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Investigating the Effect of Apolipoprotein E4 on Attention Following Repeated Mild Traumatic Brain Injuries in hAPP and hMapt mice

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder, marked by cognitive decline influenced by genetic and environmental risk factors. The APOE4 allele, the strongest genetic risk factor for late-onset AD, provides cognitive benefits in young carriers but impairs cognition in older age. APOE4 also worsens outcomes following traumatic brain injury (TBI), an environmental AD risk factor, though long-term effects are less known. Using a humanized mouse model, we assessed long-term cognitive outcomes post-TBI. Our methods included: *(i)* knock-in mice expressing wildtype APP, tau, and APOE4 without mutations; *(ii)* a repetitive closed-head mild TBI model with translatable rotational forces; and *(iii)* a rodent touchscreen continuous performance task (CPT) akin to clinical CPTs. Results showed that APOE4 mice outperformed APOE3s in attention tasks only in females, but better attention was lost post-TBI. The attentional changes indicated that while APOE4 can confer benefits, it accelerates cognitive decline when combined with TBI and female sex

Keywords

Mild traumatic brain injury, Alzheimer's disease, APOE4, antagonistic Pleiotropy, cognitive decline, attention, continuous performance task, touchscreen testing, sex-differences

Summary for Lay Audience

Alzheimer's disease (AD) is an age-related brain disorder associated with a gradual decline in mental functioning, especially in memory and attention. Many factors, including genetics and life experiences, can increase the risk of developing AD and accelerate its progression. The strongest genetic risk factor for late-onset AD is the Apolipoprotein E4 gene (APOE4), which plays a central role in pathological processes in AD, including protein aggregation and long-term inflammation. APOE4 has different effects on mental functioning across the lifespan, conferring benefits in mental functioning in young adults, especially in attention, but impairing mental abilities in older adults. Moreover, APOE4 interacts with other risk factors, such as concussive brain injuries, to accelerate AD onset further. Following mild brain injuries, those carrying the APOE4 gene experience worse recovery and poorer mental abilities in the short term; however, less is known about long-term changes in mental functioning.

To address this, we utilized a mouse model of APOE4 and brain injury while considering clinical relevance. First, as mouse AD-related proteins do not form pathological aggregates as they do in humans, we inserted two human genes that allow aggregation of such proteins without any disease-causing mutations. Second, we modeled three brain injuries after rotational forces experienced during sport-related human concussive brain injuries. Specifically, as rotational forces are shown to be more damaging to the brain, we scaled down such forces to a mouse brain to produce similar damage. Lastly, we longitudinally measured attention using a task that is nearly identical to the test given to humans in clinics. We found that without any injuries, female mice with the APOE4 gene had better attention skills than those with the non-risk factor APOE3 gene. This benefit lasted as the mice got older. However, after the mice experienced brain injuries, the APOE4 mice lost the advantage in attention. Moreover, this change was seen exclusively in female mice, with no impact on males. Taken together, these results suggest that the APOE4 gene might not be a risk factor for poor cognition by itself and may even be beneficial for women; however, in the presence of other risk factors such as brain injury, APOE4 can lead to an accelerated cognitive decline, especially for women.

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Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

Aβ	amyloid beta protein
AD	Alzheimer's disease
ANOVA	analysis of variance
APP	amyloid precursor protein
ApoE4	apolipoprotein E-ε4
BBB	blood-brain-barrier
CCI	controlled cortical impact
CHI	closed head injury
CPT	continuous performance test
d'	discrimination sensitivity
DAI	diffuse axonal injury
DAMPs	danger-associated molecular patterns
FAR	false alarm rate
GDS	global deterioration scale
GFAP	glial fibrillar acidic protein
HR	hit rate
hAPP	humanized amyloid precursor protein
hMapt	humanized microtubule associated protein tau

EOAD	early onset Alzheimer's disease
LDLR	low-density lipoprotein receptor
LOAD	late onset Alzheimer's disease
MAPT	microtubule associated protein tau
MCI	mild cognitive impairment
a-MCI	amnesic mild cognitive impairment
na-MCI	non-amnesic mild cognitive impairment
NFT	neurofibrillary tangles
PFC	prefrontal cortex
PSEN	presenilin
p-tau	hyperphosphorylated tau
TBI	traumatic brain injury
TNFα	tumor necrosis factor α
rCHI	repetitive closed head injury
rCPT	rodent continuous performance task
WT	wildtype
5-CSRTT	5-choice serial reaction time test

Chapter 1

1 Introduction

1.1 Alzheimer’s disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, characterized by an irreversible and gradual decline in cognitive function. Over 55 million individuals worldwide (World Health Organization, 2023) and half a million Canadians (Alzheimer’s Society of Canada, 2024) are living with dementia, with AD being the most prevalent cause, accounting for 60-70% of cases. With the aging population, the prevalence of AD is predicted to double every 20 years (Mayeux & Stern, 2012), making it a critical concern for public health.

Age is the leading risk factor for AD, with the incidence of AD doubling every 5 years for Canadians aged 65 and older (Canadian Institute for Health Information, 2024). AD is often categorized based on the age of onset of symptoms and can be divided into early onset (less than 65 years) or late onset (over 65 years). A small percentage of Early-onset AD (EOAD) cases are associated with autosomal dominant familial mutations, in the genes encoding Amyloid Precursor Protein (APP), Presenilin-1 (PSEN-1), and Presenilin-2 (PSEN-2) which cause a significant acceleration of the AD pathology (Rocchi et al., 2003; Mendez, 2012). EOAD, however, only accounts for 5-10% of AD patients (Mendez, 2012; Reitz et al., 2020); therefore, in the majority of patients, AD is sporadic and late onset.

Both AD symptoms and pathology are thought to develop gradually, with the pathology preceding the earliest symptoms by decades (Mintun et al., 2006; Peskind et al., 2006; Aizenstein et al., 2008; Clifford R Jack, 2010). Although AD symptom progression occurs at different rates between patients, the cognitive and behavioral progression is often clinically outlined by the 7-stage Global Deterioration Scale (GDS) (Reisberg et al., 1982; Alzheimer’s Society of Canada, 2022). In stages 1 and 2 (preclinical) there are no clinical signs of cognitive deficits, although patients may start to notice slight deficits in anterograde episodic memory. In stage 3 or Mild Cognitive

Impairment (MCI) stage (Petersen, 2004), earliest clinically observed deficits appear in one or more cognitive domains often in memory and executive function. In stages 4 to 7, cognitive impairment becomes more pronounced, significantly interfering with everyday abilities. At the late stages, individuals' quality of life and independence is severely compromised.

AD Cognition

Behaviorally, classical accounts of AD have shown earliest cognitive impairments to be in episodic memory, or memory for contextual details of personal events (Welsh et al., 1992; Artero et al., 2003). This aligns with earliest accounts of brain structural damage to be in medial temporal lobe (hippocampus and entorhinal cortex), followed by damage in neocortical regions (Braak and Braak, 1991; Pini et al., 2016). However, there is also strong evidence for deficits in other cognitive domains including semantic memory (language), visuospatial memory, working memory, and attention (Saunders and Summers, 2011; Peterson 2004; Weintraub 2012). The variability of cognitive deficits at the MCI stage between patients has motivated efforts to divide MCI further into two subtypes: amnesic-MCI (a-MCI) and non-amnesic-MCI (na-MCI) (Peterson & Morris, 2005). Interestingly, studies on the conversion rates to AD have shown an increased risk in both a-MCI and na-MCI with no difference between the two subtypes. In fact, it has been established that single-domain a-MCI is not only rare, but also less predictive of AD progression than memory deficits plus deficits in other domains (Fischer et al., 2007; Rasquin et al., 2005; Saunders and Summers, 2011). In the later stages of AD, global cognition and executive function deficits are correlated with widespread brain damage, including whole brain atrophy and decreased cortical thickness across multiple brain regions (Braskie et al., 2013).

AD Neuropathology: Aβ Plaques

The neurodegenerative aspect of AD refers to the progressive synaptic, neuronal, and axonal damage caused by AD molecular pathology. In 1907, Alois Alzheimer first characterized this pathology with the presence of senile plaques (amyloid plaques) and

neurofibrillary tangles (NFTs) that preceded neurodegeneration (Alzheimer et al., 1995). It was not until 1984 that extracellular plaques were found to be made up of amyloid beta protein (Aβ) aggregates (Glenner and Wong, 1984). Aβ monomers of 37-49 amino acids residues are formed from the proteolytic cleavage of the intermembrane APP by beta-secretase and gamma secretase. Normally, Aβ is rapidly removed and degraded; however, in AD the Aβ monomers accumulate and rapidly aggregate to form amyloid oligomers, protofibrils, and later fibrils that make up plaques. This cascade of events leading to amyloid plaques was the main explanation for the pathogenesis of AD for over 25 years – referred to as the Amyloid Hypothesis (Hardy and Higgins, 1992; Kametani and Hasegawa, 2018). Moreover, this hypothesis found support in mutations in APP or changes in chromosome 21 (containing the APP encoding region) that accelerated disease progression (Hardy and Higgins, 1992).

However, the amyloid hypothesis was later shown to not fully explain AD pathogenesis based on three main reasons: *(i)* Many mouse models generated to have Aβ deposits failed to show NFTs (Kametani and Hasegawa, 2018; Herrup, 2015); *(ii)* Many immunotherapies against Aβ while decreasing plaque load, were largely unable to improve symptoms (Long and Holtzman, 2019; Ostrowitzki et al., 2012; Giacobini and Gold, 2013); *(iii)* Lastly, later advances in imaging showed that there are many cognitively normal patients with amyloid plaques and many AD patients with very few plaques (Li et al., 2008; Edison et al., 2007). These pieces of evidence have shifted the current view of AD pathogenesis to include other key hallmarks including NFTs and inflammation (Giacobini and Gold, 2013; Bloom, 2014).

AD Neuropathology: NFTs

NFTs were first described by Alzheimer, which form intracellularly near the surface of affected neurons (Alzheimer et al., 1995). In the late 1980s, these tangles containing bundles of paired helical filaments, were found to be aggregates of phosphorylated tau protein (Brion et al., 1985; Goedert, 1996; Grundke-iqbal et al., 1986). The microtubule associated protein tau (MAPT) gene encodes tau and can be alternatively spliced on exons 2,3, and 10, giving rise to six distinct isoforms in an adult

human (Andreadis, 1992): three isoforms with three microtubule binding repeats (3R) and three with four repeats (4R). Tau protein is a microtubule-associated protein that helps promote tubulin assembly into microtubules and stabilize its structure. This is particularly important in neurons as microtubules are an essential part of cellular transport in dendrites and axons. Phosphorylation of tau is critical for its activity regulation; however, in AD, tau is three to four folds more phosphorylated (Kopke et al., 1993). This hyperphosphorylation depolymerizes microtubules, affects inter-neuron signaling, and promotes aggregation (Grundke-iqbal et al., 1986). tau pathology in AD appears first in entorhinal areas, then hippocampus and other limbic system structures, followed by the neocortex (Braak and Braak, 1991). This pattern of spreading is strongly linked with the pattern of neurodegeneration and cognitive deficits in AD (Kametani and Hasegawa, 2018).

AD Neuropathology: Chronic inflammation

More recently, chronic inflammation has been established as the third core pathology in AD. A sustained dysregulated inflammatory response is evident in AD patient's brains and preclinical models of AD (Kinney et al., 2018; Akama and Eldik, 2000). The prolonged inflammation is mainly attributed to the dysregulation of microglia (Hansen et al., 2018; Bernhardi et al., 2015) and astrocytes (Kim et al., 2024), and the changes in neuronal-glia crosstalk. The presence of chronic inflammation was once thought to be only a result of neurodegeneration; however, a large body of evidence support that inflammation facilitates and exacerbates amyloid and tau pathology (Heneka et al., 2012; Prinz et al., 2011; Lucin et al., 2009; Zotova et al., 2010; McGeer and Rogers, 1992). In fact, sustained inflammation could provide a link between amyloid pathology and the subsequent tau pathology (Kitazawa et al., 2005; Garwood et al., 2011; Rhein et al., 2009). Thus, the mentioned three core pathological hallmarks of AD are likely to collaborate either additively or synergistically, facilitating and exacerbating neurodegeneration, consequently leading to the cognitive deficits observed in AD.

AD Risk Factors

Both hereditary and environmental risk factors may play a role at various points in the interaction of the core pathologies, accelerating AD progression. The strong heritability of AD, estimated to be between 60-80% by twin studies, further emphasizes the significant impact of genetic factors on the development and progression of the disease (Gatz et al., 2006). The hereditary causes include EOAD mutations in APP—such as the Swedish, Iberian, and Arctic mutations (Citron et al., 1992; Lichtenthaler et al., 1999; Saito et al., 2014) —as well as in the PSEN-1 and PSEN-2 genes, which are rare but have nearly 100% penetrance (Janssen et al., 2003; Li et al., 2018). Other more common genes associated with LOAD, with lower risks have also been identified using large genome-wide association studies (GWAS) (Karch and Goate, 2015; Bellenguez et al., 2022). Some of these include genes involved in cholesterol metabolism (APOE, CLU, ABCA7), immune response (TREM2, CD33, CR1), and several other cellular functions implicated in AD. These genes are a gateway to study the mechanisms underlying AD pathogenesis further, and the crosstalk between the three core pathological hallmarks of AD.

Another factor that is associated with higher risk for AD is sex and gender differences in AD, which put women at a greater risk. In fact, almost two thirds of LOAD patients are women, who also exhibit more severe pathology and cognitive decline (Ferretti et al., 2018; Zhu et al., 2021). There are several mechanisms proposed to explain the sexual dimorphism in AD including significant estrogen decline post menopause (Zhu et al., 2021; German-Castelan et al., 2023), differences in glial regulation (Toro et al., 2019; Dzamba et al., 2016; Han et al., 2021), and prevalence of other risk factors including depression and cardiovascular diseases (Chene et al., 2015; Goveas et al., 2011). In addition, there are gender differences in access to education, employment, socioeconomic status that intersect to create health disparities disadvantaging women (Fitzpatrick et al., 2004; Norton et al., 2014). This creates an immediate need to address this disparity by focusing on sex difference in AD and advocating for equitable healthcare for women.

There are also environmental risk factors for AD that can be prevented or corrected. These include lifestyle-related factors such as physical inactivity, smoking, obesity, and high fat/cholesterol diet (Meng et al., 2020; Alzheimer's Association, 2024). Regular exercise, smoking cessation, and diets low in saturated fats and high fiber vegetable diets (Mediterranean, DASH, and vegetarian/vegan diets) have been associated with lower risk of AD development (Alzheimer's Association, 2024; Agarwal et al., 2023). Other factors include health-related factors such as head injuries which put individuals at a much greater risk for dementia (Alzheimer's Association, 2024; Fann et al., 2018).

1.2 Traumatic brain injury, a known environmental risk factor for AD

Traumatic Brain Injury (TBI) refers to damage caused by external forces or trauma to the brain, whether non-penetrating – such as falls, blast injuries, and sports-related incidents – or penetrating – as in the case of bone fractures and bullets (National Institute of Neurological Disorders and Stroke, n.d.). As the force and the subsequent acceleration/deceleration waves move through the brain tissue, vasculature, and supporting connective tissue, it can lead to a wide range of disruptions in normal brain function both short-term and long-term. Globally, approximately 69 million individuals, including over 165,000 Canadians, experience a TBI each year (Dewan et al., 2018; Brain Injury Canada, n.d.), marking TBI as a major cause of disability worldwide. Although the incidence rate of TBI has declined by 5% from 1990s (Guan et al., 2023), likely due to increased awareness and preventative measures, the wide range of TBI sequelae remains a critical concern for public health.

TBI effects can vary depending on the severity of the injury from mild to severe. In mild TBI, standard structural imaging typically appears normal, and there may be a brief period of consciousness loss. In moderate and severe TBIs, structural imaging may

show normal or abnormal results, with the duration of consciousness loss ranging from 30 minutes to 24 hours in moderate cases and exceeding 24 hours in severe cases (O'Neil et al., 2013). While mild TBIs, including concussions, are the least severe and temporary, they are the most prevalent form of TBI, accounting for 80-90% of TBIs (Cassidy et al., 2004), and can result in various acute symptoms. These symptoms may include, but are not limited to, physical manifestations such as headaches, photosensitivity, blurred vision, nausea, as well as cognitive challenges in concentration and shifting attention (Centre for Disease Control and Prevention, 2019).

The acute symptoms following TBI are the outcome of primary injury, comprising the immediate disruptions in the brain structure and function. The primary injuries often include diffuse axonal injury (DAI), resulting from rotational forces and forceful stretching that causes mechanical strain, axonal damage, and hematomas – bleedings caused by ruptured vasculature in and around the brain (National Institute of Neurological Disorders and Stroke, n.d.). The primary injury may trigger a large-scale complex cascade of events to protect the brain from further injury. This cascade involves inflammation and metabolic changes that clear debris and initiate repair processes (Neumann et al., 2009). However, the inflammation and metabolic changes may, over the subsequent months and years, also gradually lead to further damage, a process known as secondary injury (Needham et al., 2019).

Secondary Injury

Secondary injury manifests as a complex cascade of events subsequent to the primary injury, leading to neuronal damage (Hailer, 2007), white matter degeneration (Johnson et al., 2013), and persistent inflammation that may last for decades (Ramlackhansingh et al., 2011). Some of the key contributors to secondary injury include vascular changes, oxidative stress, and inflammatory processes in the brain, all of which adversely affect neuronal health and function. One of the most studied changes after a TBI is the vascular changes in the blood-brain-barrier (BBB), a major factor determining the progression of secondary injury (Chodobski et al., 2011). The BBB, formed by the endothelial cells connected by tight-junctions, creates a highly selective gate for blood-

borne factors, protecting the CNS. Following an injury the tight junctions are disrupted and the BBB becomes permeable in a non-selective manner, causing activation of the coagulation cascade, reduced blood flow, and secretion of proinflammatory factors (Chodobski et al., 2011). There is a growing body of evidence highlighting the disturbance of intricate glial communication– mainly astrocytes and microglia (Abbott et al., 2006; Lassmann et al., 1991) – with the brain endothelial cells as a major contributor to neuroinflammation and secondary injury (Chodobski et al., 2011).

Another mechanism leading to secondary injury is the buildup of significant oxidative stress through reactive oxygen species (ROS) and lipid peroxidation. Following the primary injury, several markers of oxidative stress have been observed to be significantly increased (Ng and Lee, 2019). This is attributed to several cellular disruptions including excessive release of NO (Hall et al., 2004), activated neutrophils, excitotoxicity from glutamate buildup (Chamoun et al 2012), and dysfunctional mitochondria (Shohami and Kohen, 2010). Another mechanism for oxidative stress is lipid peroxidation or the addition of a hydroperoxyl group into lipids, often the membrane phospholipids, as they are significantly affected by the mechanical forces in TBI. The oxidative stress created by these mechanisms is another major contributor to neuroinflammation and secondary injury (Ansari et al., 2009).

Secondary Injury: Inflammation

The intricate neuroinflammatory response following the primary injury is a complex process orchestrated mainly by the resident glia – microglia and astrocytes – and the infiltrating peripheral immune cells through the compromised BBB (Lozano et al., 2015; Xiong et al., 2018, Holmin et al., 1998). Microglia – the main agents of inflammation in the CNS – are activated and function acutely to remove debris and damaged cells (Neumann et al., 2009). This is a crucial step, as damaged cells release danger-associated molecular patterns (DAMPs) that could become proinflammatory, causing further damage to the tissue (Zhang et al., 2012). Moreover, depending on the stage after the injury and the microenvironment, microglia could be differentially stimulated, leading to distinct activation profiles with differential gene expression and

morphology (Donat et al., 2017; Loane and Kumar, 2016). Once activated, microglia may exacerbate damage by increasing their release of pro-inflammatory cytokines, and reducing release of anti-inflammatory cytokines, ROS, and glutamate which exacerbates damage (Ziebell and Morganti-Kossmann, 2010). While this activation profile could be important for repair immediately after injury, there is a strong body of evidence showing a microglial activation profile could persist for decades after even a single mild-moderate injury (Johnson et al., 2013; Ramlackhansingh et al., 2011).

