Role of Apolipoprotein E Isoform in Stress-related Changes in Behavior and Cognition

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CERTIFICATE OF APPROVAL

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Abbreviations

7KC	7-ketocholesterol
ACC	anterior cingulate cortex
AD	Alzheimer's disease
APOE	apolipoprotein E (gene)
apoE	apolipoprotein E (protein)
BBB	blood brain barrier
CAPS	clinician-administered PTSD Scale
CBT	cognitive behavioral therapy
CRH	corticotropin releasing hormone
CVS	chronic variable stress
DSM	Diagnostic and Statistical Manual of Mental Disorders
EDMR	eye movement desensitization and reprocessing
GR	glucocorticoid receptor
HDL-C	high-density lipoprotein cholesterol
HPA	hypothalamic-pituitary-adrenal
IPP	Intercultural Psychiatry Program
KO	knockout
LDL-C	low-density lipoprotein cholesterol
LDLR	low-density lipoprotein receptor
mPFC	medial prefrontal cortex
OBP-2	oxysterol binding protein-2
PCL	PTSD Checklist
PTSD	Post-Traumatic Stress Disorder
SNP	single nucleotide polymorphism
SPS	single prolonged stress
StAR	steroid acute regulatory protein
TBI	traumatic brain injury
TR	targeted replacement
vmPFC	ventromedial prefrontal cortex

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Abstract

Stress is a necessary physiological response to external pressures on homeostasis. However, extreme or chronic exposure, *i.e.* trauma, can lead to debilitating disorders such as Post-Traumatic Stress Disorder (PTSD). Symptoms of PTSD include nightmares of the trauma, general cognitive dysfunction, changes in arousal, and avoidance behavior. Previous research has demonstrated that 30-70% of the variation in PTSD can be explained by heritability. Recent work shows that the genetic factor, apolipoprotein E (apoE) may differentially influence PTSD susceptibility and symptom severity. ApoE helps regulate lipid transport and metabolism throughout the body, including the brain. It exists in humans as three isoforms: E2, E3, and E4. Both mouse models and human studies have shown that carrying the E2 allele corresponds to greater PTSD-related symptoms and in humans, greater susceptibility after trauma. However, other studies have found conflicting evidence and suggest the association between apoE isoform and PTSD is still unclear.

A plethora of literature demonstrates that women, even when controlling for rates and types of trauma exposure, are more likely to develop PTSD and experience longer, more severe symptoms. Work in the aging and Alzheimer's disease fields has shown that apoE may interact with sex; for example, E2 is protective against Alzheimer's disease and increases longevity in women but not in men. However, how sex may interact with apoE in the context of trauma has not yet been addressed.

While studies suggest that apoE may be a critical genetic factor in PTSD, the underlying biological mechanism remains unknown. In the brain, apoE transports lipid for use within cells by binding primarily to the low-density lipoprotein receptor (LDLR) after which cholesterol can be metabolized for steroidogenesis (*i.e.* glucocorticoids) and energy, and for cell/membrane structure. E2 binds to LDLR at ~1% the affinity of E3 and E4. Interestingly, LDLR regulates apoE levels in an inverse relationship and LDLR activation can attenuate glucocorticoid

secretion, potentially by providing additional cholesterol to be oxidized into oxysterols and binding to oxysterol binding proteins. This suggests that the poor binding affinity of E2 may be critical during periods of heightened stress.

In this dissertation I use mouse lines expressing human apoE isoforms via targeted replacement exposed to control conditions or chronic variable stress (CVS), a five-day paradigm including two stressors each day, to address the following three hypotheses. 1) E2 expression leads to more severe behavioral changes and cognitive impairments associated with CVS. 2) Females are more negatively impacted compared to genotype-matched males, and 3) Behavioral and cognitive changes associated with CVS exposure correspond to differences in markers of lipid metabolism.

I found evidence to support our hypothesis that E2 leads to greater impairments of spatial learning and memory after CVS, consistent with our lab's earlier work, and that suggest female E2 mice are more greatly impacted. Glucocorticoid receptor (GR) levels in the adrenal gland tissues were higher in all CVS-exposed mice compared to controls, and cortical apoE levels were highest in E2 mice with no difference due to CVS. In collaboration with Andrea DeBarber, we also developed an assay to measure 4 distinct oxysterols, including 7-ketocholesterol, in small volumes of murine brain tissue. We found that 7-ketocholesterol in female cortical tissue was higher in E3 and E4 mice exposed to CVS. In contrast, no difference was seen between E2 CVS vs control mice. These isoform-specific differences appear to be a result of downstream functions of apoE in lipid metabolism and neuroendocrine feedback that highlight the need for future studies to understand how this can be leveraged in personalized therapies for PTSD.

Chapter 1: Introduction

Stress: From heterostasis to Post-Traumatic Stress Disorder

We are all exposed to stress, or as Hans Selye defined it, the external forces that push us away from homeostasis (Selye, 1950). Oftentimes, this is an important experience because it forces us to react in a way to rectify the change through adaption in what Selye referred to as "heterostasis." However, at times these stressful situations are intense enough to reach a more severe status known as trauma, in which an individual becomes excessively distraught. Trauma can be physical, psychological, and/or emotional. Estimates suggest that upwards of 70% of the general population in the United States experience at least one traumatic event in their lifetimes while many experience more than three (Benjet et al., 2016). Such traumatic events can lead to mental health disorders, most notably Post-Traumatic Stress Disorder (PTSD). While terms such as "shell shock syndrome" and "war neurosis" floated around in the early 1900s in association with World War I, the term "post-traumatic stress disorder" began to be used in the 1970's in response to the U.S. military veterans returning from the Vietnam War. It wasn't until 1980 that the Diagnostic and Statistical Manual of Mental Disorders (DSM-3) formally recognized it as a psychiatric disorder, although it has been described in literature and studied in various degrees from the age of Hippocrates to the French Revolution to the Holocaust (Crocq & Crocq, 2000).

A brief overview of PTSD

Diagnostic criteria

Since 1980, DSM-V has been revised to include PTSD under "Trauma- and Stressor-Related Disorders" and the defining symptom categories—re-experiencing trauma, avoidance behaviors, negative alterations in cognitions and mood, and altered arousal and reactivity—are

used here. Diagnostic criteria include knowledge of the traumatic event associated with the symptoms. PTSD has been assessed with numerous rating scores. Within this dissertation, I mention two of the most commonly used tests, the Clinician Administered PTSD Scale (CAPS), the most well-used clinician-administered test consisting of 30-items, and the PTSD Checklist (PCL), which is a 20-item self-report and exists in different versions. Both address the different symptom categories or "clusters."

Symptom type and severity can vary greatly between individuals. For example, studies assessing sleep disruptions, critical for diagnosis, have demonstrated conflicting polysomnography results. A meta-analysis of 20 of these studies, including a total of 772 individuals and taking into account age, sex, and comorbid disorders, showed that when compared to controls, those with PTSD have greater amounts of stage 1 sleep and less slow-wave sleep with greater rapid-eye movement density (Kobayashi et al., 2007). PTSD has also been associated with poor performance in numerous cognitive domains, including memory, visuospatial construction, language, attention, and delayed memory (Narita-Ohtaki et al., 2018; Scott et al., 2015) . Other studies have suggested that PTSD patients are more likely to develop dementia, adding to the concerns of cognitive dysfunction. This was found even after excluding potential confounding risk factors such as head injury, depression, and substance abuse (Flatt et al., 2018; Yaffe et al., 2010).

Although 25-40% of PTSD diagnoses are expected to go into remission within one year, most individuals experience symptoms for far longer than this (Watson, 2019). In addition to primary symptoms, those with PTSD can also experience significant functional impairments including unemployment and intrapersonal difficulties (Steenkamp et al., 2015). Thus, it is unsurprising that PTSD has become a priority in public mental health on a global scale (Watson, 2019).

Trauma types

Due to the first modern reports of PTSD in combat veterans, there has been a tendency for public media to focus on PTSD in this population. Thus, it should perhaps be explicitly stated that it can occur in civilian-life as well as non-combat situations and can result from any number of types of traumatic events. There is a wealth of research that characterizes PTSD in civilian populations after natural disasters (Beaglehole et al., 2018) such as the 2011 Great East Japan Earthquake and Tsunami (Chen et al., 2020) and Hurricane Katrina in New Orleans (Kessler et al., 2008). Global events such as devastating traumas associated with the Holocaust have also been studied, with more research emerging surrounding the generational impact it leaves behind (Kellermann, 2013; Yehuda et al., 2014). Terrorists attacks such as 9/11 in the US have also led to work understanding the prevalence of PTSD and treatment outcomes in first-responders (Difede et al., 2007). The range of these events and the staggering number of traumatic events occurring show how heterogenous the initial trauma may be for individuals with PTSD and highlight a potential reason for the high variability seen within the disorder.

Prevalence

More than 70% of individuals will experience some kind of traumatic event (acute or chronic) at some point in their lives. The conditional probability of developing PTSD after trauma exposure is highly dependent on the type of trauma. PTSD develops in 65% of men and 46% of women after sexual trauma, 2% of men and 22% of women after physical assault, and 6% in men and 9% in women after an accident. This translates to a ~ 7% lifetime prevalence of PTSD in the US and affects about 13 million people in a given year (Kessler et al., 1995).

Those with PTSD are also highly likely to present with comorbid health disorders, including cardiovascular disease, diabetes, chronic pain, and additional mental health disorders (Boscarino, 2004). Among mental health concerns are depression and substance use disorder.

Major Depressive Disorder occurs in roughly half of those with PTSD (Kessler et al., 1995), and comorbid substance use disorders are found in 21-75%, the higher prevalence rates being in combat veteran populations (Breslau et al., 2003; Kessler et al., 1995; Kulka et al., 1990). Substance use disorders present a particularly challenging comorbidity due to the theory that substance use is a way to "self-medicate" to deal with trauma, yet withdrawal symptoms may exacerbate PTSD symptoms (Brady et al., 2000). Although they are critical to acknowledge and understand in terms of treatments for patients, the heterogeneity is beyond the scope of this dissertation, which will focus on the phenotype associated with PTSD alone.

In terms of sex differences, adult women are more likely to develop PTSD in general than men, although men have higher rates after sexual trauma (Shalev et al., 2017). Females tend to be more vulnerable to PTSD if the trauma occurred during childhood (Breslau et al., 1997). Women tend to also experience longer durations of the disorder (Kessler & Wang, 2008). These differences in severity and prevalence, however, are not associated with exposure rates, since men are more likely to experience traumatic events overall. Trauma type does play a large role, although it should be noted that even when controlled for, females tend to have higher prevalence rates suggesting biological influences. Specifically, lifetime prevalence of PTSD is 9.7% in females compared to 3.6% in males (Bangasser & Valentino, 2014).

Sex hormones are likely to play a role; for example, low levels of 17-beta-estradiol corresponds to worse fear extinction learning in women with PTSD (Glover et al., 2013). Estrogen has also been shown to contribute to sex differences in glucocorticoid negative feedback (Bangasser & Valentino, 2014). However, the sex differences in PTSD are complex and probably due to both biological and nonbiological differences (Ramikie & Ressler, 2018).

Children and adolescents can also be diagnosed with PTSD as a result of experiencing traumatic events (Perkonigg et al., 2005). A meta-analysis revealed that the pediatric prevalence of PTSD can be up to 15%; furthermore, children that experienced interpersonal conflict were

more likely to develop PTSD and girls exposed to interpersonal conflict experience the highest rate (33%) (Alisic et al., 2014). These rates are even higher (up to 75%) in low- and middle-income countries (Watson, 2019). Effects of childhood adversity and social context have further supported the complexity of trauma-exposure and corresponding PTSD risk and prognosis (Sareen, 2014).

Underlying biology and involved pathways

Research has long sought to understand the etiology of PTSD, even before the term "PTSD" was described. While originally characterized in terms of its psychological burden, it cannot be overemphasized that biological research and the identified markers have demonstrated that PTSD truly affects an individual on a whole body biological level (for review see: Levine et al., 2014; Pitman et al., 2012). Perhaps one of the most mystifying aspects of PTSD is that not everyone who is exposed to trauma will develop PTSD. Understanding what may cause susceptibility as well as resilience will be important in determining therapeutic targets. This idea lies at the heart of this dissertation work, and the concept of resiliency will be returned to in Chapter 3.

One of the primary pathways that have been studied in the context of PTSD is the hypothalamic-pituitary-adrenal (HPA) axis (**Figure 1.1A**). The positive and negative feedback loops of this neuroendocrine system are one of the primary physiological signaling pathways in response to stress. In brief, environmental stimuli triggers secretion of corticotropin releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus. CRH signals activation of the anterior pituitary, which then secretes adrenocorticotropic hormone to activate the adrenal cortex. The adrenal cortex then releases cortisol, or corticosterone in rodents, which sends a negative feedback signal to the hypothalamus and anterior pituitary to turn off CRH release.

It is important to note that other brain regions, such as the amygdala, medial prefrontal cortex, and hippocampus, can modulate the HPA axis. Broadly speaking, activation of the hippocampus and prelimbic cortex inhibits activation of HPA axis while amygdala stimulation induces glucocorticoid release (for review see Herman et al., 2005).

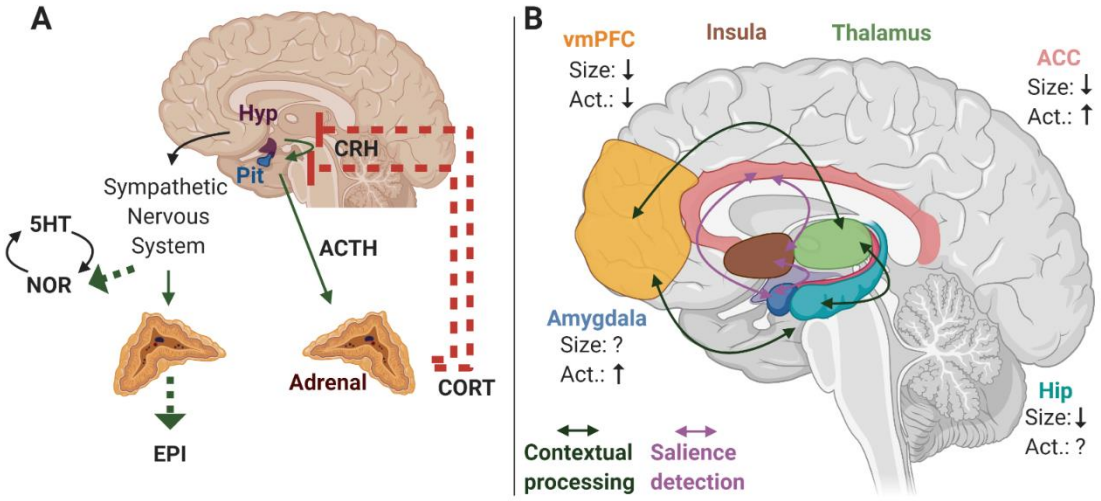

Figure 1.1 Overview of major systems altered in PTSD. A) Changes in the primary stress responses. Monoamines released from the sympathetic nervous system include epinephrine (EPI) and norepinephrine (NOR) which heavily interact with serotonin (5HT). The HPA axis consists of feedback loops of hormonal release. Dotted lines reflect changes in PTSD. See text for more information. Hyp = Hypothalamus. Pit = Pituitary. CRH = Corticotropin releasing hormone. CORT = Cortisol. B) Major brain regions of interest in PTSD and known volume and activation differences are labeled with matching font color. The amygdala and hippocampus are key structures in fear learning and memory while the ventromedial prefrontal cortex and anterior cingulate cortex are responsible for reward, emotion, decision-making, and attention. Contextual processing and salience detection networks are two networks altered in individuals with PTSD. vmPMC = ventromedial prefrontal cortex. ACC = anterior cingulate cortex. Hip = Hippocampus. Created with BioRender.com.

Dysfunction in the HPA axis, like altered levels of cortisol and catecholamine levels, has been demonstrated. While the role of corticosterone in PTSD has been equivocal (for review, see Rasmusson et al., 2003), some work suggests that low plasma cortisol level after trauma exposure is a predictor of who will develop PTSD (Yehuda, 2002), *i.e.* a depression in stress-response. Moreover, individuals diagnosed with PTSD show lower basal levels (Yehuda et al., 1994). While these results are perhaps counterintuitive since stress typically results in an acute increase in plasma cortisol levels, they align with research using the dexamethasone test for HPA axis

negative feedback function. In this test, subjects are given a low dosage of dexamethasone, an exogenous corticosteroid, which should result in suppression of cortisol levels during a stress response because it acts as a negative feedback signal. Individuals with PTSD show enhanced suppression within the HPA axis compared to healthy controls (Grossman et al., 2003). Preliminary results suggest that cortisol treatment shortly after trauma exposure may lower the risk of developing PTSD (Zohar et al., 2011). Research on cortisol have led to interests in the FK506 binding protein 5 (FKBP5), a co-chaperone of glucocorticoid receptor (GR). Mutations in *FKBP5* have been associated with greater symptom severity, specifically with regards to hyperarousal (Watkins et al., 2016). One such *FKBP5* single nucleotide polymorphism (SNP), has functional effects resulting in enhanced *FKBP5* mRNA transcription (Klengel et al., 2013). Pitman et al. reviewed findings in other notable neuropeptides and neurosteroids, such as CRH, neuropeptide Y, and allopregnanolone (Pitman et al., 2012).

Due to distinct autonomic symptoms seen in PTSD, *e.g.* hyperarousal, anxiety, tachycardia, etc., the autonomic nervous system is thought to contribute significantly to the symptoms seen in PTSD (**Figure 1.1A**) (Orr et al., 1997; Tanaka et al., 2000). Findings suggest that overly strong adrenergic responses after trauma may reinforce the traumatic memories (Pitman, 1989). Adrenergic projections of locus coeruleus neurons innervate many brain regions involved in fear and stress responses, including the hypothalamus, amygdala, and prefrontal cortex (Southwick et al., 1997). In those with PTSD, epinephrine and norepinephrine levels were higher than controls after a stressor suggesting upregulation (Blanchard et al., 1991), potentially due to decreased neuropeptide Y, which inhibits norepinephrine release (Rasmusson et al., 2000). Studies suggest the hypothesis that increased noradrenergic activity leads to hyperarousal and reexperiencing symptoms in those with PTSD (O'Donnell et al., 2004). This dysregulation may in part be due to dysfunction from the HPA axis signaling to the locus coeruleus. The serotoninergic system also greatly interacts with noradrenergic signaling making it difficult to parse out the

origin of these signals (Ninan, 1999). Serotonin has been implicated to regulate innate fear and anxiety responses in which the presynaptic serotonin transporter (*SLC6A4*) plays a key role (Hariri et al., 2002). Furthermore, serotoninergic neurons may specifically modulate basolateral amygdalar circuits during fear learning, and one polymorphism of the *SLC6A4* allele results in increased fear and anxiety-related behaviors in carriers, coinciding with greater amygdalar activation.

Glutamatergic changes are also becoming more widely thought to contribute to the psychopathology seen in PTSD (Averill et al., 2017). Glutamate is the major excitatory neurotransmitter in the brain and as such plays a key role in activation within the HPA axis. Stress and trauma have been shown to activate glutamate signaling. Potentially, when glutamate signaling extends beyond the normal threshold during chronic stress, it results in a decrease in clearance of synaptic glutamate and a corresponding excess of glutamate, *i.e.* glutamatergic excitotoxicity (Popoli et al., 2012). This excess glutamate is proposed to increase activation of extrasynaptic glutamate receptors, eventually resulting in dysregulation of the HPA axis. Alterations in glutamatergic pathways may thus lead to long-term consequences seen in PTSD including a negative feedback loop due to decreased glutamatergic tone in the medial prefrontal cortex (mPFC) and hippocampus.

