East, South, and West) in a randomized order for eight days of testing. *Testing:* On day 9, the platform was removed from the pool and the mice were run on one 60s trial from the same start point. Following training on day 9, the mice were re-trained with the platform in the pool for 4 trials from each direction (similar to the previous training days). At 9 months, the mice were not impaired at this task and did not undergo a retention probe trial one week following day 9 of testing. The mice were tested again at 12 months using a different platform location. At 12 months, the mice were impaired on this task and underwent another testing trial one week following day 9. *One-week retention probe test:* During the retention probe, the platform was removed from the pool and the mice were run on one 30s trial from the same start point. *Visible platform test:* Following the one-week retention probe trial, the platform was returned to the pool at a location in an adjacent quadrant and the mice were able to train in the pool using a visible platform. The platform was made visible by sticking a 10 cc/mL syringe covered in visible, black tape into the top of the platform so that it stuck out 10 cm from above the surface of the water. The mice were run on 4 trials from each direction (North, East, South, and West) to assess their visual skills from all start points. The purpose of the visible platform trials is to ensure that the mice still have adequate vision at the end of the MWT. The mice were run in groups of 10-15 mice. All cohorts were tested by the same experimenter.

The mice were tracked in the pool using a HVS software 28 package. The subjects were tested at both 9 and 12 months of age, however they only underwent the 1-week retention probe and visible platform testing at 12 months of age. They were tested 2 days following the conclusion of the balance beam task. The mice were trained to a different location in both rounds of testing to eliminate any bias of the previously trained location.

Fear Conditioning (FC) Task

The Fear Conditioning (FC) task is used to assess associative learning and memory, which is the process of learning and remembering relationships between previously unrelated concepts (Pavlov, 1927). This task is from the same vein as classical conditioning, wherein the subjects are exposed to a tone then shortly thereafter experiences a foot shock. Soon, the subject will begin to associate this tone with a foot shock. In this paradigm there is another factor added in the form of a context cue. As the subject always receives a shock in the same context, they will also begin to associate the context itself with the foot shock. When the context is changed and the tone is heard, they should also associate this with the experience of the foot shock. This demonstrates that both the context and the tone are important cues in the aversive memory of receiving the foot shock.

The FC task investigates both HPC- and AMG-dependent associative learning (Cheng et al., 2014; Cortez et al., 2017; Mehla et al., 2019). The AMG plays a crucial role in associative learning and memory, as it provides emotional context (LeDoux, 1993). In this case, in learning to have negative associations toward the tone and the context. The HPC also plays a role in this process as it is the brain region responsible for discriminating between the two contexts, the 'dangerous' context and the 'safe' one. The 'dangerous' context is deemed dangerous because the subjects were exposed to a foot shock stimulus while in the context, whereas the 'safe' context is deemed so as the subjects had not previously been exposed to a shock stimulus while in the context. This is why the 'safe' context is initially deemed as safe (deduced through the absence of freezing behaviours prior to the first tone interval). Whereas the 'dangerous' context is recognized as being unsafe (deduced through the presence of freezing behaviour right away after being placed in the context during testing). In an influential paper by Phillips & LeDoux (1992), they found that AMG lesions lead to impairments in conditioning to both the cue and context stimulus, whereas HPC lesions lead to impairments in context conditioning but not cue conditioning. As the HPC undergoes significant degradation throughout AD (Götz et al., 2018), it can be expected that impairments in context conditioning would align with AD progression.

In this task, mice were placed facing the wall of the front right corner of a square context (33 cm x 33 cm x 25 cm) with stainless-steel bar flooring attached to an exterior shock generator to initiate each trial. *Exposure:* On testing day 1, the mice were exposed to a 20 s 5 kHz tone every two minutes and during the last 2 s of the tone they experience a 0.5 mA foot shock. This process repeats for a total of 12 minutes as they experience five tone/shock intervals. *Context Recall:* On day 2, the mice are placed in the same 'dangerous' context they were shocked in for 5 minutes, however the shock generator was not attached, and they did not experience any foot shocks. *Tone recall:* On day 3, the mice are placed in a 'safe' novel, white, triangular context (33 cm x 33 cm x 29 cm) in a new testing room. After 2 minutes of being in this context they are exposed to the same 20 s 5 kHz tone that they experienced on day 1. They experience this tone 3 times throughout the course of testing (2 minutes apart) for a total of 7 minutes. During all testing days the chambers were covered in a Plexiglas sheet to limit scent stimuli and prevent the mice from escaping. All contexts were cleaned with Virkon in between trials to limit fear-based pheromone transfer. Testing days 1-3 were video recorded to be analyzed for freezing behaviour.

The mice were observed for freezing behaviours during the first 2 minutes of the day 1 testing (prior to receiving a shock), to establish a baseline freezing duration. This was 0 s across all subjects. This also reinforces the idea that freezing behaviour is a fear-associated response and therefore the subjects seem to be absent of fear/anxiety prior to receiving a foot shock.

The mice with functioning memory systems (including HPC and AMG) should be able to associate the context and tone with the negative shock stimulus and display a distinct freezing behaviour. During this behaviour, the mice will be completely rigid and only breathing-related movements can be observed. Mice with intact memory systems should display an increase in freezing behaviour on day 2 in the context they were shocked in, as well as during the presentation of the tone on day 3.

The mice only underwent FC testing at 12 months of age to ethically limit the number of aversive experiences. The testing was conducted in the 3 days prior to sacrifice. For all behavioural tasks, the mice were run in the same order each day to ensure that they were run on the tasks daily at the same time. The mice were run individually and held in separate rooms prior to and immediately following testing. All behavioural tests were run at least 12 hours post-treatment to ensure that acute drug effects did not influence behaviour on tasks. Furthermore, all behavioural tests were run in the first 6 h of the light phase of the light-dark cycle to limit the effect of the natural circadian rhythm on performance (Cheng et al., 2014; Coles et al., 2020). There was also a 48-h interval of rest between all behavioural tests (NOR, Motor, MWT, and FC) to allow the mice to recover and to limit testing fatigue.

Perfusions

Following the FC task, the mice were perfused at 13.5 months of age. The mice were anesthetized using isoflurane and intraperitoneally injected with 0.06-0.1 ml of Pentobarbital Sodium, then perfused using 50 ml of 1x Phosphate-Buffered Saline (PBS) solution followed immediately by 50 ml of 4% Paraformaldehyde (PFA) solution that was made from powdered PFA. Following perfusions, the heads were removed using scissors and the brains were extracted. Then, the brains were suspended in a 4% PFA solution. 24 hours following perfusions, the brains were moved from a 4% PFA solution to a 30% sucrose solution until they were sliced.

Tissue Collection

The mice were anesthetized using isoflurane then their head was removed using a guillotine. The spleen, colon, liver, kidneys, adipose fat, lungs, heart, adrenal glands, and testes or ovaries were collected. The brain was extracted and sectioned into the PFC, HPC, olfactory bulbs, neocortex, striatum, motor cortex, and brain debris.

Brain Preparation

Brains were sliced to 40 um coronal sections using an AO instrument company microtome. During slicing, samples were kept frozen using dry ice that was continually replenished. Brains were sliced using a 1 in 9 series. The brain slices were kept in PBS + 0.3% sodium azide until wet mounted prior to staining. The brain slices were wet mounted using a fine tip paint brush onto Superfrost plus microscope slides.

Histology

Nissl Staining

The histological method of Nissl staining was invented by Franz Nissl in 1894 (Nissl, 1894). This technique is widely used to study cellular morphology and brain pathology (Kádár et al., 2009). The Cresyl violet dye binds to RNA and proteins found in high concentrations in the rough endoplasmic reticulum and ribosomes (also known as Nissl substance) in the cytoplasm of cells (Kádár et al., 2009). In this project, Nissl staining is used to visualize HPC cells to quantify density.

Cresyl Violet Nissl Staining Protocol

Free floating brain sections were mounted, air-dried, and stained using a Cresyl Violet staining technique. Tissue samples were first soaked in a 1:1 100% Ethanol: Chloroform for 3 hours. Following this, the samples were stained using the following procedure: (1) 100% ethanol for 10 min, (2) 95% ethanol for 5 min, (3) 70% ethanol for 5 min, (4) RO Water for 5 min, (5) 0.5% Cresyl Violet solution for 3.5 min, (6) RO water for 0.5 min, (7) RO water for 0.5 min, (8) de-stain with a few drops of acetic acid in 70% Ethanol for 2 min, (9) 95% Ethanol for 2 min, (10) 100% Ethanol for 5 min, (11) 100% Ethanol for 5 min, (12) Hemo-De for 5 min, (13) Hemo-De for 5 min. Immediately following this procedure the slides are cover slipped with Permount. Cell counts were taken by a trained student who was blind to the experimental groups.

HPC volume was quantified using light microscopy with a 10x objective. StereoInvestigator software was paired with the Cavalieri method to estimate total HPC volume of CA1, CA3, dentate gyrus, and combined regions using an 80-grid size.

Immunohistochemistry

Immunohistochemistry (IHC) employs protein identification to confirm the physical presence and severity of AD.

APP (82E1/4G8) IHC

APP is visualized using immunohistochemical techniques. During this process antibodies are used to recognize specific amino acid sequences (Aho et al., 2010). This means that the antibody is unable to recognize the entire gene sequence but can recognize specific, unique portions to confirm the presence of genes, in this case the APP gene. This research utilizes both 82E1 and 4G8 antibodies to detect APP gene segments aa 1-16 and aa 18-22, respectively.

To visualize Aβ plaques in the brain, 82E1 and 4G8 antibodies were used. 82E1 and 4G8 are antibodies that recognize specific segments of the Aβ protein. The Aβ protein consists of 40-43 amino acids and through cleavage produces transmembrane APP (Aho et al., 2010). 82E1 stains a specific segment (aa 1-16), whereas 4G8 stains segment aa 18-22. Both segments are utilized to confirm the presence of Aβ plaques in subjects. APP-NL-G-F subjects will display this pathology whereas APP-NL subjects will not.

Inflammation (Iba1/GFAP) IHC

It is also expected that neuroinflammation will occur around Aβ plaques. There are various proteins that are specific to microglia and astrocytes; two most common markers to detect these proteins in tissue are Ionized calcium-binding adapter molecule 1 (Iba1) and Glial fibrillary acidic protein (GFAP), respectively. These proteins are extensively used as IHC markers for microglia and astrocytes in nonhuman animal studies (Ahmed et al., 2007; Casarejos et al., 2013). It should be noted that Iba1 and GFAP staining represents the staining of populations of microglia and astrocytes, respectively. Iba1 is an actin-binding protein found on the membrane cytoskeleton of microglia (Ahmed et al., 2007). Whereas GFAP is found in the intermediate filament of the astrocyte cytoskeleton (Garland et al., 2022). Thus, allowing for visualization of the entire astrocyte structure. Studies have shown that GFAP levels appear to increase with age in both rodents and humans (Garwood et al., 2017). This hints that increased inflammation is a natural consequence of aging, however AD pathology may be exacerbating this. Ultimately, Iba1 and GFAP are used to visualize neuroinflammation in the brain.

DAPI is used to stain nuclei and visualize morphology in brain tissue (Whyte et al., 2018; Wyss-Coray et al., 2003), although DAPI was not quantified in this project.

82E1 + Iba1 + DAPI Staining Protocol

For the IHC stain assessing 82E1 (Aβ), Iba1 inflammatory markers, and DAPI (for brain morphology), coronal brain sections (40 um) were mounted on Superfrost *plus* microscope slides and fixed for 4min in 4% paraformaldehyde, washed twice in 1X Tris-Buffered Saline (TBS), and washed in 70% formic acid until the tissue begin to curl (approximately 20-30 minutes), then washed twice more in 1X TBS. Following this, the tissue was washed in TBS-A (TBS + 0.1% Triton X) for 15 minutes, and TBS-B (TBS-A + 25% Bovine Serum Albumin, BSA) for 30 minutes. Following this the tissue was incubated with the primaries consisting of Anti-82E1 (Anti-β-amyloid (N), IBL,10323, mouse) 1:1000 and Anti-Iba1 (Rabbit, 019-19741, Wako) 1:1000 (both stored at -20°C) for 45 hours in a dark, humid chamber. The tissue was then washed twice in 1X TBS, once in TBS-A, and once with TBS-B. The tissue was then incubated with the secondaries consisting of anti-mouse-alexa-488 (IgG (H+ L) goat, Abcam, ab150113) 1:1000 and anti-rabbit-alexa-594 (IgG (H+L) goat, Invitrogen, A11037) 1:1000 (both stored at 4°C) for 24 hours in a dark, humid chamber. The tissue was then washed three times with 1X TBS.

Following this, the tissue was cover slipped using DAPI 1:2000 and placed in a dark, humid chamber for 1 hour. The tissue was then washed once more with 1X TBS and cover slipped using 2 drops of Vectashield (Vector Labs, H-1000). After 24 hours the slides were sealed using nail polish and stored at 4°C until analyzed.

Sections were imaged using a Nanozoomer microscope (Nanozoomer 2.0-RS, HAMAMATSU, JAPAN) at 40x resolution.

4G8 + GFAP + DAPI Staining Protocol

For the immunohistochemical stain assessing 4G8 (Aβ), GFAP inflammatory markers, and DAPI (for brain morphology), coronal brain sections (40 um) were mounted on Superfrost *plus* microscope slides and fixed for 4min in 4% paraformaldehyde, washed twice in 1X Tris-Buffered Saline (TBS), and washed in 70% formic acid until the tissue begin to curl (approximately 20-30 minutes), then washed twice more in 1X TBS. Following this, the tissue was washed in TBS-A (TBS + 0.1% Triton X) for 15 minutes, and TBS-B (TBS-A + 25% Bovine Serum Albumin, BSA) for 30 minutes. Following this the tissue was incubated with the primaries consisting of Anti-B-Amyloid, 17-24, Clone 4G8 (mouse, 800701, Biolegend) 1:1000 and Anti-GFAP (Rabbit, Ab7260, Abcam) -20C 1:2000, 1ml/slide in TBS-B for 48 hours in a dark, humid chamber. The tissue was then washed twice in 1X TBS, once in TBS-A, and once with TBS-B. The tissue was then incubated with the secondaries consisting of anti-mouse-alexa-488 (IgG (H+ L) goat, Thermofisher, A32723) 1:2000 and anti-rabbit-alexa-594 (IgG (H+L) goat, Invitrogen, A11037) 1:1000 (both stored at 4°C) (1ml/slide) in TBS-B for 24 hours in a dark, humid chamber. The tissue was then washed three times with 1X TBS. Following this, the tissue was cover slipped using DAPI 1:2000 and placed in a dark, humid chamber for 1 hour. The tissue was then washed once more with 1X TBS and cover slipped using 2 drops of Vectashield

(Vector Labs, H-1000). After 24 hours the slides were sealed using nail polish and stored at 4°C until analyzed.

Sections were imaged using a Nanozoomer microscope (Nanozoomer 2.0-RS, HAMAMATSU, JAPAN) at 40x resolution.

Cell Quantification

82E1-stained Aβ plaques, 4G8-stained Aβ plaques, Iba1-stained microglia, and GFAP-stained astrocyte images were quantified using independently trained iterations of Ilastik version 1.4.0rc2 software. Each stain was trained using 4 random brain sections from varying staining batches and experimental groups (excluding sex as a factor). Ilastik was trained using pixel classification + object classification, which allowed for specific pixels to be marked as the target object. The software was considered adequately trained once it could accurately predict the target objects with no error. The brain regions of interest (either PFC or HPC) were cropped from the Nanozoomer NDP images using Image-J and exported at the same resolution to ensure consistency. All cropped images of HPC and PFC sections were then batch processed using the trained iterations in Ilastik to obtain data from the stained images.

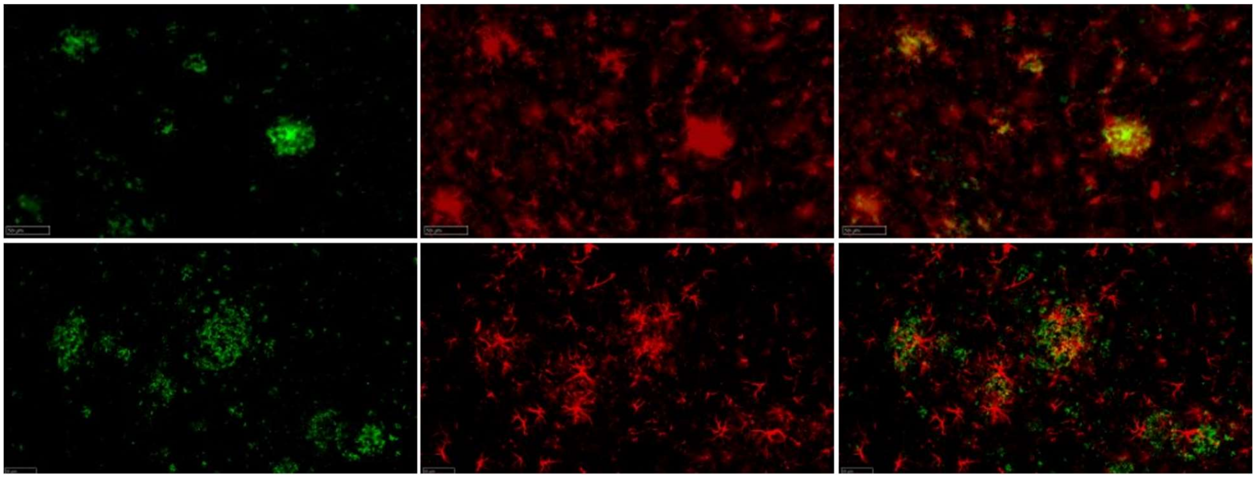


Figure 4. Immunohistochemical staining of Aβ plaques (green) and inflammatory markers (red). Shown are Nanozoomer images of 82E1-stained Aβ plaques (top left), Iba1-stained microglia (top middle), and the combined double-staining image (top right), as well as 4G8-stained Aβ plaques (bottom left), GFAP-stained astrocytes (bottom middle), and the combined double staining image (bottom right). The scale is 50 um.

In summary, various behavioural tasks (including the MWT, NOR task, and FC task) and histology stains (including HPC cells, Aβ plaques, microglia, and astrocytes) were conducted to quantify the effects of a 20:1 CBD:THC combination *cannabis* extract on AD pathology.

Results

Data Analysis

Statistical analysis was performed using GraphPad Prism statistical software (version 9.5.0). All data is presented as means +/- SEM. Any differences between means were calculated using two- or three-way Analysis of Variance (ANOVA). A p value of < 0.05 was statistically significant.

Behavioural Tests

Activity Boxes

The mice were recorded, and the testing data was analyzed using VersaDatTM software (AccuScan Instruments Inc., OH, USA). A three-way ANOVA assessed if treatment, genotype, or month influenced stereotypy time, movement time, or total distance travelled during the 10-minute testing period.