Astrocytes – a type of glia involved in several supportive roles including synapse modulation and BBB regulation – are another key contributor to neuroinflammation after an injury (Chodobski et al., 2011). Infiltration of plasma proteins (Fibrinogen) through the disturbed BBB activate inflammatory pathways such as the transforming growth factor-beta (TGF-β) signaling, which promotes astrocyte activation and astrocyte scar formation (Xiong et al., 2018). Moreover, the pro-inflammatory cytokines produced by microglia lead to astrocyte activation and further cytokine production (Xiong et al., 2018). Similar to microglia, astrocytes also show heterogeneous and progressive changes in gene expression, morphology, and function which are collectively referred to as astrogliosis (Burda et al., 2016). Astrocytes could further contribute to neuroinflammation by secreting proinflammatory factors including nitric oxide, DAMPs, and cytokines such as interleukin 33 – IL-33, a promoter of microglia/macrophage recruitment (Michinaga and Koyama, 2021).

In addition to the local response following TBI, the recruitment of immune cells in the systemic circulation is also a contributor to inflammation. This process begins with neutrophil infiltration withing the first 24 hours and progresses with monocyte/macrophage and T-cell infiltration 3-5 days post injury (Holmin et al., 1998; Jin et al., 2012). Neutrophils and macrophages, similar to other inflammatory agents, can exhibit diverse functional states based on the wide spectrum of microenvironments they encounter, ranging from proinflammatory to anti-inflammatory conditions (Murray and Wynn, 2011; Kumar et al., 2013). The function of these cells goes beyond the acute phase, as they can be detected in the brain weeks and even a year after the initial

inflammatory insult (Liu et al., 2018; Shaftel et al., 2007). This suggests a level of complexity in inflammation that could potentially explain the level of variability in TBI outcomes.

Due to the secondary injury, there is evidence from both human (McAllister et al., 2012; Casson et al., 1984; Bey and Ostick, 2009) and animal studies (Shitaka et al., 2011; Prins et al., 2010), suggesting that the brain is at a vulnerable state while recovering from TBI (Graham et al., 2014). Thus, a repeated injury during recovery may occur with less force, take longer to recover, and lead to more severe outcomes (Graham et al., 2014). Unfortunately, many military staff, athletes, and victims of violence present with a history of more than one concussion. For instance, a study of a large sample of football players over three years showed that those with three or more previous concussions had three times the risk of getting a subsequent concussion (Guskiewicz et al., 2003). This puts such individuals at an even higher risk of developing long-term cognitive deficits (Graham et al., 2014).

TBI, a known risk factor for AD: Epidemiology

TBI and its subsequent secondary injury have been shown to put individuals at a higher risk of developing a broad range of neuropsychological disorders in the long-term including mood and psychotic disorders (Didehbani et al., 2013; Molloy et al., 2011), and neurodegenerative diseases such as AD (Gupta and Sen, 2016; Gu et al., 2021). There are three main lines of evidence supporting that TBI increases the risk of AD: Longitudinal population studies, overlapping pathology, and overlapping risk factors. Several large-scale meta-analyses reviewing longitudinal population studies comparing the incidence of AD in TBI patients and matched controls have been published (Fleminger et al., 2003; Mortimer et al., 1991; Perry et al., 2016; Gu et al., 2021; Zhang et al., 2021; Li et al., 2016). Although the meta-analyses each utilized a different method of inclusion criteria and showed a broad heterogeneity in the statistics, they suggest that TBI is a moderately strong risk factor for dementia and AD. One particular study included in a recent meta-analysis (Gu et al., 2021) was a study conducted with a sample size of over 3 million individuals over the age of 50 (Nordstrom and Nordstrom, 2018). This study

demonstrated a correlation between the risk of developing dementia and the number of TBIs as well as their severity. The findings revealed that a single mild TBI was associated with a 1.63-fold increased risk for dementia, while severe TBI showed a 2.06-fold risk, and repeated TBIs were linked to a 2.81-fold increased risk (Nordstrom and Nordstrom, 2018). Notably, the overall risk of developing dementia was highest within the first year following a TBI (3.52-fold), gradually decreasing over time, and yet remaining moderately strong (1.25-fold) for over 30 years. Although the dementia associated with TBI could be either the AD type or the non-AD type, there are many observational population studies suggesting a relationship with AD (Gu et al., 2022). In addition, a history of TBI has been shown to accelerate the age of onset for AD cognitive impairments by two or more years (Li et al., 2016). Although the mentioned observational studies and meta-analyses offer valuable insights into the potential risk of AD following a TBI, they are constrained by certain design limitations. These limitations arise from various factors that confound the results, such as the clinical heterogeneity of the data due to diverse diagnostic criteria and broad TBI severity definitions. Additionally, recall bias in self-report retrospective studies and selection bias further contribute to the limitations in the study design (Gu et al., 2022; Zhang et al., 2021).

TBI, a known risk factor for AD: Pathology

TBI is also linked to the core pathologies of AD. The presence of Aβ plaques is observed in brains of up to 30% of individuals, acutely following TBI (Roberts et al., 1991; Roberts et al., 1994). These plaques were similar to those observed in early stages of AD (Johnson et al., 2010), mainly consisting of the 42-amino acid amyloid (Aβ-42) which is prone to aggregation in AD (Dekosky et al., 2007; Gentleman et al., 1997). However, unlike the gradually developing plaques in AD, TBI-associated plaques may appear within a few hours following an injury (Roberts et al., 1994; Ikonomovic et al., 2004). These plaques are likely the outcome of multiple mechanisms initiated by the primary and secondary injury, with diffuse axonal injury (DAI) being the most apparent. Characteristic to DAI is the disruptions of axonal transport, leading to accumulation of proteins such as APP at the site of axonal damage (Hamberger et al., 2003; Gentleman et

al., 1993; Lambri et al., 2001; Sherriff et al., 1994). This rapid and abundant accumulation of APP has even been used to confirm DAI in human samples (Reichard et al., 2003). In addition to APP, other enzymes and products of the APP amyloidogenic cleavage have been observed to co-localize at the site of axonal injury including the β-Site APP cleaving enzyme and Aβ (Smith et al., 1999; Smith et al., 2003). These together show a mechanistic connection between TBI and AD-like amyloid pathology in TBI.

Tau pathology has also been linked to TBI in both acute and long-term cases (Edwards et al., 2020; Johnson et al., 2012). Acutely following a TBI, there is an increase in tau levels and tau phosphorylation, with NFTs detectable as early as a few hours post injury (Ikonomovic et al., 2004; Grady et al., 1993; Smith et al., 2003). In the long-term, comparing post-mortem brains of TBI survivors showed that 34% had NFTs after a single moderate to severe injury, compared to only 9% in age-matched controls (Johnson et al., 2012). Moreover, repeated mild TBIs have been strongly linked to another tauopathy, chronic traumatic encephalopathy (CTE) (McKee et al., 2009; Goldstein et al., 2012; Gavett et al., 2010). CTE, similar to AD, is associated with phosphorylated tau and NFTs (Omalu et al., 2005; Omalu et al., 2006). However, there are notable differences in the shape and distribution of NFTs in AD and CTE (Turner et al., 2016): in AD, the NFTs tend to be present in deeper layers of the cortex (V/VI as opposed to II/III in CTE), affect different areas of the hippocampus (CA1 compared to CA1-4 in CTE), and exhibit distinct shapes (focal laminar as opposed to flame-shaped, alternating sparse-to-dense in CTE). Despite these distinctions, accumulating evidence suggests that CTE and AD can coexist (Turner et al., 2016), highlighting the presence of overlapping pathological mechanisms. These include the complex chronic neuroinflammatory response following TBI, that could make the brain more vulnerable to aggregation of Aβ and phosphorylated Tau.

TBI, a known risk factor for AD: Risk Factors

Similar to the overlapping pathology, TBI and AD share several risk factors, further highlighting the shared mechanistic pathways in their pathology. TBI risk factors are often genetic or demographic factors that lead to worse outcomes after TBI, whether

they act to influence the extent of injury or to affect repair processes (Bennett et al., 2016). The most studied gene regarding neurotrauma is the Apolipoprotein E ε4 (APOE4) isoform, implicated in both repair mechanisms (Teasdale et al., 1997) and mediating the brain inflammatory response (Laskowitz et al., 2010; Lynch et al., 2005; Wang et al., 2013). APOE4 – the strongest genetic risk factor for LOAD – plays a central role in the connection between TBI and AD pathology (Fernandez-Calle et al, 2022). Some other shared genetic factors include genetic polymorphisms in mediators of inflammation (Bennett et al., 2016), namely TGF-β, IL-6, and Tumor Necrosis Factor α (TNFα). Polymorphisms in these genes have also been shown to influence inflammation in AD and are even associated with a lower age of AD onset (Lio et al., 2006; Bosco et al., 2013).

Other factors that significantly influence TBI outcome are age and sex (Ponsford et al., 2013). Older age is a consistent predictor of higher mortality and poorer outcomes in survivors of moderate to severe TBI (Ponsford (Green et al., 2008; Chi et al., 2006; Tien et al., 2006). This is likely attributed to various mechanisms, including an exaggerated response in aged microglia (Li et al., 2023) and astrocytes (Clarke et al., 2018), leading to chronic inflammation in older adults (Andronie-Cioara et al., 2023). Given that age is also the strongest risk factor for AD, mechanisms such as chronic inflammation emerge as a potential link between TBI and AD. Gender and sex influence both the risk of experiencing TBI and the outcomes following TBI. Although epidemiological studies have reported a 2.2-fold higher risk of sustaining a TBI in men (Grost et al., 2013), many incidents of TBI in women are due to domestic violence and are significantly underreported (Costello and Greenwald et al., 2022). In fact, it is estimated that the number of women suffering TBIs related to domestic violence is 11-12 times greater than the number of TBIs experienced by military personnel and athletes combined (Lifshitz et al., 2019). In terms of TBI outcomes, both patient studies and animal models have shown higher mortality and more incidence of complications in males (Angele et al., 2006; Chaudry and Bland, 2009; Haider et al., 2009; Day et al., 2017; Bao et al., 2011). These findings led researcher to believe that female gonadal steroids such as estrogens can play a protective role in TBI (Khaksari et al., 2017; Martin-

Jimenez et al., 2019; Duncan, 2020). There are several proposed mechanisms for these findings, reflecting the widespread role of sex hormones including the anti-inflammatory and antioxidant activity of 17β-estradiol (E2), a potent estrogen (Vegeto et al., 2006; Wang et al., 2006). In postmenopausal women, the significant reduction of the neuroprotective sex hormones exacerbates the secondary injury post TBI, putting older women at a higher risk (Thompson et al., 2006; McCarthy and Raval, 2020; Blaya et al., 2022). This increased risk while creating a link between TBI and AD through shared pathological processes, also highlights a need to study the sexual dimorphisms further in TBI and allocate more resources to support women experiencing TBIs.

1.3 Apolipoprotein E4, the strongest genetic risk factor for LOAD

The APOE ε4 allele (APOE4) has been the most influential, successfully replicated, and the strongest genetic risk factor for LOAD, shown by GWAS and epidemiological studies (Betram and Tanzi, 2009; Aslam et al., 2023; Qiu et al., 2009). The ApoE4 allele with only a 13.7% frequency worldwide, is present at approximately 40% allelic frequency in AD patients (Farrer et al., 1997; Liu et al., 2013). In fact, the risk of developing AD is estimated to increase by 3-fold with a single copy and 15-fold by two copies of the APOE4 allele (Yamazaki et al., 2019; Sando et al., 2008; Strittmatter et al., 1993). The mechanistic involvement of APOE4 in AD is multi-factorial, encompassing the three core pathologies of AD, and may relate to role of ApoE in lipid transport.

As lipids are hydrophobic, they are transported in complex particles called lipoproteins. Lipoproteins contain a hydrophilic outer membrane to facilitate transport in the extracellular matrix and a hydrophobic core consisting of lipids such as cholesterol esters and triglycerides (Figure 1). Surrounding the core, the hydrophilic membrane consists of a phospholipid monolayer, free cholesterol, and apolipoproteins.

Apolipoproteins are a class of proteins that bind lipids to form lipoproteins and carryout four crucial functions: I) structural role in lipoproteins, II) initiating role in lipoprotein formation, III) ligand role for lipoprotein receptors, and IV) regulator role in lipoprotein metabolism (Feingold, 2000)

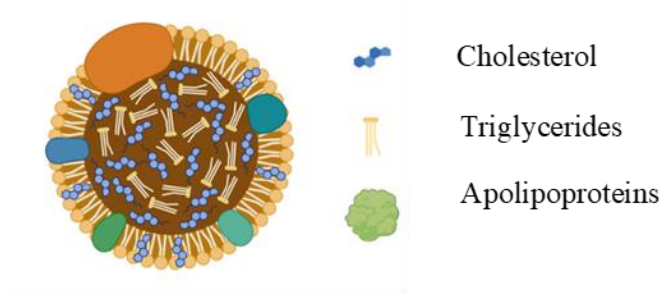

Figure 1. Lipoprotein structure. Apolipoproteins and phospholipids forming the hydrophilic membrane, and the hydrophobic core carrying cholesteryl esters and triglycerides.

There are several classes of apolipoproteins (Apo), namely ApoA, ApoB, ApoC, and ApoE which are present in different types of lipoproteins (Feinglod, 2000). ApoE is the major lipid metabolism regulator in the CNS, which along with ApoA form the lipoproteins that distribute cholesterol and phospholipids in the brain (Maheley, 2016). Under normal conditions, ApoE in the CNS is produced mainly by astrocytes, although neurons and other glial cells also produce ApoE. For instance, a fluorescent protein tracing study in mice showed that approximately 75% of astrocytes expressed ApoE, while only 6% of microglia expressed ApoE (Xu et al., 2006). The 3.7kb APOE gene on chromosome 19 encodes the 34kDa 299 amino acid ApoE protein. There are three distinct alleles of the APOE gene, the APOE2, APOE3, and APOE4 with 8.4%, 77.9%, and 13.7% allelic frequencies, respectively (Liu 2013). The three alleles give rise to three distinct isoforms of the ApoE protein, each differing by a single amino acid from ApoE3: ApoE2 (Cys112, Cys158), ApoE3 (Cys112, Arg158), and ApoE4 (Arg112, Arg158) (Fernandez-Calle et al., 2022; Liu et al., 2013). The difference between the isoforms is responsible for differential folding of the protein, leading to differential binding of the receptor binding region and the lipid binding region at the N-terminal and C-terminal,

respectively (Weisgraber et al., 1982). These differences have shown to make ApoE4 more likely to aggregate, less conformationally stable, and less lapidated than ApoE3, compromising the lipid transport between neurons and glia (Yang et al., 2023; Hubin et al., 2019). This puts ApoE4 at the center of several mechanisms in which AD pathology may be accelerated and exacerbated.

APOE4 and Amyloid Pathology

The link between APOE4 and amyloid pathology in AD has been extensively studied after it was first described (Corder et al., 1993). APOE4 allele carriers have consistently been shown to have increased plaque load and plaque density compared to non-ApoE4 carriers in both AD patients and cognitively normal individuals (Rebeck et al., 1993, Tiraboschi et al., 2004, Schmechel et al., 1993). This has also been replicated in mice, as mice expressing the human APOE alleles mimicked the isoform-specific patterns (ε4 > ε3 > ε2) seen in Aβ deposits and amyloid plaques, with more pathology in APOE4 (Fagan et al., 2002; Holtzman et al., 2000). Although the exact mechanism of ApoE4 involvement in plaque formation is poorly understood, it is likely caused by increased Aβ production, aggregation, and/or decreased clearance (Fernandez-Calle et al., 2022).

Although there is a stronger body of evidence for ApoE4-associated differences in Aβ clearance, human ApoE isoforms have been shown at multiple levels to increase Aβ production and aggregation, in an isoform-dependent manner (Fernandez-Calle et al., 2022; Kline, 2012). For instance, human ApoE has shown to stimulate APP transcription and Aβ secretion, with the highest levels being in ApoE4, followed by ApoE3 and ApoE2, respectively (Huang et al., 2016). Moreover, ApoE4 has been linked to more APP and the β-Site APP cleaving enzyme internalization, leading to increased Aβ production (He et al., 207). ApoE4 has also been shown to promote Aβ aggregation more than ApoE3, through both increasing and stabilizing Aβ oligomers to a greater degree than ApoE3 (Hashimoto et al., 2012; Cerf et al., 2011).

Decreased amyloid clearance is another mechanism that may account for increased accumulation in APOE4-carriers. In all three well-known Aβ clearance pathways—enzymatic degradation, BBB clearance, and cellular internalization by neurons and glia—ApoE4 has been linked to reduced Aβ clearance (Shi and Holtzman, 2018; Tarasoff-Conway et al., 2015). In proteolytic cleavage, proteases such as neprilysin and insulin-degrading enzyme (IDE) have been shown to work both intracellularly and extracellularly to cleave Aβ (Iwata et al., 2000; Qiu et al., 1998). Human ApoE isoforms have been shown to enhance this process in animal mice in an isoform-dependent manner, with ApoE4 exhibiting an impaired ability to enhance clearance due to its lower lipidation (Jiang et al., 2008). Moreover, lower neprilysin and IDE expression have been documented in APOE4-carriers compared to non-carriers in post-mortem brains (Miners et al., 2006; Cook et al., 2003). As for intracellular degradation through the lysosomal pathway, ApoE4 has been shown to reduce cellular clearance in microglia, astrocytes, and neurons (Jiang et al., 2008; Li et al., 2012; Koistinaho et al., 2004). In microglia, ApoE4 is associated with the slowest Aβ uptake and the least efficient Aβ degradation (Lin et al., 2018; Jiang et al., 2008). Similarly, APOE4 astrocytes show impaired ability to internalize oligomeric and fibrillar Aβ42 compared to APOE3 astrocytes (Lin et al., 2018). Although there is limited evidence for neuronal clearance of Aβ, similar patterns have been observed, with a less efficient uptake and lysosomal degradation in APOE4 neurons (Li et al., 2012). Lastly, ApoE4 has been shown to impair Aβ clearance through the BBB, although the precise mechanism is yet to be fully understood (Deane et al., 2008; Robert et al., 2017). Some known mechanisms are the impairments of the BBB in APOE4-carriers, including decreased integrity of the tight junctions (Montange et al., 2020; Bell et al., 2012) and the reduced speed of ApoE4- Aβ clearance through Very-Low-Density Lipoprotein Receptor (VLDLR) and Low-Density Lipoprotein Receptor-Related Protein 1 (LRP1) at the BBB (Deane et al., 2008).