In addition, the immune system has been implicated in PTSD similar to patients showing chronic systemic inflammation (Gill et al., 2009). Inflammatory cytokines, for example, are increased in those with chronic PTSD and may promote vulnerability to PTSD after trauma exposure. Additionally, this heightened immune response has been linked to dysregulation of cortisol. To further complicate understanding, epigenetic changes may underlie these differences in immune response (Bam et al., 2016).

Recent technological advances have allowed researchers to characterize circuits that are the underlying basis for specific behaviors (**Figure 1.1B**) (Fenster et al., 2018). These circuits

include those underlying intrusion symptoms or flashbacks, which may be a result of failure of cortical regions to inhibit limbic systems (Lanius et al., 2010). Indeed, recent work shows there is reduced functional coupling between control and memory systems, which normally act to inhibit intrusive memories compared to non-intrusive memories, in patients with PTSD. This top-down regulation of intrusive memories, arising from the dorsal lateral prefrontal cortex and targeting the hippocampus and precuneous, is notably absent in those with PTSD (Mary et al., 2020). Fear learning is another major circuit that underlies behaviors affected in those with PTSD (Raber, Arzy, et al., 2019; Tovote et al., 2015). The fear response may eventually decrease in time with the reinforcement of safety cues in what is known as fear extinction. Rather than erasing the original fear inducing memory, it appears that fear extinction is more likely the creation of new memories (Rescorla & Heth, 1975). Individuals with PTSD are able to learn fear extinction but have difficulty with retention (Milad et al., 2009; Wicking et al., 2016). Due to the importance of traumatic memories in PTSD symptoms, memory-related circuits have been argued as the "final common pathway" in PTSD (Elzinga & Bremner, 2002).

Another major circuit involved in PTSD is threat and salience detection, cognitive networks important for attention to stimuli, which may manifest as avoidance and hypervigilance behaviors (Fenster et al., 2018). One study has shown that those with PTSD tended to show stronger salience detection network activity than the typical default network (Sripada et al., 2012). Executive functioning is also impaired in PTSD (Polak et al., 2012) and may even exist prior to trauma exposure (Aupperle et al., 2012). PTSD presents additionally with dysfunction in emotional regulation/valence control. Decreased PFC activity corresponded with poorer performance within the PTSD group to downregulate negative emotional reactions (New et al., 2009). Reward circuitry is an additional area of interest, considering the anhedonia commonly seen in the disorder. A recent meta-analysis shows evidence that both reward anticipation and approach as well as hedonic responses are downregulated in PTSD (Nawijn et al., 2015).

Key anatomical regions in the aforementioned circuits have also shown PTSD-related differences in both structure and functional connectivity. The hippocampus has been shown to be significantly smaller in those with PTSD than trauma-exposed and non-trauma-exposed controls (Villarreal et al., 2002). This smaller hippocampal volume may be a predictive factor based on twin studies (Gilbertson et al., 2002). However, this relationship may be influenced by how trauma-exposed controls may still have PTSD-related symptoms without the diagnosis suggesting that smaller hippocampi may not necessarily signal as a predictor, but instead may relate to trauma exposure. The amygdala has been described as smaller in those with PTSD in a meta-analysis (Morey et al., 2012), although other research has demonstrated that larger amygdalar volumes correspond with PTSD (Kuo et al., 2012). Another brain region that appears smaller in those with PTSD is the rostral ventromedial prefrontal cortex (vmPFC) as well as the dorsal anterior cingulate cortex (ACC) (Kasai et al., 2008; Kitayama et al., 2006).

Functional neuroimaging studies using positron emission tomography (PET) or functional magnetic resonance imaging (fMRI) have shown the amygdala, hippocampus, insular cortex, vmPFC, and dorsal ACC have among the greatest levels of altered activity in those with PTSD (Pitman et al., 2012). Activity in the amygdala appears to be positively correlated with severity of symptoms as is activity in the insular cortex and dorsal ACC. The vmPFC, which inhibits the central amygdala via GABAergic intercalated cells, had reduced activation in PTSD. Findings in the hippocampus have been inconclusive as both increased and decreased activity have been reported. However, during a declarative memory task, those with PTSD showed decreased hippocampal activity compared to trauma-exposed controls (Bremner et al., 2003).

Therapeutic options for PTSD

Evidence-based therapies, supported by scientific evidence, remain the most recommended for the treatment of PTSD. Cognitive behavioral therapy (CBT) is a broad category

of therapies and is strongly recommended by the American Psychological Association. Furthermore, there are both exposure-based, *i.e.* trauma-focused which directly address the memory of the trauma, and nonexposure-based therapies. One type of CBT is prolonged-exposure therapy, perhaps the most supported trauma-focused, evidence-based therapy, which gradually teaches individuals to approach emotions and memories associated with the trauma. In addition, cognitive processing therapy is another highly recommended therapy in which patients are trained to modify challenging beliefs related to the trauma. While not as strongly recommended, another type of therapy is narrative exposure therapy, which teaches individuals to place their traumatic experiences within a coherent life narrative and is often used for refugees ("Summary of the clinical practice guideline for the treatment of posttraumatic stress disorder (PTSD) in adults," 2019). Non-trauma focused therapies, such as present-centered therapy in which individuals focus on current life situations and relations, are not as widely supported. Importantly, the nonresponse rate of CBT to PTSD may be as high as 50% in some populations, indicating the need for other therapies (Kar, 2011). Moreover, there are little data that support the efficacy of psychological therapies for those individuals with PTSD and comorbid substance use disorders (Bisson et al., 2015).

In addition to psychological interventions, the majority of patients also take pharmacological agents, mainly for targeting related depression, anxiety, insomnia, or psychosis; relapse, however, is possible even with a full therapeutic dose of 6-12 months (Shalev et al., 2017). Considering PTSD as a monoaminergic disorder, primary pharmacological agents include two selective serotonin reuptake inhibitors, paroxetine and sertraline, also common antidepressants, as the only drugs with FDA approval for PTSD (Berger et al., 2009), although there are other pharmacological treatments for the symptoms associated with PTSD (described below). While considered effective, trials have shown that 50% of those who take selective serotonin reuptake inhibitors still demonstrate significant symptoms (Asnis et al., 2004). While

the intricacies of current treatment strategies are beyond the scope of this dissertation, it should be emphasized that no single treatment strategy will work for everyone with PTSD, underscoring the need for further research in maximizing the efficacy of PTSD therapies.

Psychological, pharmacological, and "innovative" interventions have all been used with the purpose of mitigating effects after exposure to trauma. In terms of reducing PTSD prevalence after a traumatic event exposure (*i.e.* "prevention), CBT can reduce severity of symptoms although non-trauma-focused psychological therapies lack findings to support their effectiveness (Bisson et al., 2015). Other options for interventions included stress management approaches as well as pharmacological approaches. Although several pharmacological agents, including propranolol, escitalopram, temazepam, and gabapentin, have failed to be proven effective (Amos et al., 2014), there is some evidence that hydrocortisone administration after trauma may reduce symptoms (Zohar et al., 2011). Morphine and oxytocin have also been explored as ways to intervene after trauma, with reduction in prevalence and decreased symptom severity seen, respectively (Shalev et al., 2017). Despite the known changes in neuroendocrinology, selective serotonin-reuptake inhibitors seem ineffective at preventing symptoms while benzodiazepine treatment led to a counterintuitive increase in fear-related behaviors and PTSD symptoms (Amos et al., 2014).

More recently, novel therapeutics have been used to address the need to improve PTSD therapies. They include neurofeedback training and transcranial magnetic stimulation as well as novel uses for pharmacological agents, like D-cycloserine and ketamine, a glutamate NMDA receptor antagonist (Shalev et al., 2017). GABA positive modulators, such as the neurosteroid allopregnanolone, activate $GABA_A$ receptors and may ameliorate impaired neurogenesis (Pinna, 2014) as well as rapidly enhance $GABA_A$-mediated transmission in the PVN (Gunn et al., 2011). In addition, microRNA delivery is gaining traction as a method to target changes at the genomic level (Raber, Arzy, et al., 2019). Cannabinoids are a particularly promising avenue of research,

considering early studies show amelioration of insomnia, nightmares, and hyperarousal in PTSD patients (Shalev et al., 2017). Other recreational drugs, including lysergic acid diethylamide, 3,4-methylenedioxy-methamphetamine, and ketamine have long been used as therapeutic agents for those suffering from PTSD; however, research into the mechanisms and efficacy has been stymied by strict federal drug regulations (Nutt, 2019). Alternative therapies, such as trauma-sensitive yoga, animal-assisted therapy, and acupuncture have also grown in interest with promising early results, although more research is needed to determine efficacy (Wynn, 2015). Despite research on treatment, achieving efficacious treatments for individual patients can still be difficult and nonresponse rates are significant across therapies. Existing treatments focus on symptoms but do not lead to remission (Steenkamp et al., 2015).

Thus, despite significant research on treatment and prevention of PTSD, there remains a need to better understand the underlying causes of PTSD to create more targeted therapies. This would then hopefully result in higher rates of remission. My dissertation hopes to fill in part of this knowledge gap by addressing a potential genetic component of PTSD.

The genetic component of PTSD

Environmental factors have been studied heavily in the context of PTSD, in particular the type (*e.g.* accident, physical, global pandemic) and number of traumas experienced, as well as the community environment (Perrin et al., 2014). However, trauma exposure alone does not account for the variability in prevalence and symptom severity in PTSD. Previous research has demonstrated that 30-70% of the variation in PTSD can be explained by heritability, and specific genes have been identified that contribute to this (Sartor et al., 2011; Stein et al., 2002). These have included the aforementioned *FKBP5*, the functional polymorphism 5HTTLPR in *SLC6A4*, and pituitary adenylate cyclase-activating polypeptide type I receptor (*ADCYAP1R1)* among others (Chang et al., 2012; Gressier et al., 2013). Yet targeting these genes have shown

inconclusive results in terms of predicting PTSD incidence or symptom severity, as well as producing viable therapeutic targets. Considering that the majority of individuals will experience trauma and the ineffectiveness of current therapies, it is clear that more understanding of who is more likely to be affected both in terms of incidence and symptom severity is necessary to best allocate resources for intervention and treatment.

Pitman et al. suggest that gene by environment interactions further complicate the discussion since one cannot assume that biological abnormalities in PTSD necessarily result from the causative trauma. The reason for the inconsistent findings for candidate genes may be explained by epigenetic effects such as DNA methylation (Pitman et al., 2012). Recent findings show that DNA methylation profiles differ in people diagnosed with PTSD versus control populations and highlight differential expression, such as upregulation in the immune response (Uddin et al., 2010). Estradiol levels corresponded to DNA methylation in histone deacetylase 4 (*HDAC4*) and suggest that estrogen may influence epigenetic regulation and sex differences seen between men and women with PTSD (Maddox et al., 2018)

Apolipoprotein E: a pleiotropic protein in health and disease

An overview of the functional role of apolipoprotein E

Apolipoprotein E (apoE) is a protein involved with many aspects of normal cellular function, including most notably lipid transport and metabolism. Lipids are a class of biomolecule consisting of sterols (*e.g.*, cholesterol found in cell membrane and precursor to other important molecules), triglycerides (important as markers for cell recognition as well as energy, *i.e.*, "body fat"), and phospholipids (the main component of cellular membranes). As the major extracellular lipid carrier, apoE belongs to a class of apolipoproteins.

Functionally, apoE binds to particles that encapsulate fatty acids, cholesterol and cholesterol esters in what is then known as a lipoprotein. This lipid-association is necessary for

apoE to go into its receptor-active conformation (**Figure 1.2**). It then transports this lipidated vessel and binds to apoE receptors, which includes those in the low-density lipoprotein receptor (LDLR) family, including LDLR, lipoprotein receptor-related protein 1 (LRP1), lipoprotein receptor with 11 binding repeats (LR11), apolipoprotein receptor 2 (ApoER2), very low density lipoprotein receptor (VLDLR), and others (Holtzman et al., 2012). For the purpose of this dissertation, LDLR will refer specifically to the receptor and not to the receptor family. Cholesterol released by apoE has been shown *in vitro* to support synaptogenesis (Mauch et al., 2001) as well as maintenance of synapses (Pfrieger, 2003). Upon binding, apoE helps catabolize lipoproteins.

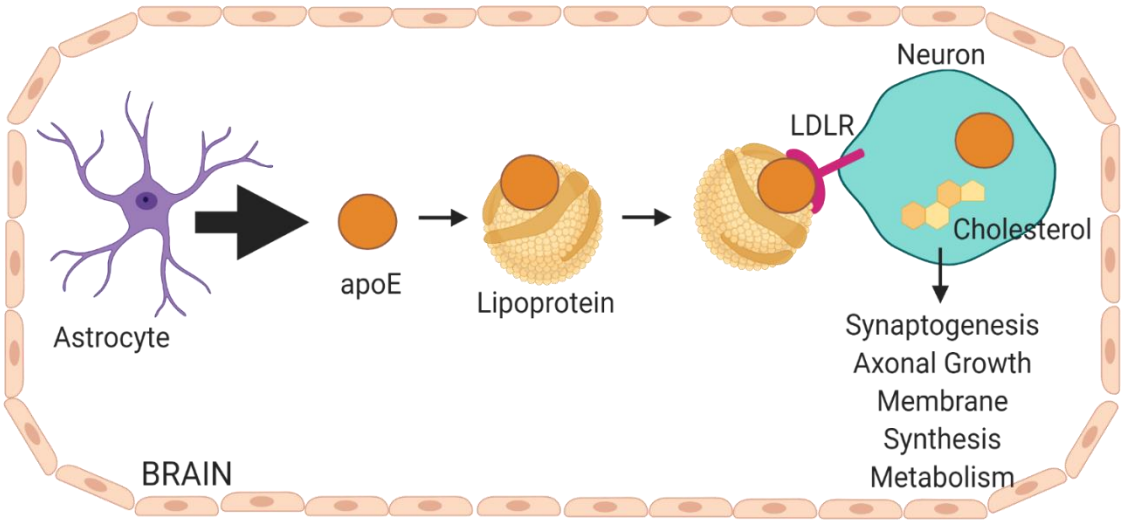

Figure 1.2 Schematic of apoE function in the brain. ApoE is primary expressed by astrocytes. It binds to lipoprotein particles and transports lipids encapsulated within. Upon binding to apoE receptors, such as LDLR, at the cell membrane, the apoE bound lipoprotein particle is internalized and cholesterol can be used for numerous cell functions. Created with BioRender.com.

ApoE is synthesized throughout the body, including the liver, brain, and kidney as well as the interstitial fluid. In the brain, astrocytes primarily produce apoE (Pitas et al., 1987), although neurons, microglia, oligodendrocytes, and ependymal cells have been demonstrated to generate apoE under distress (Boyles et al., 1985; Poirier et al., 1991; Stoll et al., 1989; Xu et al., 1999; Q.

Xu et al., 2006; Zhao et al., 2018). Both apoE mRNA and protein have been shown to be predominantly located in the soma of neurons, not in neuronal processes (Xu et al., 1999).

Considering the high percentage of body cholesterol found in the brain (25%), apoE has a critical role in healthy brain function. Sterol turnover rate in the mouse brain has been measured as 1.4 mg/day/kg body weight, suggesting that cholesterol metabolism occurs at significant amounts and alterations in this process could lead to cognitive dysfunction (Xie et al., 2003). Although primarily known to participate in cholesterol redistribution and repair following injury, apoE also has other functions outside lipid transport (Mahley, 1988; Stoll et al., 1989). ApoE knockout (KO) animals are relatively normal (Raber et al., 2000), although in response to brain injury they do show some deficits in the persistence of cholesterol-related neurodegeneration byproducts (Fagan et al., 1998). It has also recently been described that apoE can translocate to the nucleus in which it can bind to DNA and serve as a transcription factor (Theendakara et al., 2016).

In humans, apoE is found in 3 major isoforms (**Table 1.1**), E2, E3, and E4 with allele frequencies of 8.4%, 77.9%, and 13.7%, respectively (Liu et al., 2013). These allele frequencies are especially important to note regarding human studies, since inclusion of E2 and E4 carriers is often dependent on whether enough individuals are found within the experimental group. Frequently, E2 is left out altogether and individuals are characterized by whether or not they carry E4. The different isoforms exhibit similar mRNA levels in the adult human neocortex; however, protein levels revealed E2 > E3 > E4, which the authors suggest shows that E2 is resistant to post-translational modification and possibly proteolytic degradation (Conejero-Goldberg et al., 2014). This is similar to what is seen in plasma apoE protein levels (Soares et al., 2012). Furthermore, E2 is unique in that it's binding affinity to LDLR is only ~1% compared to that of E3 and E4 (Weisgraber et al., 1982). Furthermore, mice with human apoE isoforms via targeted replacement (TR) crossed with LDLR KO mice showed isoform-specific increases in apoE. E2/LDLR KO

mice were unaffected while E3/LDLR KO and E4/LDLR KO mice had 2-3 times higher levels of apoE in the cerebrospinal fluid (Fryer et al., 2005), showing that apoE levels in the CNS are regulated by LDLR in an isoform-dependent manner.

Table 1.1 Differences in amino acids at positions 112 and 158 and allele frequencies between major apoE isoforms. Modified from Liu et al. 2013.

	Isoform-specific amino acid difference		Allele Frequency (%)	
	112	158	General	Alzheimer's Disease
E2	Cys	Cys	8.4	3.9
E3	Cys	Arg	77.9	59.4
E4	Arg	Arg	13.7	36.7

Significance of apoE genotypes in cognitive health

Considering the important functions apoE has in the regulation of lipids, it has been rigorously assessed in the context of human health, initially in its associated risk in cardiovascular disease (Eichner et al., 1993; Wilson et al., 1996). Perhaps the most well-known association of apoE is the one in Alzheimer's disease (AD): E4 is the strongest genetic risk factor for late-onset AD compared to E3 (Strittmatter & Roses, 1996). In comparison, E2 has been demonstrated to confer protection against Alzheimer's disease as compared to E3 (Farrer et al., 1997). Sex may mediate the influence of apoE isoforms; female E4 carriers are more susceptible to AD during older age (Farrer et al., 1997; Neu et al., 2017). Furthermore, apoE colocalizes with amyloid plaques, a pathological hallmark of AD (Namba et al., 1991), and E4 carriers have the highest plaque density (Rebeck et al., 1993). Remarkably, a recent case study has shown homozygosity of a rare mutation in apoE3, known as the Christchurch mutation, delayed onset of familial AD by three decades in a woman despite a heavy plaque burden (Arboleda-Velasquez et al., 2019).

Previous work has implicated multiple pathogenic pathways in the association between apoE4 and AD, including impaired amyloid beta clearance (Deane et al., 2008) as well as seeding and spread of amyloid plaques, poorer cholesterol metabolism (Herz et al., 2009), the immune response (Colton et al., 2005), neuronal survival, glutamatergic signaling regulation, synaptic pruning (Chung et al., 2016; Zhuo et al., 2000), and related changes in learning and memory pathways (Weeber et al., 2002). Moreover, there may be a functional interaction between apoE and LDLR that influences AD risk (D. Cheng et al., 2005). Such diverse mechanisms involving this single genetic factor exemplify the staggering potential for apoE to disrupt healthy function.

ApoE and its relationship to stress response

The stress response is of course of particular interest in human health because of the direct impact that trauma or chronic stress can have (McEwen et al., 2015). As mentioned before, extreme stress or trauma can lead to PTSD in certain individuals. In addition, chronic stress has been shown as one of the primary risk factors for developing Major Depressive Disorder as well as Generalized Anxiety Disorder (Schmidt et al., 2008; Syed & Nemeroff, 2017). Based on human studies, an important role of apoE in mental health disorders has been argued (Gibbons et al., 2011), especially in schizophrenia where one of the prominent genetic risk factors, reelin, is also a ligand for many apoE receptors. Here I focus on PTSD, although it should be emphasized that most patients with PTSD have at least one other co-morbid health disorder, including depression, anxiety, and substance use disorders (Brady et al., 2000).