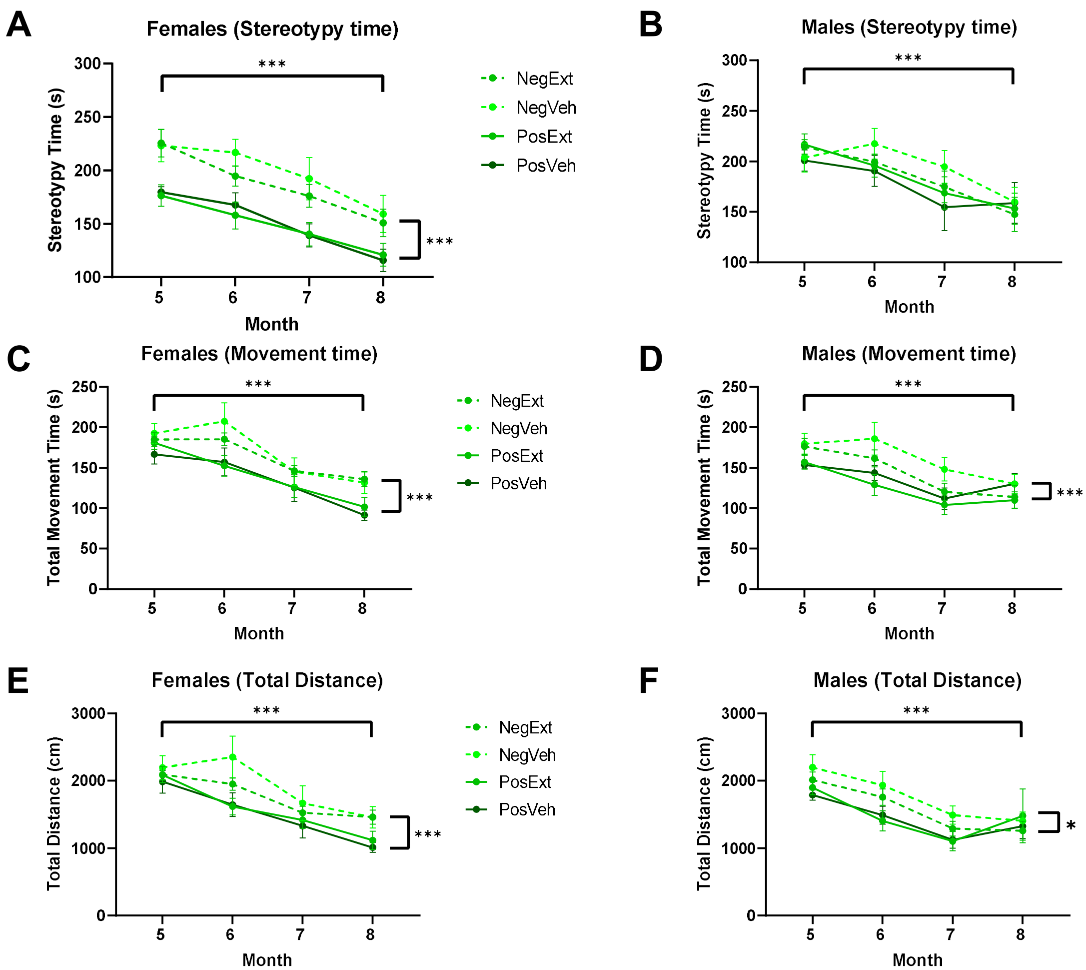


Figure 5. Activity box data collected monthly from 5-8 months of age assessing total stereotypy time (s) (A & B), total movement time (s) (C & D), and total distance travelled (cm) (E & F) during a 10-minute testing interval, separated by sex (females shown on the left and males shown on the right), in APP-NL-G-F mice and APP-NL noncarrier littermates treated with a cannabis extract or vehicle. Each experimental group contains n=10 subjects.

Figure 5 shows a decrease in stereotypy time (s) (A & B), total movement time (s) (C & D), and total distance travelled (cm) (E & F) from 5 to 8 months of age. A three-way ANOVA showed that there was a significant effect of month on stereotypy time in females (A) ($F(3, 144) = 20.13$, $p < 0.001$) and males (B) ($F(3, 140) = 11.07$, $p < 0.001$). A three-way ANOVA showed that there was a significant effect of month on total movement time in females (C) ($F(3, 144) =$

24.20, p < 0.001) and males (D) (F (3, 140) = 14.61, p < 0.001). A three-way ANOVA showed that there was a significant effect of month on total distance in females (E) (F (3, 144) = 21.96, p < 0.001) and males (F) (F (3, 140) = 15.10, p < 0.001). Furthermore, some of the data indicates that APP-NL-G-F subjects showed a reduction in stereotypy time (s), total movement time (s), and total distance travelled (cm) compared to controls. A three-way ANOVA showed that there was a significant effect of genotype on stereotypy time in females (F (1, 144) = 46.67, p < 0.001), however there was no effect of genotype in their male counterparts. Although there was a significant effect of genotype in total movement time in both females (F (1, 144) = 19.38, p < 0.001) and males (F (1, 140) = 12.97, p < 0.001). Similarly, there was also a significant effect of genotype on total distance travelled in females (F (1, 144) = 15.02, p < 0.001) and males (F (1, 140) = 6.793, p = 0.01).

Open Field Task

The testing data was analyzed using Noldus EthoVision XT version 16 software to track the movement of the mice over the 10-minute testing period.

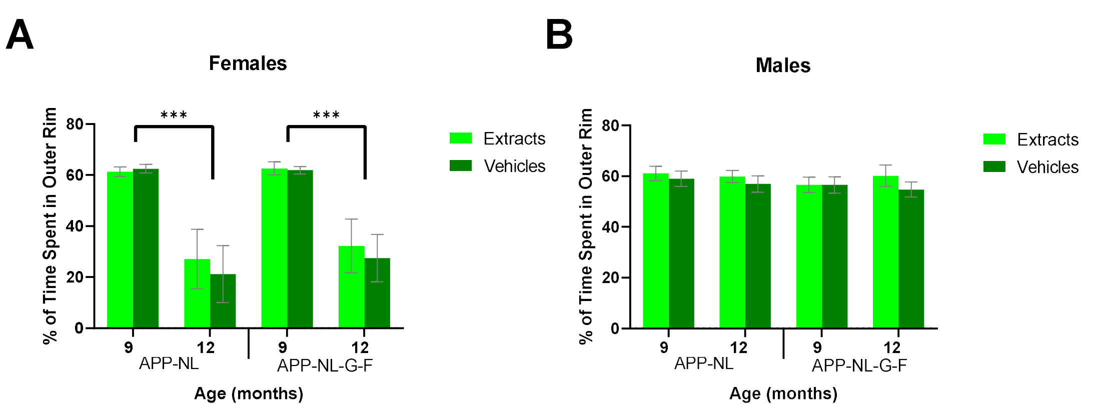


Figure 6. Percentage (%) of time spent in the outer rim of the OFT arena during a 10-minute testing period (performed during habituation day 1 of the NOR task) for female (A) and male (B) APP-NL-G-F mice and APP-NL noncarrier littermates treated with a cannabis extract or vehicle (***p < 0.001). Each experimental group contains n=10 subjects.

Figure 6 shows that the percentage of time spent in the outer rim of the arena decreased with age in the female (A), but not in the male (B) subjects. A three-way ANOVA showed that there was a significant effect of age on the percentage of time spent in the outer rim of the arena in the female subjects ($F (1, 71) = 40.92$, $p < 0.001$), but not in the male subjects ($F (1, 65) = 0.03390$, $p = 0.854$). At 9 months of age, all subjects spent a similar amount of time in the outer rim of the arena; but at 12 months the females spent less time here whereas, the males spent the same amount of time here.

Novel Object Recognition (NOR) Task

The mice were video recorded during testing and were scored based on the following parameters: 1) the mouse's nose must be within 1 cm of the object, 2) the mouse cannot have their paws touching the object, and 3) the mouse cannot be sitting on top of the object. Investigation ratio (IR) was calculated based on the amount of time spent at the novel object divided by the total amount of time spent at both objects multiplied by 100. An IR value above 0.5 shows that the group is showing a preference for the novel object, whereas an IR value below 0.5 would show the opposite. A three-way ANOVA assessed if treatment, genotype, or sex had an effect on the IR. The dotted line indicates an IR of 0.5, this indicates that any mean above the line shows a preference for the novel object.

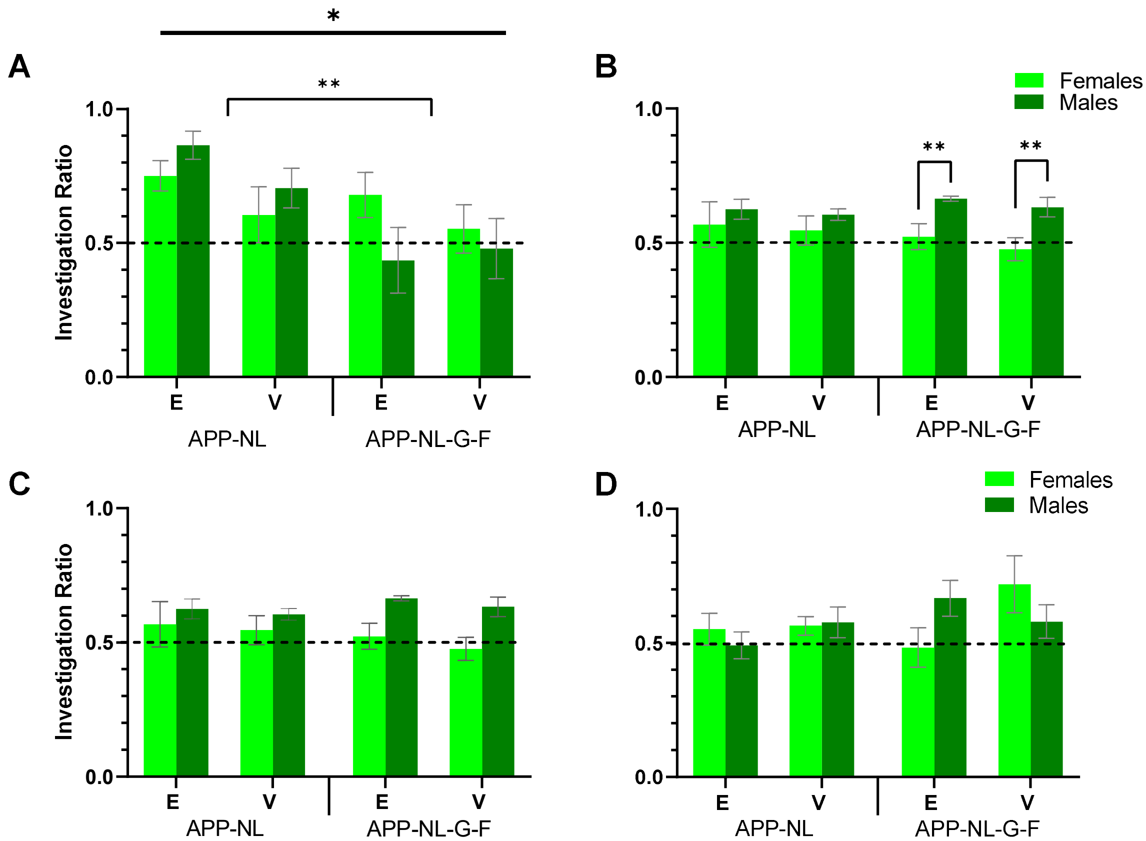


Figure 7. Investigation ratio (= time spent at novel object / total time spent at both objects) during testing day 1 of the NOR task at 9 months (A) and 12 months (B) of age and during testing day 2 of NOR task at 9 months (C) and 12 months (D) of age in female and male APP-NL-G-F mice and APP-NL noncarrier littermates treated with a cannabis extract or vehicle (*$p < 0.033$, **$p < 0.002$). The dotted line indicates an IR of 0.5. Each experimental group contains n=10 subjects.

Figure 7 indicates that the APP-NL subjects had a higher IR compared to the APP-NL-G-F subjects at 9 months (A); however, these differences disappear at 12 months of age (B). A three-way ANOVA showed that there was a significant effect of genotype on IR ($F (1, 69) = 9.051$, $p < 0.002$) during testing day 1 of NOR task at 9 months of age (A). A three-way ANOVA showed that there was a significant effect of sex on IR ($F (1, 71) = 4.127$, $p = 0.046$) in the APP-NL-G-F group during testing day 1 at 12 months of age. This shows that male APP-NL-G-F subjects were outperforming their female counterparts during testing at 12 months. The graphs show that all groups showed similar IR values during day 2 of testing at 9 and 12 months of age. There was no significant effect of genotype, sex, or treatment during NOR testing day 2.

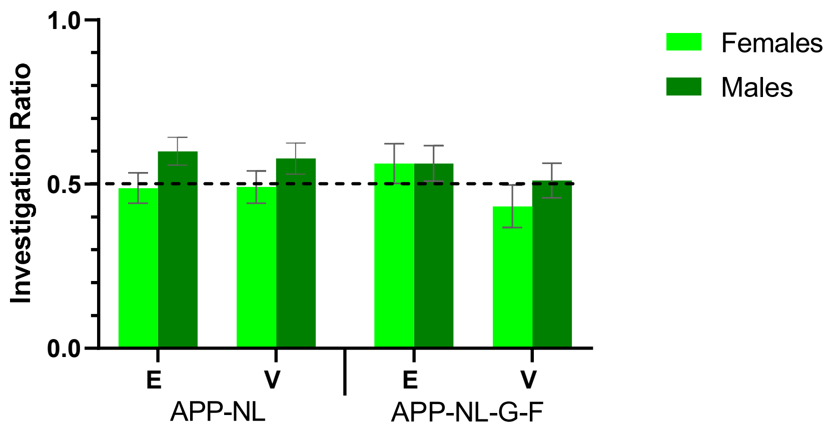


Figure 8. Investigation ratio (= time spent at novel object / total time spent at both objects) during a 5-minute testing period 1-month post training at the NOR task at 13 months of age in female and male APP-NL-G-F mice and APP-NL noncarrier littermates treated with a cannabis extract or vehicle. The dotted line indicates an IR of 0.5. Each experimental group contains n=10 subjects.

Figure 8 shows that all groups spend a similar amount of time investigating both objects (IR) during testing 1-month post training. A three-way ANOVA showed no significant effect of treatment ($F(1, 67) = 1.857$, $p = 0.178$) or genotype ($F(1, 67) = 0.3541$, $p = 0.554$) on IR during the 1-month post training test of the NOR task. A three-way ANOVA showed a trend of sex ($F(1, 67) = 3.547$, $p = 0.064$) on IR during the 1-month post training test of the NOR task. The males seem to be outperforming their female counterparts, although this is not statistically significant.

Balance Beam Task

The mice were video recorded, and latency was determined by the average amount of time it took the mice to traverse the 80 cm beam during the three trials. The mice were allowed a maximum of 3 trials/falls. At 9 months of age, all groups were able to traverse the beam. However, at 12 months of age 4 males were unable to complete the task producing groups of: Negative male extract (n= 10), negative female extract (n= 10), negative male vehicle (n= 9), negative female vehicle (n= 10), positive male extract (n= 8), positive female extract (n= 10), positive male vehicle (n= 9), and positive vehicle female (n= 10). A three-way ANOVA assessed if treatment, genotype, or sex influenced latency to cross.

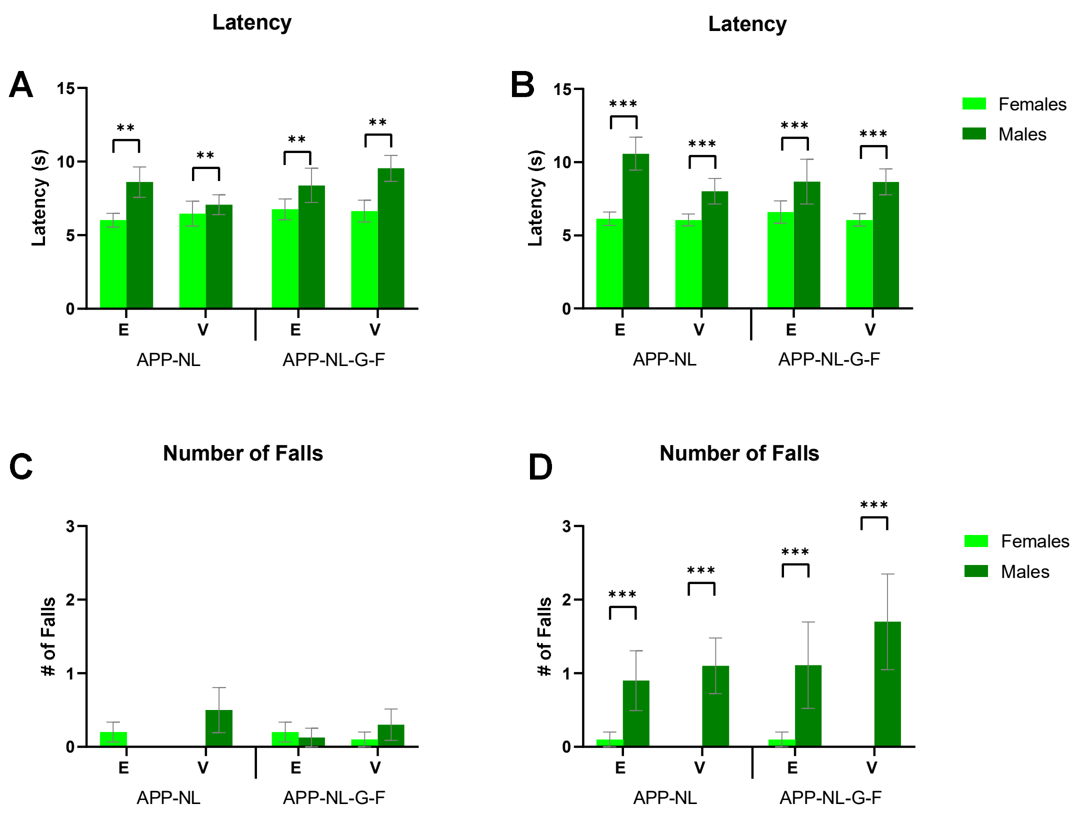


Figure 9. Average latency to cross the 80 cm beam during 3 trials on testing day at 9 months (A) and 12 months (B) of age as well as the number of falls during these trials at 9 months (C) and 12 months (D) of age in female and male APP-NL-G-F and APP-NL mice treated with a cannabis extract or vehicle (**p < 0.002, ***p < 0.001). Each experimental group contains n=10 subjects.

The graphs depicted in Figure 9 show that APP-NL-G-F and APP-NL extract and vehicle treated subjects have similar latencies to cross the balance beam at 9 (A) and 12 (B) months of age; however, the male subjects have a higher latency compared to their female counterparts. A three-way ANOVA showed that there was a significant effect of sex on latency to cross the beam at 9 ($F(1, 68) = 11.00$, $p = 0.001$) and 12 ($F(1, 68) = 21.56$, $p < 0.001$) months of age. The data shows that the male subjects took consistently longer to traverse the pole than the females. Furthermore, there was also a significant effect of sex on the number of falls during the 12-month testing period ($F(1, 71) = 20.10$, $p < 0.001$). It should be noted that the males weighed roughly 40g more than the females on average, which increased the difficulty of the task. There was no significant effect of treatment ($F(1, 68) = 0.001101$, $p = 0.974$) or genotype ($F(1, 68) = 1.851$, $p = 0.178$) on latency to cross the beam at 9 months of age. There was no significant effect of treatment ($F(1, 68) = 1.802$, $p = 0.184$) or genotype ($F(1, 68) = 0.1135$, $p = 0.737$) to cross the beam at 12 months of age.

Morris Water Task (MWT)

<u>Training</u>

The latency to find the hidden platform was determined by tracking software over the training days and the data was analyzed. The average latency for each mouse was calculated from 4 swims from each of the cardinal directions (N, E, S, and W) in a randomized order for each day of testing, although order was the same for all mice ran on the same day. A three-way ANOVA assessed if treatment, genotype, or day influenced their ability to reach the escape platform.

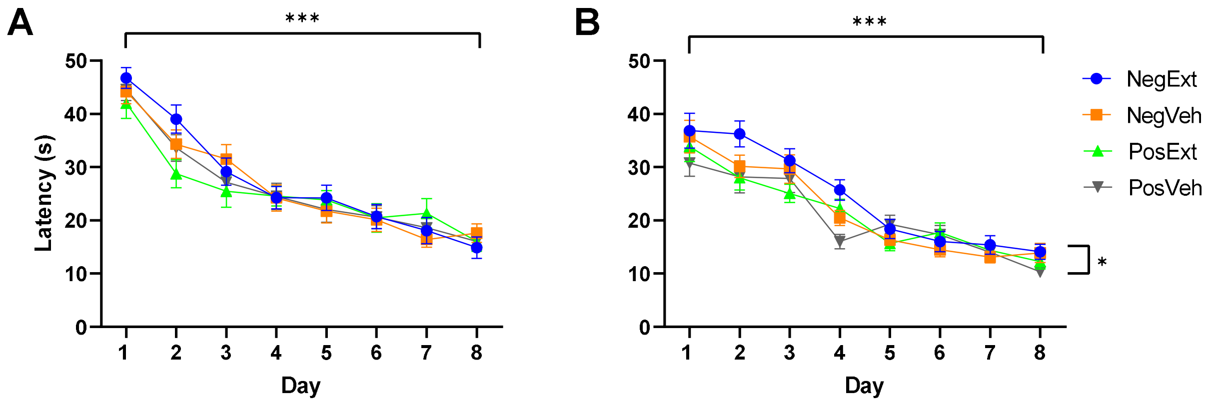


Figure 10. Latency (s) to reach escape platform during 8-day training period in MWT at 9 (A) and 12 (B) months of age in female and male APP-NL-G-F and APP-NL mice treated with a cannabis extract or vehicle (*p < 0.033, ***p < 0.001). Each experimental group contains n=20 subjects.