ApoE4 and tau Pathology

A link between ApoE4 and tau pathology independent of Aβ has been more difficult to establish and is a current topic of ongoing research. For instance, a direct

interaction between tau and ApoE has been shown *in vitro* (Shi et al., 2017; Strittmatter et al., 1994) but has not been reported *in vivo* (Fernandez-Calle et al., 2022). In the preclinical studies of tau pathology, numerous models have shown that the APOE4 genotype exacerbates tau pathogenesis, neuroinflammation, and neurodegeneration (Shi et al., 2017; Wang et al., 2021; Jablonski et al., 2021). For instance, a study by Shi et al (2017) using the P301S model – a single amino acid mutation leading to NFT development as early as 6 months and no amyloid plaques (Yoshiyama et al., 2007)– showed that ApoE4 mice have higher brain tau levels by three months, higher phosphorylated-tau by nine months, and more neuroinflammation compared to ApoE3 or no-ApoE mice (Shi et al., 2017). While the precise mechanism by which the APOE genotype might influence tau pathology remains unclear, several pathways have been proposed to explain this phenomenon partially. One mechanism could be through the two primary metabolic receptors responsible for regulating ApoE clearance – LRP1 and Low-Density Lipoprotein Receptor (LDLR), as LDLR overexpression and LRP1 knockout have been shown to reduce tau propagation in mice (Rauch et al., 2020; Shi et al., 2021). Another proposed mechanism is the aggregation of C-terminal-truncated ApoE fragments, which can cause NFT-like structures and cause neurodegeneration (Huang et al., 2001). The ApoE fragments accumulated in an age-dependent manner and in significantly higher levels in APOE4 (Huang et al., 2001; Rohn et al., 2012). Interestingly, the fragments were only observed when ApoE4 was expressed in neurons, not astrocytes, highlighting the role of neuron-specific proteolytic pathways.

ApoE4 and Neuroinflammation

Understanding the complexity of the neuroinflammatory response both acutely and long-term has been a frontier in AD and ApoE4 research. In general, ApoE4 has been associated with a dysregulated inflammatory response, contributing to pro-inflammatory changes *in vivo* and *in vitro* (Raulin et al., 2022, Zhu et al., 2012; Ulrich et al., 2018; Mhatre-winters et al., 2023; Blumenfeld et al., 2024). For instance, knock-in mice with human APOE alleles have shown increased glial activation and increased levels of pro-inflammatory cytokines including IL-1β, IL-6, TNF-α in the APOE4 genotype following

an inflammatory insult (Zhu et al., 2012; Ulrich et al., 2018). Recent findings also show that APOE4 may have cell-type specific effects in AD pathogenesis (Blumenfeld et al., 2024), particularly in the major drivers of inflammation – microglia and astrocytes. The APOE4 allele has shown to prime microglia toward a pro-inflammatory and activated state (Serrano-Pozo et al. 2021). It is known that microglia can display a range of activation profiles in vivo which could even be regionally heterogenous (Tan et al., 2020; Garcia-Revilla et al., 2019). New techniques such as RNA-Seq have identified several microglial activation phenotypes specific to AD mouse models that are not present in wildtype mice (Keren-Shaul et al., 2017; Krasemann et al., 2017; Safaiyan et al., 2021), some of these include neurodegenerative microglia (MGnD), disease associated microglia (DAM), and White matter associated microglia (WAM). Ongoing research is focusing on the role of APOE4 in each of these profiles. For instance, a study by Yin et al (2023), showed that APOE4 microglia show a dysregulated MGnD profile in an APP/PS1 mouse model of AD, and deletion of microglial APOE4 had neuroprotective effects such as reducing plaque load (Yin et al., 2023). The effects of APOE4 on microglial activation may be attributed to various mechanisms, such as its influence on inflammatory mediators like 25-hydrocholesterol (Wong et al., 2020), interactions with microglial receptors including triggering receptor expressed on myeloid cells 2 (TREM2) (Fernandez-Calle et al., 2022; Kober et al., 2020), and the ApoE4 newfound function as a transcription factor for genes implicated in AD (Yin et al., 2023). Lastly, APOE4 allele is also associated with impaired microglia- astrocyte crosstalk which is critical in orchestrating the inflammatory response in AD (Yin et al., 2023; Butovsky et al., 2014).

Astrocytes, the major producers of ApoE in the brain, play an integral role in supporting neuronal function with lipid transport which is impaired in APOE4 astrocytes (Pitas et al., 1987; Zhang and Liu, 2015). For instance, APOE4 astrocytes in vitro are shown to have increased cholesterol biosynthesis and impaired metabolism leading to intracellular accumulation of cholesterol (Tcw et al., 2022; Zhao et al., 2017; Lin et al., 2018). Similarly, the transport of toxic fatty acids to astrocytes for neutralization is thought to be disrupted in APOE4, leading to accumulation of toxic fatty acids and lipid peroxidation in neurons (Ioannou et al., 2019; Qi et al., 2021; Sienski et al., 2021).

Additionally, there is a growing body of evidence associating ApoE4 in astrocytes with BBB breakdown and metabolic changes that along with the previously mentioned mechanisms contribute to the differential role of APOE4 astrocytes in inflammation (Mhatre-Winters et al., 2023; de Leeuw et al., 2022). For instance, in a study by Arnaud et al (2022) induced pluripotent stem cell-derived astrocytes from AD patients show that the APOE4 allele primes astrocytes for a more pro-inflammatory state through higher expression of pro-inflammatory genes and cytokines. In addition to differences at the basal level, APOE4 astrocytes also show enhanced inducibility in response to cytokine induction (Arnaud et al., 2022). Similarly, in APOE4 mouse models, higher levels of astrocytic inflammatory genes have been consistently documented, including the serine protease inhibitor SerpinA3n, that is linked to inflammation following TBI (Yin et al., 2023; Zhao et al., 2020; Fissolo et al., 2021; Wang et al., 2020).

ApoE4 in TBI Outcome

The initial reports of worse outcomes following TBI in APOE4-carriers started with a seminal paper by Mayeux et al. (1995) observing 10-fold increased risk of developing AD after TBI in APOE4-carriers compared to no increase in non-carriers (Mayeux et al., 1995). Shortly after, APOE4-carriers were shown to be more than twice as likely to experience an unfavorable outcome at 6 months following TBI relative to non-carriers (Teasdale et al., 1997). Interestingly, Teasdale et al (1997) also showed a gene dose effect of APOE4, with homozygotes showing more disabilities relative to heterozygotes. Following these reports numerous studies investigated this relationship, rendering the APOE4 gene as the most extensively studied gene following TBI (Zhou et al., 2008; McFadyen et al., 2021). While there are a number of studies that show no evidence of association between APOE4 and outcomes post-TBI (Liberman et al., 2002; Millar et al., 2003; Chamelian et al., 2004), the weight of the evidence more strongly supports poorer outcomes in APOE4-carriers (Zhou et al., 2008; McFadyen et al., 2021). For instance, APOE4 has been associated with longer hospital stays and longer loss of consciousness (Chiang and Hu. 2003), higher fatality rates (Lichtman et al., 2000), higher risk of post-traumatic seizures (Diaz-Arrastia et al., 2003), and slower recovery

(Alexander et al., 2007). While many of the mentioned studies investigated the functional outcomes acutely (6 months) post-injury (Teasdale et al., 1997; Zhou et al., 2008; Chamelian et al., 2004; McFadyen et al., 2021), a study by Ponsford et al (2011) following over 600 patients detected poorer long-term outcomes 1 to 5 years post-TBI, suggesting that the detrimental effects of APOE4 become more pronounced overtime (Ponsford et al., 2011). Interestingly, they also observed a sex-difference, with poorer outcomes in female APOE4-carriers over 55 years compared to men, suggesting potential sex-specific mechanisms.

ApoE4, TBI, and AD

In line with findings of the study by Mayeux et al (1995), other studies have shown individuals with APOE4 are at a higher risk of developing AD post-TBI (O'Meara et al., 1997; Guo et al., 2000; Plassman et al., 2000; Mauri et al., 2006; Laskowitz 2010). In fact, a study looking at the age of onset for AD showed that APOE4-carriers who experienced TBI had a 5-year earlier average age of onset, compared to 2.2 years for TBI alone and 2.3 years for APOE4 (LoBue et al., 2016). Although the independent effect of both TBI and APOE4 is established, there is an ongoing debate whether these two factors operate synergistically and/or additively to increase risk for AD development (Johnson et al., 2010, Zhou et al., 2008, LoBue et al., 2016). Nevertheless, the shared mechanistic pathways implicated in AD between APOE4 and consequences of TBI have provided evidence of a synergistic relationship. For instance, more APOE4-carriers who experienced TBIs show amyloid deposition post-mortem in a gene-dose-dependent manner: 10% of patients without APOE4, 35% of heterozygous, and 100% of homozygous APOE4 patients (Nicoll et al., 1995). Similarly, APOE4 is associated with a higher tau phosphorylation and deposition of tau (Cao et al., 2017; Atherton et al., 2022; Vasilevskaya et al., 2020). There have been several mechanisms proposed to explain how APOE4 and TBI interact to increase risk of AD neuropathology, including impaired BBB repair post-TBI (Main et al., 2018; Teng et al., 2017) and chronic inflammation (Mahley and Huang, 2012; Dose et al., 2016). However, more studies are required to elucidate the

exact mechanisms and the lack of tests in translatable animal models with humanized APOE alleles is a clear gap in tools available to address this important question.

1.4 APOE4 in cognition over time

When it comes to cognition, classic accounts of AD have shown earliest cognitive impairments to be in episodic memory, or memory for contextual details of personal events (American Psychiatric Association. Task Force on DSM-IV (1994). However, there is strong evidence for changes in central executive function including attention in early stages of AD (Peterson, 2004; Weintraub et al., 2012; Backman et al., 2005). For instance, changes in attention and working memory can be detected as early as the MCI stage compared to non-impaired age-matched controls (Saunders and Summers, 2009; Belleville et al., 2007), which are strong predictors of progression to AD (Kirova 2015; Chehrehnegar 2021; Saunders and Summers, 2011). Combined with memory deficits, these deficits have shown to more reliably predict progression to the clinical stages of AD than memory deficits alone (Saunders and Summers, 2011). Given that real-world cognition requires constant interaction between attention and memory (Zimmermann et al., 2019; Summerfield et al., 2011), this creates an important need to study the progression of attention deficits and how risk factors such as APOE4 could influence this interaction.

APOE4 is associated with impaired cognition at an older age

The APOE4 genotype has been consistently associated with impaired cognitive performance in older adults (Zimmerman et al., 2019; Reitz and Mayeux, 2010; Emrani et al., 2020; Caselli et al., 2009; Gharbi-Meliani et al., 2021). For instance, a 20-year follow-up study of over 5000 individuals demonstrated that APOE4 is associated with a lower global cognition score in adults over 65 years of age, irrespective of dementia occurrence (Gharbi-Meliani et al., 2021). Moreover, the APOE4-related cognitive impairments at older age are gene-dose dependent with most studies reporting poorer

cognitive outcomes in homozygotes (Caselli et al., 2009; Rawle et al., 2018), and much fewer studies in heterozygote APOE4 carriers (Gharbi-Meliani et al., 2021). Even in cognitively healthy older adults, studies have shown APOE4-carriers have impairments in global cognitive performance (Small et al., 2004; Wisdom et al., 2011), and more specifically in measures of episodic memory and attention (Zimmermann et al., 2019; Nilsson et al., 2002; Nilsson et al., 2006).

APOE4 is associated with better cognition at a young age

Interestingly, at a younger age the opposite has been reported, with APOE4-carriers showing an advantage. APOE4 has been associated with higher likelihood of pursuing higher education (Huback et al., 2001), higher IQ at the university age (Yu et al., 2000), or even higher fertility (Trumble et al., 2023). Cognitively, a series of studies have reported APOE4-carrier's better performance in range of domains including episodic memory (Mondadori et al., 2007), verbal fluency (Alexander et al., 2007, Marchant et al., 2010), and executive function (Gharbi-Meliani et al., 2021; Marchant et al., 2010), specifically in attention (Rusted et al., 2013; Marchant et al., 2010). In the study by Gharibi-Meliani et al (2021), heterozygote APOE4-carriers had a better global cognitive score than non-carriers up to the age of 55 years (Gharibi-Meliani, 2021). Interestingly, they also showed that this cognitive advantage was mainly derived by executive function but not memory and semantic fluency. Another study by Rusted et al (2013) found that that APOE4-carriers between ages 18-30 performed significantly better on two different measures of attention – sustained and covert attention (Rusted et al., 2013).

Antagonistic Pleiotropy: Differential effect of APOE4 over time

Taken together, the cognitive advantage of APOE4 at a younger age and the adverse outcomes in the older age provide support for the antagonistic pleiotropy hypothesis of APOE4 (Tuminello and Han, 2011; Han and Bondi, 2008; Alexander, 2007). The concept of antagonistic pleiotropy, introduced by George C. Williams in 1957, refers to a gene having different and opposite effects on fitness during one's

lifetime (Williams, 1957). Although there is strong support for antagonistic pleiotropy hypothesis with regards to cognitive performance in APOE4 carriers (Rusted et al., 2013; Gharbi-Meliani et al., 2021; Wright et al., 2003; Jochemsen et al., 2012), several studies have failed to show any cognitive benefits at a young age (Bussy et al., 2019; Weissberger et al., 2018; Ihle 2012). Among these are two meta-analyses examining cognitive performance measures between APOE4 carriers and non-carriers at a young age (2-40 years of age, with average age around adolescence; Weissberger 2018; Ihle et al., 2012). There are several factors that are likely responsible for the null findings reported in these studies including genotype-related factors, sex-related factors, and cognitive task-related factors. First is the gene-dose dependent effect of APOE4, which could have different effects on cognition both in young and older adults (Gharibi-meliani et al., 2021) that was not accounted for in either of the meta-analyses. Second, is the potential sex differences that was not analyzed in both studies due to low power. Several of the studies that have demonstrated a cognitive advantage in APOE4-carriers had either all or significantly more female participants (Yu et al., 2000; Rusted et al., 2013; Stening et al., 2016). Moreover, as women are at a higher risk of developing AD and experiencing faster cognitive decline (Sohn et al., 2018), studying sex differences becomes a high priority in APOE4 research. Third, the cognitive advantage associated with APOE4 could be specific to certain cognitive domains (Gharibi-Meliani et al., 2021; Weissberger et al., 2018). As suggested by Han and Bondi (2008), the APOE4 cognitive benefits could be mediated by the compensatory recruitment of frontal-executive neural networks, supported by some but not all imaging work on young and healthy older APOE4-carriers (Filbey et al., 2006; Filbey et al., 2010; Bondi et al., 2005). Although there is much debate on whether this frontal-executive recruitment is compensatory (not task-related) or due to utilization of frontally-mediated tasks (Weissberger et al., 2018; Tuminello et al., 2011), studies have consistently shown cognitive advantage in attention tasks (Rusted et al., 2013; Marchant et al., 2010) and other executive tasks (Gharibi-Meliani 2021; Filbey et al., 2010; Weissberger et al., 2018). This task-related variation creates a need to assess cognitive domains individually over time, with a special focus on attention.

Lastly, as most of the cognition literature in APOE4-carriers has focused on older adults, there is a growing need for further studies to address the gaps in the current small and mixed literature on cognition in young APOE4-carriers. More specifically, long-term longitudinal studies such as the 20-year study by Gharibi-Meliani et al (2021) are needed to gain a better understanding of changes in cognitive performance with respect to APOE4. Even in animal models, there are only a few studies in mice that successfully replicated the APOE4 cognitive advantage and those focused solely on spatial memory (Moreau et al., 2013), creating a need for translatable cognitive studies with animal models of APOE4 and other factors that can influence cognition including TBI.

APOE4 and cognition following TBI

Three meta-analyses to date have investigated the effect of APOE4 on general functional outcomes following TBI, all pointing toward more unfavorable outcomes in APOE4-carriers (Zhou et al., 2008; Zeng et al., 2014; McFadyen et al., 2021). The focus of these analyses was mainly on broad outcomes not on cognitive function. More specific to cognition, a systemic review of 69 studies by Lawrence et al (2015) confirmed the past meta-analyses findings and concluded based on 18 studies with neuropsychological assessments that APOE4 may impair cognitive function following severe TBI (Lawrence et al., 2015). The meta-analysis by Padgett et al (2016) aimed to extend the findings by Lawrence et al (2015) in individual cognitive domains at 12 months post-injury and concluded that APOE4 does not have a detrimental effect on cognitive performance following TBI (Padgett et al., 2016). The null findings in this study could be attributed to the highly heterogenous data in each of the cognitive domains. For instance, specific to attention only three studies were included (Chamelian et al., 2004; Han et al., 2007; Liberman et al., 2002), with two of them reporting no significant difference in performance between APOE4-carriers and non-carriers 1-6 months following TBI. Interestingly, the age range for the patients was very broad in these two studies (ex: ranging from 18-81 in Liberman et al). Conversely, in the study by Han et al (2007) the results showed a better performance in APOE4-carriers and the age range was significantly younger and narrower in range (22.56 ± 3.76 years in APOE4-carriers).

Their findings support the idea that the interaction of TBI and APOE4 on cognition may differ by age. Unfortunately, little is known about the long-term changes (over 12-months) in cognition following TBI in APOE4-carriers over one's lifespan. Although it is known that APOE4-carriers with a history of TBI have an even earlier age of dementia onset (LoBue et al., 2016), there is a growing need to longitudinally assess the APOE4 interaction with consequences of TBI overtime. However, this can be difficult due to the large number of participants needed for adequate power, long-term follow-ups, and the wide range of environmental factors that could introduce alternative explanations for the results. An alternative approach is utilizing genetically engineered animal models of APOE4 and TBI to address these concerns, allowing further exploration of pathological mechanisms without other confounding variables.

Longitudinally assessing the effect of APOE4 and TBI on cognition in a mouse model requires optimizing the translatability of techniques. First, cognition needs to be measured in mice longitudinally, using a task analogous to cognitive testing in humans. More specifically, as APOE4-related benefits in cognition are more consistently reported in executive functions (Rusted et al., 2013; Marchant et al., 2010; Gharibi-Meliani 2021;), and attention is affected early in AD progression (Saunders and Summers, 2009; Belleville et al., 2007), the cognitive testing should focus on attention. Second, a mouse model of risk for LOAD should be used that expresses the human APOE4 isoform, and other AD-related genetic factors that interact with APOE4 including human Aβ and tau. Lastly, a translatable model of TBI should be used, mimicking the strain caused by the forces in the human brain following impact. More specifically, this model should focus on mild TBIs as they are the most common, and thus, representative of a larger patient population.

1.5 Measuring attention in mice

Attention is an executive cognitive domain that refers to several different attentional processes including sustained attention, selective attention, and shifting

attention (Bushnell and Strupp, 2009; James, 1950). To assess attention in rodents several different tests have been developed, focusing on different attentional processes including the 5-choice serial reaction time test (5-CSRTT) and the sustained attention task (SAT). However, these assessments had limited translatability in clinical settings (Kim et al., 2015). For instance, the 5-CSRTT requires responding to a bright light presented in one of five spatial locations, which is not assessing discrimination between stimuli and selective responding as assessed in clinical tasks.

A common clinical task is the continuous performance test (CPT), that requires subjects to attend to rapidly presented complex stimuli and selectively respond to infrequent 'target' stimuli, over many trials (Rosvold et al., 1956). Since its development, different variations of CPT have been used to detect impairments in various patient populations, including AD patients (Stopford et al., 2012) and TBI survivors (Zane et al., 2016). To overcome the limitation of previous models, Kim et al (2015) developed a variation of CPT for rodents, utilizing touchscreen systems, that allowed detection, discrimination, and selective response to complex stimuli (Kim et al., 2015). The touchscreen systems allowed large-scale, automated, and standardized cognitive testing preclinically that is minimally stress-inducing (Bussey et al., 2008) and nearly identical to human CPT in the clinic. Moreover, this test showed a high sensitivity for detection of both major and subtle changes in attentional processes following pharmacological treatment (Kim et al., 2015). The high sensitivity, reliability, and robustness of touchscreen rodent CPT make it a valuable tool to assess attentional changes in mouse models of various diseases.