Cholesterol is the precursor to steroid hormones, including those regulating stress, cortisol/corticosterone. As a critical player in lipid transport, apoE appears to influence the stress response (Raber et al., 2000). ApoE KO mice, for example, have shown age-dependent increases in anxiety-like behavior on the elevated plus maze task and have higher plasma corticosterone levels after an acute restraint stress (further described in Chapter 2) (Raber et al., 2000) as well as

greater reactivity on the acoustic startle task (Raber, 2007). In addition, direct application of glucocorticoids increase expression of apoE in cultured macrophages (Trusca et al., 2017). ApoE mRNA levels inversely correlate with steroidogenesis (Nicosia et al., 1992). Human apoE expression reduces steroidogenesis in mouse Y1 adrenal cells, arguing that apoE may modulate cholesterol utilization (Reyland et al., 1991). LDLR activation has been shown to attenuate adrenocortical secretion of glucocorticoids (van der Sluis et al., 2015). These studies suggest apoE plays a critical role as in promoting negative feedback in glucocorticoid synthesis likely via cholesterol utilization during an acute stress response; but little is known of the changes associated with long-term stress exposure.

Considering the likely role of cholesterol metabolism in PTSD, it is important to understand other potential changes in cholesterol utilization within the brain. Cholesterol and apoE are unable to cross the blood brain barrier (BBB). Thus, cholesterol synthesis as well as its metabolism must be carefully regulated. **Figure 1.3** depicts cholesterol synthesis and metabolism pathways within the brain that are of interest to this dissertation. In brief, cholesterol synthesis occurs via 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, primarily from astrocyte-secreted apoE-cholesterol complexes (lipoproteins). Cholesterol can be enzymatically and nonenzymatically metabolized via autooxidation into oxysterols, which can cross through the BBB because of their increased polarity. Although oxysterols can be toxic due to their oxidized state, they remain an important component of cholesterol metabolism (Björkhem, 2002). Previous work has shown the apoE isoforms may influence whole brain levels of oxysterols, including 7-ketocholesterol (Jenner et al., 2010). Specifically, they found that 7-ketocholesterol was lower in young E2 male mice compared to E3 and E4 mice. 7-ketocholesterol is particularly interesting in the context of stress because it serves as a ligand for oxysterol binding protein receptor-2 (OBP-2) in the adrenal gland. OBP-2 can attenuate glucocorticoid synthesis in the adrenal gland, likely by reducing expression of steroid acute regulatory protein (StAR), key in the rate limiting step of

cholesterol conversion to pregnenolone which initiates glucocorticoid synthesis (Escajadillo et al., 2016).

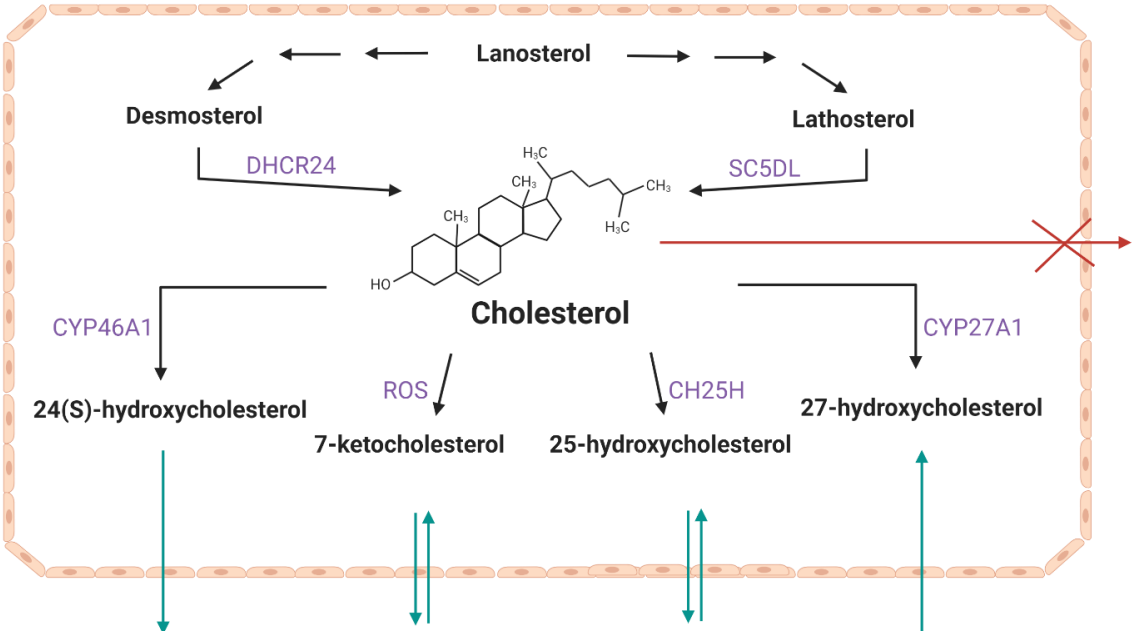

Figure 1.3 Overview of cholesterol metabolism in the brain. Most cholesterol is metabolized from astrocyte-secreted apoE-cholesterol complexes. Cholesterol does not cross the BBB although oxysterols do. Not shown here is the conversion of cholesterol to pregnenolone, the rate-liming step in steroid synthesis. Created with BioRender.com.

ApoE's potential role in PTSD thus might relate to the cholesterol metabolism providing evidence for the recent theories that metabolic dysfunction underlie the disorder (Levine et al., 2014; Michopoulos et al., 2016). In adult men, an acute, mental task (cognitive, not emotional and devised to be mildly stress-provoking) increases serum cholesterol by 0.10 mmol/L and did not reverse during a brief recovery period (Muldoon et al., 1992)**.** Patients with PTSD in a prospective study showed significantly lower high density lipoprotein cholesterol (HDL-C) and higher triglycerides at baseline compared to those that did not develop PTSD, suggesting that lipid profile at baseline may help determine who is at risk of developing PTSD (Hamazaki et al., 2014). In contrast, in a cross-sectional study, lipid profiles and stress hormones were assessed in PTSD patients and controls and revealed there was a more favorable lipoprotein profile (*i.e.* lower

LDL-C) in males and females with PTSD than controls (Vries et al., 2017). Other studies have found a lack of association between favorable lipoprotein profile and PTSD (Dennis et al., 2014; Jessica M. Gill et al., 2013). Despite the conflicting studies thus far, this research suggests that lipid metabolism may be a predictive factor as well as altered in response to trauma.

ApoE Genotype and PTSD in Human Populations

Hence, apoE genotype has been of growing interest in the field of PTSD research. In the first publication to address this association, Freeman et al. (2005) assessed 54 male Caucasian combat veterans and found a higher number of E2 homozygotes with PTSD than expected based on Hardy-Weinberg equilibrium. E2 carriers in this study also showed higher CAPS-2 re-experiencing scores (*i.e.* more severe) compared to non-E2 carriers but no difference on avoidance or arousal symptom clusters (Freeman et al., 2005). This increase in susceptibility was supported in a later, larger study assessing Korean male combat veterans from the Vietnam War with and without PTSD (Kim et al., 2013). Kim et al. also found that PTSD prevalence increased in E2 non-carriers that participated in harmful drinking behaviors but that this increase in PTSD rates was absent in E2 carriers. Interestingly, male E2 carriers showed lower mean daily alcohol intake than E4 carriers (Ward et al., 2009), suggesting that apoE isoforms may influence alcohol drinking and interact with PTSD. Although beyond the scope of this dissertation, self-medication is a prominent theory in PTSD (Khantzian, 1997), and this finding suggests apoE may influence self-medication in PTSD. In a separate cohort of male combat veterans from the Vietnam War era, it was found that those with E2 showed greater general symptom severity on the CAPS compared to E3 and E4 carriers (Johnson et al., 2015). However, the association between E2 and PTSD has been less distinct in other studies. Peterson et al. denounced the Freeman et al. findings after assessing the role of apoE genotype by quantifying cysteine residues in apoE alleles (a replacement for apoE genotype); they found that E4 was associated with greater risk for PTSD re-

experiencing symptoms (Freeman et al., 2005; Peterson et al., 2015). Why this different way of categorizing apoE genotypes may affect the relationship between apoE and PTSD remains unclear.

Other studies have further complicated the apoE genotype-PTSD association. Lyons et al. found that in a male Vietnam War era combat veteran population, E4 was associated with worse PTSD outcome after trauma compared to non E4 carriers (Lyons et al., 2013). Notably, this study did not include E2 carriers. Many subsequent studies have specifically assessed E4 carriers vs non-carriers and ignored E2 carriers altogether in their cohorts (Averill et al., 2019; Emmerich et al., 2015; Hayes et al., 2017; Mota et al., 2018; Nielsen et al., 2019; Yesavage et al., 2012). Some studies showed no significant association between apoE genotype and PTSD frequency or severity (Dretsch et al., 2016; Yesavage et al., 2012). In one study, E4 status was controlled for to assess cognitive ability in traumatic brain injury (TBI) and PTSD patients; however, no analysis directly assessed if apoE genotype influenced PTSD severity (Kaup et al., 2019). Plasma phospholipid profiles and E4 genotype may help differentiate diagnosis of mild TBI and PTSD (Emmerich et al., 2015). Specifically, lower levels of phospholipids overall were found in PTSD and TBI groups compared to controls. Monounsaturated fatty-acid containing phosphatidylcholine and phosphatidylinositol were lower in TBI and TBI + PTSD groups. E4

carriers showed higher phospholipid levels than E4 noncarriers- subjects (**Table 1.2**).

Table 1.2 Studies assessing PTSD symptom severity and/or susceptibility depending apoE genotype, including studies published as recently as April 2, 2020.

Article	Population	Sex (n)	PTSD	apoE	Finding
E2 is associated with greater PTSD severity or susceptibility					
Freeman et *al.* 2005	Caucasian veterans	Male (54)	All	First E4+ vs E4-, then E2+ vs E2-	High rate of E2 homozygotes (16.7%), E2+ had higher reexperiencing scores
Kim et *al.* 2013	Korean veterans	Male (256)	With and without	All genotypes	Greater # of E2 in PTSD group than non-PTSD, E2 results in lowered risk of substance use
Johnson et *al.* 2015	Caucasian veterans	Male (104)	All	All genotypes	E2 had higher symptom severity (CAPS and PCL), no difference in prevalence
E4 is associated with greater PTSD severity or susceptibility					
Lyons et *al.* 2013	Twin pairs, Vietnam veterans	Male (172)	With and without	E4+ vs E4-	E4 associated with worse PTSD outcome
Peterson et *al.* 2015	Veterans	Male (53), Female (6)	With and without (49 with, 10 with subthreshold PTSD)	All genotypes, cysteine residues	Lower cysteine residue (*i.e.* E4 carriers) corresponded to greater symptom severity
Kimbrel et *al.* 2015	Non-Hispanic White and Non-Hispanic Black Iran/Afghanistan veterans	Male (1291), Female (333)	With and without	E4+ vs E4-	For those with high exposure, E4 increased susceptibility in Non-Hispanic Blacks but not Whites
James et *al.* 2017	US veterans	Male (309), Female (34)	With and without	All genotypes, cysteine residue	E2 genotype related to higher resilience
Mota et *al.* 2018	2 cohorts from European Americans Veterans	2011 cohort: Male (1260), Female (126) 2013 cohort: Male (457), Female (52)	With and without	E4+ vs E4-	E4 carriers showed greater PTSD symptoms in main sample, but not in replicate sample
Merritt et *al.* 2018	Military veterans	Male (106), Female (27)	With and without	E4+ vs E4-	Trend for E4+ greater PCL score in those with TBI
Nielsen et *al.* 2019	Veterans	Male, Female (total = 87, breakdown NA)	With and without	E4+ vs E4-, excluded all E2 carriers	Plasma apoE levels correlated to PTSD symptom severity, and E4+ corresponded to increased susceptibility
Huguenard et *al.* 2020	Active duty soldiers	Male (120)	With and without	E4+ vs E4-	E4 carriers showed significant interaction with PTSD diagnosis and lipid profile
APOE genotype is NOT associated with greater PTSD severity or susceptibility					
Yesavage et *al.* 2012	Primarily not Hispanic or Latino, Vietnam veterans	Male (105)	All	E4 + vs E4-	No difference in E4 prevalence or cognitive tests, PTSD severity not directly assessed
Dretsch et *al.* 2015	Soldiers	Male (221), Female (9)	With and without	All genotypes	No differences in allele frequency compared to Hardy-Weinberg, severity was not assessed
Hayes et *al.* 2018	White, non-Hispanic U.S. combat Iraq and Afghanistan veterans	Male (149), Female (11)	With and without	All genotypes	Focused primarily on BDNF and mTBI, Did not directly test apoE and PTSD, but no differences in allele frequencies between PTSD vs not
Averill et *al.* 2019	2 cohorts: European Americans Veterans	2011 cohort: Male (1260), Female (126) 2013 cohort: Male (457), Female (52)	With and without	E4+ vs E4-	Assessed cognitive performance, did not assess PTSD severity or prevalence

The low number of women with PTSD in the previous studies precludes assessment of sex and apoE's influence on PTSD. This a salient gap considering the significant sex differences in both PTSD as well as in the associated risk between AD and apoE isoforms (noted above), which suggest that an interaction between sex and apoE in PTSD may exist. In addition, race and ethnic background modulate the differential effect of ApoE isoforms with regard to health risks (Farrer et al., 1997; Marini et al., 2019; Rajan et al., 2017). For example, Hispanic E4 carriers have lower odds of developing AD, highlighting the need for further understanding of interactions between race, ethnicity, and apoE. There may be similar effects in PTSD as evidence suggests that Hispanics may be more susceptible to PTSD and argues the importance of ethnicity, although this is a deeply complex issue (Pole et al., 2005). The influence of apoE and ethnicity remains inconclusive for PTSD, largely due to a lack of research. However, in one study, Kimbrel et al. assessed one of the larger cohorts of veterans of non-Hispanic White and non-Hispanic Black Iraq/Afghanistan-era veterans. Combat exposure, divided into high and low exposure levels, and PTSD symptom severity was determined by self-report. In high exposure levels only, E4 increased susceptibility for non-Hispanic Black but not for non-Hispanic White individuals (Kimbrel et al., 2015).

A meta-analysis of six papers on apoE effects on the incidence of PTSD concluded that E4, but not E2, contributes significant risk for developing PTSD. However, symptom severity of PTSD in relation to genotype was not assessed (Roby, 2017). More recently, as a result of E2 being studied as a protective factor in AD research, one study assessed apoE as a "resilience gene" in PTSD by quantifying the role of apoE using cysteine residue as a surrogate measure for genotype (James et al., 2017). This is similar to the approach used in the aforementioned Peterson et al. study and also found that E2 genotype corresponded to higher resilience to trauma compared to non-E2 genotypes (Peterson et al., 2015).

In summary, based on the growing number of studies that have assessed PTSD prevalence and symptom severity, it is still unclear what role apoE genotype plays in stress-related mental health disorders. This is primarily because the allele frequencies of E2 and E4 often result in underpowered studies that exclude, or group E2 carriers with E3 homozygotes, as E4 non-carriers. In addition, these studies are limited by their lack of inclusion of females, non-combat veterans, and those of other ethnic backgrounds. Childhood trauma and the influence of apoE genotype on neurocognitive function have been assessed in women, showing that E4 corresponds to worse cognitive performance in those with childhood trauma (Womersley et al., 2019). These studies support the need to expand the PTSD literature to include apoE genotype while acknowledging the numerous levels of complexity. Thus, it is critical that research takes advantage of animal models to understand the mechanisms underlying the impact of apoE isoform on the stress response.

Mouse models of PTSD

Role of mouse models in understanding human disorders

Based on the diversity of types and numbers of traumatic events as well as PTSD-related phenotypes seen in patient populations, animal models have been extremely important in better understanding the underlying biology of PTSD (described briefly above). Animal models allow researchers to control for potential confounding factors, such as diet and environmental stressors, to focus on the independent variable of interest. This then offers the ability to better determine how a particular independent variable affects the PTSD-related phenotype and what biological changes may be associated with phenotypic changes. Thus, animal models can help elucidate potential mechanisms that result in health disorders and determine novel avenues for therapeutics before continuing to clinical studies. In addition, the wide range of tools to genetically modify mice allows researchers to study specific genetic factors. TR lines allow for modification of

specific gene location unlike the random insertion used in transgenic models; when used with mouse promoters, TR lines display endogenous levels for proteins of interest. This is particularly advantageous for this dissertation since apoE isoforms are found at such disparate levels in human populations but are critical to the central question of this dissertation--what is the role of apolipoprotein E isoforms in response to stress?

Ethical concerns are inherent to in all biomedical research but are especially important with regards to the use of animals. All work completed within this dissertation was done according to institutionally approved protocols and care was taken to use the minimal number of animals to obtain enough power for statistical analyses. Further details are discussed in Chapter 2's Method section.

Human apoE targeted replacement mice

Mice do not possess multiple isoforms of apoE as humans do. Murine apoE is described to be most similar to E3, although there remain clear differences in binding patterns between mouse apoE and human E3 (Nguyen et al., 2014; Raffai et al., 2001). In order to better understand the functional differences between apoE mice that express human apoE isoforms via targeted gene replacement (TR) under the mouse apoE promoter have been created (Sullivan et al., 2004; Sullivan et al., 1997; Sullivan et al., 1998). Like in humans, apoE mRNA levels in apoE TR mice are similar across the different isoforms and also replicate the asymmetric protein levels with E2 > E3 > E4 (Bales et al., 2009; Riddell et al., 2008). This supports the use of the apoE TR lines in understanding human health concerns.

More generally speaking, the apoE TR mice have also demonstrated isoform-specific differences across behavioral tasks, both anxiety-related as well as spatial, that are age-dependent. Specifically, E2 female mice demonstrated greater anxiety-like behavior than E3 and E4 female mice. E2 mice had higher anxiety-like levels when young, middle, and old ages were assessed

together, although this was driven by the younger timepoint (Siegel et al., 2012). In addition, apoE TR mice have shown apoE-isoform specificity in response to some types of injury such as radiation for which E4 mice are more susceptible to changes (Villasana et al., 2013) while methamphetamine exposure showed no differences between E3 and E4 mice (Siegel et al., 2010).

Modeling PTSD-related behavioral and cognitive changes

In terms of modeling behavioral and cognitive performance specifically in the context of trauma exposure, perhaps the most frequently used behavioral paradigm to assess the fear learning circuitry is classical Pavlovian fear conditioning (**Figure 1.4A**). During the task, a mouse is placed into a novel environment, *i.e.* a behavioral testing chamber. After a short habituation period, the mouse is exposed to multiple, slight foot shocks separated by interstimulus intervals. The animal's average movement and percent time spent freezing, an indicator of fear behavior, are recorded and analyzed. Fear learning can be demonstrated within this acquisition trial by increases in percent time spent freezing during the interstimulus intervals over the course of the trial. Contextual fear memory can be assessed after a period of time, usually 24 hrs after fear acquisition, by placing the mouse back into the same testing chamber and measuring the percent time spent freezing without any additional foot shocks. Cued, or associative fear learning introduces an auditory cue that begins prior to and co-terminates with the foot shock during the acquisition trial (Context A). Cued memory is then tested by placing the mouse into a novel environmental (Context B, different floor type, shape of the enclosure and olfactory cues) and playing the auditory cue following a baseline period to assess associative fear memory using again the percent time spent freezing as outcome measure. A plethora of paradigms that differ on number of foot shocks, duration of the habituation, interstimulus intervals, etc. can be found in the literature. This can make incongruent findings difficult to interpret, although I would argue that consistent findings despite differences strengthen the significance of the findings.