Figure 10 shows that all groups have similar latencies to reach the escape platform across an 8-day training period at 9 (A) and 12 (B) months of age. A three-way ANOVA showed that there was a significant effect of day on average latency to reach the escape platform at 9 ($F(6.146, 461.0) = 85.69$, $p < 0.001$) and 12 ($F(4.798, 359.9) = 72.41$, $p < 0.001$) months of age in all groups. This shows that over the course of the 8-day training period, the subjects were able to acquire the location of the escape platform. There was no significant effect of treatment ($F(1, 75) = 0.01151$, $p = 0.915$) or genotype ($F(1, 75) = 0.6639$, $p = 0.418$) on the average latency to reach the escape platform at 9 months of age.

At 12 months of age the APP-NL-G-F subjects outperform their counterparts during the 8-day acquisition period of the MWT. A three-way ANOVA showed that there was a significant effect of genotype ($F(1, 75) = 6.030$, $p = 0.016$) on the average latency to reach the escape platform at 12 months of age (B). However, when path length is measured (see figure 11) this interaction disappears. There was no significant effect of treatment (F (1, 75) = 3.266, p = 0.075) on the average latency to reach the escape platform at 12 months of age.

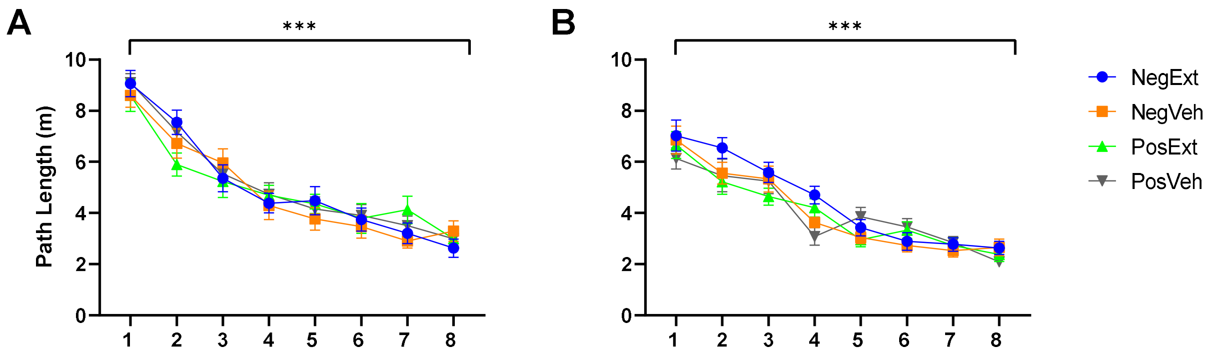


Figure 11. Path length (m) to reach escape platform during 8-day training period in MWT at 9 (A) and 12 (B) months of age in female and male APP-NL-G-F and APP-NL mice treated with a cannabis extract or vehicle (***p < 0.001). Each experimental group contains n=20 subjects.

Figure 11 shows that all groups share a similar path length to reach the escape platform across an 8-day training period at 9 (A) and 12 (B) months of age. A three-way ANOVA showed that there was a significant effect of day on average path length to reach the escape platform at 9 (F (6.119, 458.9) = 94.53, p < 0.001) and 12 (F (4.939, 370.4) = 69.94, p < 0.001) months of age. There was no significant effect of treatment (F (1, 75) = 3.345e-005, p = 0.995) or genotype (F (1, 75) = 0.09904, p = 0.754) on average path length to reach the escape platform at 9 months of age. There was no significant effect of treatment (F (1, 75) = 1.673, p = 0.200) or genotype (F (1, 75) = 2.152, p = 0.147) on average path length to reach the escape platform at 12 months of age.

Probe (1-Day Post Training/1-Week Post Training)

During the probe trial (1-day post training) the amount of time spent in each quadrant was determined by tracking software over the probe testing days and the data was analyzed. The percentage of time spent in the target quadrant was calculated based on how much time was spent in the quadrant where the platform was placed during training divided by the amount of time spent in the other 3 quadrants during a 60-second interval multiplied by 100. All the nice were run from the same cardinal direction (N), which was the furthest start point from the platform's previous location. The dotted line indicates 25% (or chance), anything above the dotted line indicates when the subjects are performing above chance. A three-way ANOVA assessed if treatment, genotype, or sex impacted quadrant preference during the probe test.

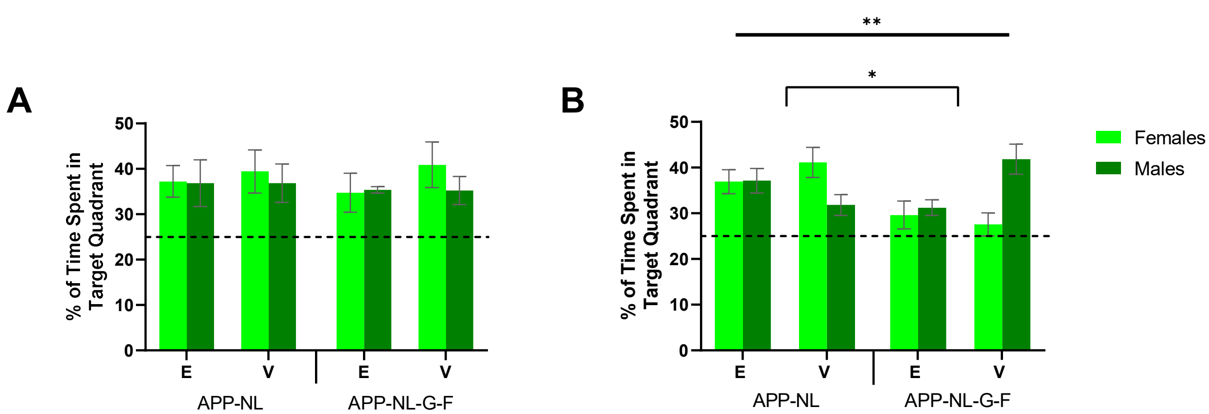


Figure 12. Percentage (%) of time spent in the previously trained target quadrant during a single probe trial (1-day post training) of the MWT in 9 (A) and 12 (B) months of age in female and male APP-NL-G-F and APP-NL mice treated with a cannabis extract or vehicle (*p < 0.033, **p < 0.002). The dotted line indicates 25% (chance). Each experimental group contains n=10 subjects.

Figure 12 shows that all groups spent a similar percentage of time in the target quadrant at 9 months of age (A); however, by 12 months of age (B) there were genotype-specific differences present. A three-way ANOVA showed that there was a significant effect of genotype at 12 months ($F(1, 71) = 4.675$, $p = 0.034$), but not at 9 months ($F(1, 71) = 0.1274$, $p = 0.722$) of age. This shows that genotype-related spatial memory impairments did not present themselves until 12 months of age. A three-way ANOVA showed that there was a significant interaction effect of genotype X sex ($F(1, 71) = 10.40$, $p = 0.002$) on percentage of time spent in the target quadrant. A Tukey's multiple comparison test showed that APP-NL-G-F vehicle-treated males outperformed their female counterparts. There was no significant effect of treatment on the percentage of time spent in the target quadrant at 9 ($F(1, 71) = 0.4931$, $p = 0.485$) or 12 ($F(1, 71) = 0.9134$, $p = 0.342$) months of age.

During the probe trial (1-week post training) the amount of time spent in each quadrant was determined by tracking software over the probe testing days and the data was analyzed. The percentage of time spent in the target quadrant was calculated based on how much time was spent in the quadrant where the platform was placed during training divided by the amount of time spent in the other 3 quadrants during a 30-second interval multiplied by 100. All subjects were run from the same cardinal direction (N), which was the furthest start point from the platform's previous location. The dotted line indicates 25% (or chance), anything above the dotted line indicates when the subjects are performing above chance. A three-way ANOVA assessed if treatment, genotype, or sex impacted the percentage of time spent in the target quadrant.

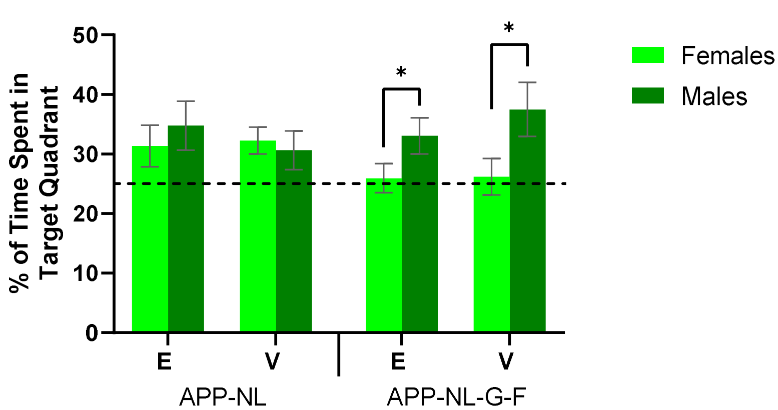


Figure 13. Percentage (%) of time spent in the previously trained target quadrant during a single probe trial (1-week post training) of the MWT in 12-month-old female and male APP-NL-G-F

and APP-NL mice treated with a cannabis extract or vehicle (*$p < 0.033$). The dotted line indicates 25% (chance). Each experimental group contains n=10 subjects.

Figure 13 shows that all APP-NL subjects are spending a similar amount of time in the target quadrant during a single probe trial (1-week post training); however, sex-specific differences are present in APP-NL-G-F individuals. The male APP-NL-G-F subjects are spending more time in the target quadrant compared to their female counterparts. A three-way ANOVA showed a significant effect of sex ($F (1, 71) = 4.500$, $p = 0.037$) on the percentage of time spent in the target quadrant in the APP-NL-G-F groups. The data shows that the APP-NL-G-F males are outperforming their female counterparts; this mirrors the sex differences found during testing day 1 of the NOR task at 12 months of age. There was no effect of treatment ($F (1, 71) = 0.02370$, $p = 0.878$) or genotype ($F (1, 71) = 0.4443$, $p = 0.507$) on the percentage of time spent in the target quadrant during the testing day (1-week post training) at 12 months of age.

Increased specificity measures including annulus crossings (Figure 21) and average proximity (Figure 22) during the 1-day post training and 1-week post training test are provided in the supplementary figures portion of the appendix.

Visible Platform

The latency to find the visible platform was determined by tracking software over 4 swims and the data was analyzed. A three-way ANOVA assessed if treatment, genotype, or sex influenced latency to reach the visible escape platform.

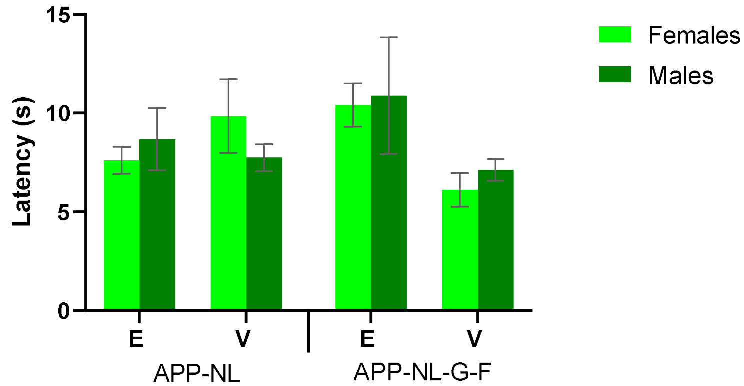


Figure 14. Average latency (s) to reach the visible escape platform during four trials at 12 months of age in female and male APP-NL-G-F and APP-NL mice treated with a cannabis extract or vehicle. Each experimental group contains n=10 subjects.

Data presented in Figure 14 shows that all groups display similar latencies to reach the visible escape platform. A three-way ANOVA showed no significant effect of treatment ($F (1, 71) = 2.752$, $p = 0.102$), genotype ($F (1, 71) = 0.02427$, $p = 0.877$), or sex ($F (1, 71) = 0.01162$, $p = 0.914$) on average latency (s) to reach the escape platform during the visible platform task.

Fear Conditioning (FC) Task

The testing days were video recorded, and the total time spent freezing was obtained by manually scoring each freezing behaviour observed in a 5-minute testing interval. A three-way ANOVA assessed if treatment, genotype, or sex influenced the total time spent freezing.

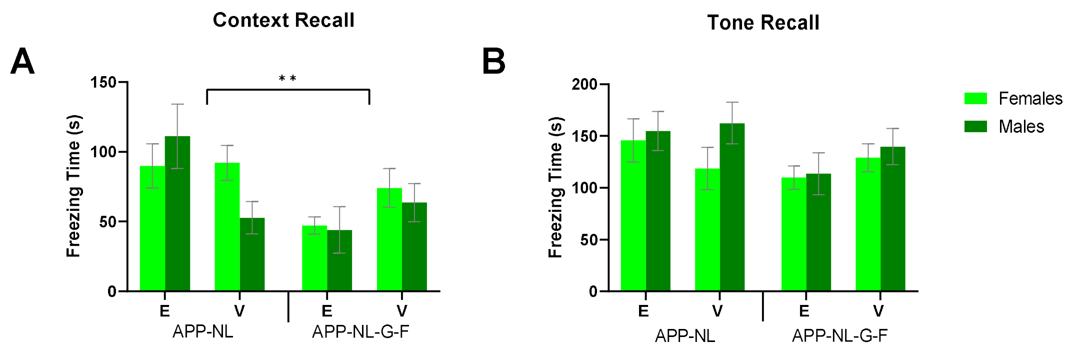


Figure 15. Average time (s) spent freezing during the 5-minute context recall trial (A) and 7-minute tone recall trial (B) of female and male APP-NL-G-F and APP-NL mice treated with a cannabis extract or vehicle during the FC task. Each experimental group contains n=10 subjects.

In Figure 15, the context recall graph (A) shows that the APP-NL-G-F subjects spent less time freezing compared to controls. A three-way ANOVA showed a significant effect of genotype ($F(1, 66) = 7.839$, $p = 0.007$) on freezing time during the 5-minute trial of context recall on testing day 2. The results show that the APP-NL subjects spent more time freezing then their APP-NL-G-F counterparts. There was no effect of treatment ($F(1, 66) = 0.05341$, $p = 0.818$) or sex ($F(1, 66) = 0.5737$, $p = 0.451$) on freezing time during the 5-miinute trial of context recall on testing day 2. In Figure 15, the tone recall graph (B) shows that the APP-NL-G-F subjects spent less time freezing compared to controls. However, there was no significant effect of genotype ($F(1, 70) = 3.000$, $p = 0.088$), treatment ($F(1, 70) = 0.2554$, $p = 0.615$), or sex ($F(1, 70) = 1.702$, $p = 0.196$) found on freezing time during the 7-minute trial of tone recall on testing day 3.

Histology

Nissl Staining

Hippocampal Volume

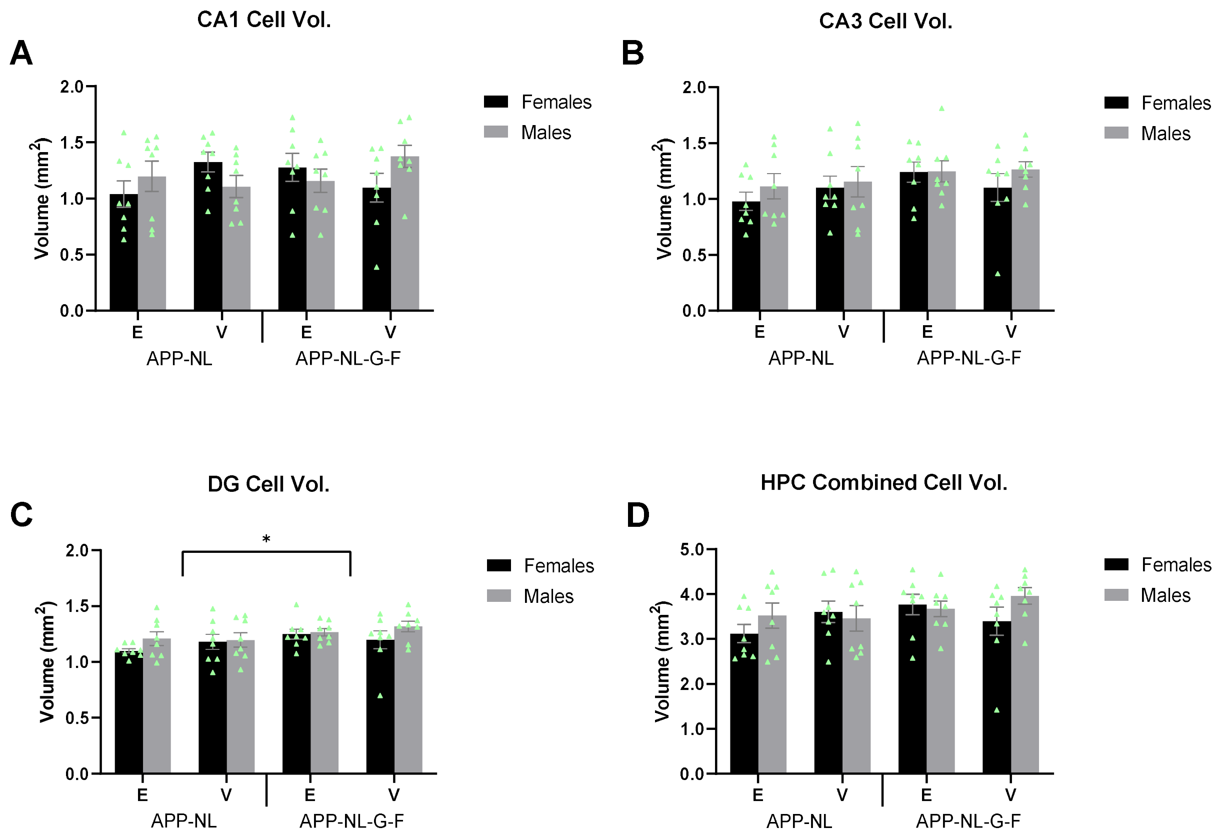


Figure 16. Volume (mm^2) of cell density of Cresyl-violet stained cells in various hippocampal regions including CA1 (A), CA3 (B), dentate gyrus (DG) (C), and the combined volumes (D) of in female and male APP-NL-G-F and APP-NL mice treated with a cannabis extract or vehicle (*$p < 0.033$). Each experimental group contains n=8 subjects.

Figure 16 indicates that various HPC regions across experimental groups have comparable cell volumes. However, APP-NL-G-F subjects have an increased cell volume in the dentate gyrus. A three-way ANOVA showed a significant effect of genotype on cell volume in the dentate gyrus ($F(1, 56) = 4.911$, $p = 0.031$) of the HPC, however there was no significant effect of genotype on cell volume in the CA1 ($F (1, 56) = 0.5497$, $p = 0.462$), CA3 ($F (1, 56) = 3.009$, $p = 0.088$) or the three combined regions ($F (1, 56) = 2.504$, $p = 0.119$) within the HPC. However, a three-way ANOVA showed a trend effect of genotype on cell volume in CA3 ($F (1, 56) = 3.009$, $p = 0.088$). There was no effect of treatment or sex on cell volume in CA1, CA3, or the three combined regions within the HPC.

Immunohistochemistry

Aβ Plaques (82E1/4G8 Markers)

The Aβ plaques were quantified using Ilastik version 1.4.0rc2 software. The percentage area covered was calculated by converting the sum of the size of the plaques in pixels to um^2 and dividing this by the size of the cropped image (cropped using Image-J software) multiplied by 100.