1.6 Mouse models of AD

There has been a great deal of interest in developing animal models to study AD pathogenesis and progression, resulting in a number of models of AD that reflect various aspects of AD pathology. The relatively short lifecycle of a mouse (18-24 months), ease of maintenance, and abundant genetic resources (Bryda, 2013), has made mice the

preferred species to study the three key pathological hallmarks of AD – namely amyloid β pathology, tau pathology, and Neuroinflammation. However, decades of preclinical drug trials targeting various AD pathologies have failed to translate successfully in the clinic (Schneider et al., 2014; Veening-Griffioen et al., 2019). One of the challenges in creating a clinically translatable mouse model has been the differences between mouse Amyloid and Tau, causing it not to form pathological aggregates like in those observed in AD patients (Saito et al., 2019). For instance, the murine APP gene differs at three amino acids within the Aβ sequence (Flood et al., 2002), shown to reduce fibril formation significantly in vitro (Atwood et al., 1998) and neural toxicity (Lv et al., 2013). Thus, many models utilize genetic tools to induce human mutations artificially in the disease-causing genes – APP, APP processing enzymes, and Tau. Unfortunately, the human genes in these transgenic lines are often overexpressed, leading to protein levels that are far beyond the normal physiological levels (Saito et al., 2019).

Mouse models of amyloid

To achieve a more translatable model of amyloid pathology while overcoming overexpression, researchers have turned to several next-generation human Aβ knock-in models of AD. The recently developed humanized APP mice better mimic the plaque pathology in humans, in an age-dependent manner (Saito et al., 2014). These models express the EOAD-associated mutations in APP—such as the Swedish, Iberian, and Arctic mutations at the close-to physiological levels (Saito et al., 2014). However, EOAD accounts for only 5-10% of AD patients (Mendez, 2012; Reitz et al., 2020), creating a need to develop models with a risk for LOAD development to represent the majority of AD patients that do not carry APP mutations.

Aiming to develop a more translatable mouse model with a risk for developing LOAD, Baglietto et al (2021) developed mice carrying a humanized version of the Aβ sequence within the mouse locus and under the control of the murine APP promoter (Baglietto-Vargas, et al., 2021). These mice expressed both human APP and Aβ at murine physiological levels when compared to Wildtype mice (C57BL/6N), which was sufficient to produce significant changes in inflammation, metabolism, and cognition in

an age-dependent manner. The humanized mice show impairments in memory by 10 to 14 months of age and increases in insoluble Aβ without extracellular deposits or plaques at 18 to 22 months (Baglietto-Vargas, et al., 2021).

Mouse models of tau

The clinical symptoms of AD strongly correlate with accumulation of tau tangles, suggesting the intricate interplay between amyloid and tau in AD pathogenesis (Bloom, 2014). Similar to APP, there are key differences between the human and murine tau, as adult mice do not express all six isoforms in humans (Brion et al., 1993). Moreover, previous models utilizing tau mutations better model different tauopathies such as frontotemporal dementia, as such mutations do not occur in AD (Saito et al., 2019). To overcome such challenges, Hashimoto et al (2019) replaced murine MAPT with human MAPT gene using homologous recombination (knock-in) in embryonic stem cells, in C57BL/6N background. These mice express all six human tau isoforms and at physiological levels as wildtype mice, without any specific tau pathology. Moreover, the humanized tau responds similarly to murine tau in response to amyloid pathology caused by APP mutations (Saito et al., 2019). Interestingly, the humanized tau mice independently do not show any tau pathology, neuroinflammation, or cognitive deficits unlike APP humanized mice.

Mouse models of APOE4

Previously studied transgenic mice expressing the human APOE isoforms were confounded by varying levels of transgene expression and by the expression of the murine APOE gene (Sullivan et al., 1997). To overcome these challenges, targeted gene replacement (knock-in) mice were generated in C57BL/6N background that express the human APOE isoforms instead of the mouse APOE gene, under the control of the mouse APOE promoter (Sullivan et al., 1997; Foley et al., 2022; Sullivan et al., 2004). The levels of human ApoE expression were similar across different APOE isoforms, specifically in hippocampal and frontal cortex samples (Sullivan et al., 2004; Foley et al., 2022). Interestingly, even in aged APOE4 knock-in mice (23 to 24 months), no

impairments in cognition were observed, suggesting that APOE4 might not drive cognitive decline in the absence of other risk factors (McLean et al., 2022).

1.7 Mouse models of TBI

Previous models of TBI utilized in animal studies include weight drop, lateral fluid percussion, and controlled cortical impact (CCI). While these models have greatly enhanced our understanding of the outcomes following severe TBI, they do not reflect the majority of human TBIs, which are mild (Cassidy et al., 2004). The previous models are often associated with extensive cortical tissue damage, neuronal loss, edema and BBB disruptions (Osier & Dixon, 2016; Smith et al., 1995). Furthermore, they induce limited diffuse axonal injury, which is a crucial aspect of the primary injury in most human TBIs, leading to many of the clinical symptoms and secondary injury (Zhang et al., 2006).

Towards a translatable model of TBI

Most human TBIs are mild, non-penetrative, and involve head and neck motion directly following impact, often resulting in no gross structural change. Thus, recent models of TBI have utilized closed head injury models, typically inducing only microstructural changes including diffuse axonal injury, neuroinflammation, and tau pathology (Hoogenboom et al., 2019). However, to produce a clinically translatable model, linear and angular acceleration that the human brain experiences following impact need to be mimicked in mice, with an emphasis on rotational acceleration, which is the main contributor to axonal injury (Smith et al., 2003).

Based on available data from hockey and football players, impacts causing rotational accelerations of 3.620 ± 2.166 krad/ s2 resulted in mild TBI (Crisco et al., 2010). In the majority of cases, the impact resulted in peak rotational accelerations around 5 krad/s2 and angular velocities of 22 rad/s (Guskiewicz & Mihalik, 2011). Our lab has collaborated with bioengineers to create a mouse close-head injury TBI model

with impact forces that result in similar rotational accelerations and velocities when scaled to humans.

1.8 Rationale and study objectives

LOAD is a neurodegenerative disorder characterized by gradual cognitive decline, influenced by the complex interplay of genetic and environmental factors over the disease progression. The APOE4 isoform, the strongest genetic risk factor for LOAD, has been shown to *(i)* accelerate the cognitive decline and AD onset (Small et al., 2004; Wisdom et al., 2011; LoBue et al., 2016), *(ii)* exacerbate the AD neuropathology (Fernandez-Calle et al., 2022; Kline, 2012) and *(iii)* interact with age, the strongest risk factor for AD, such that it provides cognitive benefit in young APOE4-carriers, and impairing effects in older years (Rusted et al., 2013; Gharibi-Meliani et al., 2021). However, there is much heterogeneity in the available data on cognitive changes in APOE4-carriers over lifespan, with more consistent trends reported in executive function and attention (Gharibi-Meliani, 2021; Rusted et al., 2013).

TBI, a known environmental risk factor for AD, has also been shown to accelerate AD onset and exacerbate AD neuropathology (LoBue et al., 2016; Cao et al., 2017). Consequences of TBI have been reported to act synergistically with APOE4 with respect to AD neuropathology – specifically in amyloid aggregation (Nicoll et al., 1995; Johnson et al., 2010), tau hyperphosphorylation (Cao et al., 2017; Atherton et al., 2022), and chronic inflammation (Mahley and Huang, 2012; Dose et al., 2016), thus increasing the risk of AD development. Acutely following TBI (6 and 12 months), several studies have reported more unfavorable outcomes – both cognitive and non-cognitive – in APOE4-carriers (Zhou et al., 2008; McFayden et al., 2021). Similarly, later in life, APOE4-carriers with TBI are at a higher risk of developing dementia (Mayeux et al., 1995; Mauri et al., 2006; Laskowitz 2010). However, little is known on how APOE4 and long-term consequences of TBI interact with age and sex to influence cognition over lifespan.

To understand the role of APOE4 in cognition over lifespan and how TBI could influence this process, this study utilized a humanized mouse model while maximizing the translatability of the methods. First, a mouse model of wildtype human Aβ, tau, and APOE4 was used without any disease-causing mutations to model the general population of APOE4-carriers better. Second, to model a mild TBI (80% of all TBI cases) more closely, a repetitive closed head injury with rotational force (rCHI-rf) was used, mimicking the forces experienced during a concussion in contact sports. Lastly, as changes in attention and executive function have been more consistently reported in APOE4-carriers, a rodent touchscreen attention task was used, identical to the test used with AD and TBI patients.

1.9 Research aims and predictions

Research Goal: To assess how Mild traumatic brain injury and APOE4 interact to affect long-term cognitive changes

Aim 1

The first aim of this study was to replicate the cognitive changes observed in APOE4-carriers in a novel mouse model homozygous for wildtype humanized *APP, MAPT,* and APOE4. Specifically, attentional processes were longitudinally assessed since *(i)* executive function and attention are among the domains affected in early stages of AD development (Peterson, 2004; Weintraub et al., 2012) and are highly associated with other cognitive domains , *(ii)* a better cognitive performance in young APOE4-carriers has been more consistently reported in executive function and attention (Rusted et al., 2013; Gharibi-Meliani et al., 2021; Marchant et al., 2010), and *(iii)* APOE4-carriers have shown to recruit executive-frontal regions responsible for attention as a compensatory mechanism (Rusted et al., 2013).

Prediction: The expression of APOE4 in mice will result in better performance in attention at early ages compared to APOE3 mice; however, as the mice age, APOE4 will result in an accelerated cognitive decline, and attention impairments at the old age.

Aim 2

The second aim of this study was to assess how APOE4 interacts with repeated mild TBIs to influence cognition. This aim had a two-part focus: First, to assess the long-term effects of mild TBIs individually, and later to investigate how these effects interact with the APOE4 genotype.

Prediction: The combination of mild TBI in APOE4 mice will accelerate and exacerbate attentional impairments, more than APOE4 or mild TBI alone

Chapter 2

2 Methods

2.1 Timeline

To address the study aims, two separate groups of mice were generated, one for behavioural experiments and one for assessing pathology. The mice in the behaviour experiment group were generated in four cohorts (Table 1) and were first assessed before injury at 6-months of age. These mice were then randomly assigned to either the injury or the sham group. Following injury or sham procedures, the mice were assessed again at 12-months and 18 months of age. After the 18-month behavioural analyses, the mice were sacrificed, and brain tissue was collected to assess pathology. The mice in the pathology group were also generated in cohorts and sacrificed at the 12-month time point for further analyses.

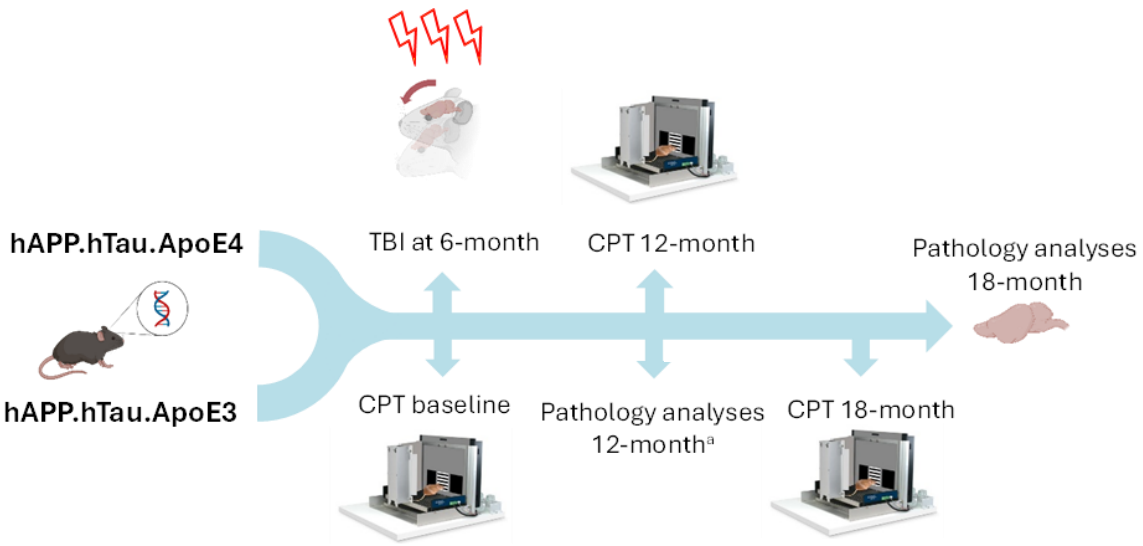

Figure 2. Experiment timeline. hAPP.hMapt.APOE4/APOE3 mice underwent CPT training at 3-4 months and baseline CPT probe testing was performed at 6 months. Immediately after 6-month CPT probes, mice experienced three TBIs or sham procedures 24 hours apart. CPT probes were assessed again at 12- and 18-months post-injury. Mice

were taken for AD-related pathology analyses at 12 and 18-months. [a] 12-month pathology mice were generated separately from the behaviour cohorts.

2.2 Animals

All mice were bred in house by intercrossing mice expressing the humanized APP (hAPP; [B6(SJL)-Apoe$^{tm1.1}$/J; Jax# 032013]; Baglietto-Vargas et al., 2021), humanized tau (hMapt; [B6.Cg-Mapt$^{tm1.1(MAPT)Tcs}$; Riken# RBRC09995]; Saito et al., 2019), and either human APOE4 or APOE3 (APOE4: [B6(SJL)-Apoe$^{tm1.1(APOE*4)adjiuj}$/J; , Jax# 027894]; APOE3: [B6.Cg-Apoe$^{em2(APOE*)adiuj}$/J; ; Jax# 029018]; Foley et al., 2022) in C57BL/6N background. This created two strains of triple knock-in mice, homozygous for the three humanized genes: hAPP.hMapt.APOE4 and hAPP.hMapt.APOE3. Throughout this thesis these strains will be referred to as APOE4 and APOE3 mice, respectively. All mice were group housed and kept on a 12hr:12hr light: dark cycle. All mice had free access to food and water unless under food restriction for touchscreen experiments described below. The mice were monitored to maintain within a healthy weight range for their age according to weight curves from Charles River. Overall, 13 mice were lost in the experiment, with no relation to genotype, sex, or injury.

2.3 TBI model

Mice were taken off food restriction 48 hours prior to the first procedure day. On each day of procedures all mice were anaesthetized with 80mg/kg ketamine and 10 mg/kg xylazine. Mice in the TBI groups received three closed head injuries each 24 hours apart, while sham mice were only anesthetized. Throughout this thesis the mice that received TBIs will be referred to as the injury mice, and those that received the sham procedure will be referred to as sham mice. The close head injuries were delivered using a controlled cortical impactor (TBI 0310, Precision Systems and Instrumentation), positioned over the intersection of bregma and the interaural line. The impact was calibrated to 3.5m/s at a

depth of 8mm and the final speed at impact was recorded for each mouse. To approximate the rotational forces experienced by people undergoing concussive injuries, the apparatus was designed for the following event to occur: i) Immediately following impact, the platform where the anesthetized mouse rested broke, allowing free rotation of head and neck; ii) The entirety of the mouse rotated following impact, landing on a cushion 8 inches below. This process was recorded using a high-speed camera, capturing 5,000 frames per second (Figure 3 B-D). The kinematic analysis demonstrated that 3.5 m/s impacts induced peak angular velocities of 277 ± 91 rad/s in males and 246 ± 66 rad/s in females. This is comparable to the peak angular velocities measured in sub-concussive impacts in football players associated with a 10% nominal injury risk (Rowson et al., 2012).

The model of injury was optimized to generate a mild TBI which does not generally exhibit gross pathological changes or skull fractures. Previous work in that lab had shown that increasing to 5 hits increased the chance of skull fracture, causing higher mortality. Similarly, the velocity used in this experiment was set to 3.5m/s as 5m/s was also tested but resulted in frequent skull fractures.

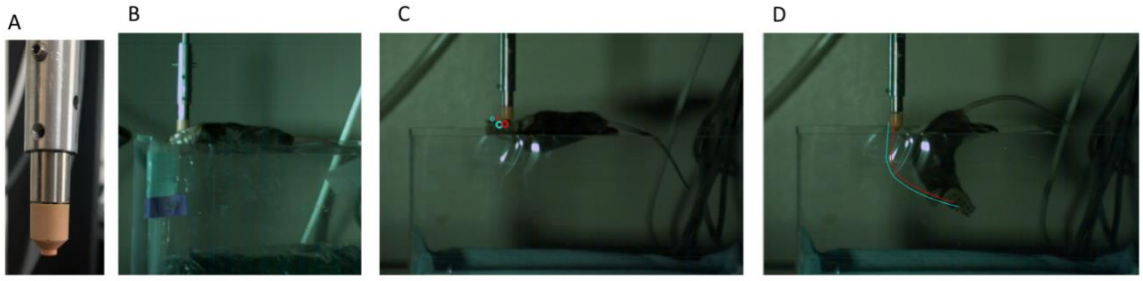

Figure 3. Set up for TBI procedures. Mice were placed on platform (B) and received impact centered on Bregma suture using a cortical impactor fitted with a silicone tip (A). Upon impactor contact with the head there is rapid rotation of the head through the platform and the mouse falls to the pillow below (C-D).

Table 1. *Summary of animals used in each behaviour cohort by sex*

Group	APOE3		APOE4	
	Sham	Injury	Sham	Injury
Behaviour cohort 1	4(4)	3(4)	4(4)	4(2)
Behaviour cohort 2	1(6)	2(6)	2(4)	4(2)
Behaviour cohort 3	5(3)	5(2)	4(3)	4(4)
Behaviour cohort 4	6(3)	5(3)	5(6)	4(6)
Behaviour Total	16(16)	15(15)	15(17)	16(14)

Note. Females (Male)

2.4 Touchscreen behavioural testing

Touchscreen behavioural testing was administered in Campden Instruments 81430 touchscreen systems (Figure 3A). Training and performance in the task were motivated by strawberry milkshake reward (Neilson Strawberry milkshake). To provide sufficient motivation, animals were subject to mild food restriction before task training at 3 months of age, equal to 2g daily food pellets (Bio-Serv Product #F0173) and free access to water. Milkshake was provided in home cage 72 hours prior to the start of training for acclimation. Touchscreen training and testing was administered between 2-7pm in the light cycle.

2.5 Rodent continuous performance task

Attention was assessed using the rodent continuous performance task (rCPT), an operant touchscreen task that is nearly identical to CPT previously used in clinical assessments (Kim et al., 2015). As CPT requires both detection and appropriate responding to target stimuli while withholding responses to non-target stimuli, a subject can make errors of omission and errors of commission. These can be used to measure two performance aspects using signal detection theory: discrimination sensitivity (d') – the subject's ability to discriminate between stimuli – and response bias (RB) – the subject's decision to respond corresponding to their response tendencies (Macmillan & Creelman, 2004). Discrimination sensitivity and response bias are parametric measures calculated under assumptions of normal distributions and equal variances in discrimination between target and non-target stimuli. For these experiments, horizontal lines were used as the target stimulus and four non-target stimuli were used as shown in Figure 3B. Correct responses to the screen were rewarded with strawberry milkshake (Neilson) accompanied by the magazine light coming on and a tone (duration 1000ms).