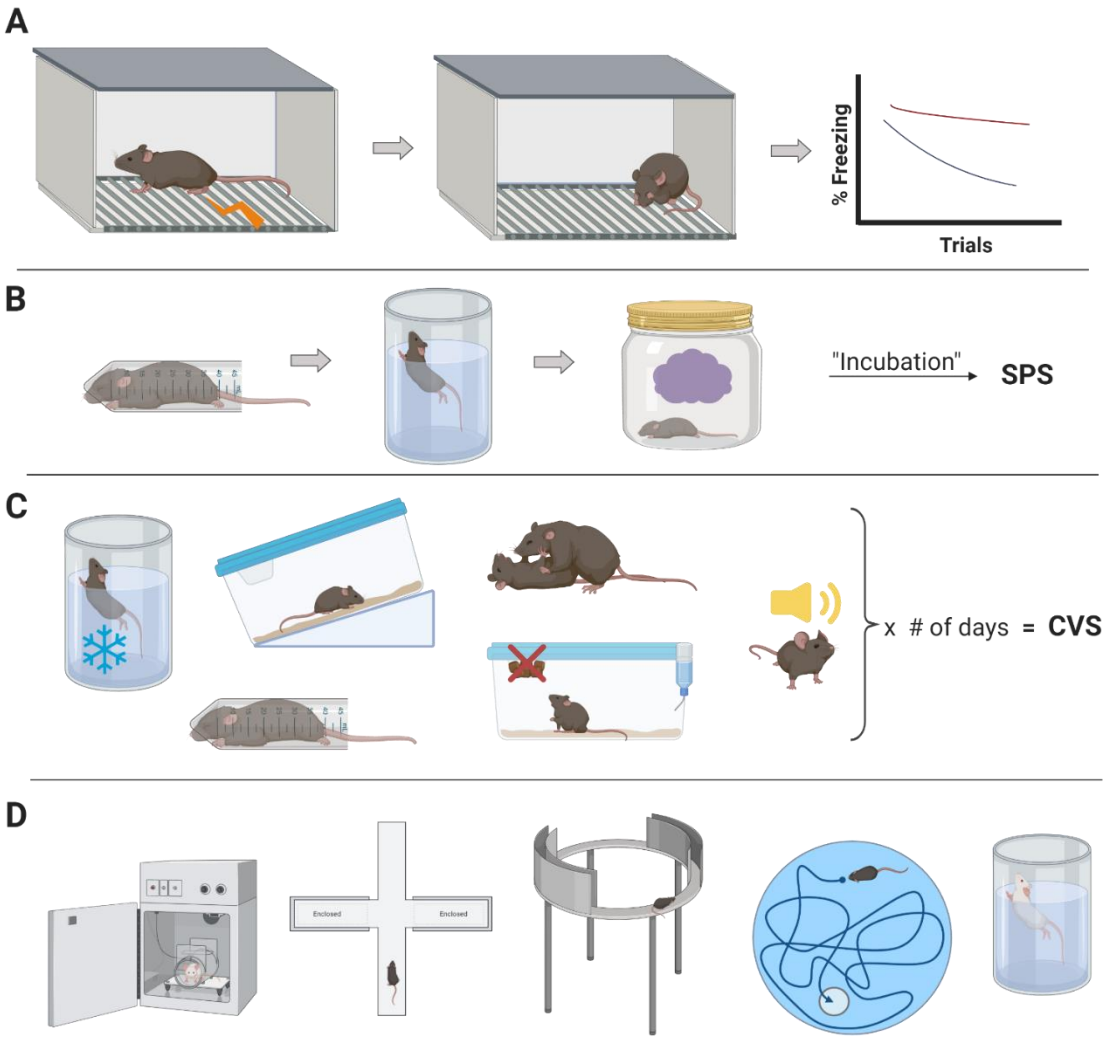

Figure 1.4 Overview of animal models of PTSD. A) Fear conditioning and extinction learning. B) Single prolonged stress exposure model (SPS). C) Example of stressors used in chronic variable stress (CVS). D) Examples of tests to assess behavioral changes associated with PTSD: acoustic startle, elevated plus maze, elevated zero maze, water maze, forced swim test. Created with BioRender.com.

The apoE TR mice display differential responses in fear extinction learning; E2 male and female mice fail to demonstrate extinction learning in a paradigm which E3 and E4 mice do (Olsen et al., 2012).This is of particular interest, considering that extinction learning closely parallels the learning in exposure therapy. Indeed, fear conditioning has been extremely informative, not just for modeling fear circuits in rodents and assessing a phenotype associated

with stress, but also in developing better extinction-based therapies and anatomical predictions for anatomical studies in patients (Milad et al., 2006).

However, as a model for PTSD, fear conditioning alone has been considered inadequate because "The psychological effects of trauma are more pervasive than accounted for by a conditioning model" (Pitman et al., 1993). Such thinking has led to additional protocols such as stress-enhanced fear learning, during which prior stress exposure (repeated foot shocks) produces increased fear memory that is resistant to extinction learning (Rau et al., 2005). Another model is the Single Prolonged Stress (SPS) exposure model (**Figure 1.4B**), in which rats experience a series of stressors—cold water swim, restraint stress, and induced unconsciousness by ether dosing—originally described by Liberzon and Young and resulting in a multitude of variations in the length and timing of stressors (Liberzon & Young, 1997; Yamamoto et al., 2009). This model includes a "sensitization" or "incubation" period in which animals are left untouched for 7 days after the stress exposure day in order to facilitate memory of the event.

Similar to the SPS model is the chronic variable or unpredictable stress (CVS) paradigm (**Figure 1.4C**). This model incorporates several of the stressors in the SPS exposure model in addition to others but extends exposure over 5+ days depending on each lab's protocol. During this time, rodents are exposed to one or more stressors each day at different times throughout the day. The perceived "randomness" and unpredictability ensures the animal will not habituate to the stress, leading to long-term upregulation of the HPA axis. It has been used extensively in both depression and PTSD research (Goswami et al., 2013). It is the model used for this dissertation work because of the previous apoE isoform-specific differences found in behavioral, cognitive, and physiological measures after exposure (Johnson et al., 2015). Mechanistically, CVS has also been shown to result in sex-specific differences. For example, females showed greater presynaptic innervation than male rats (Carvalho-Netto et al., 2011) as well as lower CRFR1/c-

Fos in the bed nucleus of the stria terminalis (Rosinger et al., 2020). This line of research may help elucidate sex differences in disorders such as PTSD.

In addition to models to induce PTSD-like changes, there are also numerous behavioral and cognitive tests that have been used to characterize changes (**Figure 1.4D**) (Deslauriers et al., 2018). While the extent of a rodent's ability to feel emotions compared to a human is debated (LeDoux, 2012; Raber, Arzy, et al., 2019), these tests allow researchers to understand facets of the disorder. For example, acoustic startle tests that directly corresponds to arousal tests in patients. In non-human primates, the fear potentiated startle test has been adapted to be used for repeated measures design and testing for responses to safety-signals (Davis, 2006; Kazama et al., 2013). Anxiety tests involve a balance between a rodent's innate drive to explore novel environments versus staying in a safer (enclosed) area (approach-avoidance scenario) and measure how much a rodent explores an anxiogenic area such as the open areas in the elevated plus/zero mazes. Cognitive tasks such as object recognition, spatial and emotional learning, and memory can also be used in a mouse model. Animal models in general are a precursor to translating findings to human patients.

Dissertation studies

Based on these previous findings that suggest that apoE genotype influences PTSD symptom severity, the following questions remain: 1) Does apoE isoform influence the behavioral and cognitive changes associated with stress exposure? 2) How does sex modulate the apoE isoform-dependent response to stress? 3) Are these apoE isoform-specific changes related to lipid metabolism and signaling within the stress response?

The purpose of the experiments described in this dissertation were to address these questions. As mentioned previously, this dissertation involves the human apoE TR lines in the CVS model of stress exposure as a means to assess underlying biological changes associated with

an animal model of PTSD. 1) Due to the significantly lower LDLR binding affinity of E2 compared to E3 and E4, and LDLR's critical role in cholesterol in metabolism and potential role in stress, I hypothesized that in the context of chronic stress, an animal's ability to compensate for the low binding affinity of E2 to LDLR is weakened, resulting in less functional output of LDLR binding leading to the E2-specific phenotypes seen. Thus, I hypothesized that E2 (vs E3 or E4) does result in greater behavioral changes as a consequence of chronic stress exposure that is dependent on the downstream uses of cholesterol. 2) Females were expected to have greater anxiety-like behavior and exacerbated spatial cognitive impairments compared to males. 3) Furthermore, I hypothesized that the behavioral and cognitive changes seen would correspond to changes in downstream markers of apoE function, namely utilization of cholesterol.

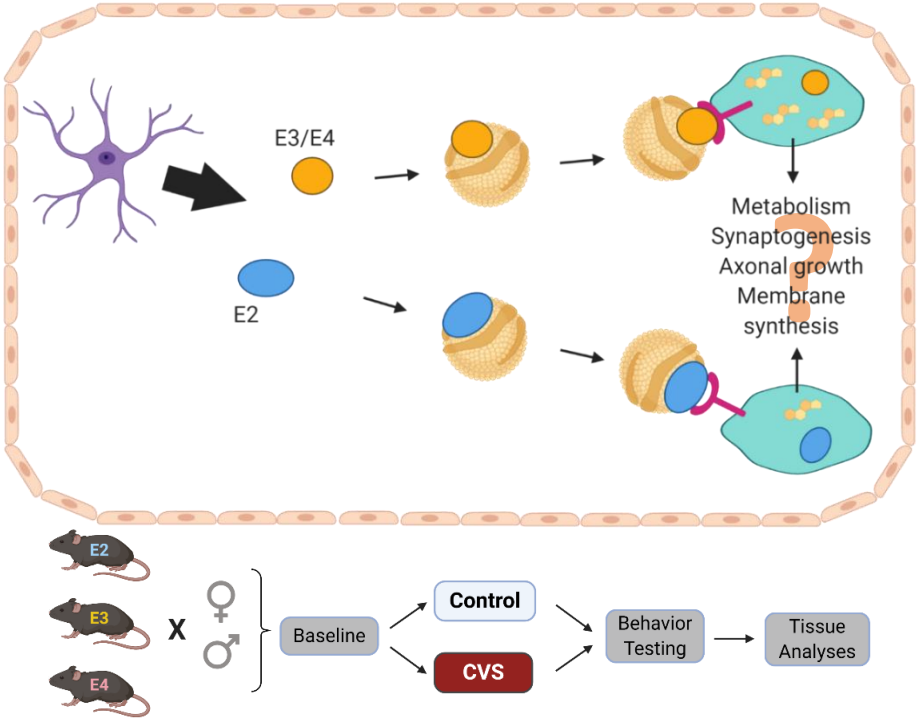

Figure 1.5 Summary of dissertation experiments. I hypothesized that differences between E2 and E3/E4 to LDLR in binding affinity is exacerbated during severe stress exposure that leads to changes in downstream products of cholesterol utilization. These changes then result in differences in behavior. To test this, E2, E3, and E4 male and female mice were included in either control or CVS-exposed groups and tested for behavioral and changes associated with changes in tissue markers. Created with BioRender.com.

To assess these hypotheses, I proposed the experiments contained within this dissertation (**Figure 1.5**). Briefly, male and female E2, E3, and E4 mice were included to assessed sex differences in addition to the effects of apoE genotype. Home cage activity and body weights were recorded throughout the experiment. Anxiety-like behaviors were assessed at baseline as well as after exposure to control conditions or CVS. I also measured spatial learning and memory after control or CVS conditions. Tissues and plasma were collected after the last behavioral test and used to measure corticosterone, apoE, and cholesterol metabolite concentrations, and to assess protein and lipid markers of LDLR function. The design and findings of these proposed experiments are described more fully in Chapter 2.

Chapter 2: ApoE-isoform specific changes after CVS

[This chapter has been reformatted for inclusion for this dissertation from: Torres ERS, Luo J, DeBarber A, and Raber J. In preparation.]

Abstract

Post-Traumatic Stress Disorder (PTSD) is a highly prevalent mental health disorder affecting 3.6% of adults in the US in a given year. However, because of the high level of variability in susceptibility, symptoms and severity, PTSD therapies are still insufficient for complete recovery and highlight a need to better understand PTSD. One prominent genetic risk factor is *apolipoprotein E*, which encodes the protein (apoE) that is functionally involved in cholesterol transport and metabolism. ApoE exists in 3 major isoforms in humans: E2, E3, and E4. Compared to E3, the E2 isoform results in more severe PTSD-related symptoms, although the reason why is still unclear. Female and male mice (3-5 months of age) expressing human E2, E3, or E4 were used in an animal model of PTSD to assess potential mechanisms contributing to this E2-related phenotype. Mice were either placed into control groups or exposed to chronic variable stress (CVS), which has been shown to induce PTSD-like behavioral and neuroendocrine changes. E2 mice showed a unique response to CVS compared to E3 and E4 mice that included poorer spatial learning and memory, increased adrenal gland weight, and no increase in glucocorticoid receptor protein levels (normalized to apoE levels). In addition, the cholesterol metabolite 7-ketocholesterol was elevated in the cortex after CVS in E3 and E4, but not E2 female mice. Altogether, these data show that E2 confers unique changes in behavioral, cognitive, and biomarker profiles after stress exposure and identify 7-ketocholesterol as a possible novel biomarker of the traumatic stress response.

Introduction

Although the majority of individuals are exposed to at least one traumatic event at some point in their lifetime, only 7.8% will develop Post-Traumatic Stress Disorder (PTSD), one of the most prevalent trauma- and stress-related mental health disorders (Kessler et al., 1995). Symptoms of PTSD are categorized into re-experiencing trauma, negative alterations in cognitions (*e.g.* visuospatial construction, attention, and delayed memory) and mood, altered arousal and reactivity, and avoidance (DSM V). Additionally, many patients also suffer from comorbid disorders, including other mental health disorders like depression and anxiety**,** but also diabetes, hypertension, and cardiovascular disease (Levine et al., 2014; Shalev et al., 2017). These symptoms range widely in severity and many patients endure them for decades (Watson, 2019). This heterogeneity presents challenges for therapies, which are still lacking.

In terms of risk factors for development of the disorder, previous research supports that both environmental (*e.g.*, types and number of traumas) (Perrin et al., 2014) and genetic factors (Sartor et al., 2011; Stein et al., 2002) influence diagnosis. *Apolipoprotein E* is one genetic factor that has been implicated in PTSD (Freeman et al., 2005). The gene encodes the protein apolipoprotein E (apoE), which exists in 3 major isoforms in humans: E2, E3, and E4. Allelic frequencies for E2, E3, and E4 are 8.4%, 77.9%, and 13.7%, respectively (Liu et al., 2013). The imbalance can make it challenging to study differences between these isoforms in human populations (See Chapter 1 for further discussion). In addition, apoE is a major player in lipid transport and metabolism and is the strongest genetic risk factor of Alzheimer's disease (AD). This risk for AD is greater in women than men suggesting an interaction between sex and apoE (Farrer et al., 1997). Prior to this association to AD, apoE genotype had been associated with cardiovascular disease (Eichner et al., 1993; Wilson et al., 1996). ApoE is a particularly interesting genetic factor because in addition to its lipid transport functions, studies involving apoE KO mice have shown that apoE plays an important modulatory role in hypothalamic-

pituitary-adrenal (HPA) axis function by tonic inhibition of steroidogenesis and activity in the adrenal cortex, likely via altering cholesterol metabolism (Raber et al., 2000). ApoE mRNA also inversely correlates with steroidogenesis in the adrenal gland (Nicosia et al., 1992). Considering that dysregulation of the HPA axis has been heavily studied as the cause of PTSD (Dunlop & Wong, 2019), these reports argue that apoE may be an important modulator in PTSD severity. In humans, Freeman et *al.* found a significant association between E2 carriers and disease severity (Freeman et al., 2005). Specifically, E2 was associated with significantly worse reexperiencing symptoms and impaired memory function. However, additional studies suggest the association between apoE and PTSD symptom severity is still equivocal (See Chapter 1).

Many animal models have been used to better understand PTSD, and have included the use of physical trauma, such as foot shocks (Schöner et al., 2017). While physical traumas may also induce PTSD, they induce different neurological response than psychological traumas (Kogler et al., 2015). Chronic variable stress (CVS) includes both such types of traumas and has been previously used to instill PTSD-related symptoms in rodent model (Deslauriers et al., 2018; Schöner et al., 2017) and will be employed in this dissertation.

Our previous studies have assessed the association between PTSD-related symptoms and E2 genotype by comparing mouse lines expressing human apoE isoforms via TR in fear conditioning and CVS paradigms. Specifically, E2 homozygote mice showed impaired fear extinction learning that was not seen in E3 or E4 mice (Johnson et al., 2015; Olsen et al., 2012). After exposure to CVS, E2 mice demonstrated increased levels of anxiety-like behavior, impaired spatial learning, higher corticosterone levels and increased home cage activity, compared to control E2 mice. Clinically, E2-carrying PTSD patients also demonstrated more severe symptoms as well as blunted changes associated with circadian rhythm in levels of salivary cortisol than non-E2 carriers (Johnson et al., 2015). However, other research indicated that E4 is associated with PTSD symptom severity (Lyons et al., 2013; Mota et al., 2018; Peterson et al., 2015) and

susceptibility (Kimbrel et al., 2015; Nielsen et al., 2019). It is important to note that there are some limits associated with these studies, including grouping E2 carriers with the E4-noncarrier group for analysis (See Chapter 1, Table 1.2). Low allelic frequencies of E2 and E4 also makes it challenging to obtain enough individuals for sufficient power to determine differences. These studies also did not assess sex differences, highly relevant considering that women are more likely to develop PTSD even though men experience higher rates of trauma (Ramikie & Ressler, 2018). Furthermore, the underlying molecular mechanisms for this apoE isoform specificity has yet to be determined.

One unexplored potential mechanism for apoE isoform-specific effects is through its major receptor in the CNS, the low-density lipoprotein receptor (LDLR) (Holtzman et al., 2012). LDLR activation can reduce secretion of glucocorticoids from the adrenal cortex (van der Sluis et al., 2015) and reduces apoE levels within cerebrospinal fluid (Fryer et al., 2005). Of interest, E2 has a lower binding affinity (~1%) to the low-density lipoprotein receptor LDLR compared to E3 and E4, which is normally compensated by other apoE-binding receptors in the LDLR family (Weisgraber et al., 1982). We thus hypothesized that the E2-specific changes seen in patients and the apoE TR mice may be a result of dysregulated cholesterol metabolism in part due to the ineffectiveness of LDLR activation. This then may increase glucocorticoid synthesis in adrenal glands and dysregulate normal HPA axis activity.

In addition to LDLR's effects on glucocorticoid secretion, apoE and LDLR's subsequent cholesterol transport and metabolism may also result in changes in the utilization of cholesterol, *i.e.* what cholesterol is metabolized into. Oxysterols, oxidized metabolites of cholesterol, are of interest because unlike cholesterol, they can cross through the blood-barrier and act as signaling molecules for cholesterol metabolism. One such oxysterol, 7-ketocholesterol, inhibits glucocorticoid action in adipocytes by reducing GR activation via substrate competition at the enzyme activity level and subsequently influences GR transcriptional activity (Wamil et al.,

2008). It can also serve as a ligand for oxysterol binding protein receptors, which attenuate glucocorticoid synthesis, in the adrenal gland (Escajadillo et al., 2016). Moreover, silencing of oxysterol binding protein 2 resulted in enhanced mRNA expression of StAR, key in the conversion of cholesterol to pregnenolone and subsequent glucocorticoid synthesis. This did not correspond to increased protein levels but may instead promote glucocorticoid output by assisting in proper mitochondrial localization of StAR.