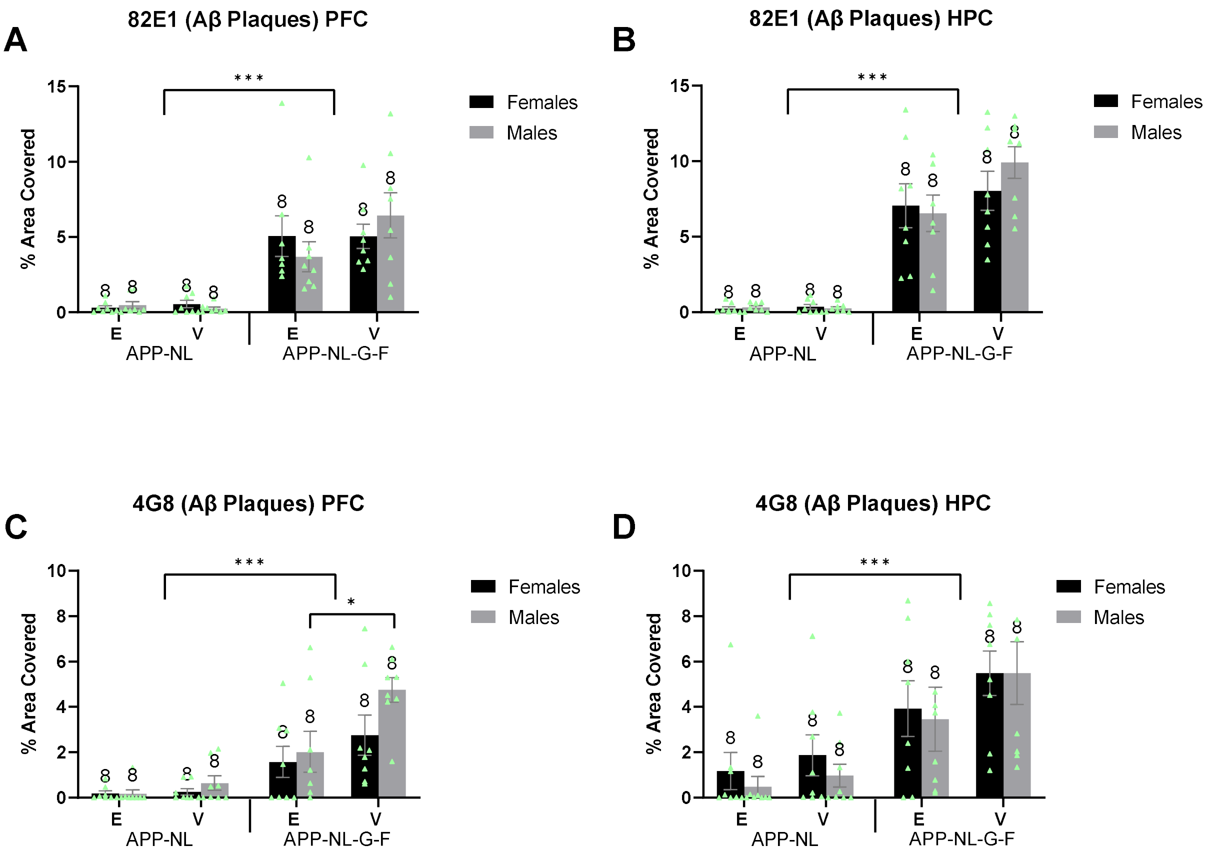


Figure 17. The surface area percentage (%) of Aβ plaques stained by A$\beta_{1\text{-}16}$ antibody, 82E1 in the PFC and HPC (A & B, respectively) and A$\beta_{18\text{-}22}$ antibody, 4G8 in PFC and HPC (C & D, respectively) of female and male APP-NL-G-F and APP-NL mice treated with a cannabis extract or vehicle (*$p < 0.033$, ***$p < 0.001$). Each experimental group contains n=8 subjects.

Figure 17 shows that the APP-NL-G-F subjects have a higher level of 82E1 and 4G8-stained Aβ plaques compared to APP-NL controls. A three-way ANOVA confirmed a significant effect of genotype on the percentage of area covered by 82E1-stained Aβ plaques in PFC (A) (F (1, 56) = 59.49, $p < 0.001$) and HPC (B) (F (1, 56) = 145.1, $p < 0.001$). A three-way ANOVA also showed a significant effect of genotype on the percentage of area covered by 4G8-stained Aβ plaques in PFC (C) (F (1, 56) = 38.19, $p < 0.001$) and HPC (D) (F (1, 56) = 23.09, $p < 0.001$). Furthermore, the graph shows that APP-NL-G-F extract-treated subjects have a fewer Aβ plaques compared to their vehicle-treated counterparts. A three-way ANOVA showed a significant effect of treatment ($F (1, 56) = 7.874$, $p = 0.007$) on the percentage of area covered by 4G8-stained Aβ plaques in APP-NL-G-F subjects' PFC (C). Similarly, a three-way ANOVA showed a trend of treatment on 82E1-stained Aβ plaques in HPC (B) ($F (1, 56) = 3.045$, $p = 0.086$). There was no effect of sex on 82E1-stained or 4G8-stained Aβ plaques in PFC or HPC.

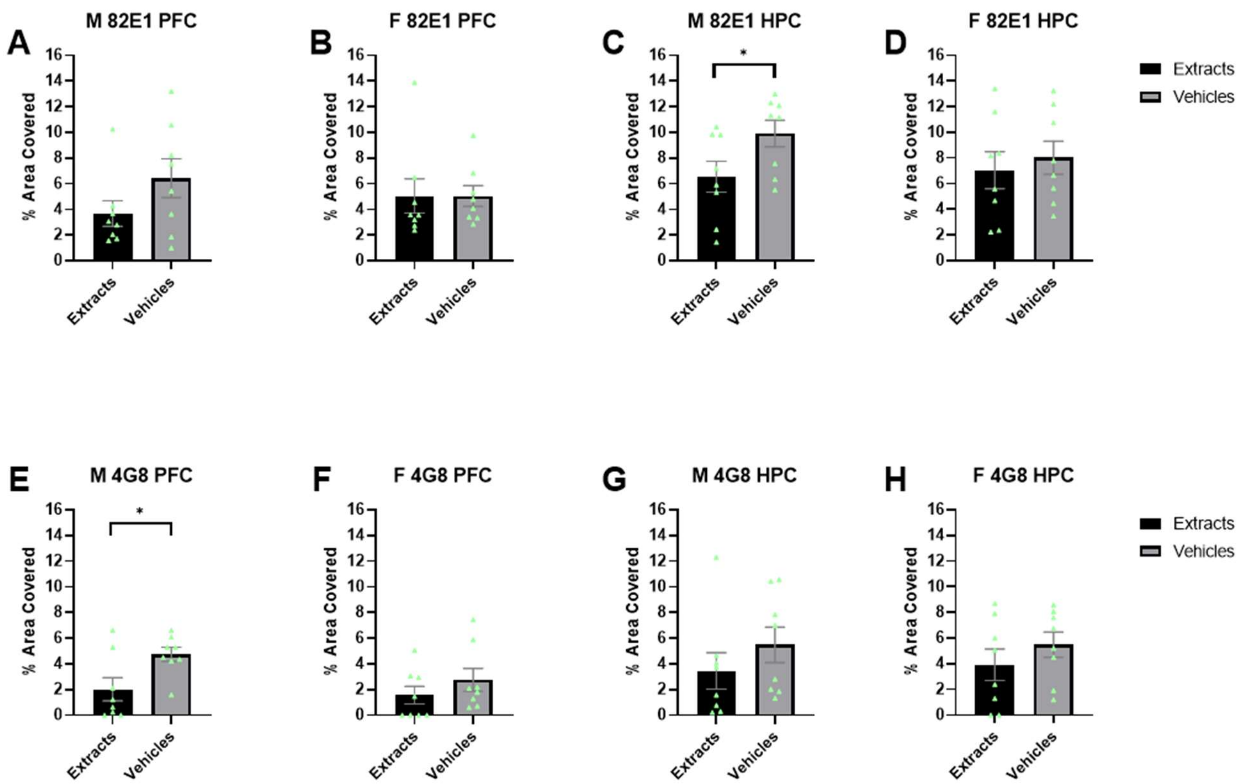


Figure 17b. The surface area percentage (%) of Aβ plaques stained by Aβ1-16 antibody, 82E1 in (1) PFC in males (A) and females (B) and in (2) HPC in males (C) and females (D) along with Aβ18-22 antibody, 4G8 in (3) PFC in males (E) and females (F) and in (4) PFC in males (G) and females (H) in APP-NL-G-F mice treated with a cannabis extract or vehicle (*$p < 0.033$). Each experimental group contains n=8 subjects.

Figure 17b shows that APP-NL-G-F males treated with the cannabis extract had significantly fewer 82E1-stained Aβ plaques in HPC and 4G8-stained Aβ plaques in PFC compared to vehicle-treated controls. Multiple t-tests using nonparametric Mann-Whitney measures revealed a significant effect of treatment in male 82E1-stained Aβ plaques in HPC (p = 0.038) and 4G8-stained Aβ plaques in PFC (p = 0.050).

Inflammation (Iba1/GFAP Markers)

Inflammatory markers were quantified using Ilastik version 1.4.0rc2 software. The percentage area covered was calculated by converting the sum of the size of the plaques in pixels to um^2 and dividing this by the size of the cropped image (cropped using Image-J software) multiplied by 100.

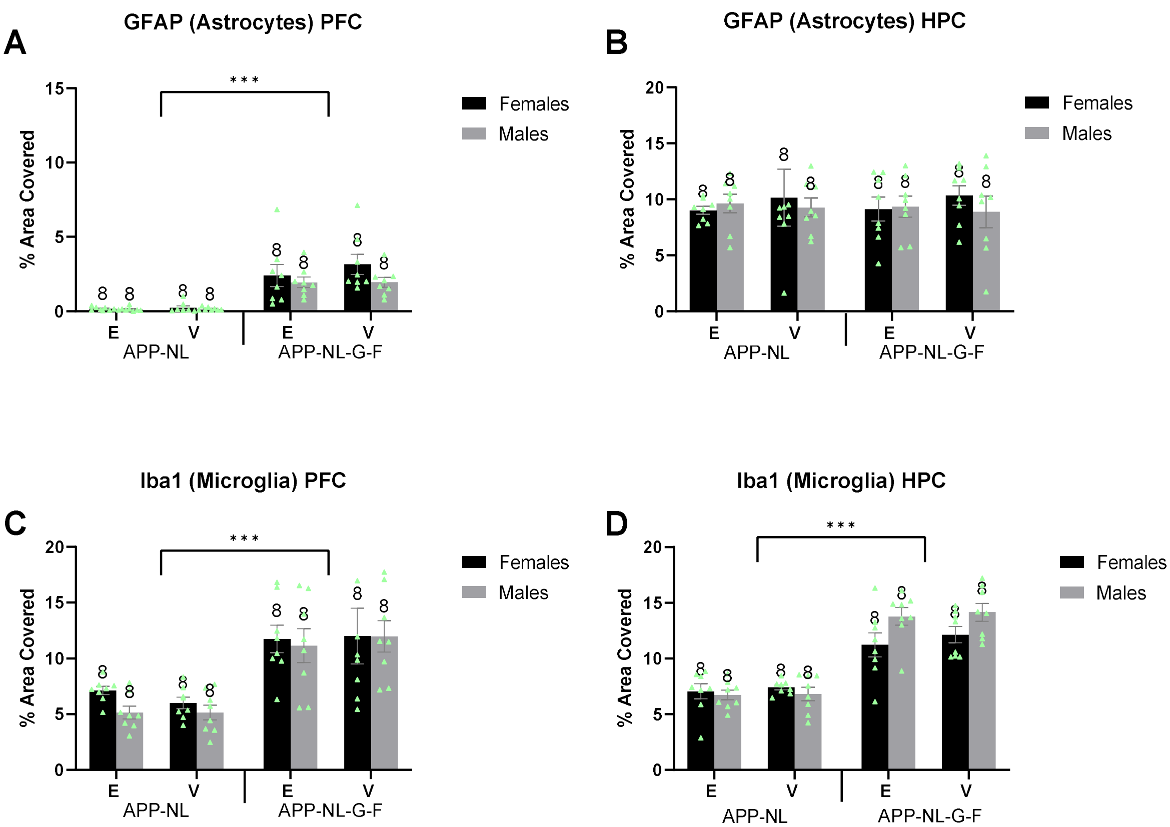


Figure 18. The percentage (%) of GFAP in the PFC (A) and HPC (B) and Iba1 in PFC (C) and HPC (D) of female and male APP-NL-G-F and APP-NL mice treated with a cannabis extract or vehicle (***p < 0.001). Each experimental group contains n=8 subjects.

Figure 18 shows that the APP-NL-G-F subjects have a higher average percentage of GFAP and Iba1 in both the PFC and HPC compared to controls. A three-way ANOVA showed a significant effect of genotype on the percentage of area covered by GFAP-stained astrocytes in PFC (A) ($F(1, 56) = 61.13$, $p < 0.001$), however there was no effect on HPC (B) ($F(1, 56) = 0.01121$, $p = 0.916$). A three-way ANOVA showed a significant effect of genotype on the percentage of area covered by Iba1-stained microglia in PFC (C) ($F(1, 56) = 41.26$, $p < 0.001$) and HPC (D) ($F(1, 56) = 135.7$, $p < 0.001$). There was no significant effect of treatment or sex on the percentage of area covered by GFAP-stained astrocytes or Iba1-stained microglia in PFC or HPC.

Cytology

Estrus Cycle

Cytology data was collected via lavage samples from female mice daily at the same time from 14 days prior to the initial testing period at 9 months and 12 months of age. The estrus stage of each sample was determined by using a Zeiss Axioskop-2 Mot Plus Upright Fluorescence Microscope to determine which cell type was most present. As previously mentioned, the cell type that is most present in each sample determines the estrus stage.

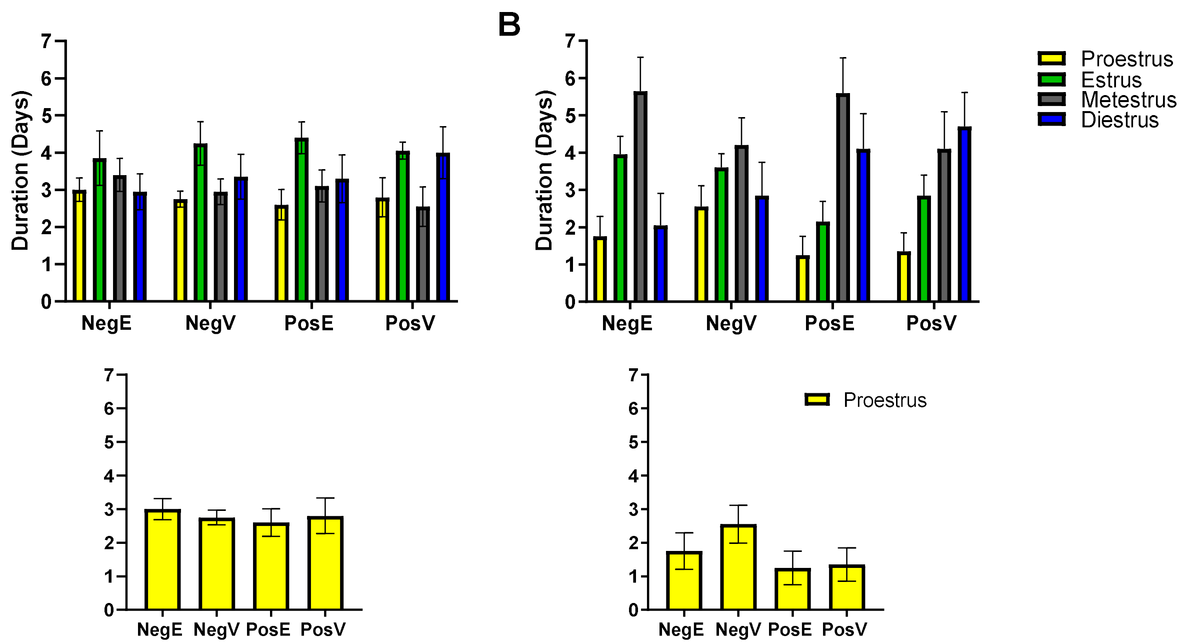


Figure 19. Stage duration in the estrus cycle of female subjects (including APP-NL-G-F and APP-NL extract and vehicle-treated subjects) during a 14-day period at 9 months (A) and 12 months (B) of age (the proestrus phase is emphasized under each respective graph).

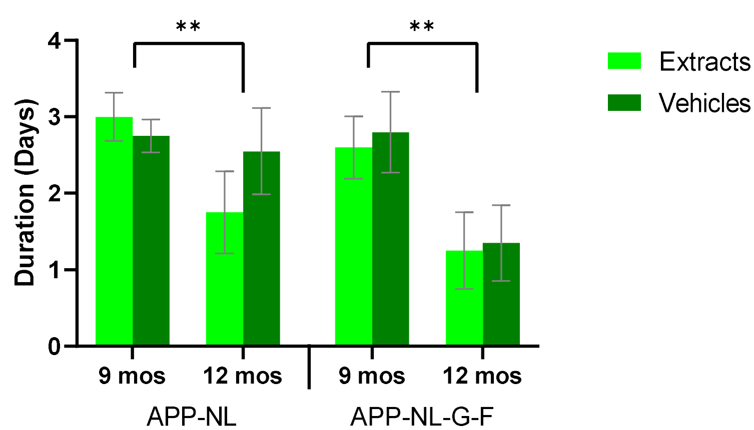


Figure 20. Proestrus phase duration in the estrus cycle of female subjects over a 14-day period at 9 and 12 months of age (**p < 0.002). Each experimental group contains n=10 subjects.

As can be seen in Figure 20, all female subjects show a longer duration of time spent in the proestrus phase of their estrus cycle at 9 months compared to 12 months. A three-way

ANOVA confirmed a significant effect of age ($F(1, 72) = 10.65$, $p = 0.002$) on the duration of the proestrus phase during a 14-day period. This shows that the duration of proestrus has significantly decreased in all female groups from 9 to 12 months of age. There was no significant effect of treatment ($F(1, 72) = 0.4260$, $p = 0.516$) or genotype ($F(1, 72) = 2.478$, $p = 0.120$) on the duration of proestrus.

Discussion

The aim of the present research project was to assess the effectiveness of a combination *cannabis* extract at treating pathology and memory impairments related to AD using an APP-NL-G-F mouse model. This preclinical mouse model was of particular interest as it displays Aβ plaques only, which allowed for a more accurate assessment of early intervention treatment. The subjects began receiving a daily oral dose of either a *cannabis* extract or control at 5 months of age until sacrifice at roughly 13.5 months of age. The *cannabis* extract consisted of a 20:1 CBD:THC extract that has been previously shown to improve memory impairments and reduce chronic inflammation (Nidadavolu et al., 2021; Sánchez-Sarasúa et al., 2020); two main insults found in AD. As memory processes occur in the MTL and associated areas, they become heavily affected throughout AD progression and thus, were regions of interest in this research project. The subjects were tested on a variety of tasks that evaluated AD-related memory impairments including the MWT, NOR task, and FC task that assess the HPC, PRC, and AMG/HPC, respectively. Post-mortem brain samples were collected to analyze upregulated inflammatory markers and Aβ plaque accumulation known to be present in AD pathology. To ensure that any behavioural differences found between groups was not due to different abilities, various tasks were employed including the balance beam task, visible platform test, and activity box assessments which assess motor skills, vision, and overall activity levels, respectively. Combined, these measures provide a clearer picture of the multimodal nature of AD pathology.

The findings show little to no effect of treatment on improving symptoms of AD. However, results show a trend of treatment-specific effects on certain measures. Overall, the results of the present research project are varied. The behavioural data suggests that genotype-specific differences begin to emerge at 12 months of age. Unfortunately, there were few significant findings showing that the combination *cannabis* extract improved performance on the memory-based behavioural tasks. However, there were findings that sex-related differences were present on various measures assessing AD pathology. Ultimately, there were multiple significant findings of genotype and sex related effects; although, there were few showing any treatment-related changes in brain and behaviour pathology related to AD. In the following sections I will 1) discuss novel and interesting findings in this research and 2) acknowledge important issues and considerations raised by the present studies.