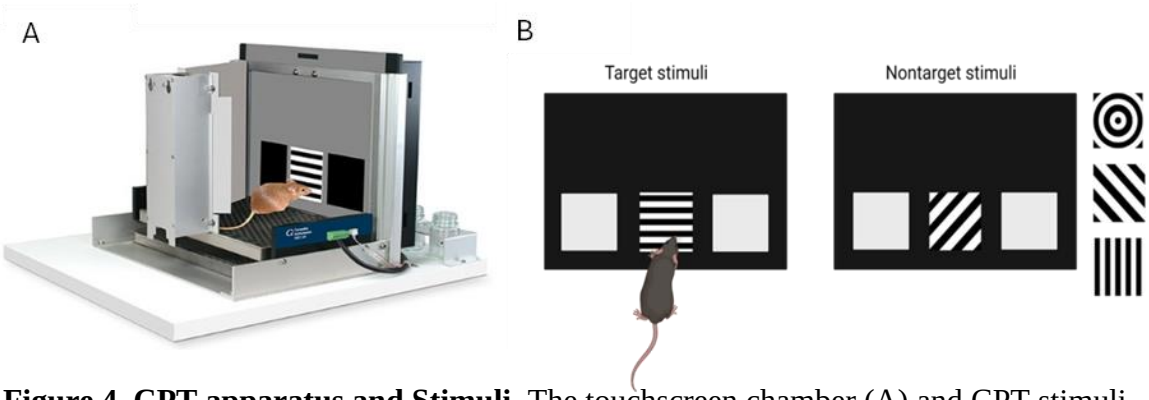

Figure 4. CPT apparatus and Stimuli. The touchscreen chamber (A) and CPT stimuli (B).

Touchscreen parameters

Hit rate (HR): correct responses to target stimuli, indicates attentiveness.

$$HR = \frac{Hit}{Hit+Miss}$$

False alarm rate (FAR): incorrect responses to non-target stimuli, indicates impulsivity.

$$FAR = \frac{Mistake}{Mistake+Correct\ rejection}$$

Discrimination sensitivity (d'): indicates the ability to discriminate between target and non-target, higher score indicates increased ability to respond selectively to target stimuli.

$$d' = z(HR) - z(FAR)$$

Response criterion/ response bias (RB): decision to respond corresponding to subjects' response tendencies, motivation or task strategy, higher score indicates more conservative responding while lower score indicates more liberal responding.

$$RB = \frac{z(HR) - z(FAR)}{2}$$

CPT training

Mice were first habituated to the touchscreen chamber, food magazine, and milkshake reward, followed by training to touch the screen when a square appeared to receive reward (stage 1). In stage 2 mice were trained to touch the screen when the target stimulus (horizontal lines) appeared. Stage three training added one-non-target stimuli (white snowflake) and mice were rewarded only when they responded to the target stimuli; incorrect responding to the non-target resulted in correction trial where non-target stimuli would appear until mouse correctly withheld response. The final stage of training contained all four non-targets presented at a target probability of 25% (i.e., 3 non-targets: 1 target). Mice were trained until they reached $d' > 1.5$, $HR > 0.6$ and $FAR < 0.2$ a minimum of 7 days for stage 3 and 10 days at stage 4 (Figure 4). Upon

completing training mice were placed on maintenance (identical to stage four training) every 1-2 weeks, until probe testing timepoint.

Training stages Stimulus set	Stimulus duration	Criterion
Stage 1 White square 	10s	Achieving 60 rewards Minimum 1 day
Stage 2 S+ introduction 	2s	Achieving 60 rewards Minimum 1 day
Stage 3 S+ (50%) One S- (50%) 	2s	d' > 1.5, HR > 0.6, FAR< 0.2, for two consecutive days Minimum 7 days
Stage 4 S+ (33%) Four S- (66%) 	2s	d' > 1.5, HR > 0.6, FAR< 0.2, for two consecutive days Minimum 10 days

Figure 5. CPT training progression and criteria.

CPT variable stimulus duration probe testing

Each testing period started with 4 consecutive days of maintenance stage (identical to stage 4), to ensure stable performance on 2s stimulus duration. As shown by Kim et al (2015), reducing stimulus duration is a robust method to increase attentional load. Thus, the CPT probe sessions assessed performance across four stimulus durations: 2s, 1s, 0.5s, and 0.2s. In each session, the four stimulus duration conditions were randomly presented, and performance was averaged for each stimulus duration. Performance was assessed daily for four days and averaged for each mouse.

CPT flanker distractor probe testing

Following the last day of variable stimulus duration probes at the 18-month timepoint, performance was tested in CPT flanker distractor probes. To test the distractibility in mice, stimuli were shown on both sides of the center panel to distract the mice, creating three conditions: Congruent trials, where the distractors match the stimulus presented in the center panel (i.e. target stimulus at the center with target stimuli on both sides); Incongruent trials, where the distractors do not match the stimulus presented in the center panel (i.e. target stimulus at the center with non-target stimuli on both sides); and non-distractor trials. On the first two days stimuli at the center were shown for a 2s duration and flanker distractors for 0.6s. Shorter distractor stimuli duration was selected to assess distractibility in perception of stimuli but not response. Following the two days, center stimulus duration was changed to 1s, while distractor duration remained at 0.6s and data was collected for four consecutive sessions. Performance under each condition was averaged for each mouse over the four sessions.

2.6 Statistical analysis

All statistics were performed using Graphpad Prism 10.2 software. Comparisons between groups were analyzed by two-way ANOVA with repeated measures. CPT behaviour data was analyzed by two-way ANOVA followed by Šidák's multiple comparison test when warranted. In all probes the probe conditions were set as the row factor, and column factor was the groups corresponding to the question at hand (main effect of injury, genotype, sex, or time). Values are considered statistically significant with alpha threshold of 0.05. Data are presented as mean ± SEM.

Chapter 3

3 Results

The present study aims to assess the interaction between the APOE4 genotype and repeated mild TBIs in male and female mice on attentional performance longitudinally. There are four major independent variables that can influence attentional performance: injury, genotype, sex, and age. Each of these factors is evaluated using pairwise comparisons, such that for each comparison, only two groups are compared at a time, keeping three out of four factors constant.

The findings of the present study are organized in this section in the following order: (i) baseline performance at 6 months of age before injury; (ii) the effect of injury, determined first in male mice and then in female mice; (iii) the effect of the APOE4 genotype, determined first in male mice and then in female mice; (iv) the effect of sex, determined in both sham and injured mice; and (v) changes in performance over the three time points, evaluated within each group.

3.1 Baseline performance in APOE4 and APOE3 mice, immediately prior to receiving TBI or sham procedures

All mice underwent baseline CPT testing at 6-months of age, prior to receiving three consecutive TBIs. In each probe session, performance was assessed under varying attentional demands by shortening stimulus duration from 2s to 1s, 0.5s, and 0.2s. APOE4 male mice showed no differences in performance from APOE3 males in discrimination sensitivity (d'), the main measure of attention in CPT (Figure 6A; $F(1, 66) = 0.7642$, $p = 0.3852$). Similarly, in hit rate and response bias, there are no significant differences (Figure 6B, D; HR: $F(1, 66) = 0.6760$, $p = 0.4139$; RB: $F(1, 66) = 2.905$, $p = 0.0930$;). In false alarm rate, however, APOE4 male mice seem to make fewer mistake than APOE3 males ($F(1, 66) = 2.905$, $p = 0.0395$).

In females, APOE4 mice have a better d' score compared to APOE3 mice at baseline (**Figure 6E;** F (1, 66) = 4.412, p =0.0395). Further post-hoc analysis reveals that the difference only reaches significance threshold at the 2s stimulus duration (p =0.0071), suggesting the better performance in APOE4 female mice disappears under higher attentional demand. This difference seems to be predominantly driven by the false alarm rate, as APOE4 females seem to make fewer mistakes than APOE3 females, although it does not reach the significance threshold (**Figure 6G**; F (1, 67) = 3.635; p =0.0609). In hit rate and response bias there are no differences between APOE4 and APOE3 females (**Figure 6F, H;** HR: F (1, 67) = 0.1317, p =0.7178; RB: F (1, 67) = 0.4275, p =0.5154).

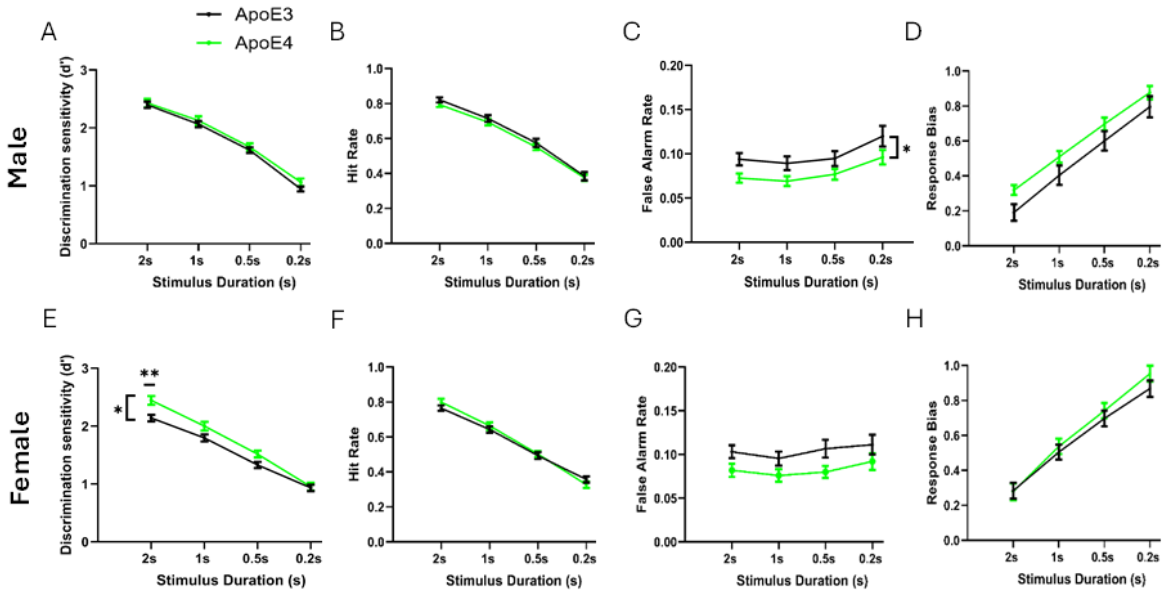

Figure 6. CPT baseline performance. Performance of 6-month old mice was averaged for each stimulus duration (2s, 1s, 0.5s, 0.2s) over the four sessions. Males (A-D) and females (E-H). Discrimination sensitivity (A,E), hit rate (B,F), false alarm rate (C,G), and response bias (D,H). hApp.hMapt.APOE4 (APOE4), hApp.hMapt.APOE3 (APOE3). Animal numbers per group are as follows: male APOE3 n = 33, male APOE4 n = 35, female APOE3 n = 36, female APOE4 n = 33. All data are mean ± SEM, *p<0.05. (Two-way ANOVA, Sidak's multiple comparison test).

3.2 Effect of repeated mild TBI on long-term CPT performance: Male mice

To investigate the long-term effects of mild TBI in APOE4 and APOE3 males, CPT performance was reassessed at 12 and 18 months of age, comparing between groups that received TBI and sham procedure. Looking at d' in both APOE4 and APOE3 males, the sham groups seem to perform slightly better than injury groups at 12 months of age, although not reaching the significance threshold (**Figure 7A;** APOE4: $F(1, 29) = 3.353$, $p = 0.0774$; APOE3: $F(1, 30) = 2.381$, $p = 0.1333$). In all other measures of CPT, there are no significant differences between sham and injury groups in APOE4 and APOE3 males (**Figure 7B-C;** HR (APOE4: $F(1, 29) = 3.241$, $p = 0.0822$; APOE3: $F(1, 30) = 0.3054$, $p = 0.5846$); FAR (APOE4: $F(1, 29) = 0.01850$, $p = 0.8927$; APOE3: $F(1, 30) = 0.5337$, $p = 0.4707$); RB (APOE4: $F(1, 29) = 1.078$, $p = 0.3077$; APOE3: $F(1, 30) = 0.0599$, $p = 0.8083$)). The slight performance difference between sham and injury is no longer observed at 18 months of age in both APOE3 and APOE4 males (**Figure 7E-H;** d'(APOE4: $F(1, 29) = 0.0014$, $p = 0.9695$; APOE3: $F(1, 30) = 0.1215$, $p = 0.7300$); HR (APOE4: $F(1, 29) = 0.7775$, $p = 0.3854$; APOE3: $F(1, 30) = 0.0012$, $p = 0.9715$); FAR (APOE4: $F(1, 29) = 0.5963$, $p = 0.4462$; APOE3: $F(1, 30) = 0.0459$, $p = 0.8317$); RB ($F(1, 29) = 1.719$, $p = 0.2001$; APOE3: $F(1, 30) = 0.0305$, $p = 0.8627$)).

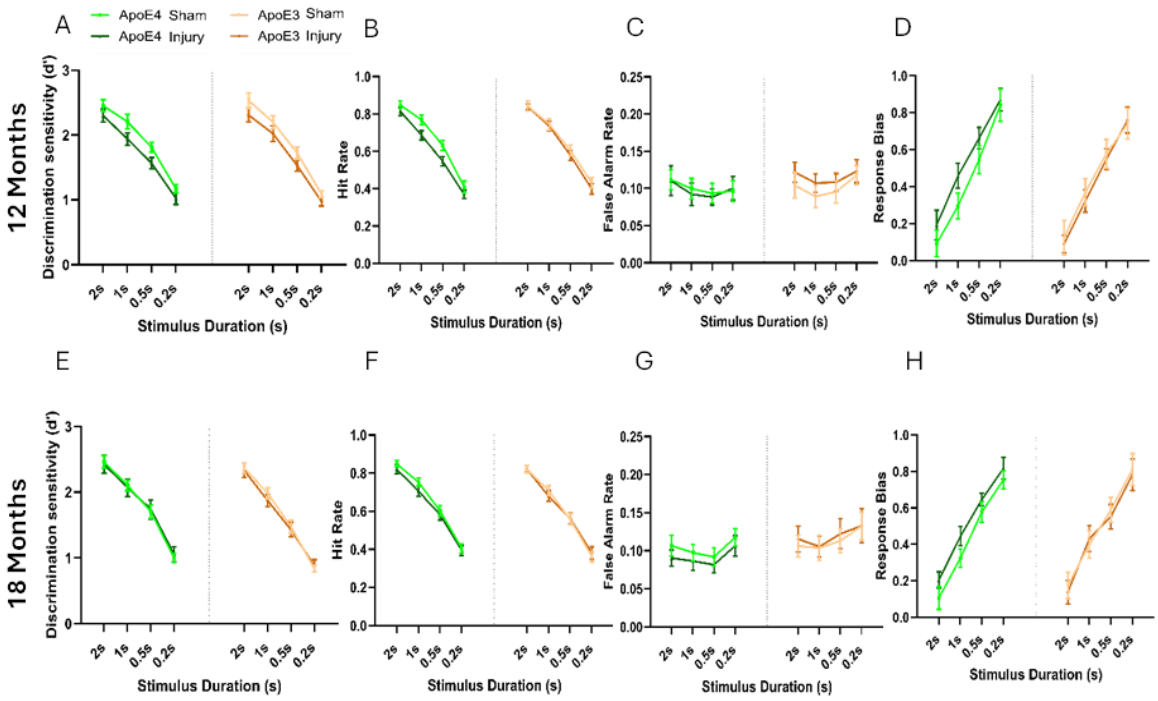

Figure 7. Long-term effects of TBI on CPT performance in male mice. CPT Performance in variable stimulus duration probes was compared between sham and injury in both APOE4 and APOE3 males. Performance was averaged for each stimulus duration (2s, 1s, 0.5s, 0.2s) over the four sessions. 12 month (A-D) and 18 month (E-H). Discrimination sensitivity (A,E), hit rate (B,F), false alarm rate (C,G), and response bias (D,H). Animal numbers per group are as follows: APOE4 sham n = 17, APOE4 injury = 14, APOE3 sham n = 16, APOE3 injury n = 15. All data are mean ± SEM, *p<0.05. (Two-way ANOVA, Sidak's multiple comparison test).

3.3 Effect of repeated mild TBI on long-term CPT performance: Female mice

To evaluate the long-term effect of repeated mild TBIs in females, CPT performance at 12 and 18 months was compared between mice that received TBI and sham procedure in both genotypes. At 12 months, there was no effect of injury on d' and

hit rate in either of the genotypes (**Figure 8 A-B;** d': (F (1, 30) = 0.2098, p =0.6502; APOE3: F (1, 30) = 0.6520, p =0.4254); HR (APOE4: F (1, 30) = 1.164, p =0.2893; APOE3: F (1, 31) = 3.623, p =0.0663)). In false alarm rate however, while there is no injury effect in APOE4 mice, sham APOE3 females make significantly less mistakes than APOE3 injured females (**Figure 8C;** APOE4: F (1, 30) = 0.08244, p =0.7760); APOE3: F (1, 31) = 4.694, p =0.0381). This difference in false alarm rate is likely driven by a more impulsive responding strategy, as there is no effect of injury in APOE4 females, but APOE3 females that received TBI show a lower response bias (**Figure 8D;** APOE4: F (1, 30) = 0.5945, p =0.4467); APOE3: F (1, 31) = 6.118, p =0.0191). Interestingly, at 18 months of age, there is no longer an injury effect in either of the genotypes in any of the CPT measures (**Figure 8E-H;** d'(APOE4: F (1, 29) = 2.302, p =0.1400; APOE3: F (1,29) = 1.279, p =0.2674); HR (APOE4: F (1, 29) = 2.806, p =0.1046; APOE3: F (1,29) = 3.469, p =0.0727); FAR (APOE4: F (1, 29) = 0.1472, p =0.7040; APOE3: F (1, 29) = 0.2719, p =0.6060); RB (F (1, 29) = 1.033, p =0.3178; APOE3: F (1, 29) = 2.343, p =0.1367)).

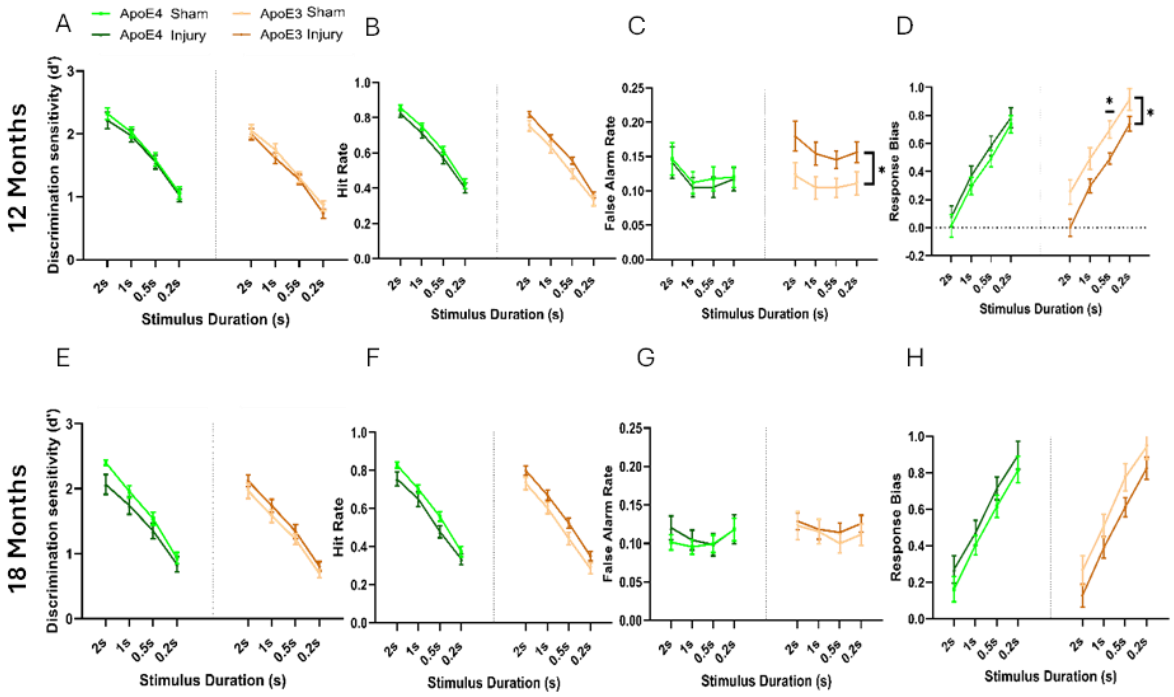

Figure 8. Long-term effects of TBI on CPT performance in female mice. CPT performance in variable stimulus duration probes was compared between sham and injury in both APOE4 and APOE3 females. Performance was averaged for each stimulus duration (2s, 1s, 0.5s, 0.2s) over the four sessions. 12 month (A-D) and 18 month (E-H). Discrimination sensitivity (A,E), hit rate (B,F), false alarm rate (C,G), and response bias (D,H). Animal numbers per group are as follows: APOE4 sham n = 16, APOE4 injury = 15, APOE3 sham n = 16, APOE3 injury n = 15. All data are mean ± SEM, *p<0.05. (Two-way ANOVA, Sidak's multiple comparison test).