Furthermore, apoE mRNA has been shown to increase with corresponding levels of 7-ketocholesterol suggesting that there may be a positive feedback loop between the two (Cader et al., 1997). 7-ketocholesterol is a relatively understudied oxysterol and data have largely focused on peripheral tissues, although studies in postmortem frontal cortical tissue of Alzheimer's disease patients showed that 7-ketocholesterol levels increased with disease progression (Testa et al., 2016). This, however, has not been assessed in terms of apoE isoform in the context of the stress responses.

ApoE thus regulates the HPA axis, which may contribute in an isoform-dependent manner due to differences in metabolism of cholesterol and consequent glucocorticoid synthesis. This is hypothesized to result in PTSD-related symptoms after trauma associated with metabolism and cognitive dysfunction. The goals of this study were to 1) expand the current understanding of the role of apoE genotype in PTSD, 2) examine sex differences interacting with apoE genotype in PTSD-related symptoms, and 3) better understand underlying neurochemical and neuroendocrine changes. We used male and female apoE TR mice and a CVS paradigm to assess behavioral and cognitive performance differences associated with CVS. Neurochemical markers, such as LDLR levels as well as components of the HPA axis (GR and corticosterone) were measured as a function of sex, genotype, and CVS exposure. Protein levels of microtubule associated protein 2 (MAP-2), a marker of synaptic plasticity and microtubule stability (Harada et al., 2002) which is also responsive to chronic stress (Martin et al., 2017), were also examined. To better understand

cholesterol metabolism and utilization in the context of stress exposure, we measured cholesterol precursors and metabolites by developing an assay to measure sterols and oxysterols in low volumes of murine tissue.

We found that 7-ketocholesterol was increased in E3 and E4 mice exposed to CVS compared to their genotype-matched control counterparts but this CVS-related difference was absent in E2 mice. CVS-related change in E2 mice was also absent for GR normalized to apoE levels. Spatial learning and memory were impaired in E2 mice exposed to CVS versus E2 controls and were more severe in female E2 mice. E2 male, but not female mice, also lost body weight during the week of CVS. These changes were unique to E2 mice and not seen in E3 or E4 mice similarly treated. Thus, E2 resulted in sex-specific behavioral and cognitive changes that may be due to a dysregulated stress response corresponding to changes in cholesterol metabolism. These data support apoE genotype as an important factor to consider in stress-related disorders such as PTSD that also demonstrate significant metabolic changes.

Methods

Animals

All housing and experimental procedures were carried out according to OHSU IACUC policy. Male and female mice expressing human apoE isoforms—E2, E3, or E4 homozygotes—under the mouse apoE promoter were included in this study and were originally generated by Dr. Patrick Sullivan (Sullivan et al., 2004; Sullivan et al., 1997; Sullivan et al., 1998). Mice were bred in house and were 3-5 months of age at the start of the experiments. Food and water were available ad libitum except for when noted below. Lights in the housing room were set to 12 hr light : 12 hr dark cycle (on at 6am, off at 6pm during Daylight Savings Time). All behavioral tests and procedures took place during the light phase, except for home cage activity which occurred continuously throughout the experiment. One mouse was treated for malocclusion throughout the

testing and two were euthanized for health reasons. None of these health concerns appeared linked to CVS.

Experimental Design

In order to address the goals of this experiment listed above, apoE TR mice were pair-housed at the start of the experiment. Body weights were recorded weekly throughout the experiment and home cage activity was recorded during the entire experiment. A within-subject design was used to assess anxiety-like behavior; mice were tested during Week 1 after a one-week habituation period (Baseline Week) and again as the final behavioral measure before tissue and blood collection (Week 4). During Week 2, control mice remained in their home cages without additional interactions from the tester while CVS mice were exposed to stressors. Additional details are described below. Mice were tested among 6 cohorts with sex and genotype distributed throughout. Group sizes of each sex within genotypes for CVS exposure were n = 12-16. Control and CVS mice were tested in separate cohorts due to concerns of the groups influencing each other's behavioral changes and physiological (see Appendix: Additional Methodology Notes for further details). The same experimenter handled and tested all mice and was kept blind to the genotype and sex throughout the experiment.

Home cage activity monitoring

Home cage activity was recorded with noninvasive home cage monitors (Biobserve, Germany) throughout the entire experiment, as a measure for circadian activity and general locomotion. This occurred while control mice were pair-housed. CVS mice were pair-housed for the entire study except during CVS exposure in Week 2. Activity was recorded every second over the course of the entire experiment (MLog, Biobserve) and was compiled as 30-min averages

using R (R Foundation for Statistical Computing, Vienna, Austria). Activity during the light and dark phases were assessed separately.

Chronic Variable Stress

Exposure to CVS took place over the course of 5 days and was modeled on a previously published paradigm (Johnson et al., 2015). Stressors included social deprivation (*i.e.*, single housing), 30° cage tilt for 3 hrs, wet home cage for 3 hrs, overnight food deprivation, 3-min cold swim (10-12°C), and 15-min restraint. Mice were exposed to 2 unique stressors at random times throughout each day (details on the timing and tests are provided in **Suppl. Figure 2.1**. Stressors within the home cage (white noise, wet cage, cage tilt, and food deprivation) took place in the housing room while stressors that required mice be moved from the home cage (cold swim and restraint) occurred in the testing room adjacent to the housing room. Mice were not individually dried after the wet cage and cold swim stressors, and all mice appeared to be dry after 30 mins.

Behavioral testing

Elevated plus maze

The elevated plus maze has been previously used to model anxiety-like behavior in rodent models based on differential exploration. It consists of two opposing open arms and two opposing closed arms. Mice are placed in the intersection of the arms of the maze and allowed to explore for a single 10-min trial. Two mice were tested at a time and 0.05% acetic acid was used to clean the mazes after each test. Testing occurred in the morning and light levels was 80-100 lux. Time in the center, open arms, and closed arms are measured separately using infrared photo beams to track movement (Kinder Scientific, Poway, CA).

Water maze

The water maze is a spatial memory task that consists of a 140 cm diameter pool filled with water (21°C ± 2°C). The maze was surrounded by large spatial cues. A small circular platform (10 cm in diameter) was placed within the pool so that it was 1 cm below the surface of the water. White chalk was added to the water so that the location of this platform was hidden. Mice were given 2 trials per session starting in the morning. Trials were approximately 10 min apart from each other; 2 daily sessions were spaced 4 hr apart. Mice were first trained to locate a visible flag to learn to escape from the maze by stepping onto the hidden, submerged platform. After remaining on the platform for 3 s, the tester gently picked up the mouse and returned it to its home cage. No mice appeared to have issues with drying off on its own by the next trial. Each trial was 60 s; if the mouse did not locate the platform, it was led to the platform by the experimenter. The platform location was changed after each session to avoid procedural learning.

After 2 days of visible training, the flag was removed so that the platform became hidden from view. There was a total of 2 hidden locations during the water maze testing. The first location remained the target for 4 sessions before switching to the reversal trials, a secondary hidden location. The next morning after the last session of each hidden location, mice were tested for search strategy in a probe trial. During these probe trials, the escape platform was removed completely from the water maze and mice were left in the pool for a total of 60 s after which they were gently picked up and returned to their home cage.

During all trials, swim speed, latency to last target location, cumulative distance to the target, time spent in each quadrant, and time spent in the periphery were recorded using an overhead camera and Ethovision 7.1 XT software (Noldus, Netherlands).

Elevated zero maze

In order to assess post-CVS changes in anxiety-like behavior and exploration, mice were tested on the elevated zero maze. Similar to the elevated plus maze, mice are allowed to explore the arena for 10 min. The elevated zero maze has 4 sections, 2 opposing "open" areas, considered to be anxiety-provoking, and 2 opposing "closed" areas. Time in each area and total distance traveled are measured using infrared beams.

Plasma and tissue collection and preparation

Immediately after completion of the elevated zero maze, blood was collected from the mandibular vein in unanesthetized mice as per the approved IACUC protocol. Whole blood (~50-200 µL depending on the speed of blood flow and the time to apply pressure to stop bleeding) was collected with 0.5M EDTA and centrifuged at 4000G for 10 min at 4°C to collect plasma.

Three hours after blood collection, mice were anesthetized with a lethal ketamine cocktail and then perfused with 1x PBS. PBS perfusion was done to remove blood from the brain for sterol and oxysterol analyses, described further below. Brain regions, including the frontal cortex, medial prefrontal cortex, and hippocampus (Pflibsen et al., 2015; Raber, Yamazaki, et al., 2019), along with the adrenal gland, liver, and kidney were dissected and flash frozen using liquid nitrogen. Tissues were kept at -80°C until further use. Tissues were homogenized in a lysis buffer consisting of 1M Tris-Cl, 6M NaCl, 10% SDS, and 0.5M EDTA, 1% Triton-X, and protease inhibitor, (Roche, Sigma Aldrich, catalog #11836170001, St. Louis, MO). Total protein amounts were determined by BCA protein assay kit (Pierce, Thermo Scientific, catalog #23225, Waltham, MA).

Radioimmune assay for corticosterone

Corticosterone was measured in plasma samples using a radioimmune assay kit (MP Biomedicals, catalog #7120102, Irvine, CA). All samples were measured in singlet using the same kit while duplicates were run for the standards. Intra-assay coefficient of variation was 10% and the inter-assay coefficient of variation was 7%. The best-fit curve for the standard readouts was calculated, and sample results were interpolated based on the standard curve for each assay.

Protein measurements

Western blot was used to measure apoE, LDLR, and GR in tissues. Pilot studies were done to ensure that target protein levels measured were within the linear range of the fluorescence signal; 15 mg of total protein for each sample was loaded for all tissues except for the mPFC, for which 10 mg of total protein per sample was loaded. Samples were run in duplicate and all groups were included on each individual blots. Tissue homogenates were prepared with a Laemmli sample buffer and β-mercaptoethanol mixture and run on SDS-PAGE gels (Bio-Rad, 4-15% TGX Total Protein Stain-Free gels, 26 wells, Hercules, CA) for 42 min at 200 V. Proteins were then transferred to low fluorescence PVDF membranes using a wet transfer system with 1x Tris/Glycine with 20% methanol buffer solution for 30 min at 100 V. Total protein was measured on each blot using the Bio-Rad Total Protein Stain-Free gel system with UV 302 nm visualization as imaged on the Azure c600 (San Francisco, CA). See **Supplementary Figure 2.2** for an example of total protein. Blots were rinsed and then blocked with Azure fluorescent blocking buffer for 1 hr before being cut so to avoid cross-reactivity with between primary antibodies raised in goat. Primary antibody solutions (Millipore Calbiochem catalog #178479 goat anti-apoE 1:4000, Burlington, MA; R&D catalog #AF2255 goat anti-LDLR 1:1000, Minneapolis, MN; and Cell-Signaling, catalog #12041S rabbit anti-GR 1:2000, Danvers, MA) were then used for overnight incubation at 4ºC. Blots were rinsed and incubated in secondary antibody solutions

(LiCor IRDye 680RD Donkey anti-rabbit 1:10,000, LiCor IRDye 800CW donkey anti-goat 1:10,000, Lincoln, NE) before being rinsed again. Images of target proteins were acquired using an Azure c600 at the corresponding fluorescent wavelength. Target proteins and total protein were analyzed using AzureSpot software. All target proteins were normalized to the total protein for each sample.

ApoE (Millipore Sigma, RAB0613-1KT, Burlington, MA) and MAP-2 (MyBioSource, MBS725632, San Diego, CA) were also measured using ELISA using the manufacturer's protocol. Briefly, test dilutions were run first to determine the dilution to be used based on the standard curve. For the apoE ELISA, a quantitative sandwich ELISA, samples were diluted with 1x PBS according to the test run results and loaded into assigned wells on the coated plate along with standards. The plate was incubated at 37°C for 2.5 hr before the incubation solution was discarded. The plate was then washed five times with the provided washing buffer. ApoE antibody solution was added and the plate was incubated for 1 hr at room temperature. Incubation solution was discarded and the plate was washed three times. HRP-conjugated streptavidin solution was added for a 45-min incubation. After the plate was washed, TMB-substrate solution was added for a 15-min incubation. The enzymatic reaction was stopped using a dilute sulfuric acid solution. The procedure for the MAP-2 ELISA, also a quantitative sandwich ELISA, was similar to the apoE ELISA procedure except that there was no initial incubation of samples alone in the plate wells, balance solution was added to tissue homogenates, and MAP-2 amounts were visualized using an HRP-substrate enzymatic reaction that was stopped with dilute sulfuric acid. Both types of ELISA plate were then spectrophotometrically measured at 450 nm using a SpectraMax iD5 microplate reader (Molecular Devices, San Jose, CA). All samples for each tissue type were run in the same assay. Standards were run in duplicate and samples run in singlets. For the apoE ELISA, the intra-assay CV is <10% and the sensitivity is 1.5 ng/mL. The inter-assay CV for the MAP-2 ELISA is < 9% and the sensitivity is 0.1 ng/mL. Graphpad Prism

software (Prism, La Jolla, CA, USA) was used to calculate the best-fit curve for the standard readouts and sample results were interpolated based on the standard curve for each assay.

Cholesterol & related metabolite analyses

Oxysterol panel in cortical tissue

Cortical tissue (30 mg) were homogenized in 1x PBS using homogenization beads. Total (free plus esterified) 7-ketocholesterol, 24S-hydroxycholesterol, 25-hydroxycholesterol and 27-hydroxycholesterol were determined in these homogenized samples by LC-MS/MS following sample saponification, extraction with hexane and derivatization with N,N-dimethylglycine (DMG) (Jiang et al., 2007; Pataj et al., 2016). Samples (75 µL) were spiked with 5 µL of internal standard mixture containing 7-ketocholesterol-d7 1ng/µl, 25-hydroxycholesterol-d6 2 ng/µl and 27-hydroxycholesterol-d6 20 ng/µl in methanol. Standards were prepared in homogenization buffer. Saponification was accomplished by diluting sample with 2 mL of ethanol followed by 0.120 mL of 33% KOH. Samples were vortexed then heated at 37ºC for 1 hr. After saponification, each sample was diluted with 2 mL of water and extracted twice with 4 mL of hexane. The combined hexane extracts were dried under vacuum. After drying the tubes were rinsed with 0.4 mL of hexane and dried again. The dried sample was treated with 25 µl mixture of DMG at 0.5M and 4-(N,N-dimethylamino)pyridine at 1M in chloroform and 25 µl 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide at 1M chloroform, then heated at 45°C. After 1 hr, 50 µl of methanol was added to deactivate the excess derivatizing agent. Samples were dried down in speed vacuum, suspended in 100 µl of methanol, vortexed, centrifuged and filtered prior to analysis of 5 µl injection with LC-MS/MS.

DMG Derivatives were analyzed using a 4000 Q-TRAP hybrid/triple quadrupole linear ion trap mass spectrometer (SCIEX, Framingham, MA) with electrospray ionization (ESI) in positive mode. The mass spectrometer was interfaced to a Shimadzu (Columbia, MD) SIL-20AC

XR auto-sampler followed by 2 LC-20AD XR LC pumps. The instrument was operated with the following settings: source voltage 4000 kV, GS1 40, GS2 30, CUR 40, TEM 500 and CAD gas medium. Compounds transitions were quantified with multiple reaction monitoring (MRM) with peak retention times as described in **Table 2.1**.

Table 2.1 Oxysterol MRM transitions. Quantifying (quan) and qualifying (qual) ion information. *Note that 25OH-C-d6-DMG quan internal standard was used for quantification of 24SOH-C-DMG quan.

Q1 mass	Q3 mass	Retention time (min)	Analyte	DP (V)	EP (V)	CE (V)	CXP (V)
488.4	385.4	4.4	24SOH-C-DMG quan*	81	10	23	12
488.4	367.4	4.4	24SOH-C-DMG qual*	81	10	29	12
488.4	104.1	4.7	27OH-C-DMG quan	101	10	39	20
488.4	58.2	4.7	27OH-C-DMG qual	101	10	91	10
494.4	58.2	4.7	27OH-C-d6-DMG quan	101	10	91	10
488.4	367.4	4.0	25OH-C-DMG quan	81	10	29	12
488.4	385.4	4.0	25OH-C-DMG qual	81	10	23	12
494.4	373.4	4.0	25OH-C-d6-DMG quan	81	10	29	12
486.4	58.2	7.2	7-KC-DMG quan	76	10	87	10
486.4	104.1	7.2	7-KC-DMG qual	76	10	37	18
493.4	390.2	7.2	7-KC-d7-DMG quan	76	10	27	12

Separation was achieved using an ACE Excel 3 µm C18-PFP 100x2.1mm (ACE, part # EXL-1110-1002U) column kept at 18°C using a Shimadzu CTO-20AC column oven. The gradient mobile phase was delivered at a flow rate of 0.4 mL/min between 0-6.5 min, 0.8 mL/min between 6.6-10 min and 0.4 mL/min between 10.1-12 min, and consisted of two solvents, A: 0.1% formic acid, 2mM ammonium acetate in water:methanol at 95:5 v/v, B: 0.1% formic acid, 2 mM ammonium acetate in methanol:acetonitrile at 10:90 v/v. The initial concentration of solvent B was 55% followed by a linear increase to 70% B in 3 min, then to 100% B in 2.5 min, held for 4 min, decreased back to starting 55% B over 0.1 min, and then held for 2.5 min. Data were

acquired using Analyst 1.6.2 and analyzed with Multiquant 3.0.3 software (SCIEX, Framingham, MA). Sample values were calculated from standard curves generated from the peak area ratio of the analyte to internal standard versus the analyte concentration that was fit to a linear equation with 1/x weighting. Analytical measurement range was 5-1,000 ng/mL homogenate.

7-Ketocholesterol in liver and plasma

After determining that 7-ketocholesterol was of primary interest from the cortical tissue results, liver tissue samples, homogenized similarly in 1x PBS, and plasma samples were assayed for free 7-ketocholesterol levels, similarly to the above method but without the saponification step due to matrix interference determined in pilot liver and plasma samples.

Hippocampal cholesterol analyses

For hippocampal cholesterol analyses, lipids were extracted from hippocampal homogenates by chloroform:methanol (2:1) extraction. Internal standard was added and the samples were dried and saponified with alcoholic KOH and extracted into hexane. Samples were derivatized with N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Thermo-Scientific, Rockford, IL) and cholesterol concentration was measured by capillary column gas chromatography on an Agilent (Santa Clara, CA) gas chromatograph (Model 6890N) with a ZB1701 column (30 m, 0.25 mm ID, 0.25 μm film; Phenomenex, Torrance, CA) and a FID detector. An internal standard (epicoprostanol; Sigma, St. Louis, MO) and an authentic cholesterol standard (Steraloids, Newport, RI) were used for calibration.