Novel and Interesting Findings

Memory Assessments

Morris Water Task (MWT)

The MWT was used to assess spatial learning and memory in rodents (Barnhart et al., 2015; Rudy, 2021; Sakakibara et al., 2019). Spatial memory arises when animals (including humans) acquire map-like mental representations of their environment to navigate and learn where objects are in space (O'Keefe & Nadel, 1978). Lesion studies have shown that the HPC is heavily implicated in spatial memory (Morris, 1984). The HPC is one of the main brain areas that undergoes significant atrophy in the progression of AD (Kril et al., 2002). Therefore, investigating how *cannabis* extracts can improve HPC-related memory processes is essential to evaluating therapeutic benefits.

Looking at the behavioural data, there seems to be uniform results. In the MWT data, there was a uniform pattern of acquisition during training at 9 and 12 months of age across all groups. This shows that all groups, regardless of sex, genotype, or treatment type learned the task relatively the same at both time points. There was no improved performance in the extract-treated individuals compared to their vehicle-treated counterparts. However, as there were also no genotype-specific differences that emerged, this suggests that the APP-NL-G-F subjects were not impaired at the acquisition measured in this task at these timepoints. Furthermore, during the probe trial (1-day post training), the APP-NL subjects showed a stronger preference for the trained quadrant compared to the APP-NL-G-F subjects at 12, but not 9 months of age. This shows a genotype-specific emergence during the probe trial (1-day post training), suggesting that a genotype-specific effect is present at 12 months of age. Ultimately, this reveals that APP-NL-G-F subjects begin to show AD behavioural symptoms at 12 months of age, which mirrors results found in the literature (Mehla et al., 2019). As behavioural symptoms arise well after brain pathology begins, it can be assumed that the subjects have begun to descend into AD around 12 months of age. Additionally, there seemed to be no genotype-specific difference present during the second probe trial (1-week post training), which assesses long-term memory retention. This suggests that short-term, but not long-term memory is affected in the subjects, which mirrors deficits seen in the initial stages of AD in humans.

Novel Object Recognition (NOR) Task

The NOR task was used to assess recognition memory in rodents (Berlyne, 1950). Recognition memory is the ability to recognize previously encountered objects, places, or individuals through recollection and familiarity (Suzuki & Naya, 2014). Lesion studies have shown that the PRC is heavily implicated in recognition memory (Ennaceur et al., 1996; Warburton & Brown, 2015). The PRC has been shown to undergo significant atrophy through AD progression (Juottonen et al., 1998). Therefore, similar to HPC, assessing how *cannabis* extracts can improve PRC-related memory processes is crucial to examining therapeutic benefits.

In the NOR task data, there was a genotype-specific difference found at 9 months of age during the first testing day, however this difference disappeared at 12 months of age. At 9 months of age, the genotype-specific differences mirror those found in the MWT, suggesting that AD-related pathology may be evolving in the APP-NL-G-F subjects. At 12 months of age a sex-specific difference emerged in the APP-NL-G-F subjects, as the males outperformed their female counterparts on recognition memory. This suggests that the females may be showing accelerated, more subtle AD-related behavioural symptoms. This aligns with data showing that all the female subjects uniformly enter post-estrus around 12 months of age. As females lose the protective cognitive effects of estrogen, it makes sense that they would show greater cognitive decline than their male counterparts. Furthermore, there seemed to be no genotype, sex, or treatment-related effects on recognition memory during the second testing day at 9 or 12 months. I suspect that this task diminishes in effectively assessing recognition memory with repeated exposures. During the trial 1-month post training the subjects showed no significant differences between genotype, sex, or treatment-related performance. This mirrors the results found during the 1-week retention probe trial in the MWT, this reinforces the idea that short-term, but not long-term memory is affected in the initial stages of AD.

Fear Conditioning (FC) Task

The contextual FC task was used to assess associative learning and memory in rodents (Mehla et al., 2019). Associative learning and memory is the ability to learn and remember relationships between previously unrelated concepts (Pavlov, 1927). Lesion studies have shown that both the AMG and HPC are heavily involved in this process (Phillips & LeDoux, 1992). The HPC is one of the main brain areas that undergoes significant atrophy in the progression of AD

(Götz et al., 2018; Kril et al., 2002). Therefore, investigating how *cannabis* extracts can improve HPC-related memory processes is essential to evaluating therapeutic benefits.

In the FC task data, there were genotype-specific differences found during the context recall testing day, however there were no differences found during the tone recall day. During, the context recall testing day, the APP-NL-G-F subjects spent significantly less time freezing than the controls. This suggests that HPC-AMG processes are breaking down in the positive subjects, as they display weaker negative associations with the 'dangerous' context. The differences seen between the context recall and tone recall day may be because the context recall test was performed one day post shock training and the tone recall test was performed two days post shock training, making the shock-related memory more salient during context recall testing. This also reinforced the idea that HPC-AMG related memory plays a significant role in the FC task. The context-related memory of the shock is seemingly stronger than the tone-related memory. There were no treatment or sex-related effects found during the context recall or tone recall testing days.

Brain Pathology Assessments

Hippocampal Cell Volume

In the HPC cell volume data, there was no genotype, sex, or treatment-specific effect found in the CA1, CA3 or combined HPC regions of the subjects. However, there was a genotype-specific effect found in the dentate gyrus of the subjects, the APP-NL-G-F subjects had an increased cell volume compared to the APP-NL subjects. This is contradictory to AD-related pathology that displays a decrease in combined HPC cell volume over time (Ardekani et al., 2016). Overall, we should be seeing atrophy of HPC regions in APP-NL-G-F subjects. A reason we are not seeing sufficient HPC atrophy may be due to the relatively young age of the subjects (13.5 months at sacrifice).

<u>Aβ Plaques (82E1/4G8 Markers)</u>

Aβ plaques are a main pathological hallmark of AD as they develop early on in disease progression and are present throughout (Aso et al., 2016; Aso & Ferrer, 2014; Bloom, 2014; Cooray et al., 2020; Mooko et al., 2021; Sasaguri et al., 2017; Zhao et al., 2020). Thus, the amount of Aβ plaques can be correlated with disease evolution.

In the Aβ plaques (82E1/4G8) data, there was a significant effect of genotype found in the amount of 82E1 and 4G8-stained Aβ plaques in the PFC and HPC of the subjects. There were significantly more Aβ plaques stained for 82E1 and 4G8 in the PFC and HPC of APP-NL-G-F subjects compared to APP-NL subjects. This is what we would expect as APP-NL subjects did not display Aβ plaques related to AD-pathology. This is reinforced by studies that confirm no presence of Aβ plaques in APP-NL control subjects (Saito et al., 2014). Additionally, there was a significant effect of treatment found in 4G8-stained Aβ plaques found in the PFC of APP-NL-G-F subjects. The data showed fewer Aβ plaques in the PFC of APP-NL-G-F subjects treated with the *cannabis* extract. Similarly, the data revealed a trend of treatment on the surface area covered in 82E1-stained Aβ plaques in HPC ($p = 0.086$). This suggests that the *cannabis* extract is having a positive effect on downregulating Aβ plaques in AD-related pathology. However, this may not yet be fully revealed due to the relatively young age of the mice.

<u>Inflammation (Iba1/GFAP Markers)</u>

Chronic brain inflammation is heavily associated with AD progression and severity (Spangenberg & Green, 2017). Microglia and astrocytes are two cells that become active in the presence of inflammation and thus are key facilitators of inflammatory responses and mechanisms. Microglia and astrocyte activation provide insights into disease progression as they cluster around Aβ plaques in a futile attempt to remove them.

In the inflammation-related (GFAP/Iba1) data, there was a genotype-specific effect found in the GFAP expression in the PFC and Iba1 expression in PFC and HPC. The APP-NL-G-F subjects showed an increased expression of inflammatory markers, GFAP and Iba1 in the PFC and PFC/HPC regions, respectively. This suggests that AD-related pathology is present in the APP-NL-G-F subjects at sacrifice, at roughly 13.5 months of age. There was no effect of genotype found in GFAP expression in the HPC, this may be due to the fact that astrocytes show fewer physical differences between 'active' and 'non-active' states compared to microglia. When microglia become 'activated' their cell bodies increase substantially in size, however when astrocytes become 'activated' their processes increase slightly in size, which means that the change in surface area of the cells is more substantial in microglia. Furthermore, the PFC showed little to no GFAP expression in the APP-NL subjects, suggesting that any GFAP expression shown in the APP-NL-G-F subjects would be significantly more regardless. Overall, inflammatory-related processes were upregulated in APP-NL-G-F subjects, which corresponds with AD-related pathology.

Comparative Assessments

Various assessments and measure were used to ensure that any differences found between groups were not the result of variable motor or visual abilities, activity levels, or hormones.

Motor Function

In terms of motor function, the subject was assessed on two measures: the balance beam task and the visible platform test. In the balance beam task, there was a sex-specific effect found at 9 and 12 months of age. This is likely due to the fact that the male subjects weighed on average 40g heavier than the females, which made this task unfavourably difficult for the males. As there is no genotype or treatment-specific effect found it can be suggested that performance is related to sex-specific differences, such as body weight. Additionally, there was no genotype, sex, or treatment-specific effect on performance during the visible platform test. This shows that all groups displayed comparable motor and visual abilities across all tasks. Overall, no one experimental group showed a physical advantage over the others.

Activity Levels

To ensure that the *cannabis* extract was not producing changes in activity level, the subjects were assessed on various activity-related measures from when they first began treatments at 5 months of age to when they began behavioural testing at 9 months of age. In terms of activity, the subjects uniformly showed a decrease in activity (using measurements in stereotypy time, movement time, and total distance travelled) across time from 5 to 8 months of age. The males showed no genotype or treatment related differences; however, the females showed a genotype-specific effect in stereotype time, movement time, and total distance travelled. The female APP-NL subjects showed an increased level of stereotypy time, movement time, and total distance travelled compared to their APP-NL-G-F counterparts. This shows that APP-NL female subjects were more active and exhibited more exploratory behaviour than the APP-NL-G-F female subjects. Similarly, in the OFT the male subjects did not display any significant genotype, age, or treatment-related differences between groups; however, the females did show a significant age-related increase in exploratory behaviours between 9 and 12 months of age. Although an increase in exploratory behaviour may be due to increased familiarity with the arena as the subjects receive repeated exposures throughout the OFT and NOR tasks.

Cytology

Female mice experience an estrus cycle (similar to that of a menstrual cycle in humans) in which they undergo fluctuations in hormone levels (see Figure 1). These hormones may lead to alterations in cognitive abilities. Mice enter a period of post estrus (similar to menopause in humans) around 12 months of age (Brinton, 2012). There was no significant effect of genotype or treatment on proestrus stage duration which suggests that the extract treatments did not influence the natural estrous cycle of the mice.

In the estrus-related cytology data, there was no genotype or treatment-related effect on proestrus stage duration; however, there was a significant effect of age. The data shows that the duration of proestrus significantly decreased with age across all groups. At 9 months of age, the groups spent an average of 2.51 days in proestrus in a 14-day period, whereas at 12 months of age, the groups only spent an average of 2.00 days in proestrus during a 14-day period. If this trend were to continue, then the females would proceed to show a decreased level of protective estrogen with advanced age. Overall, this indicates that protective estrogen levels were decreasing over time.

In summary, the data overwhelming supported genotype-specific differences across various behavioural measures, including the NOR task, FC task, and MWT; as well as pathology-related analysis of 82E1 and 4G8-stained Aβ plaques and inflammatory markers, GFAP and Iba1. However, the subjects displayed few treatment-related effects on behavioural and pathology measures. Although, in all assessments the extract-treated subjects did not have significantly poorer outcomes than their vehicle-treated counterparts. This is a crucial finding as it demonstrates that the *cannabis* extract is not damaging important memory processes. In fact, certain trends indicate that the *cannabis* extract is beginning to improve pathology. Specifically, the data revealed a trend of treatment on HPC surface area covered in 82E1-stained Aβ plaques ($p = 0.086$). This suggests that there may still be subtle positive changes occurring in the brain as a result of the extract treatments.

Caveats

Familial v. Sporadic AD

AD presents itself in two forms: familial AD (FAD) (<5% of cases) and, the more common, sporadic AD (SAD) (>95% of cases) (Watt & Karl, 2017). Recently, genome-wide association studies (GWAS) have confirmed the importance of specific genes that increase the risk of developing AD pathology; namely ApoE, APP, PS1, and PS2 (Karl et al., 2012). Apolipoprotein E (ApoE), the most prevalent genetic risk factor for late-onset AD, is a polymorphic lipoprotein that mediates the transport and delivery of cholesterol and other lipids through ApoE receptors on the surface of cells (Newcombe et al., 2018). ApoE exists in three forms: ApoE4 (the highest risk form), ApoE3, and ApoE2 (Newcombe et al., 2018; O'Brien & Wong, 2011). ApoE is responsible for various functions in the CNS including repairing neurons and synaptic-dendritic connections, acting as a modulator of immune responses, and scavenging toxins such as Aβ (Newcombe et al., 2018). The ApoE4 is the highest risk allele of the ApoE gene as it seems to be the worst at removing Aβ (Nebel et al., 2018; O'Brien & Wong, 2011). Amyloid precursor protein (APP), presenilin-1 (PS1), and presenilin-2 (PS2), the most prevalent genetic risk factors for early-onset AD, are responsible for the cleavage of APP into smaller fragments (Sasaguri et al., 2017; Zhao et al., 2020). When this process goes awry and larger segments of Aβ are formed, they cannot be broken down by normal processes (Sasaguri et al., 2017). These processes are key areas of study as they allow researchers to gain insight into the molecular mechanisms that go awry during AD progression. Early-onset AD is an interesting topic of study as it allows for investigation into the initial progression of the disease, before pathology has become more advanced and perhaps more difficult to remedy.

Overall, these genes significantly contribute to the development of AD, however environmental factors (such as, diet, activity levels, stress, and cognitive load) also play important roles as they have a compounding effect on the likelihood of developing dementia. SAD occurs spontaneously and seemingly without cause; however, it presents itself pathologically like FAD which allows for FAD animal models, like APP-NL-G-F, to be developed and used to better encompass all aspects of AD pathology.

Treatment Issues

Ultimately, only one specific *cannabis* extract was used in this research project. There are hundreds of different combinations and dosages that can be created from *cannabis*. This means that the therapeutic potential will continue to remain largely unexplored until more extracts are synthesized and used. As no adverse effects were found at a 20:1 CBD:THC ratio, I would suggest increasing the dosage. Administration route also may be a reason more significant results were not found. Oral administration has a very low absorption rate and bioavailability compared to inhalants and oral mucosal routes (Bruni et al., 2018). This suggests that inhalants and/or oral mucosal administration may help penetrate the BBB enough to produce significant effects on neuronal processes and, resultantly, cognition. The lack of treatment-related effects observed in this project may be due, in part, to the oral doses not reaching the brain. However, recent findings in our lab show that 20 mg/kg CBD treatments in 18-month-old, aged mice do improve HPC-mediated memory deficits; suggesting that the treatments are reaching the brain (unpublished data).

Alternatively, our findings may indicate that the subjects were assessed too early in the progression of the disease, thus not allowing the disease to fully present itself; as the natural lifespan of a mouse is 18-24 months and AD symptoms are at their worst just prior to death (Sarne et al., 2018). By treating AD later in progression, we may see more significant differences arise between treatment groups.

It is also important to consider the fact that a tolerance to THC develops with repeated, prolonged exposure to CB1 agonists, leading to a decreased effect (as the receptor develops decreased reactivity or desensitization) (Kendall & Yudowski, 2017). As the extracted-treated subjects continually received THC over a nearly 9-month period from 5 to 13.5 months of age the effects of THC likely reduced over time. To combat this issue, the treatments could be administered less frequently, at an older age, or at an increasing dosage as they age.

Furthermore, other brain areas (other than the ones assessed in this research project) are involved in AD pathology. A more in-depth analysis of all brain areas may be required to see the early effects of treatment. Additionally, the mPFC was not explicitly assessed in any of our behavioural assays. This could be improved in future studies.

Furthermore, various sex differences arose on a variety of behavioural measures. These differences may be the result of a lowered amount of protective estrogen in female subjects. Studies have found that ERT does improve AD outcomes in human subjects (Song et al., 2020). This suggests that poorer outcomes in females may be remedied by hormonal intervention.

In conclusion, there was no overwhelming evidence showing that *cannabis* extracts improve AD pathology and memory-related impairments in APP-NL-G-F mice. However, the data did reveal treatment-related trends, suggesting that a modified method may be required to see behavioural and pathological improvements.

Other Issues

Sex differences remain a crucial area of inquiry in AD research, as well as vulnerable groups in society such as Indigenous groups. According to findings in the *Canadian Journal of Public Health*, First Nation populations have a 34% higher rate of dementia than non-Indigenous populations (Jacklin et al., 2013). Furthermore, a recent study found that Canadian Indigenous populations showed a disproportionately high prevalence of modifiable risk factors for AD including, diabetes, obesity, physical inactivity, low education, and smoking compared to non-Indigenous populations (these findings were especially true for Indigenous people who reside on reserves) (MacDonald et al., 2015). Furthermore, their findings also indicated that these modifiable risk factors may account for over 75% of AD cases among First Nations peoples in Canada (MacDonald et al., 2015). Combined this suggests that genetic and environmental factors are heavily influencing this at-risk population. Although, there are few scientific studies investigating the effects and etiology of AD in Canadian Indigenous populations. This stresses the importance of researching how societal impacts (such as wealth disparity, racism, stress, and access to healthcare and other basic needs) play a role in Indigenous AD outcomes; as it is essential to treating and combating the incessant rise of all cases of AD across the country and the globe. Advocating for significant and drastic societal changes may be fundamental in changing the overall narrative of AD.

References

Ahmed, Z., Shaw, G., Sharma, V. P., Yang, C., McGowan, E., & Dickson, D. W. (2007). Actin-binding proteins coronin-1a and IBA-1 are effective microglial markers for immunohistochemistry. *Journal of Histochemistry and Cytochemistry*, *55*(7), 687–700. https://doi.org/10.1369/jhc.6A7156.2007

Aho, L., Pikkarainen, M., Hiltunen, M., Leinonen, V., & Alafuzoff, I. (2010). Immunohistochemical visualization of amyloid-β protein precursor and amyloid-β in extra- and intracellular compartments in the human brain. *Journal of Alzheimer's Disease*, *20*(4), 1015–1028. https://doi.org/10.3233/JAD-2010-091681

Alzheimer, A. (1907) Über eine eigenartige erkankung der hirnrinde. *Clinical Anatomy, 8*(6), 429-431.