3.4 Effect of APOE4 genotype on long-term CPT performance: Male mice

To investigate the long-term effects of APOE genotype on attentional functioning in male mice, CPT performance was compared between APOE4 and APOE3 males in both sham and injury groups at 12 and 18 months of age. There was no difference in performance observed between the genotypes in either the sham or the injury groups at 12 months (**Figure 9A-D**; d' (Sham: F (1, 31) = 0.06218, p =0.8047; Injury: F (1, 28) = 6.66e-7, p =0.9994); HR (Sham: F (1, 31) = 0.06322, p =0.8031; Injury: F (1, 28) = 1.231, p =0.2767); FAR (Sham: F (1, 31) = 0.004380, p =0.9477; Injury: F (1, 28) = 0.8775, p =0.3569); RB (Sham: F (1, 31) = 0.0073, p =0.9324; Injury : F (1, 28) = 2.023, p =0.1659)).

Compared to 12 months, at 18 months of age there is a slightly larger difference in performance between APOE3 and APOE4 injured males, although not reaching the significance threshold (**Figure 9E**; Sham: F (1, 31) = 2.277, p =0.1414; Injury: F (1, 28) = 1.660, p =0.2085). This is driven by the slightly higher but not significantly different false alarm rate in injured APOE3 males compared to APOE4 injured males (**Figure 9G**; Sham: F (1, 31) = 0.2945, p =0.5912; Injury: F (1, 28) = 1.799, p =0.1910). There were no differences in performance between genotypes in the other measures of CPT performance (**Figure 9F**, **H**; HR (Sham: F (1, 31) = 1.766, p =0.1935; Injury: F (1, 28) =

0.1852, p =0.6704); RB (Sham: F (1, 31) = 0.3675, p =0.5488; Injury: F (1, 28) = 0.4138, p =0.5255)).

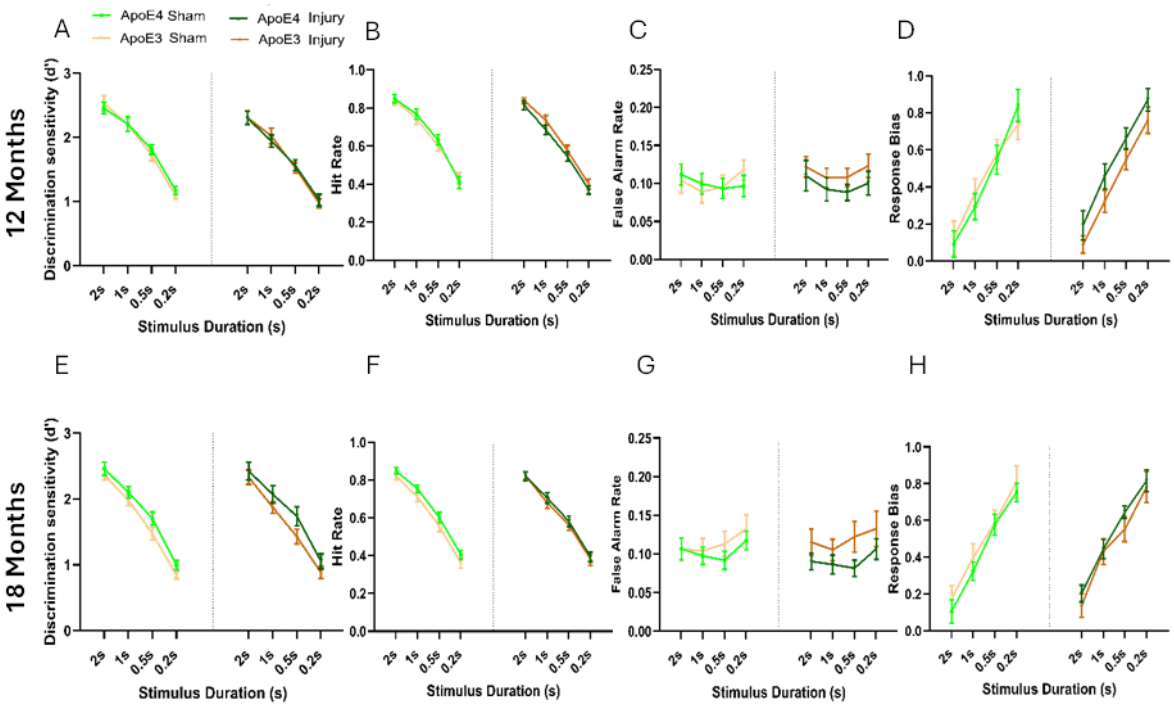

Figure 9. Long-term effects of APOE4 genotype on CPT performance in male mice. CPT performance in variable stimulus duration probes was compared between APOE4 and APOE3 males in both sham and injury groups. Performance was averaged for each stimulus duration (2s, 1s, 0.5s, 0.2s) over the four sessions. 12 month (A-D) and 18 month (E-H). Discrimination sensitivity (A,E), hit rate (B,F), false alarm rate (C,G), and response bias (D,H). Animal numbers per group are as follows: APOE4 sham n = 17, APOE4 injury = 14, APOE3 sham n = 16, APOE3 injury n = 15. All data are mean ± SEM, *p<0.05. (Two-way ANOVA, Sidak's multiple comparison test)

3.5 Effect of APOE4 genotype on long-term CPT performance: Female mice

To investigate the effects of APOE genotypes in attentional functioning in female mice, CPT performance was compared between genotypes in both sham and injury groups. At 12 months of age, APOE4 females show a higher discrimination sensitivity compared to APOE3 female mice, irrespective of the injury (**Figure 10A;** Sham: $F(1, 31) = 5.051$, $p = 0.0319$; Injury: $F(1, 31) = 5.345$, $p = 0.0276$). Further post-hoc analysis indicated significant differences at the 1s and 0.5s stimulus durations in both sham and injury groups. However, the difference only passes the significance threshold at the 1s stimulus duration, when comparing between APOE4 and APOE3 injured females ($p = 0.0402$). Looking at other CPT measures suggests that the better performance in APOE4 sham female mice is driven by a more liberal responding strategy, resulting in higher hit rate and response bias (**Figure 10B-D first panels;** HR: $F(1, 30) = 11.50$, $p = 0.0020$; FAR: $F(1, 30) = 0.3182$, $p = 0.5769$; RB: $F(1, 30) = 4.633$, $p = 0.0395$). In contrast, the better performance in APOE4 injured females compared to APOE3 injured females is predominantly driven by fewer mistakes in the APOE4 injury group, although not reaching the significance threshold (**Figure 10B-D second panels;** HR: $F(1, 31) = 0.5891$, $p = 0.4486$); FAR: $F(1, 31) = 3.562$, $p = 0.0685$); RB: $F(1, 31) = 0.8428$, $p = 0.3657$)).

At 18 months of age, APOE4 sham females continue to outperform APOE3 sham females in d' (**Figure 10E first panel;** d': $F(1, 30) = 9.672$, $p = 0.0041$). Further post-hoc analyses show that the higher d' in APOE4 sham females is mainly at the 2s and 1s stimulus durations (2s: $p = 0.0032$; 1s: $p = 0.0135$). This difference is mainly driven by a higher hit rate in APOE4 sham female mice (**Figure 10F-H first panels;** HR (Sham: $F(1, 30) = 8.282$, $p = 0.0073$); FAR (Sham: $F(1, 30) = 1.799$, $p = 0.1910$); RB (Sham: $F(1, 30) = 1.907$, $p = 0.1775$)). In contrast, there is no differences in d' between APOE4 injured female mice and APOE3 injured females at 18 months of age (**Figure 10E second panel;** $F(1, 28) = 0.00029$, $p = 0.9865$). Similarly in other measures of CPT, there are no differences between APOE4 injured females and APOE3 injured females (**Figure**

10F-H second panels; HR (F (1, 28) = 0.6299, p =0.4341); FAR (F (1, 28) = 0.4034, p =0.5305); RB (F (1, 28) = 1.373, p =0.2512)).

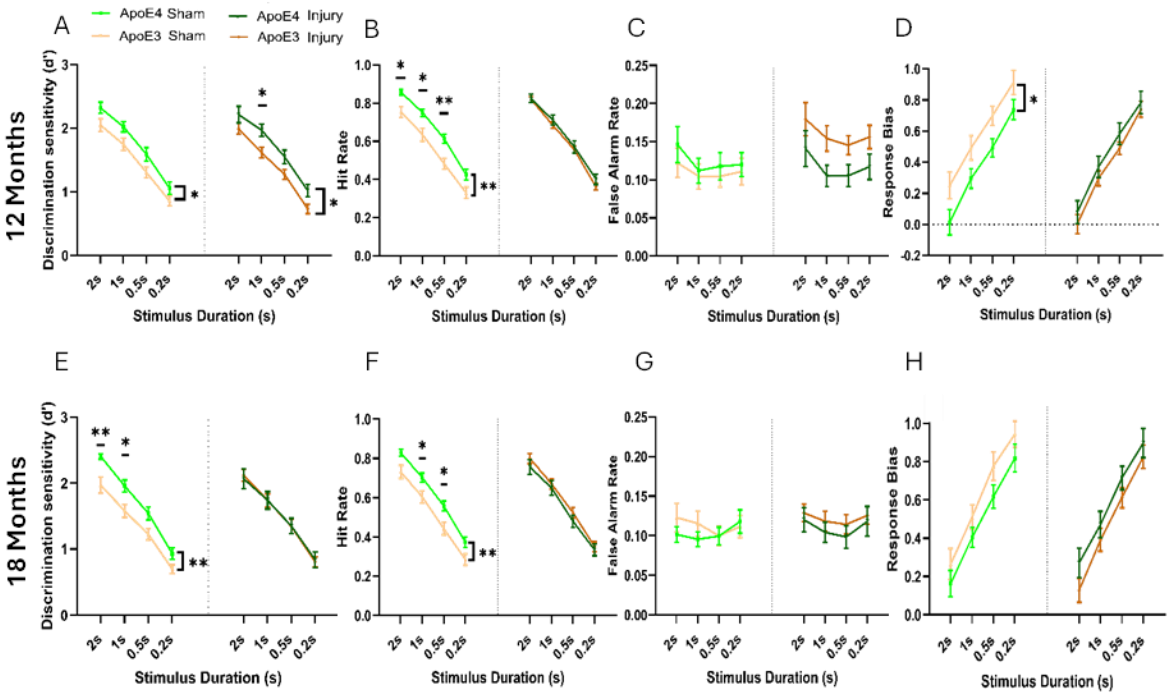

Figure 10. Long-term effects of APOE4 genotype on CPT performance in female mice. CPT performance in variable stimulus duration probes was compared between APOE4 and APOE3 females in both sham and injury groups. Performance was averaged for each stimulus duration (2s, 1s, 0.5s, 0.2s) over the four sessions. 12 month (A-D) and 18 month (E-H). Discrimination sensitivity (A,E), hit rate (B,F), false alarm rate (C,G), and response bias (D,H). Animal numbers per group are as follows: APOE4 sham n = 16, APOE4 injury = 15, APOE3 sham n = 16, APOE3 injury n = 15. All data are mean ± SEM, *p<0.05. (Two-way ANOVA, Sidak's multiple comparison test).

3.6 Effect of APOE4 genotype on CPT distractor flanker probe performance at 18 months of age

To challenge attentional processes further and assess distractibility, all mice were also tested on the CPT flanker distractor probes at 18 months of age. In this task, distracting stimuli were presented on both sides of the center touchscreen panel, creating three conditions: congruent, incongruent, and non-distractor. Similar to variable stimulus duration probes, no difference between genotypes was observed in discrimination sensitivity, hit rate, or response bias in males across both sham and injury groups (**Figure 11A, B, D;** d' (Sham: $F(1, 31) = 0.4704$, $p = 0.4979$; Injury: $F(1, 27) = 3.618$, $p = 0.0679$); HR (Sham: $F(1, 31) = 0.6210$, $p = 0.4367$; Injury: $F(1, 27) = 0.1707$, $p = 0.6828$); RB (Sham: $F(1, 31) = 1.881$, $p = 0.1801$; Injury: $F(1, 27) = 1.076$, $p = 0.3088$)). Although not passing the significance threshold, the APOE4 injured males seem to perform better than APOE3 injured males, which is mostly driven by the lower false alarm rate in APOE4 injured males (**Figure 11C;** Sham: $F(1, 31) = 3.051$, $p = 0.0906$; Injury: $F(1, 27) = 5.941$, $p = 0.0217$).

In females, similar to the variable stimulus duration probes, APOE4 sham female mice perform better than APOE3 sham female mice, which is predominantly driven by a higher hit rate in APOE4 sham females ((**Figure 11E-H first panels;** d' (Sham: $F(1, 30) = 9.799$, $p = 0.0039$); HR (Sham: $F(1, 30) = 9.924$, $p = 0.0037$); FAR (Sham: $F(1, 30) = 1.799$, $p = 0.1910$); RB (Sham: $F(1, 30) = 0.4388$, $p = 0.5128$)). In contrast to the genotype difference in sham groups, there is no difference between genotypes in any of the CPT measures in injured females (**Figure 11E-H second panels;** d' (Sham: $F(1, 28) = 0.2126$, $p = 0.6483$); HR (Sham: $F(1, 28) = 0.6534$, $p = 0.4257$); FAR (Sham: $F(1, 28) = 2.792$, $p = 0.1059$); RB (Sham: $F(1, 28) = 1.969$, $p = 0.1715$)).

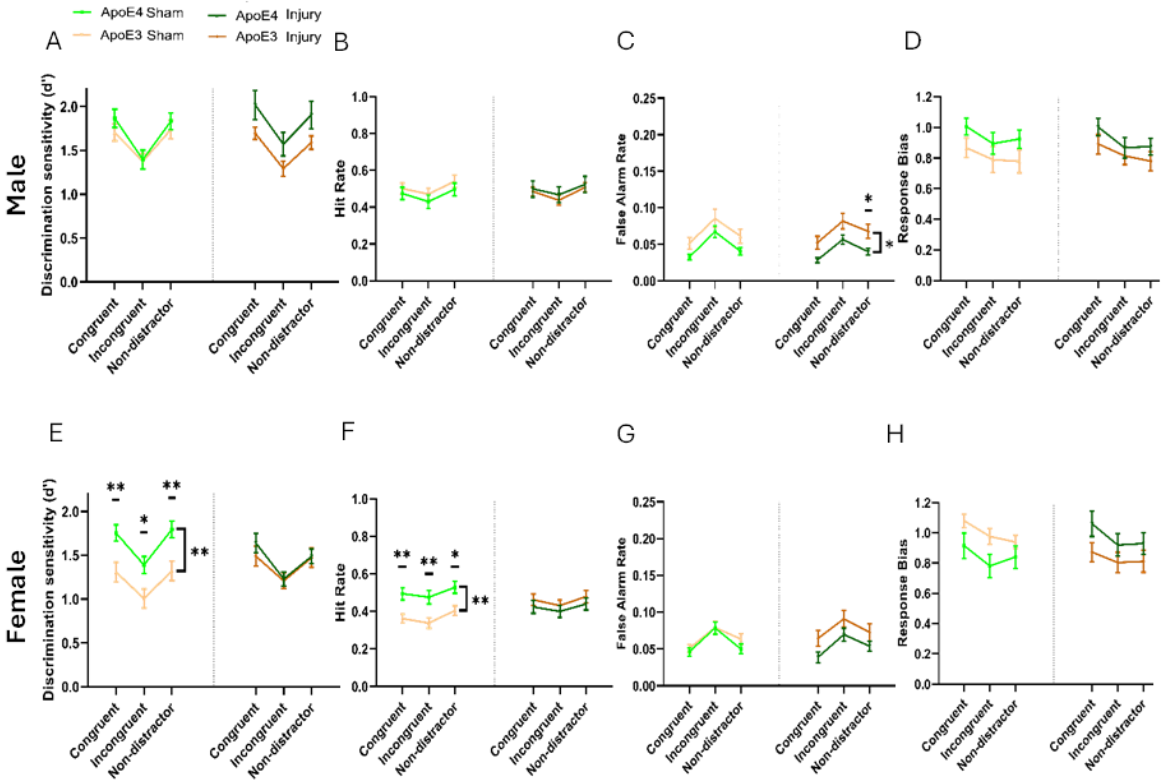

Figure 11. Effect of APOE4 genotype on CPT distractor probe performance. CPT Performance in flanker distractor probes was compared between APOE4 and APOE3 male and female mice in both sham and injury groups. Performance was averaged for condition (congruent, incongruent, and non-distractor) over four sessions. Male (A-D) and Female (E-H). Discrimination sensitivity (A,E), hit rate (B,F), false alarm rate (C,G), and response bias (D,H). Animal numbers per group are as follows: APOE4 sham n = 16, APOE4 injury = 15, APOE3 sham n = 16, APOE3 injury n = 15. All data are mean ± SEM, *p<0.05. (Two-way ANOVA, Sidak's multiple comparison test).

Table 2. Summary of CPT results from long-term behavioural study

Time	Comparison	d'	HR	FAR	RB
Baseline					
Male	APOE4 Vs APOE3	↔	↔	↓	↔
Female	APOE4 Vs APOE3	↑	↔	↔	↔
Male					
What is the effect of TBI in APOE4 males?					
12 months	APOE4 sham vs injury	↔	↔	↔	↔
18 months	APOE4 sham vs injury	↔	↔	↔	↔
What is the effect of TBI in APOE3 males?					
12 months	APOE3 sham vs injury	↔	↔	↔	↔
18 months	APOE3 sham vs injury	↔	↔	↔	↔
What is the effect of APOE4 in sham males?					
12 months	APOE4 sham vs APOE3	↔	↔	↔	↔
18 months	APOE4 sham vs APOE3	↔	↔	↔	↔
What is the effect of APOE4 in injured males?					
12 months	APOE4 injury vs APOE3	↔	↔	↔	↔
12 months	APOE4 injury vs APOE3	↔	↔	↔	↔
Female					
What is the effect of TBI in APOE4 females?					
12 months	APOE4 sham vs injury	↔	↔	↔	↔
18 months	APOE4 sham vs injury	↔	↔	↔	↔
What is the effect of TBI in APOE3 females?					
12 months	APOE3 sham vs injury	↔	↔	↓*	↑*
18 months	APOE3 sham vs injury	↔	↔	↔	↔
What is the effect of APOE4 in sham females?					
12 months	APOE4 sham vs APOE3	↑*	↑**	↔	↓*
18 months	APOE4 sham vs APOE3	↑**	↑**	↔	↔
What is the effect of APOE4 in injured females?					
12 months	APOE4 injury vs APOE3	↑*	↔	↔	↔
18 months	APOE4 injury vs APOE3	↔	↔	↔	↔

Note. The table displays results of two-way ANOVA comparisons followed by Šidák's multiple comparison test where warranted. ↑ = increase; ↓ = decrease; ↔ = no effect, comparisons are shown relative to first group in comparison. d' = discrimination

sensitivity; HR = hit rate; FAR = False alarm rate; APOE4 = hAPP.hMapt.APOE4; APOE3 = hAPP.hMapt.APOE3. * indicates $p<0.05$; ** indicates $p<0.05$

3.7 Effect of sex in long-term CPT performance

To evaluate the sex differences in CPT variable stimulus duration probes, performance is compared between males and females in each group at every timepoint. At baseline, there is no difference in performance between the sexes in APOE4 mice; however, APOE3 males perform better than APOE3 females (**Figure 12A;** APOE4: $F(1, 66) = 1.133$, $p = 0.2911$; APOE3: $F(1, 67) = 10.82$, $p = 0016$). Further post-hoc analysis reveals that the differences are predominantly at higher stimulus durations, and performance differences disappear at very high attentional demand (0.2s). At 12-months of age, in both sham and injury groups, similar performance patterns could be observed, with APOE3 males performing better in d' compared to APOE3 females, but no sex-difference in APOE4 groups (**Figure 12B, D;** Sham (APOE4: $F(1, 31) = 2.156$, $p = 0.1521$; APOE3: $F(1, 31) = 13.14$, $p = 0.001$); Injury (APOE4: $F(1, 28) = 0.02026$, $p = 0.8878$; APOE3: $F(1, 31) = 7.168$, $p = 0.0118$)). At 18-months of age, in shams similar patterns could be seen, with a sex difference in APOE3 but not APOE4 genotype (**Figure 12C;** APOE4: $F(1, 31) = 1.027$, $p = 0.3188$; APOE3: $F(1, 30) = 8.253$, $p = 0.007$). In contrast in the injury group, there is no sex-difference in either the APOE4 or the APOE3 groups (**Figure 12E;** APOE4: $F(1, 27) = 3.469$, $p = 0.0735$; APOE3: $F(1, 28) = 1.082$, $p = 0.3072$). APOE4 injured males perform better than APOE4 injured females, suggesting a potential drop in performance from 12 months to 18 months in APOE4 injured females.