Sterol panel in hippocampal tissue

Gas chromatography-mass spectrometry (GC-MS) was used to measure cholestanol, desmosterol, and lanosterol. An internal standard (epicoprostanol) was added to hippocampal

homogenates and sterols were extracted by chloroform:methanol (2:1) extraction. Extracts were dried and sterols were saponified by the addition of ethanol/KOH, incubated at 37°C for one hour and the aqueous phase was extracted twice with hexane. Concentrations of the trimethylsilyl ether derivatives of sterols were measured using GC performed with a ZB1701 column (Phenomenex, Torrance, CA) coupled to a mass spectrometer (Agilent GC 6890N and MS 5975; Santa Clara, CA). Mass spectra were collected in selected ion mode with m/z= 355 and 370 ions monitored for epicoprostanol internal standard (quantifying and qualifying ions respectively), m/z = 393.2 and 498.2 ions for lanosterol, m/z = 343.3 and 441.5 ions for desmosterol, and m/z = 458.5 and 255.3 ions for lathosterol. Calibrants were generated using authentic standards (cholestanol, desmosterol, and lathosterol from Avanti Polar Lipids, Alabaster, AL). Analyte concentrations were calculated across the range 0.04–3.2 mg/dL using calibration curves generated by performing a least-squares linear regression for peak area ratios plotted against specified calibrant concentration. The lower limit of quantification was determined as the lowest spiked concentration in matrix for which the signal-to-noise ratio was ≥ 5. The between-run precision of the assay was determined to be < 20% relative standard deviation. Due to method sensitivity and sample amount limitations, sterols were assessed only in male hippocampal tissues and oxysterols were only analyzed in female cortical tissues.

Statistical analyses

The experimenter was blind to genotype and sex throughout the experiment. Behavioral and biochemical data are reported as mean ± standard error of the mean and were analyzed using SPSS v25 (IBM, Armonk, NY, USA). GraphPad software v.8.2.0 was used to visualize findings (Prism, La Jolla, CA, USA). Multi-factorial analyses of variance were used, including genotype, sex, and stress exposure as factors with Sidak's correction for multiple comparisons. Although stress exposure was not applicable during baseline measures because these measures were taken

before CVS exposure, it was still included in analyses to account for incidental baseline differences between groups. For activity monitoring data, sexes were collapsed during Week 2 (*i.e.* the week of CVS exposure) when software malfunction reduced group sizes. For water maze training data, mean swim speed was included as a covariate. For Western blot data, blot was included as a covariate for analyses to account for differences in staining. Other tissue analyses were assessed within the same assay run. Estimated marginal means were used for pairwise comparisons. Repeated measures were used when necessary and when Mauchly's test of sphericity was significant, Greenhouse-Geisser corrections were applied. Significance was set at $p \leq 0.5$.

Results

Statistical analyses for the main figures are listed in **Supplementary Tables 2.1 – 2.7**. Significant effects are discussed further below.

Home cage activity and body weights

Due to the impact of PTSD symptoms such as nightmares and altered arousal levels, we measured home cage activity as a non-invasive way to assess circadian activity throughout the experiment (experimental timeline shown in **Figure 2.1A**). Since mice are nocturnal and primarily sleep in the light phase while more active in the dark phase, we assessed home cage activity during the light and dark phases as separate outcomes. Average activity during the light phase of baseline week showed significant sex x genotype interactions (**Suppl. Figure 2.3A.** $F_{2,63} = 4.052$, $p = 0.022$). During the dark phase, females moved more than males (**Suppl. Figure 2.3B**, $F_{1,64} = 4.078$, $p = 0.048$). In addition, E3 mice were more active than E4 mice in the dark phase ($F_{2,64} = 4.20$, $p = 0.019$; pairwise comparison E3 vs E4: $p = 0.032$) and E3 females moved more than E3 males in the dark phase (sex x genotype $F_{2,64} = 3.49$, $p = 0.036$). Although prior to

CVS exposure, mice in the CVS group moved more during the dark phase than control mice ($F_{1,64}$ = 8.00, p = 0.006).

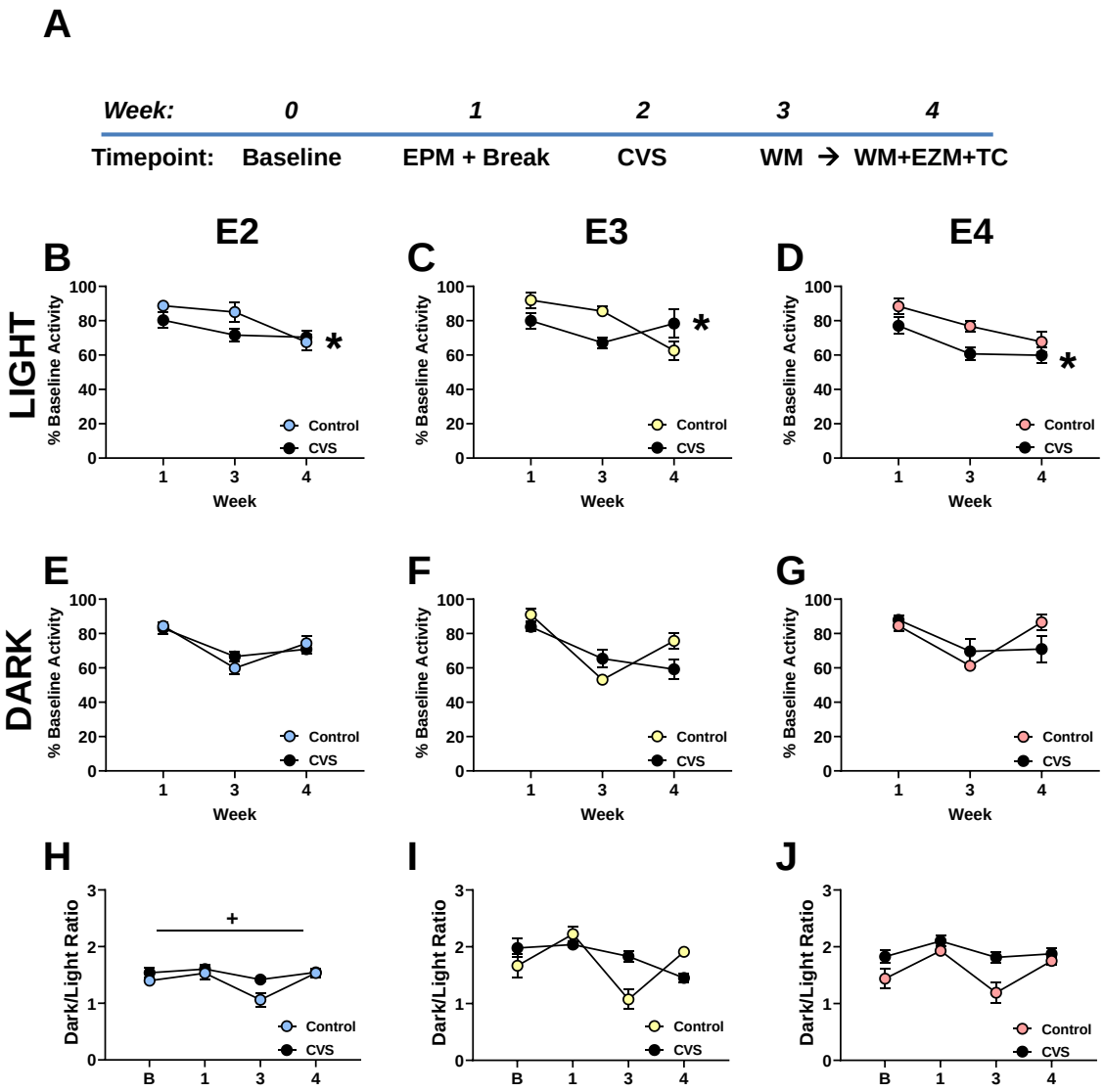

Figure 2.1 A) Experimental design. EPM = Elevated Plus Maze. CVS = Chronic Variable Stress. WM = Water Maze. EZM = Elevated Zero Maze. TC = Tissue Collection. **B-D**) Percent baseline home cage activity during the light cycle throughout the experiment shows that CVS exposure resulted in overall less activity compared to controls (*p < 0.05). **E-G**). Percent baseline home cage activity during the dark cycle was not altered by genotype or CVS exposure. **H-J**) Activity was also assessed a ratio of activity during the light and dark cycles, which showed E2 mice overall had lower dark/light ratios (+p < 0.05). B = Baseline. Sexes were collapsed for the figure. Symbols: + refers to pairwise comparison of genotype effect, * refers to CVS effect.

Due to these baseline differences, activity was further assessed by normalizing to baseline activity so that individual mice could serve as their own control (**Figure 2.1 B-G**). Since

CVS exposure occurred during the light phase of Week 2 and involved disruptions to the mice's home cage in the CVS groups, Week 2 was left out of analysis. During the light phase, mice overall showed decreased activity over time as measured by the weekly average as a continuous variable ($F_{1.74, 78.13} = 10.54$, $p < 0.001$) suggesting habituation to the change in housing conditions, which was also influenced by sex x genotype ($F_{3.48, 69.60} = 2.88$, $p = 0.035$); this is driven by a within-subjects effect of sex in which the E2 females showed less habituation over the experiment than E2 males. CVS-exposed mice were less active than controls ($F_{1, 40} = 7.68$, $p = 0.008$). There were no other significant main effects due to sex or genotype. During the dark phase, there was a decrease in activity during week 3, but also a significant increase in activity at the next time point ($F_{1.50, 59.87} = 89.24$, $p < 0.001$). This was influenced by genotype as activity in E3 mice remained lower even during week 4 (Week x Geno: $F_{2.99, 59.89} = 3.98$, $p = 0.012$).

Activity was also assessed as a ratio of dark to light activity (**Figure 2.1 H-J**). Analysis showed an effect of week ($F_{2.28, 104.90} = 9.81$, $p = 0.001$) that was affected by genotype ($F_{4.56, 104.90} = 3.37$, $p = 0.009$) as well as by group ($F_{2.28, 104.90} = 3.447$, $p = 0.030$). Interestingly, activity level in all CVS groups did not vary across weeks, whereas the controls decrease in activity during Week 3 and recover by Week 4. This may be due in part to the novelty of the increased handling for the control mice during water maze testing after previously being undisturbed while the CVS groups experienced more handling due to CVS exposure. E2 mice exposed to CVS appeared to show less change in dark phase activity over the weeks compared to E2 controls although this week x genotype x group interaction did not reach significance ($F_{4.56, 104.90} = 2.092$, $p = 0.078$). E2 mice also showed lower dark/light ratios overall ($F_{2, 46} = 22.942$, $p < 0.001$: pairwise comparisons E2 vs E3: $p < 0.001$, E2 vs E4: $p < 0.001$). These data show that CVS exposure altered activity during the light phase overall and that E2 mice demonstrated lower overall activity, *i.e.* dark/light ratio, compared to E3 and E4 mice.

Previously, male apoE KO mice have shown increases in food intake and body weights as well as decreased brown and fat deposits that were seen in 18 month old but not 6 month old mice (Raber et al., 2000). People with PTSD also tend to have problems with weight gain and obesity (Suliman et al., 2016), but this has not yet been assessed for apoE isoform-dependence. We thus assessed body weights throughout the experiment. Baseline body weights (**Suppl. Figure 2.4**) were significantly higher in males than females as expected ($F_{1, 140} = 213.56$, $p < 0.001$) and E2 mice weighed more than E3 mice, which weighed significantly more than E4 mice regardless of sex (main effect of genotype: $F_{2, 140} = 57.35$, $p < 0.001$; pairwise comparisons: E2 vs E3: $p < 0.001$, E2 vs E4: $p < 0.001$, E3 vs E4: $p < 0.001$). There was also a significant interaction between sex x genotype x group ($F_{2, 140} = 10.16$, $p < 0.001$). Consequently, we analyzed % baseline body weight over the rest of the experiment (**Figure 2.2**). Males and females showed different changes in % baseline body weight over the course of the 5 weeks of the experiment (**Figure 2.2 A-F**. Week x sex: $F_{2.97, 410.43} = 7.29$, $p < 0.001$) as well as overall different % baseline bodyweights across the experiment ($F_{1, 138} = 6.90$, $p = 0.010$) and were hence assessed separately.

Females increased in body weight throughout the experiment (**Figure 2.2 A**,**C**,**E**. $F_{3.08, 206.37} = 21.59$, $p < 0.001$), which was influenced by genotype ($F_{6.16, 206.37} = 8.89$, $p < 0.001$). Specifically, E2 mice showed the least growth over time (**Figure 2.2 A**. $F_{2, 67} = 5.85$, $p = 0.005$, pairwise comparisons E2 vs E3: $p = 0.004$). Males on the other hand demonstrated less consistent growth over weeks of the experiment, *i.e.* all groups dramatically shift during Week 2, (**Figure 2.2 B**,**D**,**E**. $F_{2.59, 183.80} = 7.075$, $p < 0.001$) that was affected by genotype ($F_{5.18, 183.80} = 2.89$, $p = 0.014$), group ($F_{2.59, 183.80} = 14.15$, $p < 0.001$), and a genotype x group interaction ($F_{5.18, 183.80} = 3.95$, $p = 0.002$). There was also a sex x genotype x group interaction in the overall statistical model ($F_{2, 71} = 5.40$, $p = 0.007$).

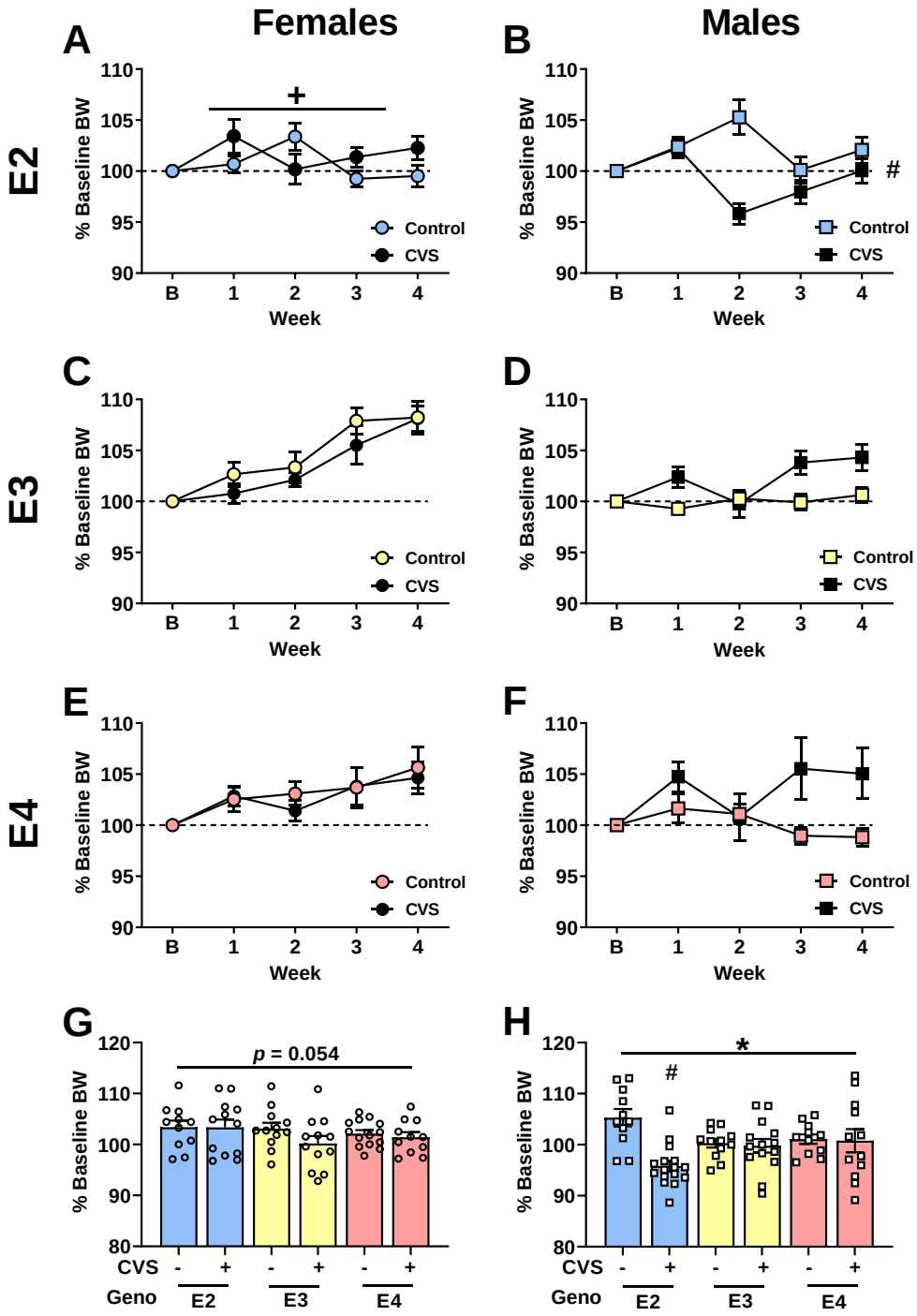

Figure 2.2 Body weight measurements throughout the experiment. B = Baseline. **A,C,E**) Female mice overall gained body weight throughout the experiment; however, this growth was not seen in either female E2 group (+$p < 0.05$). **B,D,F**). In males, percent baseline bodyweights changed throughout the weeks of testing. These within-subject changes over the weeks were influenced by genotype and CVS exposure, as well as by an interaction between genotype and CVS exposure (# $p < 0.05$). Percent baseline bodyweight during week 2, the week of CVS exposure, was assessed separately to better understand these interactions. While females exposed to CVS showed a trend to lower percent baseline bodyweights ($p = 0.054$) (**G**), CVS exposed males showed lower percent baseline bodyweights compared to controls (*$p < 0.05$) which was due to the lower percent baseline bodyweight in E2 CVS males compared to E2 control males (**H**. genotype x group interaction: #$p < 0.05$). Symbols: + refers to pairwise comparison of genotype effect, * refers to CVS effect, # refers to genotype x CVS interaction.

Due to these interactions, we further analyzed the % baseline body weights with the sexes separated during week 2, which was the week during CVS exposure. Female mice showed no significant genotype differences, but there was a trend towards decreased weight in CVS groups overall (**Figure 2.2 G**. $F_{1, 67} = 3.84$, $p = 0.054$). Males showed both a significant difference between groups (**Figure 2.2 H**. $F_{1, 71} = 6.90$, $p = 0.011$) as well as a genotype x group interaction ($F_{2, 71} = 7.39$, $p = 0.001$) driven by the difference between lower % baseline bodyweight in E2 CVS mice compared to E2 control mice.

Spatial learning and memory

After all groups were exposed to CVS or left alone as controls, mice were tested for spatial learning and memory using the water maze. The testing paradigm used is shown in **Figure 2.3 A**. Since swim speed during a timed training trial may alter the interpretation of a mouse's performance in learning the task, average swim speeds were assessed through each of the different training session types: visible, hidden, reversal 1 and reversal 2 (**Figure 2.3 B).** Sex was not a significant factor ($F_{1,138} = 1.744$, $p = 0.189$), so data in panels B-E are shown collapsed across sex. Session type affected average swim speed ($F_{2.67, 369.029} = 43.87$, $p < 0.001$) with mice faster during hidden and reversal sessions than visible training sessions. E4 mice swam slower than E3 mice (Genotype: $F_{2,138} = 5.48$, $p = 0.005$, pairwise comparisons: E3 vs E4: $p = 0.004$). There were no significant differences between E2 mice and the E3 or E4 mice. Swim speed during each session type was thus used as a covariate when latency to find the target platform was analyzed as a performance measure. During all types of training sessions (visible, hidden, reversal 1, reversal 2), there was a main effect of genotype (Visible: $F_{2, 137} = 3.78$, $p = 0.025$; Hidden: $F_{2,137} = 5.590$, $p = 0.005$; Reversal 2: $F_{1,137} = 6.28$, $p = 0.013$; Reversal 2: $F_{1,137} = 5.45$, $p = 0.005$).