Anderson, L. L., Low, I. K., Banister, S. D., McGregor, I. S., & Arnold, J. C. (2019). Pharmacokinetics of Phytocannabinoid Acids and Anticonvulsant Effect of Cannabidiolic Acid in a Mouse Model of Dravet Syndrome. *Journal of Natural Products*, *82*(11), 3047–3055. https://doi.org/10.1021/acs.jnatprod.9b00600

Antunes, M., & Biala, G. (2012). The novel object recognition memory: Neurobiology, test procedure, and its modifications. *Cognitive Processing*, *13*(2), 93–110. https://doi.org/10.1007/s10339-011-0430-z

Ardekani, B. A., Convit, A., & Bachman, A. H. (2016). Analysis of the MIRIAD Data Shows Sex Differences in Hippocampal Atrophy Progression. *Journal of Alzheimer's Disease*, *50*(3), 847–857. https://doi.org/10.3233/JAD-150780

Aso, E., Andrés-Benito, P., Carmona, M., Maldonado, R., & Ferrer, I. (2016). Cannabinoid receptor 2 participates in amyloid-β processing in a mouse model of Alzheimer's disease but plays a minor role in the therapeutic properties of a cannabis-based medicine. *Journal of Alzheimer's Disease*, *51*(2), 489–500. https://doi.org/10.3233/JAD-150913

Aso, E., Andrés-Benito, P., & Ferrer, I. (2016). Delineating the Efficacy of a Cannabis-Based Medicine at Advanced Stages of Dementia in a Murine Model. *Journal of Alzheimer's Disease*, *54*(3), 903–912. https://doi.org/10.3233/JAD-160533

Aso, E., & Ferrer, I. (2014). Cannabinoids for treatment of alzheimer's disease: Moving toward the clinic. In *Frontiers in Pharmacology: Vol. 5 MAR* (Issue March, pp. 1–11). https://doi.org/10.3389/fphar.2014.00037

Aso, E., & Ferrer, I. (2016). CB2 Cannabinoid Receptor As Potential Target against Alzheimer's Disease. *Frontiers in Neuroscience*, *10*(May), 1–10. https://doi.org/10.3389/fnins.2016.00243

Aso, E., Sánchez-Pla, A., Vegas-Lozano, E., Maldonado, R., & Ferrer, I. (2015). Cannabis-based medicine reduces multiple pathological processes in AβPP/PS1 mice. *Journal of Alzheimer's Disease*, *43*(3), 977–991. https://doi.org/10.3233/JAD-141014

Astur, R. S., Taylor, L. B., Mamelak, A. N., Philpott, L., & Sutherland, R. J. (2002). Humans with hippocampus damage display severe spatial memory impairments in a virtual Morris water task. *Behavioural Brain Research*, *132*(1), 77–84. https://doi.org/10.1016/S0166-4328(01)00399-0

Ativie, F., Komorowska, J. A., Beins, E., Albayram, Ö., Zimmer, T., Zimmer, A., Tejera, D., Heneka, M., & Bilkei-Gorzo, A. (2018). Cannabinoid 1 Receptor Signaling on Hippocampal GABAergic Neurons Influences Microglial Activity. *Frontiers in Molecular Neuroscience*, *11*(August), 1–14. https://doi.org/10.3389/fnmol.2018.00295

Awasthi, M., Singh, S., Pandey, V. P., & Dwivedi, U. N. (2016). Alzheimer's disease: An overview of amyloid beta dependent pathogenesis and its therapeutic implications along with in silico approaches emphasizing the role of natural products. *Journal of the Neurological Sciences*, *361*, 256–271. https://doi.org/10.1016/j.jns.2016.01.008

Azam, S., Haque, M. E., Jakaria, M., Choi, D.-K., Jo, S.-H., Kim, I.-S., & Choi, D.-K. (2020). G-Protein-Coupled Receptors in CNS : A Potential Neurodegenerative Disorders and Associated. *Cells*, *9*, 506.

Bahr, B. A., Karanian, D. A., Makanji, S. S., & Makriyannis, A. (2006). Targeting the endocannabinoid system in treating brain disorders. *Expert Opinion on Investigational Drugs*, *15*(4), 351–365. https://doi.org/10.1517/13543784.15.4.351

Barnhart, C. D., Yang, D., & Lein, P. J. (2015). Using the Morris water maze to assess spatial learning and memory in weanling mice. *PLoS ONE*, *10*(4), 1–16. https://doi.org/10.1371/journal.pone.0124521

Basavarajappa, B. S., Shivakumar, M., Joshi, V., & Subbanna, S. (2017). Endocannabinoid system in neurodegenerative disorders. *Journal of Neurochemistry*, *142*(5), 624–648. https://doi.org/10.1111/jnc.14098

Benito, C., Núñez, E., Pazos, M. R., Tolón, R. M., & Romero, J. (2007). The endocannabinoid system and Alzheimer's disease. *Molecular Neurobiology*, *36*(1), 75–81. https://doi.org/10.1007/s12035-007-8006-8

Ben-Shabat, S., Fride, E., Sheskin, T., Tamiri, T., Rhee, M. H., Vogel, Z., Bisogno, T., De Petrocellis, L., Di Marzo, V., & Mechoulam, R. (1998). An entourage effect: inactive endogenous fatty acid glycerol esters enhance 2-arachidonoyl-glycerol cannabinoid activity. *European Journal of Pharmacology*, *353*(1), 23–31. https://doi.org/10.1016/S0014-2999(98)00392-6

Berlyne, D. . (1950). Novelty and Curosity as Determinants of Exploratory Behaviour. *Physical Review*, *80*(113), 580–594.

Bie, B., Wu, J., Foss, J. F., & Naguib, M. (2018). An overview of the cannabinoid type 2 receptor system and its therapeutic potential. *Current Opinion in Anaesthesiology*, *31*(4), 407–414. https://doi.org/10.1097/ACO.0000000000000616

Bilkei-Gorzo, A., Albayram, O., Draffehn, A., Michel, K., Piyanova, A., Oppenheimer, H., Dvir-Ginzberg, M., Rácz, I., Ulas, T., Imbeault, S., Bab, I., Schultze, J. L., & Zimmer, A. (2017). A chronic low dose of Δ9-tetrahydrocannabinol (THC) restores cognitive function in old mice. *Nature Medicine*, *23*(6), 782–787. https://doi.org/10.1038/nm.4311

Bizon, J. L., LaSarge, C. L., Montgomery, K. S., McDermott, A. N., Setlow, B., & Griffith, W. H. (2009). Spatial reference and working memory across the lifespan of male Fischer 344 rats. *Neurobiology of Aging*, *30*(4), 646–655. https://doi.org/10.1016/j.neurobiolaging.2007.08.004

Bliss, T. V., & Lømo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *The Journal of physiology, 232*(2), 331-356.

Bloom, G. S. (2014). Amyloid-β and tau: The trigger and bullet in Alzheimer disease pathogenesis. *JAMA Neurology*, *71*(4), 505–508. https://doi.org/10.1001/jamaneurol.2013.5847

Booth, J. K., & Bohlmann, J. (2019). Terpenes in Cannabis sativa – From plant genome to humans. *Plant Science*, *284*(March), 67–72. https://doi.org/10.1016/j.plantsci.2019.03.022

Brinton, R. D. (2012). Minireview: Translational animal models of human menopause: Challenges and emerging opportunities. *Endocrinology*, *153*(8), 3571–3578. https://doi.org/10.1210/en.2012-1340

Brown, A. J. (2007). Novel cannabinoid receptors. *British Journal of Pharmacology*, *152*(5), 567–575. https://doi.org/10.1038/sj.bjp.0707481

Brown, M. W., & Aggleton, J. P. (2001). Recognition memory: What are the roles of the perirhinal cortex and hippocampus? *Nature Reviews Neuroscience*, *2*(1), 51–61. https://doi.org/10.1038/35049064

Bruni, N., Pepa, C. Della, Oliaro-Bosso, S., Pessione, E., Gastaldi, D., & Dosio, F. (2018). Cannabinoid delivery systems for pain and inflammation treatment. *Molecules*, *23*(10). https://doi.org/10.3390/molecules23102478

Burwell, R. D., & Amaral, D. G. (1998). Cortical afferents of the perirhinal, postrhinal, and entorhinal cortices of the rat. *Journal of comparative neurology, 398*(2), 179-205.

Byers, S. L., Wiles, M. V, Dunn, S. L., & Taft, R. A. (2012). Mouse estrous cycle identification tool and images. *PLoS ONE*, *7*(4), 1–5. https://doi.org/10.1371/journal.pone.0035538

Calabrese, E. J., & Rubio-Casillas, A. (2018). Biphasic effects of THC in memory and cognition. *European Journal of Clinical Investigation*, *48*(5), 1–9. https://doi.org/10.1111/eci.12920

Caligioni, C. S. (2009). Assessing reproductive status/stages in mice. *Current Protocols in Neuroscience*, *SUPPL. 48*. https://doi.org/10.1002/0471142301.nsa04is48

Calina, D., Buga, A. M., Mitroi, M., Buha, A., Caruntu, C., Scheau, C., Bouyahya, A., El Omari, N., El Menyiy, N., & Docea, A. O. (2020). The Treatment of Cognitive, Behavioural and Motor Impairments from Brain Injury and Neurodegenerative Diseases through Cannabinoid System Modulation—Evidence from In Vivo Studies. *Journal of Clinical Medicine*, *9*(8), 2395. https://doi.org/10.3390/jcm9082395

Carlini, E. A., Karniol, I. G., Renault, P. F., & Schuster, C. R. (1974). Effects of marihuana in laboratory animals and in man. *British journal of pharmacology, 50*(2), 299. https://doi.org/10.1111/j.1476-5381.1974.tb08576.x

Carter, R. J., Morton, J., & Dunnett, S. B. (2001). Motor Coordination and Balance in Rodents. *Current Protocols in Neuroscience*, *15*(1), 1–14. https://doi.org/10.1002/0471142301.ns0812s15

Casarejos, M. J., Perucho, J., Gomez, A., Muñoz, M. P., Fernandez-Estevez, M., Sagredo, O., Fernandez Ruiz, J., Guzman, M., De Yebenes, J. G., & Mena, M. A. (2013). Natural cannabinoids improve dopamine neurotransmission and tau and amyloid pathology in a mouse model of tauopathy. *Journal of Alzheimer's Disease*, *35*(3), 525–539. https://doi.org/10.3233/JAD-130050

Cassano, T., Villani, R., Pace, L., Carbone, A., Bukke, V. N., Orkisz, S., Avolio, C., & Serviddio, G. (2020). From Cannabis sativa to Cannabidiol: Promising Therapeutic Candidate for the Treatment of Neurodegenerative Diseases. *Frontiers in Pharmacology*, *11*(March), 1–10. https://doi.org/10.3389/fphar.2020.00124

Cheng, D., Low, J. K., Logge, W., Garner, B., & Karl, T. (2014). Chronic cannabidiol treatment improves social and object recognition in double transgenic APPswe/PS1ΔE9 mice. *Psychopharmacology*, *231*(15), 3009–3017. https://doi.org/10.1007/s00213-014-3478-5

Cheng, D., Spiro, A. S., Jenner, A. M., Garner, B., & Karl, T. (2014). Long-term cannabidiol treatment prevents the development of social recognition memory deficits in alzheimer's disease transgenic mice. *Journal of Alzheimer's Disease*, *42*(4), 1383–1396. https://doi.org/10.3233/JAD-140921

Cholerton, B., Gleason, C. E., Baker, L. D., & Asthana, S. (2002). Estrogen and Alzheimer's disease: The story so far. *Drugs and Aging*, *19*(6), 405–427. https://doi.org/10.2165/00002512-200219060-00002

Coles, M., Watt, G., Kreilaus, F., & Karl, T. (2020). Medium-Dose Chronic Cannabidiol Treatment Reverses Object Recognition Memory Deficits of APP Swe /PS1ΔE9 Transgenic Female Mice. *Frontiers in Pharmacology*, *11*(December), 1–15. https://doi.org/10.3389/fphar.2020.587604

Cooray, R., Gupta, V., Suphioglu, & C., & Suphioglu, C. (2020). Current Aspects of the Endocannabinoid System and Targeted THC and CBD Phytocannabinoids as Potential Therapeutics for Parkinson's and Alzheimer's Diseases: a Review. *Molecular Neurobiology*, *57*(11), 4878–4890. https://doi.org/10.1007/s12035-020-02054-6

Cora, M. C., Kooistra, L., & Travlos, G. (2015). Vaginal Cytology of the Laboratory Rat and Mouse:Review and Criteria for the Staging of the Estrous Cycle Using Stained Vaginal Smears. *Toxicologic Pathology*, *43*(6), 776–793. https://doi.org/10.1177/0192623315570339

Cortez, I. D., Bulavin, D. V., Wu, P., McGrath, E. L., Cunningham, K. A., Wakamiya, M., Papaconstantinou, J., & Dineley, K. T. (2017). Aged dominant negative p38α MAPK mice are resistant to age-dependent decline in adult-neurogenesis and context discrimination fear conditioning. *Behavioural Brain Research*, *322*, 212–222. https://doi.org/10.1016/j.bbr.2016.10.023

Costa, B., Trovato, A. E., Comelli, F., Giagnoni, G., & Colleoni, M. (2007). The non-psychoactive cannabis constituent cannabidiol is an orally effective therapeutic agent in rat chronic inflammatory and neuropathic pain. *European Journal of Pharmacology*, *556*(1–3), 75–83. https://doi.org/10.1016/j.ejphar.2006.11.006

Daubner, S. C., Le, T., & Wang, S. (2011). Tyrosine hydroxylase and regulation of dopamine synthesis. *Archives of Biochemistry and Biophysics*, *508*(1), 1–12. https://doi.org/10.1016/j.abb.2010.12.017

De Oliveira Alvares, L., De Oliveira, L. F., Camboim, C., Diehl, F., Pasqualini Genro, B., Bispo Lanziotti, V., & Quillfeldt, J. A. (2005). Amnestic effect of intrahippocampal AM251, a CB1-selective blocker, in the inhibitory avoidance, but not in the open field habituation task, in rats. *Neurobiology of Learning and Memory*, *83*(2), 119–124. https://doi.org/10.1016/j.nlm.2004.10.002

Ehrhart, J., Obregon, D., Mori, T., Hou, H., Sun, N., Bai, Y., Klein, T., Fernandez, F., Tan, J., & Shytle, D. (2005). Stimulation of cannabinoid receptor 2 (CB2) suppresses microglial activation. *Journal of Neuroinflammation*, *2*, 1–14. https://doi.org/10.1186/1742-2094-2-29

Ennaceur, A., Neave, N., & Aggleton, J. P. (1996). Neurotoxic lesions of the perirhinal cortex do not mimic the behavioural effects of fornix transection in the rat. *Behavioural Brain Research*, *80*(1–2), 9–25. https://doi.org/10.1016/0166-4328(96)00006-X

Esposito, G., Scuderi, C., Savani, C., Steardo, L., De Filippis, D., Cottone, P., Iuvone, T., Cuomo, V., & Steardo, L. (2007). Cannabidiol in vivo blunts β-amyloid induced neuroinflammation by suppressing IL-1β and iNOS expression. *British Journal of Pharmacology*, *151*(8), 1272–1279. https://doi.org/10.1038/sj.bjp.0707337

Eubanks, L. M., Rogers, C. J., Beuscher IV, A. E., Koob, G. F., Olson, A. J., Dickerson, T. J., & Janda, K. D. (2006). A molecular link between the active component of marijuana and Alzheimer's disease pathology. *Molecular Pharmaceutics*, *3*(6), 773–777. https://doi.org/10.1021/mp060066m

Euston, D. R., Gruber, A. J., & McNaughton, B. L. (2012). The Role of Medial Prefrontal Cortex in Memory and Decision Making. *Neuron*, *76*(6), 1057–1070. https://doi.org/10.1016/j.neuron.2012.12.002

Farkhondeh, T., Khan, H., Aschner, M., Samini, F., Pourbagher-Shahri, A. M., Aramjoo, H., Roshanravan, B., Hoyte, C., Mehrpour, O., & Samarghandian, S. (2020). Impact of Cannabis-Based Medicine on Alzheimer's Disease by Focusing on the Amyloid β-Modifications: A Systematic Study. *CNS & Neurological Disorders - Drug Targets*, *19*(5), 334–343. https://doi.org/10.2174/1871527319666200708130745

Fisher, A. (2008). Cholinergic Treatments with Emphasis on M1 Muscarinic Agonists as Potential Disease-Modifying Agents for Alzheimer's Disease. *Neurotherapeutics*, *5*(3), 433–442. https://doi.org/10.1016/j.nurt.2008.05.002

Fowler, C. J. (2021). The endocannabinoid system – current implications for drug development. *Journal of Internal Medicine*, *290*(1), 2–26. https://doi.org/10.1111/joim.13229

Gallily, R., Yekhtin, Z., & Hanuš, L. O. (2018). The Anti-Inflammatory Properties of Terpenoids from Cannabis . *Cannabis and Cannabinoid Research*, *3*(1), 282–290. https://doi.org/10.1089/can.2018.0014

Garland, E. F., Hartnell, I. J., & Boche, D. (2022). Microglia and Astrocyte Function and Communication: What Do We Know in Humans? *Frontiers in Neuroscience*, *16*(February), 1–21. https://doi.org/10.3389/fnins.2022.824888

Garwood, C. J., Ratcliffe, L. E., Simpson, J. E., Heath, P. R., Ince, P. G., & Wharton, S. B. (2017). Review: Astrocytes in Alzheimer's disease and other age-associated dementias: a supporting player with a central role. *Neuropathology and Applied Neurobiology*, *43*(4), 281–298. https://doi.org/10.1111/nan.12338

Gomez-Arboledas, A., Davila, J. C., Sanchez-Mejias, E., Navarro, V., Nuñez-Diaz, C., Sanchez-Varo, R., Sanchez-Mico, M. V., Trujillo-Estrada, L., Fernandez-Valenzuela, J. J., Vizuete, M., Comella, J. X., Galea, E., Vitorica, J., & Gutierrez, A. (2018). Phagocytic clearance of presynaptic dystrophies by reactive astrocytes in Alzheimer's disease. *Glia*, *66*(3), 637–653. https://doi.org/10.1002/glia.23270

Gonçalves, E. C. D., Baldasso, G. M., Bicca, M. A., Paes, R. S., Capasso, R., & Dutra, R. C. (2020). Terpenoids, cannabimimetic ligands, beyond the cannabis plant. *Molecules*, *25*(7), 1–47. https://doi.org/10.3390/molecules25071567

Götz, J., Bodea, L. G., & Goedert, M. (2018). Rodent models for Alzheimer disease. *Nature Reviews Neuroscience*, *19*(10), 583–598. https://doi.org/10.1038/s41583-018-0054-8

Gould, T. D., Dao, D. T., & Kovacsics, C. E. (2009). Mood and Anxiety Related Phenotypes in Mice. *Neuromethods*, *42*, 1–20. https://doi.org/10.1007/978-1-60761-303-9

Gowran, A., Noonan, J., & Campbell, V. A. (2011). The multiplicity of action of cannabinoids: Implications for treating neurodegeneration. *CNS Neuroscience and Therapeutics*, *17*(6), 637–644. https://doi.org/10.1111/j.1755-5949.2010.00195.x

Grayson, B., Leger, M., Piercy, C., Adamson, L., Harte, M., & Neill, J. C. (2015). Assessment of disease-related cognitive impairments using the novel object recognition (NOR) task in rodents. *Behavioural Brain Research*, *285*, 176–193. https://doi.org/10.1016/j.bbr.2014.10.025

Guo, T., Zhang, D., Zeng, Y., Huang, T. Y., Xu, H., & Zhao, Y. (2020). Molecular and cellular mechanisms underlying the pathogenesis of Alzheimer's disease. *Molecular Neurodegeneration*, *15*(1), 1–37. https://doi.org/10.1186/s13024-020-00391-7

Gustavsson, A., Norton, N., Fast, T., Frölich, L., Georges, J., Holzapfel, D., Kirabali, T., Krolak-Salmon, P., Rossini, P. M., Ferretti, M. T., Lanman, L., Chadha, A. S., & van der Flier, W. M. (2023). Global estimates on the number of persons across the Alzheimer's disease continuum. *Alzheimer's and Dementia*, *19*(2), 658–670. https://doi.org/10.1002/alz.12694

Hanuš, L. O., & Hod, Y. (2020). Terpenes/Terpenoids in Cannabis: Are They Important? *Medical Cannabis and Cannabinoids*, *3*(1), 25–60. https://doi.org/10.1159/000509733

Hemonnot, A. L., Hua, J., Ulmann, L., & Hirbec, H. (2019). Microglia in Alzheimer disease: Well-known targets and new opportunities. *Frontiers in Cellular and Infection Microbiology*, *9*(JUL), 1–20. https://doi.org/10.3389/fnagi.2019.00233

Hooli, B. V, & Tanzi, R. E. (2009). A current view of Alzheimer's disease. *F1000 Biology Reports*, *1*(July), 3–6. https://doi.org/10.3410/b1-54