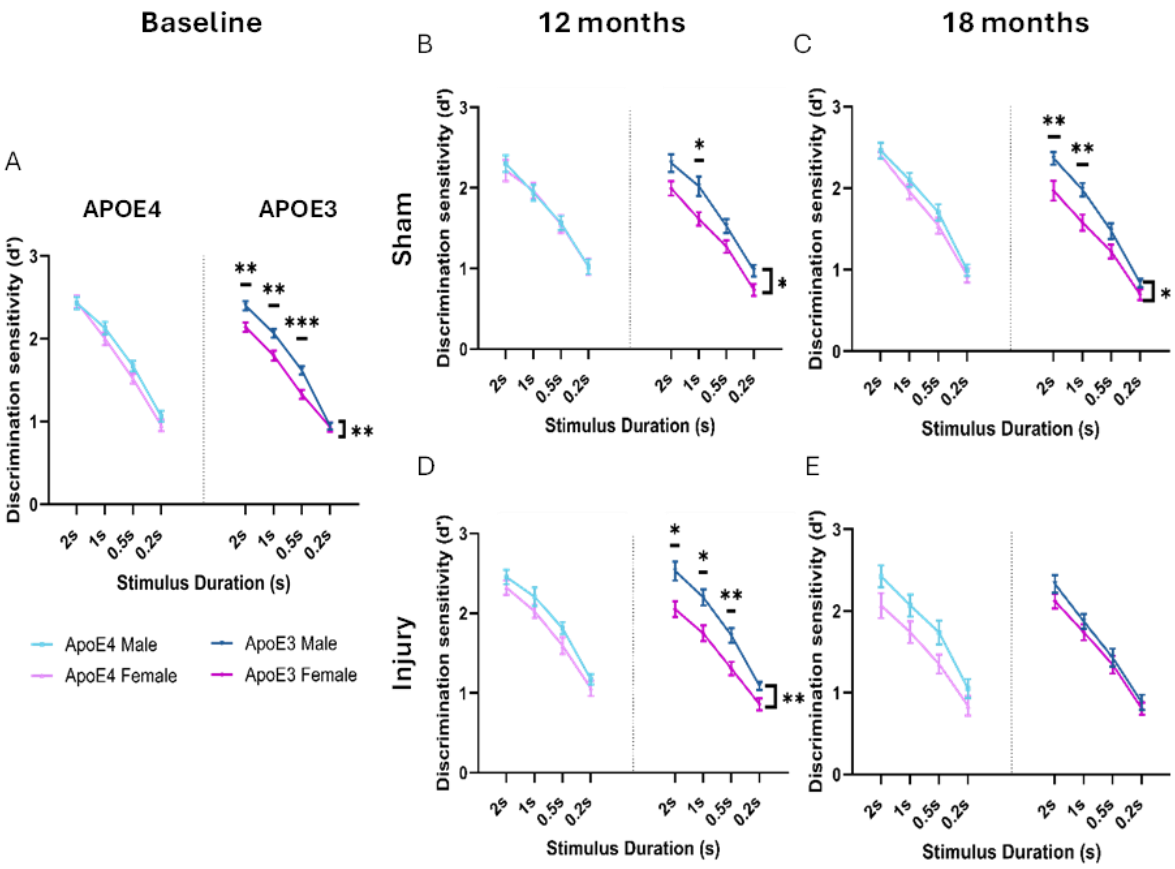

Figure 12. Sex differences in CPT performance. CPT performance in variable stimulus duration probes was compared between sexes in both genotypes and sham/injury groups. Performance was averaged at each stimulus duration (2s, 1s, 0.5s, 0.2s) over four sessions. Sham (B,C) and Injury (D, E). Baseline (A), 12 months (B,D), and 18 month (C, E). Animal numbers per group are as follows: Baseline APOE4 Male n = 35, Baseline APOE4 female = 33, Baseline APOE3 Male n = 33, Baseline APOE3 female = 36. APOE4 Male sham n = 17, APOE4 Male Injury n = 14, APOE4 Female sham n = 16, APOE4 Female Injury n = 15, APOE3 Male sham n = 16, APOE3 Male Injury n = 15, APOE3 Female sham n = 16, APOE3 Female Injury n = 15. All data are mean ± SEM, *p<0.05. (Two-way ANOVA, Sidak's multiple comparison test).

3.8 Changes in CPT performance over time

To evaluate whether there are changes in attention over time, performance in variable stimulus duration probes data was compared within each group across three timepoints: baseline, 12 months, and 18 months of age. In APOE4 sham male and female mice, CPT performance was very consistent over time with no sign of improvement or decline over time (**Figure 13A**, E; Male: F (1.747, 27.95) = 0.7289, p =0.4738; Female: F (1.779, 26.69) = 0.1428; p =0.8443). In the APOE4 injury groups, particularly the APOE4 injured females, there is a wider gap in performance over time, although not reaching significance threshold (**Figure 13B**, F; Male: F (1.781, 23.16) = 0.8688, p =0.4212; Female: F (1.536, 21.51) = 1.967; p =0.1709). Further post-hoc analysis reveals that at the 2s stimulus duration, performance is significantly reduced from baseline (12 months: p =0.0075; 18 months: p =0.0123). Looking into the other stimulus durations, the lowest d' is observed at the 18-month timepoint, although not reaching the significance threshold. In APOE3 shams, in both males and females the lowest performance is at 18-months, although only reaching statistical significance in males (**Figure 13C**, **G**; Male: F (1.986, 29.80) = 3.448, p =0.0453; Female: F (1.839, 27.58) = 2.821, p =0.0808). In the APOE3 injured males, there is a decline in age with the lowest performance at 18-months of age (**Figure 13D**; F (1.902, 26.63) = 0.9454, p =0.3970). Interestingly, in the APOE3 injured females, the performance at the 12-month time point is the lowest, confirming a transient effect of injury in this group (**Figure 13H**; F (1.945, 27.22) = 3.569, p =0.0432).

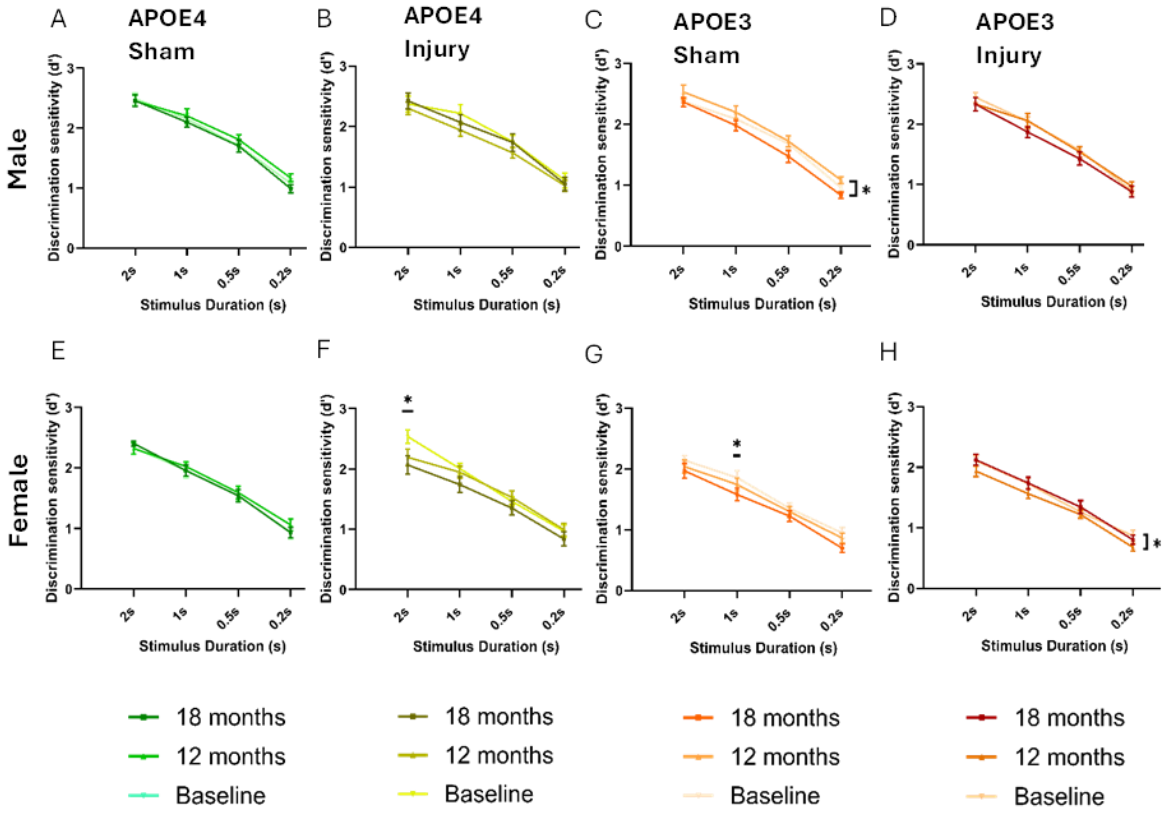

Figure 13. CPT performance in all groups across three timepoints. CPT performance in variable stimulus duration probes was compared within each group over three timepoints baseline, 12 month, and 18 month of age. Performance was averaged at each stimulus duration (2s, 1s, 0.5s, 0.2s) over four sessions. Sham (B,C) and Injury (D, E). Baseline (A), 12 months (B,D), and 18 month (C, E). Animal numbers per group are as follows: APOE4 Male sham n = 17, APOE4 Male Injury n = 14, APOE4 Female sham n = 16, APOE4 Female Injury n = 15, APOE3 Male sham n = 16, APOE3 Male Injury n = 15, APOE3 Female sham n = 16, APOE3 Female Injury n = 15. All data are mean ± SEM, *p<0.05. (Two-way ANOVA, Dunnett's multiple comparison test).

Chapter 4

4 Discussion

Alzheimer's disease is a multifaceted neurodegenerative disorder, influenced by the complex interplay of genetic and environmental factors. The APOE4 gene, being the strongest genetic risk factor for LOAD, plays a central role in many mechanisms involved in AD pathology including amyloid pathology, tau pathology, and inflammation (Fernandez-Calle et al., 2022; Kline, 2012). Traumatic brain injury has been shown to work synergistically to exacerbate the same underlying pathologies as AD, increasing the risk for AD (Johnson et al., 2010; Cao et al., 2017; Mahley and Huang, 2012). This study investigated the interplay of APOE4 and TBI in the development of cognitive deficits over time in a humanized mouse model. The mice in the present study were homozygous for three human wildtype genes— *APP*, *MAPT*, and either *APOE4* or *APOE3*—each expressed under the murine promoter to ensure murine physiological expression levels. Inclusion of the wildtype *APP* and *MAPT* genes – expressing human APP and tau, respectively – for the following reasons: *(i)* both amyloid and tau pathology are key pathological hallmarks of AD and have been associated with cognitive decline (Hanseeuw et al., 2019; Ossenkoppele et al., 2022); *(ii)* murine forms of these genes do not form the pathological aggregates as observed in humans (Saito et al., 2019). For instance, the murine *App* differs at three amino acids within the Aβ sequence (Flood et al., 2002), shown to reduce fibril formation significantly *in vitro* (Atwood et al., 1998) and neural toxicity (Lv et al., 2013). Similarly for tau, adult wildtype mice only express three out of six isoforms observed in humans (Brion et al., 1993) and have demonstrated slower tau propagation than mice with humanized *MAPT* (Saito et al., 2019). Thus, by humanizing *APP* and *MAPT*, the mice more closely mimicked human genetics involved in LOAD, placing them at a higher risk for developing AD-related pathology and deficits.

As these mice were optimized to model genetic risk for LOAD better, we predicted that in the presence of APOE4 and TBI, two AD risk factors, the mice would show similar trends in attention as observed in humans. In longitudinal studies with cognitively unimpaired individuals, APOE4 carriers show higher cognitive performance

in young adulthood, specifically in executive function, but greater cognitive impairments in later adulthood (Rusted et al., 2013; Gharbi-Meliani et al., 2021; Tuminello and Han, 2011; Han and Bondi, 2008; Alexander, 2007). These patterns of cognition in APOE4-carrying individuals led us to anticipate similar patterns over time in the humanized mice. Additionally, since APOE4 carriers tend to have worse cognitive outcomes after TBI and exhibit increased neuropathology (Zhou et al., 2008; McFadyen et al., 2021), we expected an accelerated cognitive decline in APOE4 mice post-TBI. More specifically, as attention deficits are observed in AD and following TBI (Peterson, 2004; Weintraub et al., 2012; Chamelian et al., 2004), the present study longitudinally assessed how the interaction between APOE4 and TBI results in long-term attentional changes with age.

To model changes in cognition observed following TBI more accurately, the present study was optimized for clinical relevance in both the TBI model and cognitive testing. Firstly, the TBI model was mild, developed to represent the rotational forces experienced during human concussive impacts without causing fractures or gross anatomical changes. Secondly, the attentional testing was nearly identical to testing using CPT in humans, investigating discrimination between complex stimuli over a prolonged time. These methods allowed the assessment of changes in cognition over the lifespan in the humanized mice at three timepoints: 6 months (baseline), 12 months, and 18 months of age.

Few studies in the past have experimentally assessed the interaction between APOE4 and TBI. These studies either included only males and did not see an effect of APOE4 genotype in mice (Mannix et al., 2013) or focused on the acute cognitive outcomes following injury (Giarratana et al., 2020). To our knowledge only one other study has experimentally investigated the long-term interaction of APOE4 and TBI with samples including both males and females (Mannix et al., 2011). Although they only used a single mild controlled cortical impact, they observed spatial memory deficit in APOE4 mice at 6-months post-injury when compared to wildtype C57BL/6J mice. Interestingly, when the same mice were tested before injury at 2-4 months of age, no difference was observed between APOE4 and wildtype mice performance. However, there are key

limitations in the study by Mannix et al (2011) that were addressed in this experiment: *(i)* utilizing triple knock-in humanized mice expressing wildtype human APP, Tau, and APOE4 at murine physiological levels, *(ii)* utilizing a repeated closed-head mild TBI model that mimics forces experienced in sport concussion injuries, and *(iii)* utilizing a cognitive testing that is highly sensitive to subtle changes in cognition.

4.1 In the absence of Injury, APOE4 female mice outperform APOE3 females at all ages tested

Studies investigating cognitive function in mouse models expressing APOE4 have been limited and inconsistent, with some showing impairments in domains such as spatial memory (Bour et al., 2008; Grootendorst et al., 2005; Raber et al., 1998; Raber et al., 2002), while others have demonstrated better cognitive performance in APOE4-carriers compared to APOE3-carriers (Siegel et al., 2010; Villasana et al., 2008; Moreau et al., 2013). One explanation for the inconsistent findings may lie in how APOE4 expression is regulated in the mouse models. For instance, many of the studies reporting cognitive impairments in APOE4 mice have been conducted using mice expressing APOE4 under the control of the Glial Fibrillary Acidic Protein (GFAP) promoter (Raber et al., 1998; Raber et al., 2002; van Meer et al., 2007). While this model has the advantage of specific APOE4 expression in cells that express GFAP, mainly astrocytes in the CNS and Schwann cells in the periphery (Yang and Wang, 2015), it lacks the physiological expression profile of murine *Apoe*. Knock-in models of APOE4 expressed under the control of murine APOE promoter, have addressed this limitation, more closely mimicking the cognitive benefits of APOE4 at an early age (Siegel et al., 2010; Moreau et al., 2013). Yet, there are still a number of studies utilizing these mice that have demonstrated deficits in APOE4 mice compared to APOE3 mice (Bour et al., 2008; Grootendorst et al., 2005).

To model cognition in APOE4-carriers better, the interaction between APOE4 and other factors such as APP and tau need to also be considered, as they are highly

correlated with cognition in AD (Hanseeuw et al., 2019; Ossenkoppele et al., 2022). The only other available study that has used the APOE4 knock-in mice crossed with a transgenic human APP model, demonstrates better cognitive performance in 2-5 months-old APOE4 mice compared to APOE3 controls (Moreau et al., 2013). This is consistent with the findings in the present study, showing higher attentional performance at 6 months of age in APOE4 females, and human data showing a beneficial cognitive role of APOE4 in early adulthood (Rusted et al., 2013; Gharbi-Meliani et al., 2021).

The findings in the present study also suggest that the better performance in APOE4 females persists even at 18 months of age in uninjured female animals. This finding might appear inconsistent with the human data that suggests the APOE4 genotype is beneficial early in life but is associated with cognitive deficits in late adulthood. There are two possible explanations for the difference in the human data and findings in this study. First is the possibility that the mice did not age long enough to show age-related cognitive decline. According to the survival data for 300 C57BL/6J mice collected by the Jackson Laboratory team, the relative age of a mouse compared to a human is not linear and changes as the animal ages, such that the 6-, 12-, and 18-month timepoints in this study correspond to 30, 42.5, and 56 years of age, respectively (Hagan, 2017; The Jackson Laboratory, 2017). However, several studies have not observed any cognitive deficits in APOE4-carriers by the age 56 (Alexander et al., 2007; Gharbi-meliani et al., 2021). In fact, a study by Caselli et al (2011) estimated the beginning of cognitive decline in preclinical APOE4-carriers to be around age 55-60 years of age (Caselli et al., 2011). This suggests that the addition of a later time point at 24-28 months of age might show a decline in cognition in the APOE4 mice in this study. However, at this time point the survival rate in mice would significantly decrease with age, reaching only 50% by 28 months (The Jackson Laboratory, 2017), significantly limiting power to detect subtle cognitive changes.