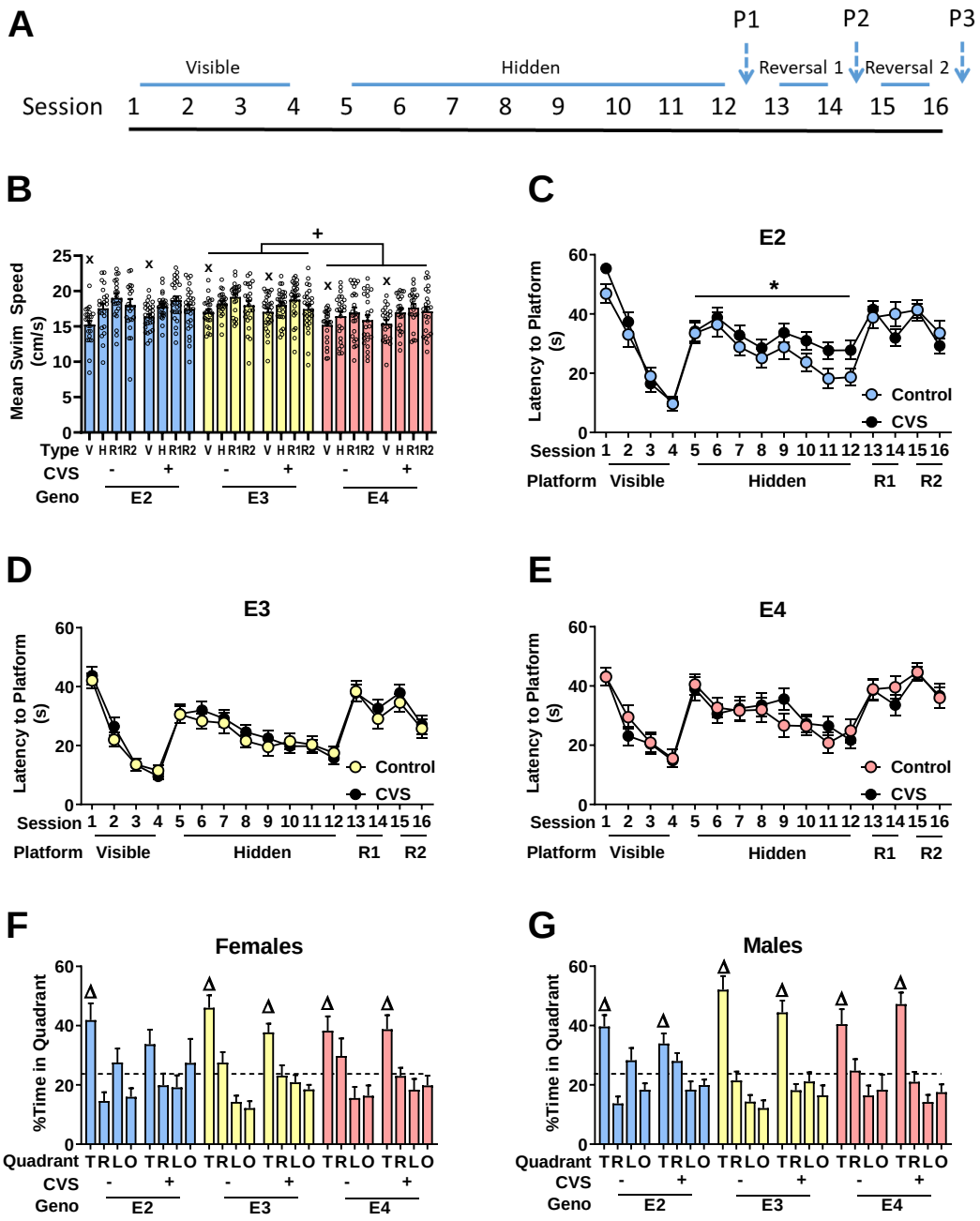

Figure 2.3 Spatial learning and memory were assessed in the water maze. **A**) Timeline of session types over the 9 days of testing. P = Probe. **B**) E4 mice swam the slowest throughout testing (+$p < 0.05$). Mice overall swam slower during visible platform trials compared to hidden and reversal trials ($^{x}p < 0.05$). V = Visible, H = Hidden, R= Reversal. **C-E**) Latency to locate the target platform is shown for the different genotypes. Sexes are shown collapsed. E2 mice (**C**) were the only genotype to be significantly affected by CVS throughout any of the sessions. E2 CVS exposed mice improved less over hidden training session compared to controls (*$p = 0.05$) and E2 control mice did not improve during the first reversal location testing, compared to E2 CVS mice (*$p < 0.05$). E3 (**D**) and E4 (**E**) mice did not show significant differences due to CVS. **F,G**). Percent time in each quadrant is shown for probe 1. T = Target, R = Right, L = Left, O = Opposite. Female E2 mice exposed to CVS was the only group that failed to show a preference for the target quadrant (Δ $p < 0.05$). Data for **B-E** are shown with sexes collapsed. Symbols: + refers to pairwise comparison of genotype effect, * refers to CVS effect, ^{x} refers to effect of session type, Δ refers to effect of quadrant within subjects.

In order to simplify the resulting interactions, further analyses were done with genotypes separated. During visible training sessions, there was an interaction between session x sex x group in E2 mice in the latency to target platform ($F_{2.52,171.118} = 3.62$, $p = 0.022$). Neither the E3 nor the E4 mice showed significant differences in the latency to the target platform due to sex or group. (**Figure 2.3 C-E**). During hidden training sessions for E2 mice, swim speed was a significant covariate ($F_{1,45} = 4.72$, $p = 0.035$). There was also a trending effect of CVS ($F_{1,45} = 3.96$, $p = 0.053$) and no effect of sex ($F_{1,45} = 0.67$, $p = 0.42$). One E2 female control mouse failed to learn the task and timed out during 29 of the 32 trials, the most in all groups. To see if this individual influenced the data, we removed it and collapsed the sexes. CVS E2 mice showed poorer performance overall compared to E2 controls (**Figure 2.3 C.** $F_{1,45} = 4.055$, $p = 0.050$). While E3 males did slightly better than E3 females ($F_{1,47} = 4.064$, $p = 0.050$), neither E3 nor E4 mice showed any differences due to CVS exposure. There were no significant differences during either reversal 1 or reversal 2 within each genotype group.

During the first probe trial, each group was assessed separately for preference to the target quadrant using the percent total time spent in each quadrant to assess spatial memory retention (**Figure 2.3 F-G**). Except for female E2 CVS mice, all mice showed a significant effect of quadrant (ANOVA: E2 female controls: $F_{3,30} = 4.32$, $p = 0.012$; E3 female controls $F_{3,33} = 18.15$, $p < 0.001$; E4 female controls $F_{3,33} = 4.31$, $p = 0.011$; E3 female CVS: $F_{3,39} = 7.78$, $p < 0.001$; E4 female CVS: $F_{3,30} = 4.86$, $p = 0.007$; E2 male controls: $F_{3,30} = 9.41$, $p < 0.001$; E3 male controls $F_{3,33} = 24.36$, $p < 0.001$; E4 male controls; $F_{1.71, 18.76} = 4.60$, $p = 0.028$; E2 male CVS: $F_{3,45} = 5.27$, $p = 0.003$; E3 male CVS: $F_{3, 39} = 12.64$, $p < 0.001$; E4 male CVS: $F_{3, 33} = 17.34$, $p < 0.001$). Pairwise comparisons within each group demonstrated that the significant effect of quadrant in each group (again, except the female E2 CVS mice) was due to more time being spent within the target quadrant (**Suppl. Table 2.4**). Mice across all groups failed to show preference

for the target quadrant during Probes 2 and 3 perhaps due to inadequate training for the new locations (**Suppl. Table 2.4**).

Changes in anxiety-like behavior and related physiological measures

Baseline anxiety-like behavior was measured in the elevated plus maze as avoidance of the open arms. E2 mice explored the open arms in the maze less than E3 mice suggesting higher levels of anxiety (**Figure 2.4 A**, $F_{2, 137} = 6.73$, $p = 0.002$; E2 vs E3: $p = 0.001$). Within genotype, sex differences were only seen in E3 mice, with females spending less time in the open arms compared to males (Sex x genotype interaction: $F_{2, 137} = 4.011$, $p = 0.020$).

After CVS exposure, there was again a difference due to genotype with E2 mice spending less time in the open areas of the zero maze overall (**Figure 2.4 B**, $F_{2, 137} = 5.75$, $p = 0.004$; E2 vs E3: $p = 0.007$, E2 vs E4: $p = 0.023$). Female mice explored the open areas of the zero maze less than males ($F_{1, 137} = 6.23$, $p = 0.014$). There were no significant effects associated with CVS exposure in how long the mice spent in the open arms of the elevated zero maze.

Corticosteroid response after stress exposure is lower in those with PTSD than controls (Grossman et al., 2003). This is in contrast to the normally elevated levels seen in healthy controls after an acute stressor. Plasma corticosterone, the major corticosteroid in rodents, was measured in samples taken immediately after mice completed the elevated zero maze, intended as a minor stressor (**Figure 2.4 C**). There was an interaction between sex and CVS condition; female CVS mice had lower plasma corticosterone levels compared to controls whereas male CVS mice had higher plasma corticosterone levels compared to controls ($F_{1, 68} = 4.82$, $p = 0.032$). E2 mice had higher plasma corticosterone levels compared to E3 and E4 ($F_{2, 68} = 8.70$, $p < 0.001$; pairwise comparisons: E2 vs E3 $p = 0.001$, E2 vs E4 $p = 0.005$), and E2 females had significantly higher plasma corticosterone levels than other groups (sex x genotype interaction: $F_{2, 68} = 5.55$, $p = 0.006$). There was also a sex x CVS condition interaction ($F_{1, 68} = 5.25$, $p = 0.025$) driven by

decreased levels of corticosterone in female CVS-exposed mice. This suggests that E2 mice respond to an anxiety-provoking maze in a sex-dependent manner.

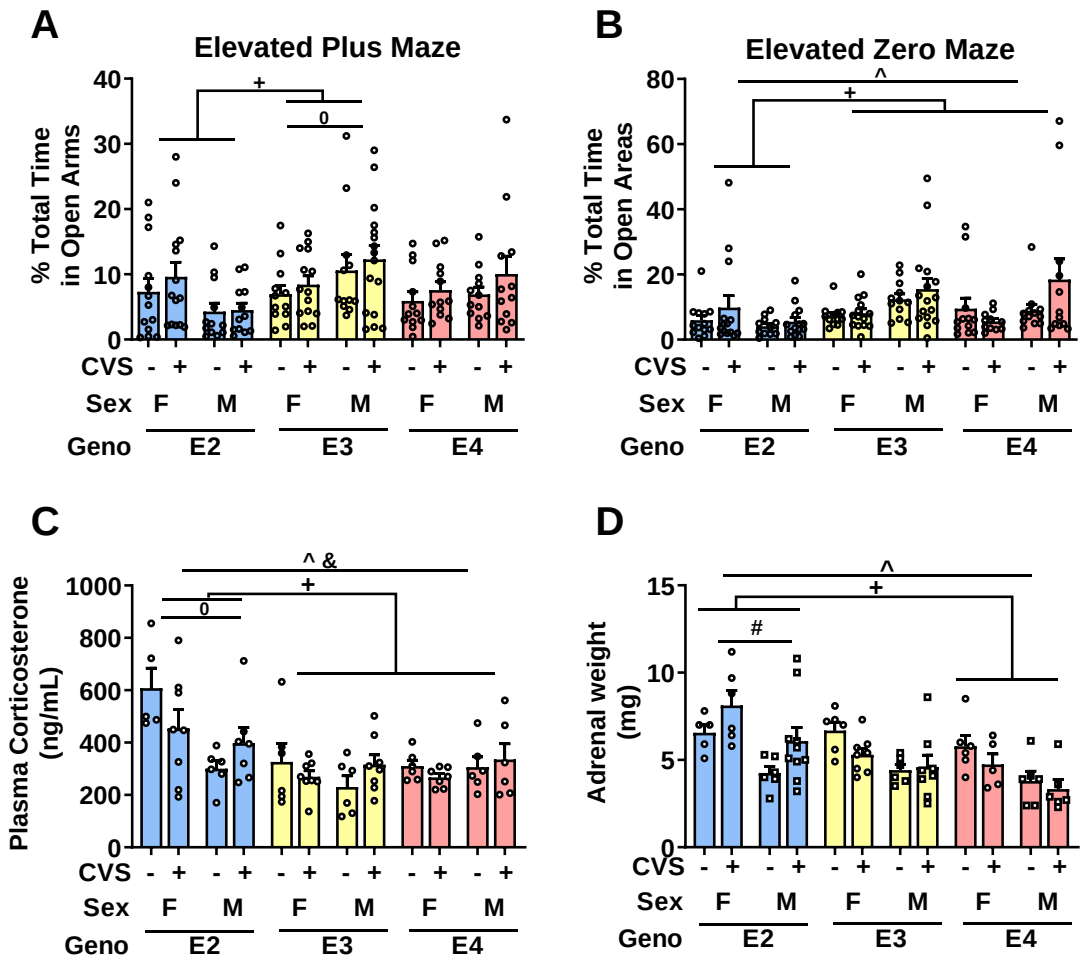

Figure 2.4 Behavioral and physiological anxiety-related measures. **A**) E2 mice spent significantly less time in the open arms of the elevated plus maze compared to E3 mice ($+p < 0.05$). E3 females also spent less time than E3 males ($\wedge p < 0.05$). **B**) E2 mice again explored the least time in the open areas of the elevated zero maze ($+p < 0.05$). Males explored the open areas more than females ($\wedge p < 0.05$). **C**) Plasma corticosterone levels after a mild stressor (elevated zero maze) showed that females had higher levels compared to males ($\wedge p < 0.05$) which was driven by the difference in E2 mice ($^{0}p < 0.05$). levels seen in female mice exposed to CVS ($^{\&}p < 0.05$). **D**) Adrenal glands were dissected after all behavioral testing. Females had heavier adrenal glands than males ($\wedge p < 0.05$). E2 mice had the heaviest overall ($+p < 0.05$) and CVS exposed E2 mice had larger glands compared to their genotype-matched controls ($*p < 0.05$). Symbols: + refers to pairwise comparison of genotype effect, ^ refers to sex effect, * refers to CVS effect, # refers to genotype x CVS interaction, ^{0} refers to sex x genotype interaction, $^{\&}$ refers to sex x CVS interaction.

Following behavioral and cognitive testing, adrenal glands were removed and weighed as a correlate measure of glucocorticoid secretion (J. P. Herman et al., 1995; Ulrich-Lai et al., 2006)

(**Figure 2.4 D**). Females had heavier adrenal glands (both sides) compared to males ($F_{1, 66}$ = 23.67, $p < 0.001$) and E2 mice had larger adrenal glands compared to E4 mice ($F_{2, 66} = 8.08$, p = 0.001; pairwise comparison E2 vs E4: $p < 0.001$). Furthermore, E2 mice exposed to CVS showed larger adrenal glands compared to their genotype-matched controls (ANOVA: Genotype x group interaction: $F_{2, 66} = 4.67$, $p = 0.013$).

Level of target proteins

In order to address the potential influence of apoE isoform as a primary component of cholesterol metabolism in the brain as well as its influence on the stress response, protein levels of apoE, LDLR, and GR were analyzed in brain regions and peripheral tissues of interest and normalized to total protein (see methods). Although sex was included in the statistical model, as is true for the other measures, it was largely insignificant within our protein analyses. In the frontal cortex, apoE, LDLR, and GR levels were not significantly different due to sex, genotype, or CVS **(Suppl. Table 2.8)**.

Since LDLR levels influence apoE and apoE appears to modulate the glucocorticoid system, we normalized both LDLR and GR to apoE protein levels to assess if there was a relationship between these markers and CVS exposure that was dependent on the amount of apoE. There was an apoE isoform-dependent effect on GR/apoE in the cortex (**Figure 2.5 A-B**. $F_{2, 47} = 18.26$, $p < 0.001$; pairwise comparisons E2 vs E3: $p = 0.003$; E2 vs E4: $p < 0.001$; E3 vs E4: $p = 0.052$). Furthermore, CVS mice had higher GR/apoE ratios versus controls ($F_{1, 47} = 19.70$, $p < 0.001$) as observed in E3 and E4 mice (genotype x group: $F_{2, 47} = 4.33$, $p = 0.019$). Analyses of LDLR/apoE ratios revealed similar differences in the cortex, specifically that E2 showed the lowest ratios (**Figure 2.5 C**. $F_{2,47} = 4.85$, $p = 0.012$: E2 vs E4 $p = 0.009$). In addition, CVS mice had larger ratios compared to control counterparts ($F_{1,47} = 13.39$, $p = 0.001$) which was again absent in E2 mice ($F_{2, 47} = 3.85$, $p = 0.028$).

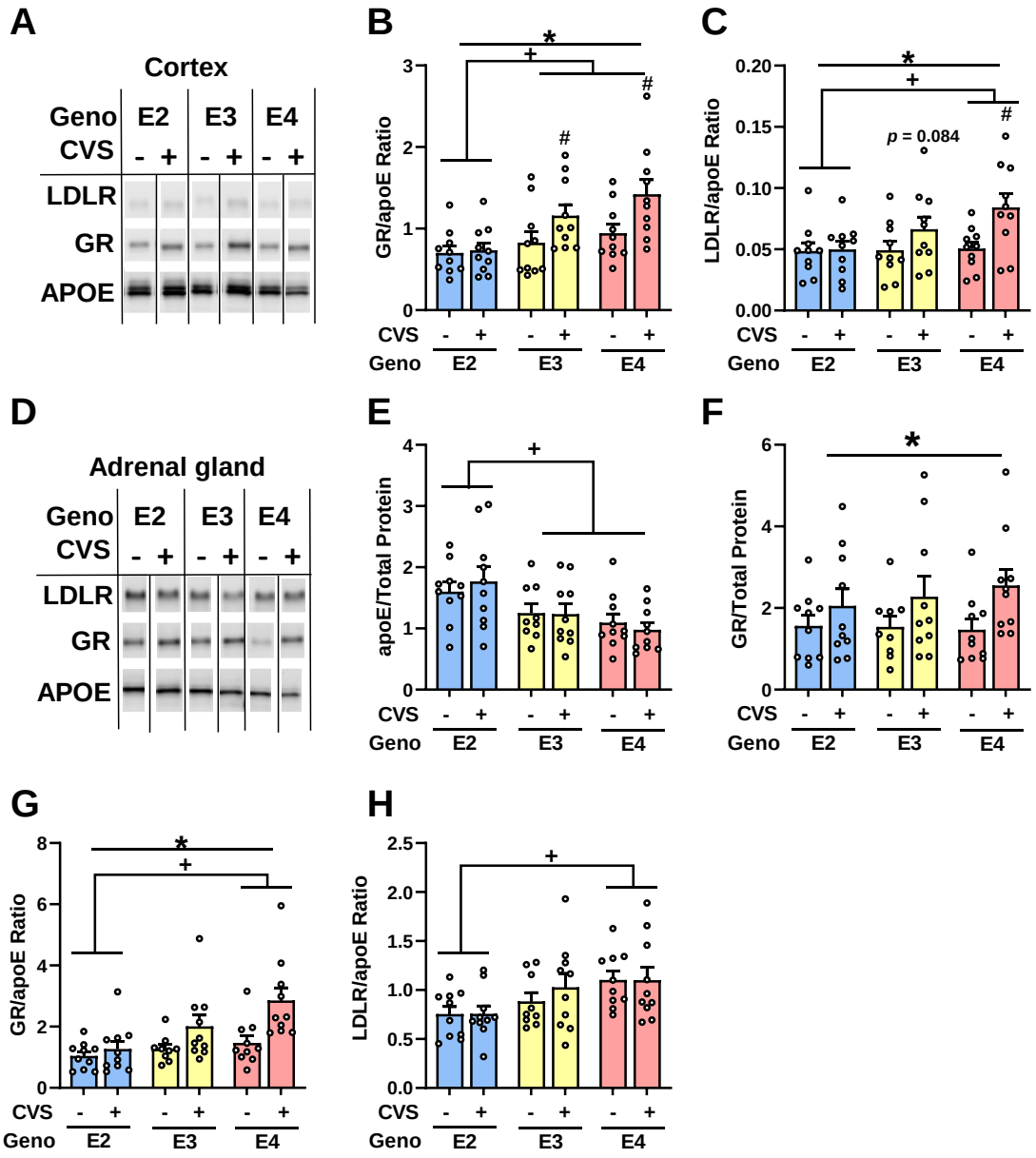

Figure 2.5 Western blot analyses of APOE, LDLR, GR in cortex and adrenal gland tissue. Representative blot showing target proteins (LDLR and apoE were imaged in the same channel, GR in another). Bands of interest were sliced and placed next to each other for clarity. Full-length blots, including total protein blots, are presented in **Supplementary Fig. 2.2 A**) Representative cortex blot. **B**) E2 mice had the lowest GR/APOE ratio (+$p < 0.05$) and E3 and E4 CVS-exposed mice showed larger ratios compared to controls (#$p < 0.05$). **C**) LDLR/apoE ratios had similar differences to the GR/apoE ratios (+$p < 0.05$, #$p < 0.05$). **D**) Representative adrenal gland blot. **E**) E2 mice had the highest apoE levels in adrenal glands (+$p < 0.05$). **F**) GR in the adrenal gland was higher in CVS-exposed mice (*$p < 0.05$). **G**) GR/apoE ratios were lowest in E2 mice (+$p < 0.05$). CVS-exposure resulted in higher GR/apoE ratio compared to controls (*$p < 0.05$). **H**) E2 mice had the lowest LDLR/apoE ratios (+$p < 0.05$). Symbols: + refers to pairwise comparison of genotype effect, * refers to CVS effect, # refers to genotype x CVS interaction.