Hoshi, M., Takashima, A., Murayama, M., Yasutake, K., Yoshida, N., Ishiguro, K., Hoshino, T., & Imahori, K. (1997). Nontoxic amyloid β peptide1-42 suppresses acetylcholine synthesis. Possible role in cholinergic dysfunction in Alzheimer's disease. *Journal of Biological Chemistry*, *272*(4), 2038–2041. https://doi.org/10.1074/jbc.272.4.2038

Hossain, S. R., Karem, H., Jafari, Z., Kolb, B. E., & Mohajerani, M. H. (2023). Early tactile stimulation influences the development of Alzheimer's disease in gestationally stressed APP

NL-G-F adult offspring NL-G-F/NL-G-F mice. *Experimental Neurology*, *368*(July), 114498. https://doi.org/10.1016/j.expneurol.2023.114498

Howlett, A. C., Breivogel, C. S., Childers, S. R., Deadwyler, S. A., Hampson, R. E., & Porrino, L. J. (2004). Cannabinoid physiology and pharmacology: 30 Years of progress. *Neuropharmacology*, *47*, 345–358. https://doi.org/10.1016/j.neuropharm.2004.07.030

Huestis, M. A. (2007). Human cannabinoid pharmacokinetics. *Chemistry and Biodiversity*, *4*(8), 1770–1804. https://doi.org/10.1002/cbdv.200790152

Hughes, B., & Herron, C. E. (2019). Cannabidiol Reverses Deficits in Hippocampal LTP in a Model of Alzheimer's Disease. *Neurochemical Research*, *44*(3), 703–713. https://doi.org/10.1007/s11064-018-2513-z

Hyman, B. T., Van Hoesen, G. W., Damasio, A. R., & Barnes, C. L. (1984). Alzheimer's disease: cell-specific pathology isolates the hippocampal formation. *Science, 225*(4667), 1168-1170

Idriss, H. T., & Naismith, J. H. (2000). TNFα and the TNF receptor superfamily: Structure-function relationship(s). *Microscopy Research and Technique*, *50*(3), 184–195. https://doi.org/10.1002/1097-0029

Jankowsky, J. L., & Zheng, H. (2017). Practical considerations for choosing a mouse model of Alzheimer's disease. *Molecular Neurodegeneration*, *12*(1), 1–22. https://doi.org/10.1186/s13024-017-0231-7

Jiang, W., Zhang, Y., Xiao, L., Van Cleemput, J., Ji, S. P., Bai, G., & Zhang, X. (2005). Cannabinoids promote embryonic and adult hippocampus neurogenesis and produce anxiolytic- and antidepressant-like effects. *Journal of Clinical Investigation*, *115*(11), 3104–3116. https://doi.org/10.1172/JCI25509

Jin, K., Xie, L., Kim, S. H., Parmentier-Batteur, S., Sun, Y., Mao, X. O., Childs, J., & Greenberg, D. A. (2004). Defective adult neurogenesis in CB1 cannabinoid receptor knockout mice. *Molecular Pharmacology*, *66*(2), 204–208. https://doi.org/10.1124/mol.66.2.204

Jobson, D. D., Hase, Y., Clarkson, A. N., & Kalaria, R. N. (2021). The role of the medial prefrontal cortex in cognition, ageing and dementia. *Brain Communications*, *3*(3). https://doi.org/10.1093/braincomms/fcab125

Johnson, J. R., Burnell-Nugent, M., Lossignol, D., Ganae-Motan, E. D., Potts, R., & Fallon, M. T. (2010). Multicenter, Double-Blind, Randomized, Placebo-Controlled, Parallel-Group Study of the Efficacy, Safety, and Tolerability of THC:CBD Extract and THC Extract in Patients with Intractable Cancer-Related Pain. *Journal of Pain and Symptom Management*, *39*(2), 167–179. https://doi.org/10.1016/j.jpainsymman.2009.06.008

Juottonen, K., Laakso, M. P., Insausti, R., Lehtovirta, M., Pitkänen, A., Partanen, K., & Soininen, H. (1998). Volumes of the entorhinal and perirhinal cortices in Alzheimer's disease. *Neurobiology of Aging*, *19*(1), 15–22. https://doi.org/10.1016/S0197-4580(98)00007-4

Kádár, A., Wittmann, G., Liposits, Z., & Fekete, C. (2009). Improved method for combination of immunocytochemistry and Nissl staining. *Journal of Neuroscience Methods*, *184*(1), 115–118. https://doi.org/10.1016/j.jneumeth.2009.07.010

Kar, S., Slowikowski, S. P. M., Westaway, D., & Mount, H. T. J. (2004). Interactions between β-amyloid and central cholinergic neurons: Implications for Alzheimer's disease. *Journal of Psychiatry and Neuroscience, 29*(6), 427–441.

Kar, S., & Quirion, R. (2004). Amyloid β peptides and central cholinergic neurons: Functional interrelationship and relevance to Alzheimer's disease pathology. *Progress in Brain Research, 145*, 261–274. https://doi.org/10.1016/S0079-6123(03)45018-8

Karl, T., Cheng, D., Garner, B., & Arnold, J. C. (2012). The therapeutic potential of the endocannabinoid system for Alzheimer's disease. *Expert Opinion on Therapeutic Targets*, *16*(4), 407–420. https://doi.org/10.1517/14728222.2012.671812

Karl, T., Garner, B., & Cheng, D. (2017). The therapeutic potential of the phytocannabinoid cannabidiol for Alzheimer's disease. *Behavioural Pharmacology*, *28*(2-3 Special Issue), 142–160. https://doi.org/10.1097/FBP.0000000000000247

Karniol, I. G., & Carlini, E. A. (1973). Pharmacological interaction between cannabidiol and δ9-tetrahydrocannabinol. *Psychopharmacologia*, *33*(1), 53–70. https://doi.org/10.1007/BF00428793

Karniol, I. G., Shirakawa, I., Kasinski, N., Pfeferman, A., & Carlini, E. A. (1974). Cannabidiol interferes with the effects of Δ9-tetrahydrocannabinol in man. *European Journal of Pharmacology, 1*(1), 303–335.

Kendall, D. A., & Yudowski, G. A. (2017). Cannabinoid receptors in the central nervous system: Their signaling and roles in disease. *Frontiers in Cellular Neuroscience*, *10*(January), 1–10. https://doi.org/10.3389/fncel.2016.00294

Kim, S., Kim, M. J., Kim, S., Kang, H. S., Lim, S. W., Myung, W., Lee, Y., Hong, C. H., Choi, S. H., Na, D. L., Seo, S. W., Ku, B. D., Kim, S. Y., Kim, S. Y., Jeong, J. H., Park, S. A., Carroll, B. J., & Kim, D. K. (2015). Gender differences in risk factors for transition from mild cognitive impairment to Alzheimer's disease: A CREDOS study. *Comprehensive Psychiatry*, *62*, 114–122. https://doi.org/10.1016/j.comppsych.2015.07.002

Kim, S. H., Yang, J. W., Kim, K. H., Kim, J. U., & Yook, T. H. (2019). A review on studies of Marijuana for Alzheimer's disease - Focusing on CBD, THC. *Journal of Pharmacopuncture*, *22*(4), 225–230. https://doi.org/10.3831/KPI.2019.22.030

Klein, C., Karanges, E., Spiro, A., Wong, A., Spencer, J., Huynh, T., Gunasekaran, N., Karl, T., Long, L. E., Huang, X. F., Liu, K., Arnold, J. C., & McGregor, I. S. (2011). Cannabidiol potentiates Δ 9-tetrahydrocannabinol (THC) behavioural effects and alters THC pharmacokinetics during acute and chronic treatment in adolescent rats. *Psychopharmacology*, *218*(2), 443–457. https://doi.org/10.1007/s00213-011-2342-0

Kolb, B. (1984). Functions of the frontal cortex of the rat: A comparative review. *Brain Research, 320*(1), 65-98. https://psycnet.apa.org/doi/10.1016/0165-0173(84)90018-3

Kolb, B., & Cioe, J. (2004) Organization and plasticity of the prefrontal cortex of the rat. *Prefrontal cortex: From synaptic plasticity to cognition,* 1-32. Springer US. https://doi.org/10.1007/1-4020-7949-4_1

Koppel, J., & Davies, P. (2008). Targeting the endocannabinoid system in Alzheimer's disease. *Journal of Alzheimer's Disease*, *15*(3), 495–504. https://doi.org/10.3233/JAD-2008-15315

Kril, J. J., Patel, S., Harding, A. J., & Halliday, G. M. (2002). Neuron loss from the hippocampus of Alzheimer's disease exceeds extracellular neurofibrillary tangle formation. *Acta Neuropathologica*, *103*(4), 370–376. https://doi.org/10.1007/s00401-001-0477-5

Kruk-Slomka, M., Dzik, A., Budzynska, B., & Biala, G. (2017). Endocannabinoid System: the Direct and Indirect Involvement in the Memory and Learning Processes—a Short Review. *Molecular Neurobiology*, *54*(10), 8332–8347. https://doi.org/10.1007/s12035-016-0313-5

Latif-Hernandez, A., Sabanov, V., Ahmed, T., Craessaerts, K., Saito, T., Saido, T., & Balschun, D. (2020). The two faces of synaptic failure in App NL-G-Fknock-in mice. *Alzheimer's Research and Therapy*, *12*(1), 1–15. https://doi.org/10.1186/s13195-020-00667-6

Latif-Hernandez, A., Shah, D., Craessaerts, K., Saido, T., Saito, T., De Strooper, B., Van der Linden, A., & D'Hooge, R. (2019). Subtle behavioral changes and increased prefrontal-hippocampal network synchronicity in APP NL−G−F mice before prominent plaque deposition. *Behavioural Brain Research*, *364*(November 2017), 431–441. https://doi.org/10.1016/j.bbr.2017.11.017

LeDoux, J. E. (1993). Emotional memory systems in the brain. *Behavioural Brain Research*, *58*(1–2), 69–79. https://doi.org/10.1016/0166-4328(93)90091-4

Lueptow, L. M. (2017). Novel object recognition test for the investigation of learning and memory in mice. *Journal of Visualized Experiments*, *2017*(126), 1–9. https://doi.org/10.3791/55718

Luong, T. N., Carlisle, H. J., Southwell, A., & Patterson, P. H. (2011). Assessment of motor balance and coordination in mice using the balance beam. *Journal of Visualized Experiments*, *49*, 5–7. https://doi.org/10.3791/2376

MacDonald, J. P., Barnes, D. E., & Middleton, L. E. (2015). Implications of risk factors for Alzheimer's disease in Canada's indigenous population. *Canadian Geriatrics Journal CGJ, 18*(3), 152-158. https://doi.org/10.5770/cgj.18.159

Maresz, K., Carrier, E. J., Ponomarev, E. D., Hillard, C. J., & Dittel, B. N. (2005). Modulation of the cannabinoid CB2 receptor in microglial cells in response to inflammatory stimuli. *Journal of Neurochemistry*, *95*(2), 437–445. https://doi.org/10.1111/j.1471-4159.2005.03380.x

Martín-Moreno, A. M., Brera, B., Spuch, C., Carro, E., García-García, L., Delgado, M., Pozo, M. A., Innamorato, N. G., Cuadrado, A., & de Ceballos, M. L. (2012). Prolonged oral cannabinoid administration prevents neuroinflammation, lowers β-amyloid levels and improves cognitive performance in Tg APP 2576 mice. *Journal of Neuroinflammation*, *9*(1), 8. https://doi.org/10.1186/1742-2094-9-8

Martín-Moreno, A. M., Reigada, D., Ramírez, B. G., Mechoulam, R., Innamorato, N., Cuadrado, A., & De Ceballos, M. L. (2011). Cannabidiol and other cannabinoids reduce microglial activation in vitro and in vivo: Relevance to alzheimer's disease. *Molecular Pharmacology*, *79*(6), 964–973. https://doi.org/10.1124/mol.111.071290

Masliah, E., & Selman, B. (2008). Alzheimer's in real time A hard statistical view. *Nature*, *451*(February), 638–639.

McDonald (Unpublished). *[Unpublished manuscript]*. Department of Neuroscience, University of Lethbridge, Canada.

McLean, A. C., Valenzuela, N., Fai, S., & Bennett, S. A. L. (2012). Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. *Journal of Visualized Experiments*, *67*, 4–9. https://doi.org/10.3791/4389

Mechoulam, R., & Parker, L. A. (2013). The endocannabinoid system and the brain. *Annual Review of Psychology*, *64*, 21–47. https://doi.org/10.1146/annurev-psych-113011-143739

Mehla, J., Deibel, S. H., Karem, H., Hossain, S., Lacoursiere, S. G., Sutherland, R. J., Mohajerani, M. H., & McDonald, R. J. (2022). Dramatic impacts on brain pathology, anxiety, and cognitive function in the knock-in APPNL-G-F mouse model of Alzheimer disease following long-term voluntary exercise. *Alzheimer's Research and Therapy*, *14*(1), 1–20. https://doi.org/10.1186/s13195-022-01085-6

Mehla, J., Lacoursiere, S. G., Lapointe, V., McNaughton, B. L., Sutherland, R. J., McDonald, R. J., & Mohajerani, M. H. (2019). Age-dependent behavioral and biochemical characterization of single APP knock-in mouse (APPNL-G-F/NL-G-F) model of Alzheimer's disease. *Neurobiology of Aging*, *75*, 25–37. https://doi.org/10.1016/j.neurobiolaging.2018.10.026

Miller, L. L., & Branconnier, R. J. (1983). Cannabis: Effects on memory and the cholinergic limbic system. *Psychological Bulletin*, *93*(3), 441–456. https://doi.org/10.1037/0033-2909.93.3.441

Mooko, T., Bala, A., Tripathy, S., Kumar, C. S., Mahadevappa, C. P., Chaudhary, S. K., & Matsabisa, M. G. (2021). Cannabis Sativa L. Flower and Bud Extracts inhibited In vitro Cholinesterases and b-Secretase Enzymes Activities: Possible Mechanisms of Cannabis use in Alzheimer Disease. *Endocrine, Metabolic & Immune Disorders - Drug Targets*, *21*, 1–13. https://doi.org/10.2174/1871530321666210222124349

Morena, M., & Campolongo, P. (2014). The endocannabinoid system: An emotional buffer in the modulation of memory function. *Neurobiology of Learning and Memory*, *112*, 30–43. https://doi.org/10.1016/j.nlm.2013.12.010

Morris, R. G. M., Garrud, P., Rawlins, J. N. P., & O'Keefe, J. (1982). Place navigation impaired in rats with hippocampal lesions. *Nature (London), 297*(5868), 681-683. https://doi.org/10.1038/297681a0

Morris, R. (1984). Developments of a water-maze procedure for studying spatial learning in the rat. *Journal of Neuroscience Methods*, *11*(1), 47–60. https://doi.org/10.1016/0165-0270(84)90007-4

Mumby, D. G., & Pinel, J. P. J. (1994). Rhinal cortex lesions and object recognition in rats. Behavioral Neuroscience, 108(1), 11-18. https://doi.org/10.1037/0735-7044.108.1.11

Nebel, R. A., Aggarwal, N. T., Barnes, L. L., Gallagher, A., Goldstein, J. M., Kantarci, K., Mallampalli, M. P., Mormino, E. C., Scott, L., Yu, W. H., Maki, P. M., & Mielke, M. M. (2018). Understanding the impact of sex and gender in Alzheimer's disease: A call to action. *Alzheimer's and Dementia*, *14*(9), 1171–1183. https://doi.org/10.1016/j.jalz.2018.04.008

Newcombe, E. A., Camats-Perna, J., Silva, M. L., Valmas, N., Huat, T. J., & Medeiros, R. (2018). Inflammation: The link between comorbidities, genetics, and Alzheimer's disease 11 Medical and Health Sciences 1109 Neurosciences 11 Medical and Health Sciences 1107 Immunology. *Journal of Neuroinflammation*, *15*(1), 1–26. https://doi.org/10.1186/s12974-018-1313-3

Nidadavolu, P., Bilkei-Gorzo, A., Krämer, M., Schürmann, B., Palmisano, M., Beins, E. C., Madea, B., & Zimmer, A. (2021). Efficacy of Δ9 -Tetrahydrocannabinol (THC) Alone or in Combination With a 1:1 Ratio of Cannabidiol (CBD) in Reversing the Spatial Learning Deficits in Old Mice. *Frontiers in Aging Neuroscience*, *13*(August), 1–11. https://doi.org/10.3389/fnagi.2021.718850

Nissl F. (1894). Ueber eine neue Untersuchungsmethode des Centralorgans zur Feststellung der Localisation der Nervenzellen. *Neurologisches Centralblatt*, *13*, 507–8.

Nixon, A.G. (2022). *The effects of cannabinoid compounds on the brain and behavior of mouse models of Alzheimer's disease.* University of Lethbridge, Department of Neuroscience.

O'Brien, R. J., & Wong, P. C. (2011). Amyloid precursor protein processing and alzheimer's disease. *Annual Review of Neuroscience*, *34*, 185–204. https://doi.org/10.1146/annurev-neuro-061010-113613

O'Keefe J. & Nadel L. (1978). *The hippocampus as a cognitive map*. Clarendon Press; Oxford University Press.

Oveisgharan, S., Arvanitakis, Z., Yu, L., Farfel, J., Schneider, J. A., & Bennett, D. A. (2018). Sex differences in Alzheimer's disease and common neuropathologies of aging. *Acta Neuropathologica*, *136*(6), 887–900. https://doi.org/10.1007/s00401-018-1920-1

Pavlov, I. P. (1927). *Conditioned reflexes: an investigation of the physiological activity of the cerebral cortex*. Oxford University Press.

Pellati, F., Borgonetti, V., Brighenti, V., Biagi, M., Benvenuti, S., & Corsi, L. (2018). Cannabis sativa L. and Nonpsychoactive Cannabinoids: Their Chemistry and Role against Oxidative Stress, Inflammation, and Cancer. *BioMed Research International*, *2018*. https://doi.org/10.1155/2018/1691428

Penke, B., Bogár, F., & F l, L. (2017). β-amyloid and the pathomechanisms of Alzheimer's disease: A comprehensive view. *Molecules*, *22*(10). https://doi.org/10.3390/molecules22101692

Perry, V. H., Nicoll, J. A. R., & Holmes, C. (2010). Microglia in neurodegenerative disease. *Nature Reviews Neurology*, *6*(4), 193–201. https://doi.org/10.1038/nrneurol.2010.17

Phillips, R. G., & LeDoux, J. E. (1992). Differential Contribution of Amygdala and Hippocampus to Cued and Contextual Fear Conditioning. In *Behavioral Neuroscience* (Vol. 106, Issue 2, pp. 274–285). https://doi.org/10.1037/0735-7044.106.2.274

Poulin, S. P., Dautoff, R., Morris, J. C., Barrett, L. F., & Dickerson, B. C. (2011). Amygdala atrophy is prominent in early Alzheimer's disease and relates to symptom severity. *Psychiatry Research - Neuroimaging*, *194*(1), 7–13. https://doi.org/10.1016/j.pscychresns.2011.06.014

Preston, A. R., & Eichenbaum, H. (2013). Interplay of hippocampus and prefrontal cortex in memory. *Current Biology*, *23*(17), R764–R773. https://doi.org/10.1016/j.cub.2013.05.041

Puighermanal, E., Marsicano, G., Busquets-Garcia, A., Lutz, B., Maldonado, R., & Ozaita, A. (2009). Cannabinoid modulation of hippocampal long-term memory is mediated by mTOR signaling. *Nature Neuroscience*, *12*(9), 1152–1158. https://doi.org/10.1038/nn.2369

Puzzo, D., Gulisano, W., Palmeri, A., & Arancio, O. (2015). Rodent models for Alzheimer's disease drug discovery. *Expert Opinion on Drug Discovery*, *10*(7), 703–711. https://doi.org/10.1517/17460441.2015.1041913

Quirion, R. (1993). Cholinergic markers in Alzheimer disease and the autoregulation of acetylcholine release. *Journal of Psychiatry and Neuroscience, 18*(5), 226–234.