Another explanation for the differences in cognition between APOE4 sham animals in this study and the human data could be that APOE4 is only a risk factor for an accelerated cognitive decline under environmental stress (Siegel et al., 2010). One line of

evidence for this explanation comes from human post-mortem studies. For instance, a recent study of over 45,000 individuals with APOE genotyping data and post-mortem brain autopsy showed that APOE4 genotype is associated with decreased mortality in those with low AD-associated neuropathology, but increased mortality in individuals with high AD-associated pathology (Pirraglia et al., 2023). Another line of evidence for this explanation is in investigations of experimentally induced pathology in APOE4 mice. For instance, using ^{137}Cesium irradiation to induce brain tissue damage resulting in cognitive deficits in mice, Villasana and colleagues (2008) showed that sham APOE4 mice performed similar or slightly better than APOE3 mice in Morris Water maze; however, the irradiated APOE4 groups showed an increased cognitive decline compared to APOE3 mice. These lines of evidence support the contention that APOE4 may potentiate cognitive performance, especially in attention, in healthy brains and may only lead to cognitive deficits under stressful conditions, including experiencing repeated TBIs.

4.2 Repetitive mild TBI alone does not cause long-term attentional changes in APOE4 or APOE3 mice, only a transient increase in impulsivity in APOE3 female mice

In general, long-term follow-up after mild TBI in the present study did not reveal attentional deficits in discrimination sensitivity, irrespective of age, sex, or genotype. However, at 12 months the injury resulted in a more liberal response strategy in APOE3 injured females, leading to more mistakes, but also more correct choices. Interestingly this change in behaviour was not present at 18 months, suggesting it is likely caused by transient and time-specific changes following TBI. This is consistent with previous studies reporting higher impulsivity and increased risky decision making following TBI in rats, without any cognitive deficits (Haar et al., 2017; Shaver et al., 2019). Moreover, this increase in impulsivity has been reported to be transient and observed up to 4 months post-injury in rats following mild TBI (Haar et al., 2016). Similarly in human studies, increased accounts of impulsivity and risky decision making has been reported in TBI

patients, with a large impact on daily functioning (Rochet et al., 2010; James et al., 2014).

Looking further into potential contributing factors for this behavioral change, the study by Haar et al (2017) demonstrated that the increase in impulsivity was correlated with a substantial increase in interleukin 12 (IL-12; an inflammatory cytokine) following both mild and more severe TBIs. Although the time course of IL-12 expression with respect to impulsivity changes is yet to be elucidated, it can be predicted that the cognitive changes occur in parallel to the inflammatory consequences of TBI. Thus, changes in impulsivity in the APOE3 female mice in this study could indicate a short-term inflammatory response as a result of repeated mild TBIs for up to 6 months following injury. This short-term inflammatory response could have a protective role against long-term changes in attention.

Interestingly, these impulsivity changes observed in APOE3 females are absent in APOE4 injured females. While this seems surprising at first, APOE4 has been linked to inefficient repair processes following TBI and an inefficient inflammatory response (Fernandez-Calle et al., 2022). Regarding IL-12, little is known about outcomes following injury; however, after inflammatory stress induced by LPS injection, APOE4 target replacement mice have shown a significantly higher increase in IL-12 levels (2-fold) and several other inflammatory factors compared to APOE3 mice (Vitek et al., 2009). While paradoxically this indicates higher inflammation, it could also indicate a dysregulated inflammation profile that is inefficient in carrying out repair processes following TBI (Giartanna et al., 2020). However, as the present study does not have the corresponding pathology, specifically inflammatory markers, this explanation cannot be fully supported. Further research is required to elucidate the complex relationship between acute and long-term inflammation and their role in long-term cognitive changes.

4.3 Repeated mild TBI interacts with APOE4, such that following Injury APOE4 female mice no longer outperform APOE3 females

The sham mice in the present study demonstrated a better attentional performance that continued up to 18 months of age in the absence of injury; however, following three mild TBIs at 6-months of age, this was no longer apparent in female APOE4 mice at 18 months of age. At the 12-month time-point, the lower false alarm rate in APOE4 injured females – although not significantly different – drove the attentional differences observed in discrimination sensitivity, which was no longer apparent at 18 months. This finding was further confirmed by the flanker distractor probes, showing a better performance in APOE4 sham mice that is not apparent in the APOE4 injured mice. The observed change in performance could have two explanations: (i)APOE4 injured females have dropped in performance over time or (ii)APOE3 injured females have improved over time. Although there is support for both in our data, there is a stronger support for the first explanation, as there appears to be a larger drop in performance in APOE4 injured females when comparing d' at 12 and 18 months compared to gain in performance in APOE3 injured females. The slight performance improvement in APOE3 mice could be due to training effect or task-specific learning. As APOE4 injured female mice do not show an improvement overtime like APOE3 female mice, we can predict that either the effect of cognitive decline in APOE4 is larger than the training effect, or there is no training effect. Both of these potential explanations suggest that there is a decline in cognition in APOE4 injured female mice relative to the APOE3 injured female mice.

The difference in attentional performance at 18 months between APOE4 sham females and injured females, supports the hypothesis that APOE4 expression may only result in cognitive decline in the presence of environmental stress or pathology. Although this study lacks the neuropathological analyses to support this hypothesis at the moment, there is supporting evidence from other projects in the lab and previous studies to complement these results. The pathology following repeated mild TBIs, is thought to follow synergistically with AD pathology, through shared mechanistic pathways involving amyloid pathology, tau pathology, and neuroinflammation (Nicoll et al., 1995;

Cao et al., 2017; Mahley and Huang, 2012; Dose et al., 2016). Concerning amyloid pathology, unpublished data from the Prado lab – specifically work of Madison Longmuir, Aya Arrar–indicated the absence of amyloid plaques in the hAPP.hMapt.APOE4 or APOE3 mice using Thioflavin S immunohistology staining. Moreover, looking into earlier steps of the amyloid cascade with enzyme-linked immunosorbent assays (ELISAs) no differences in soluble or insoluble Aβ levels have been observed between APOE3 and APOE4 mice. Thus, it can be predicted that in the absence of injury there is little or no amyloid pathology in these mice, and similar to the sham female APOE4 mice in this study, APOE4 female mice perform better than APOE3 female mice even at an older age. Interestingly, in similar strains of mice where the only genetic difference is the addition of human mutations in the *APP* gene—namely the Swedish and Iberian mutations (Saito et al., 2014) instead of wildtype *APP* — both amyloid pathology and an accelerated cognitive decline are observed in female APOE4 mice. These results further support that APOE4 may only lead to cognitive deficits in the presence of stress or pathology, in this case amyloid pathology.

As for tau pathology and inflammation, it has been more difficult to assess the long-term effect independent of amyloid pathology in APOE4-related cognitive changes. For instance, looking at tau pathology in non-demented elderly participants, several studies have demonstrated an association of APOE4 genotype and tau pathology with poor cognition (Wang et al., 2023; Weigand et al., 2020; Paradela et al., 2023). Moreover, Weigand and colleagues (2020) demonstrated that this association occurs even in the absence of amyloid pathology, detected through positron emission tomography. In mouse model studies, Shi and colleagues (2017) demonstrated that APOE4 knock-in mice that also carry the P310S mutation – a tauopathy model – show higher levels of hyperphosphorylated tau, tau-mediated neurodegeneration, and neuroinflammation independent of amyloid pathology (Shi et al., 2017). However, whether the presence of tau pathology alone is sufficient to accelerate cognitive decline in APOE4-carriers, is yet to be experimentally determined. Similarly, there is limited information on whether neuroinflammation independently contributes to APOE4-associated cognitive changes. Future work is needed to assess the effect of AD pathological hall marks experimentally

– namely amyloid pathology, tau pathology, and neuroinflammation – individually with respect to cognitive changes in APOE4-carriers. Additionally, it is equally important to look at the interplay between these pathologies together, as they likely reciprocally influence each other in a time-specific manner.

4.4 Female mice are more susceptible to APOE4-mediated changes in attention

In general, the cognitive differences observed in this study whether due to genotype, injury, or their interaction were almost exclusively observed in females, with little or no cognitive changes in males. This suggests that there are sex-specific effects at multiple levels in this study. First, looking at baseline and uninjured animals in this study, it is only in females that APOE4 mice perform better than APOE3 mice. Similarly, in the limited studies available on APOE4 and cognition, a female-specific trend can be observed, such that APOE4-mediated cognitive impairments or benefits are more prominent in female mice (Siegel et al., 2010; Bour et al., 2008; Raber et al., 1998; Raber et al., 2002). This could suggest that female mice are more susceptible to the effects of APOE4 on cognition (Siegel et al., 2010; Villasana et al., 2008); however, whether this effect is in female mice in general or APOE4-specific needs to be considered. A study comparing sexes in wildtype C57BL/6J mice, concluded that there are no differences between sexes in measures of learning, memory, or anxiety-like behaviors at 8-12 weeks of age (Tsao et al., 2023). However, as the mice age – especially at 12 to18-month timepoints – female mice present with a more pronounced cognitive decline in domains including spatial memory and passive avoidance memory (Benice et al., 2006; Frick et al., 2000). While these findings suggest that there is a general sex-difference in cognitive changes over time, our study demonstrates APOE4-specific sex-differences when comparing attention performance between APOE4 and APOE3 mice. For instance, APOE3 sham male mice consistently score higher than APOE3 sham females in discrimination sensitivity across all time points. This is consistent with previous unpublished findings from other lab members, seeing a better CPT performance in male

hAPP.hMapt or hAPP.hMapt.APOE3 mice compared to female mice. In contrast, APOE4 sham females perform as well as APOE4 sham males, suggesting a sex-difference in performance that is genotype-dependent.

As for the sex-difference in cognition following injury, APOE3 injured males continue to have a better performance than females, up to 12 months. At 18 months, it is interestingly the APOE3 injured males that seem to drop performance as they age, although not significantly. On the other hand, in APOE4 injured mice, female performance drops over time, widening the difference in discrimination sensitivity compared to APOE4 injured males at 18 months of age. Comparing these findings with the TBI literature, only Giarratana et al (2020) have investigated sex differences following TBI in APOE4-carriers and did not observe any differences between sexes (Giarratana et al., 2020). This difference in the findings can be mainly attributed to the age of testing and time following injury, as Giarratana et al (2020) focused on juvenile TBI and acute effects in mice (less than a month) following TBI.

Even in studies on wildtype mice that have investigated the long-term cognitive outcomes following TBI, most of the literature has focused on male mice (Rubin et al., 2019; Gupte et al., 2019). The studies that have investigated sex-differences, have reported highly variable results, with more studies reporting worse outcomes in males (Rubin et al., 2019). This is in direct contrast with human studies that, like the findings in our study, suggest that there are worse outcomes in females (Gupte et al., 2019; Rubin et al., 2019). One reason contributing to this discrepancy between human and animal studies is the models utilized. For instance, the majority of previous models have focused on more severe forms of injury. However, the number of studies showing parallel results with human studies significantly increased when focusing on mild TBIs (Gupte et al., 2019). Moreover, more studies utilizing the closed-head injury models have shown worse outcomes in females compared to studies utilizing controlled cortical impact models (Gupte et al., 2019). This suggests that as the TBI models more closely mimic human mild TBIs, they also better mimic sex differences observed in cognitive outcomes following TBI, as observed in this study.

Another factor that contributes to the discrepancy in sex-differences between human studies and animal models are the differences in hormonal changes over time. In postmenopausal women for instance, the significant decline in female hormones, especially estrogens that play a neuroprotective role, puts women at a higher risk for AD-related cognitive decline (Ferretti et al., 2018; Zhu et al., 2021) and worse outcomes following TBI (Thompson et al., 2006; McCarthy and Raval, 2020; Blaya et al., 2022). As most women experience menopause by the age of 55 years (World Health Organization), it is important to consider how this might affect long-term cognitive changes. As female mice do not experience menopause, there is a need to create better models to study long-term cognitive outcomes following TBI.

4.5 Limitations and future directions

Longitudinal touchscreen training and food restriction

While longitudinal assessment of cognition at multiple timepoints allows to identify subtle cognitive changes over time, it also requires repeated cognitive testing. As the mice were on maintenance training between probe sessions, the training might have influenced their cognition overtime. Although cognitive training was consistently performed for all mice, it might have had varying effects on different groups. For instance, it has been shown that behavioural testing can affect AD-neuropathology markers in APOE4 and APOE3 target replacement mice, such that the isoform-specific differences are reduced (Salomon-Zimri et al., 2014). Similarly, following TBI, environmental enrichment has been reported to reduce neuropathology, and improve spatial learning and memory (Tapias et al., 2022). Thus, repeated cognitive testing could act as a protective factor (Shepherd et al., 2021), masking the long-term impairments in the mice, especially in the APOE4 injured mice. Moreover, the mice in this study were maintained on food restriction to ensure staying within a healthy weight range while also motivating performance on touchscreen tasks. As caloric restriction in rodents has been reported to have neuroprotective effects in the brain such as reduced AD pathology (J.

Lee et al., 2002; Patel et al., 2005), food restriction could have also masked impairments in our mice. To understand how these factors can influence cognition in APOE4 mice following TBI, another cohort of mice should be studied that is raised without food restriction and is only tested at one timepoint.

Modeling mild TBI

Modeling mild TBIs continues to be a challenge due to the heterogeneity of human TBIs and their symptoms. Many athletes, military personnel, or domestic violence victims may experience several TBIs in their lifetime with varying severities, impact directions, or time between TBIs. These factors may not be best represented by our TBI model that delivered three mild TBIs with similar kinematics and direction 24 hours apart. All these factors need to be considered in future research, toward reaching a translatable model of TBI. Additionally, as the mice were under anesthesia during TBIs, two additional factors need to be considered that separate our model from human TBIs. First, as the mice are under anesthesia and have lost voluntary control of their head and neck muscles, the head movement likely differs from head movement following impact in humans. Secondly, anesthesia has been linked to cognitive impairment in rodents (Bianchi et al., 2008; Zhang et al., 2012). Although anesthesia was identically done in both sham and injured groups, the interaction between anesthesia effects with sex, APOE isoform, and TBI should be considered in future research.

Implications for animal models in AD research

To model LOAD risk better, this study aimed to evaluate cognitive changes in mice with two risk factors – APOE4 and repeated TBIs – in mice that did not express any genetic mutations that caused AD. However, this presented a challenge, as in the absence of injury, the mice did not show any cognitive decline up to 18 months of age, and likely did not develop amyloid plaques or tau tangles. This reflects a challenge in the field, as

many LOAD models present with increases in soluble Aβ and phospho-tau, that do not develop pathological hallmarks of AD (Baglietto-Vargas et al., 2021; Saito et al., 2019). This could be due to two possible reasons: *(i)* the mice are not old enough to develop pathology or *(ii)* there are other genetic/environmental factors that contribute to pathology in humans that are absent in these mice.

With regards to insufficient aging in mice, including later timepoints in the study allows exploring whether cognitive deficits or AD-pathology occurs at a later age. For instance, including a 24-month timepoint would more accurately reflect AD symptom onset. As for including other factors that might contribute to disease progression, this study showed that in the presence of an environmental risk factor such as TBI, a cognitive decline can be observed in APOE4 female mice. Other factors that can help better model a risk for LOAD could be a high-fat high-sugar diet (Reagen et al., 2022) or TREM2 genetic risk factor. However, with inclusion of such factors, it is important to include appropriate controls. For instance, in this study inclusion of APOE4 required APOE3 as a control, as all human APOE isoforms have shown to be more protective against amyloid formation compared to mouse apoE (Fagan et al., 2002; Liao et al., 2015). However, inclusion of APOE knock out mice would have also been useful in better understanding the differential effects of APOE4 in cognition.

Implications for humans research, especially for females

With all the limitations considered, this study still demonstrated a strong sex-specific effect of both APOE4 and TBI on cognition. Although APOE4 was associated with better cognitive performance in the absence of injury, following TBI an accelerated decline was predominantly observed in females. Bridging this finding to human studies, this study highlights the need to focus more attention on several aspects of human research on cognition in APOE4 carriers and cognitive outcomes following TBI.

First, studies need to assess cognition longitudinally in APOE4 carriers to understand better cognitive changes over a lifetime, as APOE4 could have differential

effects on cognition over time. Second, future studies need to have adequate power to investigate sex differences in APOE4 carriers. Lastly, when investigating the effect of APOE4 on cognitive outcomes following TBI, it is important to have adequate power to consider sex differences, time following injury, and the impact of severity.

If human cognitive studies align with the findings of this study, and APOE4 is only a risk factor in the presence of environmental or pathological stress in females, the next step is to identify APOE4-specific mechanisms through which such stressors influence cognition. Exploring these APOE4-specific mechanisms and the timing of their action may suggest targets for prevention strategies and pharmacological treatments. Moreover, this would create a stronger need to develop healthcare and research policies that specifically promote better health outcomes for females.

Chapter 5

5 Conclusion and relevance

In conclusion, this study demonstrates that in the absence of injury, APOE4 female mice show a better attention performance compared to APOE3 that persists to old age. However, following repeated mild TBIs – which do not lead to long-term attentional changes alone – the better performance is lost. Moreover, these attentional changes are more prominent in females. These results suggest that while female sex, APOE4 genotype, and TBI individually might not pose a risk for long-term cognitive decline, their combination poses a risk for accelerated cognitive decline.

These findings have research implications for our current understanding of the role APOE4 plays in cognition, suggesting that the differential effects observed over a lifetime in human participants (antagonistic pleiotropy) may alternatively be explained by the interaction of APOE4 and accumulated AD neuropathology. Furthermore, this study has implications for healthcare and health policies, indicating that women with an APOE4 genotype and a history of mild TBIs are at a significantly higher risk for cognitive decline.

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Appendices

Appendix A : Animal use protocol

AUP Number: 2022-174
PI Name: Brown, Arthur
AUP Title: Investigations of CNS Injury and Regenerative Therapies - Mouse
Yearly Renewal Date: 03/01/2025

The **annual renewal** to Animal Use Protocol (AUP) 2022-174 has been approved by the Animal Care Committee (ACC), and will be approved through to the above review date.

Please at this time review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1. This Animal Use Protocol is in compliance with:
    - Western's Senate MAPP 7.12 [PDF]; and
    - Applicable Animal Care Committee policies and procedures.
2. Prior to initiating any study-related activities—as per institutional OH&S policies—all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have:
    - Completed the appropriate institutional OH&S training;
    - Completed the appropriate facility-level training; and
    - Reviewed related (M)SDS Sheets.

Submitted by: McInnis, Jennifer on behalf of the Animal Care Committee

Curriculum Vitae

Khashayar Khasheeipour

Academic Background

M.Sc Neuroscience 2022-present
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Awards and Scholarships:

Canada Graduate Scholarship-Master's program 2023
Canadian Institutes of Health Research
Valued at $17500 for students who demonstrate a high standard of achievements

Highly Commended Entrant 2022
Global undergraduate Awards
2022 international summit, Dublin, Ireland

Related Work Experience

Pre-graduate research assistant 2024
Robarts Research Institute
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Abstracts and Presentations:

Khasheeipour K, Geremia N, Xu K, Prado M, Brown A. July 2023
Next-Generation Humanized Mice to Investigate Traumatic Brain Injury's Interaction with APOE4, the Strongest Genetic Risk Factor for Dementia.
Abstract and virtual Poster presentation, Alzheimer's Association International Conference 2023, Amsterdam, Netherlands

Khasheeipour K, Geremia N, Xu K, Prado M, Brown A. May 2023
Next-Generation Humanized Mice to Investigate Traumatic Brain Injury's Interaction with APOE4, the Strongest Genetic Risk Factor for Dementia.
Abstract and Poster presentation, Canadian Association for Neuroscience Conference 2023, Montreal, Quebec, Canada

Khasheeipour K, Shahshahani L, Diedrichsen J. May 2022
Chunking in Backward Recall of Digits: An fMRI study., Presentation, The Global Undergraduate Awards Summit, Dublin, Ireland