Ramírez, B. G., Blázquez, C., Gómez Del Pulgar, T., Guzmán, M., & De Ceballos, M. L. (2005). Prevention of Alzheimer's disease pathology by cannabinoids: Neuroprotection mediated by blockade of microglial activation. *Journal of Neuroscience*, *25*(8), 1904–1913. https://doi.org/10.1523/JNEUROSCI.4540-04.2005

Rom, S., & Persidsky, Y. (2013). Cannabinoid receptor 2: Potential role in immunomodulation and neuroinflammation. *Journal of Neuroimmune Pharmacology*, *8*(3), 608–620. https://doi.org/10.1007/s11481-013-9445-9

Romero-Sandoval, E. A., Horvath, R. J., & DeLeo, J. A. (2008). Neuroimmune interactions and pain: Focus on glial-modulating targets. *Current Opinion in Investigational Drugs*, *9*(7), 726–734.

Rosenberg, R. N., Lambracht-Washington, D., Yu, G., & Xia, W. (2020). Genomics of Alzheimer's disease. *Rosenberg's Molecular and Genetic Basis of Neurological and Psychiatric Disease*, *73*(7), 3–18. https://doi.org/10.1016/b978-0-12-813866-3.00001-1

Rudy J. W. (2021). *The neurobiology of learning and memory* (Third). Sinauer Associates: Oxford University Press.

Russo, E. B. (2008). Cannabinoids in the management of difficult to treat pain. *Therapeutics and Clinical Risk Management*, *4*(1), 245–259. https://doi.org/10.2147/tcrm.s1928

Russo, E. B. (2019). The case for the entourage effect and conventional breeding of clinical cannabis: No "Strain," no gain. *Frontiers in Plant Science*, *9*(January), 1–8. https://doi.org/10.3389/fpls.2018.01969

Russo, E. B. (2011). Taming THC: Potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. *British Journal of Pharmacology*, *163*(7), 1344–1364. https://doi.org/10.1111/j.1476-5381.2011.01238.x

Russo, E. B. (2018). Cannabis therapeutics and the future of neurology. *Frontiers in Integrative Neuroscience*, *12*(October), 1–11. https://doi.org/10.3389/fnint.2018.00051

Russo, E., & Guy, G. W. (2006). A tale of two cannabinoids: The therapeutic rationale for combining tetrahydrocannabinol and cannabidiol. *Medical Hypotheses*, *66*(2), 234–246. https://doi.org/10.1016/j.mehy.2005.08.026

Saito, T., Matsuba, Y., Mihira, N., Takano, J., Nilsson, P., Itohara, S., Iwata, N., & Saido, T. C. (2014). Single App knock-in mouse models of Alzheimer's disease. *Nature Neuroscience*, *17*(5), 661–663. https://doi.org/10.1038/nn.3697

Sakakibara, Y., Sekiya, M., Saito, T., Saido, T. C., & Iijima, K. M. (2019). Amyloid-β plaque formation and reactive gliosis are required for induction of cognitive deficits in App knock-in mouse models of Alzheimer's disease. *BMC Neuroscience*, *20*(1), 1–14. https://doi.org/10.1186/s12868-019-0496-6

Sánchez-Sarasúa, S., Fernández-Pérez, I., Espinosa-Fernández, V., Sánchez-Pérez, A. M., & Ledesma, J. C. (2020). Can we treat neuroinflammation in alzheimer's disease? *International Journal of Molecular Sciences*, *21*(22), 1–23. https://doi.org/10.3390/ijms21228751

Sarne, Y., Toledano, R., Rachmany, L., Sasson, E., & Doron, R. (2018). Reversal of age-related cognitive impairments in mice by an extremely low dose of tetrahydrocannabinol. *Neurobiology of Aging*, *61*, 177–186. https://doi.org/10.1016/j.neurobiolaging.2017.09.025

Sasaguri, H., Nilsson, P., Hashimoto, S., Nagata, K., Saito, T., De Strooper, B., Hardy, J., Vassar, R., Winblad, B., & Saido, T. C. (2017). APP mouse models for Alzheimer's disease preclinical studies. *The EMBO Journal*, *36*(17), 2473–2487. https://doi.org/10.15252/embj.201797397

Schubert, D., Kepchia, D., Liang, Z., Dargusch, R., Goldberg, J., & Maher, P. (2019). Efficacy of Cannabinoids in a Pre-Clinical Drug-Screening Platform for Alzheimer's Disease. *Molecular Neurobiology*, *56*(11), 7719–7730. https://doi.org/10.1007/s12035-019-1637-8

Scoville, W. B., & Milner, B. (1957). Loss of recent memory after bilateral hippocampal lesions. *The Journal of Neuropsychiatry and Clinical Neurosciences*, *20*(11), 11–21. https://doi.org/10.1176/jnp.12.1.103-a

Shaywitz, S. E., Shaywitz, B. A., Pugh, K. R., Fulbright, R. K., Skudlarski, P., Mencl, W. E., Constable, R. T., Naftolin, F., Palter, S. F., Marchione, K. E., Katz, L., Shankweiler, D. P., Fletcher, J. M., Lacadie, C., Keltz, M., & Gore, J. C. (1999). Effect of estrogen on brain activation patterns in postmenopausal women during working memory tasks. *American Medical Association*, *281*(13), 1197–1202. https://doi.org/10.1001/jama.281.13.1197

Simón, A. M., Frechilla, D., & Del Río, J. (2010). Perspectives on the amyloid cascade hypothesis of Alzheimer's disease. *Revista de Neurologia*, *50*(11), 667–675. https://doi.org/10.33588/rn.5011.2010211

Sipos, E., Kurunczi, A., Kasza, Á., Horváth, J., Felszeghy, K., Laroche, S., Toldi, J., Párducz, Á., Penke, B., & Penke, Z. (2007). β-Amyloid pathology in the entorhinal cortex of rats induces memory deficits: Implications for Alzheimer's disease. *Neuroscience*, *147*(1), 28–36. https://doi.org/10.1016/j.neuroscience.2007.04.011

Siracusa, R., Fusco, R., & Cuzzocrea, S. (2019). Astrocytes: Role and functions in brain pathologies. *Frontiers in Pharmacology*, *10*(SEP), 1–10. https://doi.org/10.3389/fphar.2019.01114

Song, Y., Li, S., Li, X., Chen, X., Wei, Z., Liu, Q., & Cheng, Y. (2020). The Effect of Estrogen Replacement Therapy on Alzheimer's Disease and Parkinson's Disease in Postmenopausal Women: A Meta-Analysis. *Frontiers in Neuroscience*, *14*(March), 1–13. https://doi.org/10.3389/fnins.2020.00157

Spangenberg, E. E., & Green, K. N. (2017). Inflammation in Alzheimer's disease: Lessons learned from microglia-depletion models. In *Brain, Behavior, and Immunity* (61), 1–11. Elsevier Inc. https://doi.org/10.1016/j.bbi.2016.07.003

Squire, L. R., & Zola-Morgan, S. (1991). The medial temporal lobe memory system. In *Science* 253(5026), 1380–1386. https://doi.org/10.1126/science.1896849

Stelzmann, R. A., Norman Schnitzlein, H., & Reed Murtagh, F. (1995). An english translation of alzheimer's 1907 paper, "über eine eigenartige erkankung der hirnrinde." *Clinical Anatomy*, *8*(6), 429–431. https://doi.org/10.1002/ca.980080612

Sutherland, R. J., Kolb, B., & Whishaw, I. Q. (1982). Spatial mapping: Definitive disruption by hippocampal or medial frontal cortical damage in the rat. *Neuroscience Letters, 31*(3), 271. https://doi.org/10.1016/0304-3940(82)90032-5

Sutherland, R. J., Whishaw, I. Q., & Kolb, B. (1983). A behavioural analysis of spatial localization following electrolytic, kainate- or colchicine-induced damage to the hippocampal formation in the rat. *Behavioural Brain Research, 7*(2), 133-153. https://doi.org/10.1016/0166-4328(83)90188-2

Suzuki, W. A., & Naya, Y. (2014). The perirhinal cortex. *Annual Review of Neuroscience*, *37*, 39–53. https://doi.org/10.1146/annurev-neuro-071013-014207

Takehara-Nishiuchi, K. (2014). Entorhinal cortex and consolidated memory. *Neuroscience Research*, *84*, 27–33. https://doi.org/10.1016/j.neures.2014.02.012

Thathiah, A., & De Strooper, B. (2009). G protein-coupled receptors, cholinergic dysfunction, and Aβ toxicity in alzheimer's disease. *Science Signaling*, *2*(93). https://doi.org/10.1126/scisignal.293re8

Toro, C. A., Zhang, L., Cao, J., & Cai, D. (2019). Sex differences in Alzheimer's disease: Understanding the molecular impact. *Brain Research*, *1719*(May), 194–207. https://doi.org/10.1016/j.brainres.2019.05.031

Tulving, E., & Markowitsch, H. J. (1998). Episodic and declarative memory: Role of the hippocampus. *Hippocampus, 8*(3), 198-204.

Van Rossum, D., & Hanisch, U. K. (2004). Microglia. *Metabolic Brain Disease*, *19*(3–4), 393–411. https://doi.org/10.1023/B:MEBR.0000043984.73063.d8

Varvel, S. A., Wiley, J. L., Yang, R., Bridgen, D. T., Long, K., Lichtman, A. H., & Martin, B. R. (2006). Interactions between THC and cannabidiol in mouse models of cannabinoid activity. *Psychopharmacology*, *186*(2), 226–234. https://doi.org/10.1007/s00213-006-0356-9

Vieira, A. J., Beserra, F. P., Souza, M. C., Totti, B. M., & Rozza, A. L. (2018). Limonene: Aroma of innovation in health and disease. *Chemico-Biological Interactions*, *283*(February), 97–106. https://doi.org/10.1016/j.cbi.2018.02.007

Viravathana, P., & Marr, D. W. M. (2001). Differential effects of Δ9-THC on spatial reference and working memory in mice. *Psychopharmacology*, *157*(2), 142–150. https://doi.org/10.1007/s002130100780

Vogt, L. J. K., Hyman, B. T., Van Hoesen, G. W., & Damasio, A. R. (1990). Pathological alterations in the amygdala in Alzheimer's disease. *Neuroscience*, *37*(2), 377–385. https://doi.org/10.1016/0306-4522(90)90408-V

Wang, Y., Hong, Y., Yan, J., Brown, B., Lin, X., Zhang, X., Shen, N., Li, M., Cai, J., Gordon, M., Morgan, D., Zhou, Q., & Cao, C. (2022). Low-Dose Delta-9-Tetrahydrocannabinol as Beneficial Treatment for Aged APP/PS1 Mice. *International Journal of Molecular Sciences*, *23*(5). https://doi.org/10.3390/ijms23052757

Warburton, E. C., & Brown, M. W. (2015). Neural circuitry for rat recognition memory. *Behavioural Brain Research*, *285*, 131–139. https://doi.org/10.1016/j.bbr.2014.09.050

Waters, A., & Laitner, M. H. (2021). Biological sex differences in Alzheimer's preclinical research: A call to action. *Alzheimer's and Dementia: Translational Research and Clinical Interventions*, *7*(1), 5–7. https://doi.org/10.1002/trc2.12111

Watt, G., Shang, K., Zieba, J., Olaya, J., Li, H., Garner, B., & Karl, T. (2020). Chronic Treatment with 50 mg/kg Cannabidiol Improves Cognition and Moderately Reduces AB40 Levels in 12-Month-Old Male APPswe/PS1E9 Transgenic Mice. *Journal of Alzheimer's Disease*, 937–950. https://doi.org/10.1007/978-3-031-07753-1_7

Watt, G., & Karl, T. (2017). In vivo evidence for therapeutic properties of cannabidiol (CBD) for alzheimer's disease. *Frontiers in Pharmacology*, *8*(FEB), 1–7. https://doi.org/10.3389/fphar.2017.00020

Watt, G., Shang, K., Zieba, J., Olaya, J., Li, H., Garner, B., & Karl, T. (2020). Chronic Treatment with 50 mg/kg Cannabidiol Improves Cognition and Moderately Reduces Aβ40 Levels in 12-Month-Old Male AβPPswe/PS1ΔE9 Transgenic Mice. *Journal of Alzheimer's Disease*, *74*(3), 937–950. https://doi.org/10.3233/JAD-191242

Webster, S. J., Bachstetter, A. D., Nelson, P. T., Schmitt, F. A., & Van Eldik, L. J. (2014). Using mice to model Alzheimer's dementia: An overview of the clinical disease and the preclinical behavioral changes in 10 mouse models. *Frontiers in Genetics*, *5*(APR), 1–23. https://doi.org/10.3389/fgene.2014.00088

Wegiel, J., Wang, K. C., Imaki, H., Rubenstein, R., Wronska, A., Osuchowski, M., Lipinski, W. J., Walker, L. C., & LeVine, H. (2001). The role of microglial cells and astrocytes in fibrillar plaque evolution in transgenic APPSW mice. *Neurobiology of Aging*, *22*(1), 49–61. https://doi.org/10.1016/S0197-4580(00)00181-0

Whyte, L. S., Hemsley, K. M., Lau, A. A., Hassiotis, S., Saito, T., Saido, T. C., Hopwood, J. J., & Sargeant, T. J. (2018). Reduction in open field activity in the absence of memory deficits in the AppNL−G−F knock-in mouse model of Alzheimer's disease. *Behavioural Brain Research*, *336*(August 2017), 177–181. https://doi.org/10.1016/j.bbr.2017.09.006

Wu, J., Bie, B., Yang, H., Xu, J. J., Brown, D. L., & Naguib, M. (2013). Activation of the CB2 receptor system reverses amyloid-induced memory deficiency. *Neurobiology of Aging*, *34*(3), 791–804. https://doi.org/10.1016/j.neurobiolaging.2012.06.011

Wyss-Coray, T., Loike, J. D., Brionne, T. C., Lu, E., Anankov, R., Yan, F., Silverstein, S. C., & Husemann, J. (2003). Adult mouse astrocytes degrade amyloid-β in vitro and in situ. *Nature Medicine*, *9*(4), 453–457. https://doi.org/10.1038/nm838

Yim, T. T., Hong, N. S., Ejaredar, M., McKenna, J. E., & McDonald, R. J. (2008). Post-training CB1 cannabinoid receptor agonist activation disrupts long-term consolidation of spatial memories in the hippocampus. *Neuroscience*, *151*(4), 929–936. https://doi.org/10.1016/j.neuroscience.2007.08.037

Yoo, K. Y., & Park, S. Y. (2012). Terpenoids as potential anti-alzheimer's disease therapeutics. *Molecules*, *17*(3), 3524–3538. https://doi.org/10.3390/molecules17033524

Zhao, J., Liu, X., Xia, W., Zhang, Y., & Wang, C. (2020). Targeting Amyloidogenic Processing of APP in Alzheimer's Disease. *Frontiers in Molecular Neuroscience*, *13*(August). https://doi.org/10.3389/fnmol.2020.00137

Zimmer, A., Zimmer, A. M., Hohmann, A. G., Herkenham, M., & Bonner, T. I. (1999). Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(10), 5780–5785. https://doi.org/10.1073/pnas.96.10.5780

Zimmer, A., Zimmer, A. M., Hohmann, A. G., Herkenham, M., Zimmer, A., Zimmer, A. M., Hohmannt, A. G., Herkenhamt, M., & Bonner, T. O. M. I. (1999). Increased Mortality , Hypoactivity, and Hypoalgesia in Cannabinoid CB1 Receptor Knockout Mice. *Proceedings of the National Academy of Sciences*, *96*(10), 5780–5785.

Appendix

Supplementary Figures

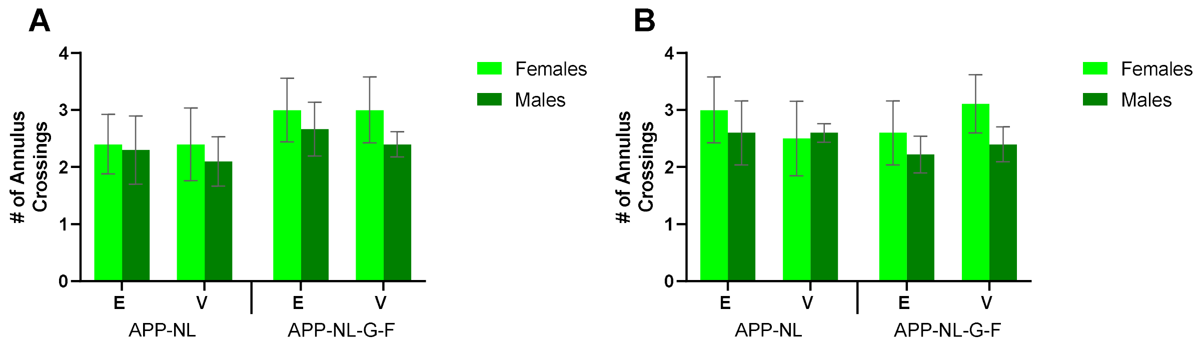


Figure 21. The average number of annulus crossings of the previously trained platform location during a single probe trial (1-day post training) of the MWT at 9 (A) and 12 (B) months of age in female and male APP-NL-G-F and APP-NL mice treated with a cannabis extract or vehicle. Each experimental group contains n=10 subjects.

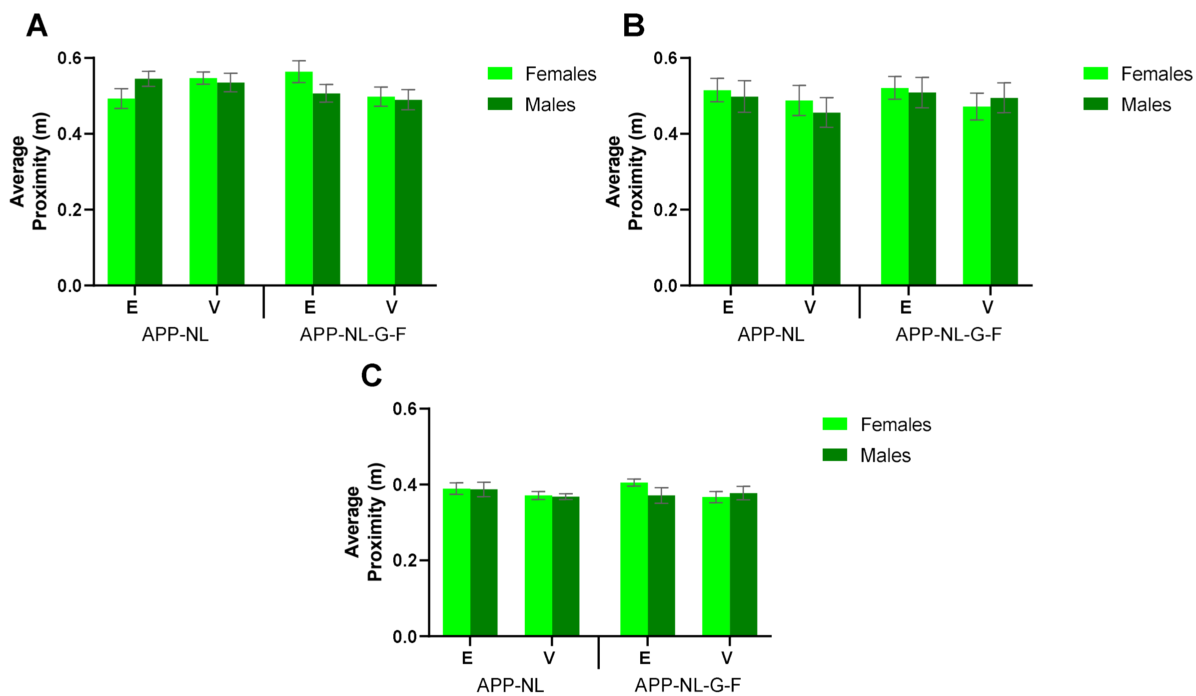


Figure 22. The average proximity distance to the previously trained platform location during a single probe trial (1-day post training) of the MWT at 9 (A) and 12 (B) months of age and 1-week post training at 12 (C) months of age in female and male APP-NL-G-F and APP-NL mice treated with a cannabis extract or vehicle. Each experimental group contains n=10 subjects.