Western blot analysis of adrenal glands (**Figure 2.5 D**) showed apoE-isoform dependent differences in apoE (**Figure 2.5 E.** $F_{2, 46} = 9.568$ $p < 0.001$: pairwise comparisons: E2 vs E3: $p = 0.018$, E2 vs E4: $p < 0.001$). Groups did not show differences in adrenal LDLR levels (**Suppl. Table 2.8**). GR levels were higher in CVS exposed mice compared to control mice (**Figure 2.5 F**. $F_{1, 46} = 6.37$, $p = 0.015$). GR/apoE ratios were also dependent on apoE isoform (**Figure 2.5 G**. $F_{2, 46} = 8.17$, $p = 0.001$: E2 vs E4 $p = 0.001$) with E2 levels being lower than E4. CVS groups were higher than controls ($F_{1, 46} = 13.74$, $p = 0.001$). There was also a sex x genotype interaction ($F_{1, 46} = 4.30$, $p = 0.019$). LDLR/apoE ratios were again dependent on apoE isoform (**Figure 2.5 H**. $F_{2, 46} = 8.29$, $p = 0.001$: pairwise comparison: E2 vs E3 $p = 0.001$). Hippocampal, medial prefrontal cortical, and liver tissues did not show significant differences between genotypes or CVS conditions (**Suppl. Table 2.8**).

Plasma apoE levels were analyzed, based on evidence for a positive correlation between plasma apoE levels and PTSD symptom severity (Nielsen et al., 2019). E2 mice showed higher levels of plasma apoE compared to E3 and E4 mice (**Figure 2.6 A**. $F_{2,52} = 81.780$, $p < 0.001$; E2 vs E3: $p < 0.001$, E2 vs E4: $p < 0.001$). Furthermore, females had higher apoE levels although this appears to be driven by E2 females (Sex: $F_{1, 52} = 4.59$, $p = 0.037$; Sex x genotype: $F_{2,52} = 3.70$, $p = 0.031$). CVS mice had lower levels compared to controls (Group: $F_{1,52} = 6.89$, $p = 0.011$), which was also driven by the decrease seen in E2 mice ($F_{2,52} = 6.16$, $p = 0.004$).

Within the cortex, all CVS groups had lower apoE levels compared to controls (**Figure 2.6 B**. $F_{1,58} = 48.91$, $p < 0.001$); sex and genotype were not found to be significant. This is in contrast to what we found using Western blot using the same samples which may be due to the denaturization of the protein used in Western blotting. MAP-2, a measure of synaptic density, levels showed no significant main effect of sex ($F_{1, 59} = 0.116$, $p = 0.735$). When sexes were collapsed, MAP-2 levels were higher in mice exposed to CVS ($F_{1, 65} = 58.57$, $p < 0.001$). There

was also a genotype x group interaction ($F_{2,65} = 3.34$, $p = 0.041$) from a markedly lesser increase in E2 CVS-exposed mice compared to E2 controls.

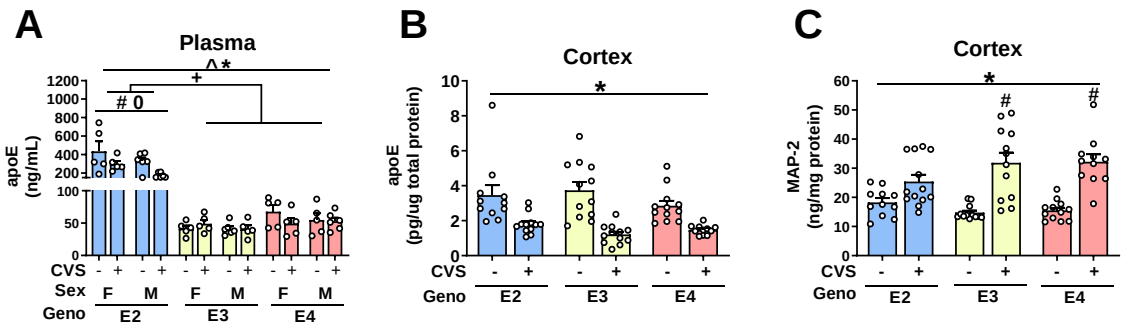

Figure 2.6 ELISA measures in plasma and cortex. **A**) Plasma levels of apoE were highest in E2 mice (+$p < 0.05$). Furthermore, E2 mice exposed to CVS had lower apoE levels compared to controls (*$p < 0.05$). Female E2 mice had more plasma apoE than male E2 mice ($^{0}p < 0.05$). **B**) In cortical tissue, CVS exposure led to lower apoE levels (*$p < 0.05$). **C**) Meanwhile, MAP-2 levels in the cortex were higher in mice exposed to CVS (*$p < 0.05$). Symbols: + refers to pairwise comparison of genotype effect, * refers to CVS effect, ^ refers to sex effect, # refers to genotype x CVS interaction, ^{0} refers to sex x genotype interaction.

Assessment of cholesterol metabolism

To examine the effect of apoE genotype and the interaction of stress (CVS) on cholesterol metabolism, we assessed cholesterol as well as 7 different sterol (cholestanol, desmosterol, and lathosterol) and oxysterols (24S-hydroxycholesterol, 25-hydroxycholetserol, and 27 hydroxycholesterol) in brain tissue of these mice (**Figure 2.7, Suppl. Tables 2.7 and 2.9**). This was done to better understand part of the changes in cholesterol metabolism associated with CVS exposure. Sterols were assessed only in male hippocampal tissues and oxysterols were only analyzed in female cortical tissues due to technical limitations. Of these sterols and oxysterols, 7-ketocholesterol was the only one significantly affected by CVS exposure (**Figure 2.7 A**). Two-way ANOVA revealed a significant main effect of CVS exposure in which CVS corresponded to higher 7-ketocholesterol cortical levels ($F_{1,26} = 6.53$, $p = 0.017$). There was also an interaction between genotype and group ($F_{2,26} = 4.72$, $p = 0.018$), which when evaluated using Sidak's multiple comparisons showed that E4 mice exposed to CVS had higher 7-ketocholesterol levels

than their genotype-matched controls ($p = 0.013$). This CVS-related difference was absent in E2 mice and trending in E3 mice ($p = 0.11$).

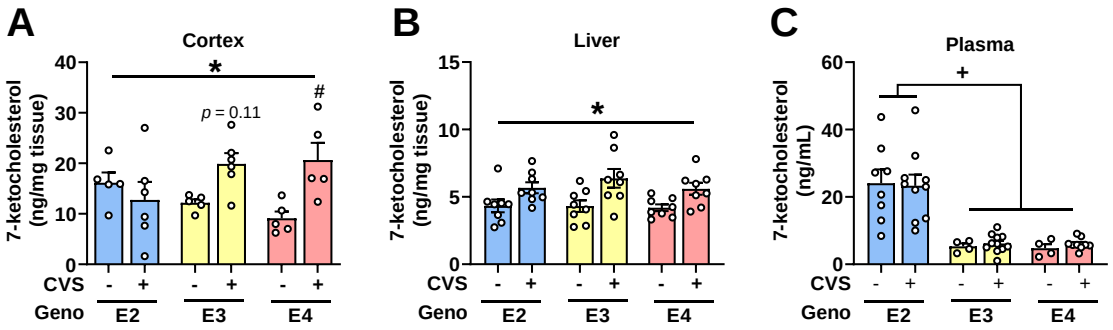

Figure 2.7 Levels of 7-ketocholesterol throughout the body. **A**). Female cortical tissue showed a genotype x group interaction in which only E4 mice exposed to CVS showed higher levels of 7-ketocholesterol compared to controls (#$p < 0.05$). **B**) CVS exposure was associated with higher 7-ketocholesterol levels (*$p < 0.05$) regardless of genotype or sex (shown collapsed). **C**) Plasma levels were highest in E2 mice (+$p < 0.05$, shown with sexes collapsed). Symbols: + refers to genotype effect, * refers to CVS effect, # refers to genotype x CVS interaction.

This striking genotype x CVS interaction led us to explore 7-ketocholesterol levels in liver, a major organ for cholesterol metabolism, and in the plasma. Exposure to CVS corresponded to higher levels of free 7-ketocholesterol in the liver regardless of genotype or sex (**Figure 2.7 B**. $F_{1, 36} = 16.13$, $p < 0.001$). This effect of CVS exposure was not seen in plasma samples; however, E2 mice regardless of sex or CVS exposure showed greater levels of 7-ketcholesterol compared to E3 and E4 mice (**Figure 2.7 C.** $F_{2,34} = 24.42$, $p < 0.001$). There were no significant effects of sex or CVS exposure on plasma levels of free 7-ketocholesterol.

Discussion

This study expands on previous work highlighting the role of apoE isoforms in modulating the behavioral and cognitive changes associated with stress exposure. Experimental aims included determining if sex differences exist in this association between apoE and stress and exploring what molecular mechanism may be related. Our findings show that CVS exposure results in long-term changes, specifically lower home cage activity during the light phase, the

inactive phase in mice, compared to controls, indicative of circadian rhythm disruptions. It is important to note that this reflects activity of both mice within a home cage; thus, it suggests that there may be more inactivity and less social interactions during the light phase that is punctuated by bouts of sleep. CVS exposure was also associated with downstream effects associated with lipid transport and metabolism, including decreased levels of apoE in cortical tissue and increased levels of 7-ketocholesterol in the liver providing evidence of changes related to cholesterol metabolism throughout the body.

Since LDLR and apoE have demonstrated inverse expression levels (Fryer et al., 2005), we analyzed whether the amount of apoE affected the relationship between LDLR and apoE genotype. LDLR normalized to apoE was lowest in adrenal glands of E2 mice. In the cortex, LDLR/apoE ratios were higher in E3 and E4 CVS-exposed mice compared to genotype matched controls, but this CVS difference was absent in E2 mice. Taken with the changes in 7-ketocholesterol, these data suggest that LDLR may be functionally important in the stress response. Consistent with this notion, identification of the *LDLR* SNP, rs5925, showed predictive value of PTSD symptom severity and prevalence 6 months after the 2008 Wenchuan earthquake in adolescents (Chen et al., 2020). Furthermore, the functional effects of LDLR, such as lipid internalization and cholesterol utilization, may be more relevant to study. Recent studies show that lipoprotein profile, in particular low-density lipoprotein cholesterol (LDL-C), is higher in those with PTSD than healthy patients (Jergovic et al., 2015) although other data suggests it may be decreased in PTSD (Vries et al., 2017). While these studies do not assess apoE genotype in relation to LDLR, previous studies showing that LDLR KO increases apoE suggest that these two factors are intrinsically tied together. I further discuss how my findings relate to these clinical studies in Chapter 3.

Human apoE expression in mouse Y1 adrenal cells results in decreases in glucocorticoid secretion and suggests that apoE may modulate cholesterol utilization (Reyland et al., 1991). This

has been further supported with findings using apoE KO mice that demonstrate increased corticosterone levels after an acute restraint stress compared to wildtype (Raber et al., 2000). We found that plasma corticosterone levels in E2 mice were overall higher than E3 and E4 mice. Moreover, female E2 mice exposed to CVS showed lower corticosterone levels compared to genotype-matched controls after a mild stressor (a novel maze). This is consistent with the excessive negative feedback found in PTSD patients (Yehuda, 2002; Zohar et al., 2011). However, this genotype-specific response has not been characterized in humans thus far to our knowledge. Furthermore, this is in contrast to previous research showing that E2 male mice exposed to CVS showed higher levels of apoE after an acute restraint stress (Johnson et al., 2015). This may be due to the type of stressor used (*i.e.* restraint vs novel maze) or the length of time between CVS and the additional stressor.

The increases in GR/apoE in E3 and E4 mice exposed to CVS suggests that more GR may be expressed in response to CVS and that this may be dependent on apoE isoform. LDLR normalized to apoE levels showed a similar difference corresponding to CVS further suggesting a CVS-dependent response. Similar patterns in the adrenal gland emphasize this relationship between GR and apoE is mediated for apoE isoform and that E2 mice may lack the increase in GR relative to apoE needed to signal additional feedback.

This study supports previously noted differences in the adrenal weights of apoE TR mice exposed to CVS (Johnson et al., 2015) that may be a result of decreased feedback inhibition. In the water maze, E2 mice are also susceptible to CVS-related impairments in spatial learning E2 females, but not males, fail to show spatial memory retention. In our previous study, male E2 mice lacked target preference assessed immediately after exposure to fear stress (Johnson et al., 2015), suggesting that this effect may be transient. Consistent with transient effects, male E2 mice decreased in percent baseline body weight during the week of CVS but regained their body weight afterwards. Both E3 and E4 males showed increased body weight after CVS exposure.

These data suggest that weight gain seen in PTSD patients may be apoE genotype-dependent. Food intake was not measured in this particular study but would be informative to help determine if these weight changes are due to feeding behaviors or metabolism. These peripheral effects may result from separate mechanisms than what occurs in the brain, since both apoE and cholesterol do not cross the BBB and exist largely as separate pools in the periphery and the CNS. It may also result from top-down activity in the brain either as changes in behavior or signaling cascades, although a direct link will need to be explored in additional future experiments.

Our findings in female cortical tissue highlight another downstream product formed by nonenzymatic autooxidation of cholesterol, 7-ketocholesterol, that may serve as a signaling molecule that crosses the BBB and modulate glucocorticoid synthesis. Jenner et al. show that the composition of levels in total (free and unesterified) sterols and oxysterols in young (8 weeks old) male apoE TR mice is fairly similar among E2, E3, and E4 mice but changes dependent on genotype by 1 year of age (Jenner et al., 2010). Young E2 mice had higher whole brain levels of lathosterol but lower levels of 7-ketocholesterol compared to E3 and E4 mice at 8 weeks of age. While the difference in lathosterol levels persisted at 1 year of age, the difference in 7-ketocholesterol did not, although the level appeared higher in E2 than E3 and E3 than E4. They conclude that E2 has a significant effect on cholesterol synthesis and metabolism (perhaps via upregulation of the lathosterol pathway) as well as cholesterol oxidative damage. Take with our current study findings, the stress response of cholesterol metabolism in young animals may follow a unique pathway compared to what is seen in the context of aging. That is to say, cortical 7-ketocholesterol does not increase in E2 female mice in response to stress as it does with age while in E3 and E4 levels of 7-ketocholesterol are only changed in the stress response in young animals.

Plasma analysis did not follow the same pattern seen in cortical tissue but showed that E2 mice having the highest levels regardless of CVS. In the liver, CVS exposure corresponded to

increased 7-ketocholesterol regardless of genotype, which may contribute to systemic levels of 7-ketocholesterol. While these measures reflect free 7-ketocholesterol compared to total 7-ketocholesterol in cortical tissue, previous data suggest there are esterification of 7-ketocholesterol is poor and thus contributes little to total levels (Lee et al., 2015). Furthermore, 7-ketocholesterol can move through the BBB, but there are additional mechanisms regulating its degradation and excretion that differ between tissues and circulation arguing for more research to understand the relationship between peripheral and central levels of 7-ketocholesterol.

Differences in E2 mice vs E3 and E4 mice regardless of CVS exposure highlight baseline differences in the apoE TR mice. After CVS, male E2 mice were reported to have higher activity during the light phase compared to E3 and E4 mice and greater anxiety-like behavior (Johnson et al., 2015). This was not replicated in the current study. Salient differences between the studies may have contributed to these divergent results: 1) This study involved males and females, which were tested simultaneously, whereas only males were assessed previously. 2) Mice were pair-housed for the entire duration of this study with a littermate except for when CVS mice were singly housed for 5 days during exposure to stressors. Females may be more affected by the effects of social isolation (Matsuda et al., 2018; Senst et al., 2016), which may play a role in the severe memory impairment seen in females (see Chapter 3 for further discussion). In this study, we used two common, similar tests of anxiety-like behavior (elevated plus and zero mazes) to assess baseline and post-CVS anxiety-like behavior. Both mazes were used to avoid the potential confound of habituation to the maze itself. Results from both mazes showed that E2 mice spent less percent total time in the open areas of both mazes and detected sex differences. Individual data also positively correlated with each other (Spearman's $r = 0.390$, $p < 0.001$). Considering the consistency of these results, CVS did not elicit anxiety-like behavior in this study, but this may be due to floor effect (*i.e.* that all mice demonstrated anxiety-like behavior) that limited our ability to detect CVS-dependent differences.

Although chronic stress has been shown to reduce dendritic complexity, we found evidence that CVS corresponds to increases in cortical MAP-2 levels. Chronic restraint stress, but not chronic variable stress, resulted in increased dendritic arborization in the hippocampus and amygdala (Vyas et al., 2002). In aged mice and nonhuman primates, MAP-2 was increased in association with age (Haley et al., 2010). Our present findings with MAP-2 in the cortex suggest that there may be regionally-specific differences in these changes; however, this will need to be addressed with future studies.

In summary, these data highlight that apoE TR mice show isoform-specific responses to CVS, behaviorally, cognitively, and physiologically. E2 mice exposed to CVS showed impaired spatial learning and memory compared to their genotype-matched controls. Female E2 mice showed greater memory impairment when exposed to CVS, which was not seen in male E2 mice. This sex difference however, was not correlated with LDLR and GR levels and cholesterol-related measures, and oxysterols were only assessed in the female cortex. This incongruency between protein measures and cognitive impairment argues that there are other factors contributing the exacerbated phenotype in females that need to be explored further. Female E2 mice appeared to lack the typical response, *i.e.* an increase corresponding to CVS compared to control conditions, seen in E3 and E4 mice in cortical 7-ketcholesterol levels as well as GR/apoE levels in the cortex and adrenal glands. Future studies are warranted to assess how 7-ketocholesterol is influenced by apoE isoform and how this could be targeted for more personalized therapies in stress-related disorders like PTSD.

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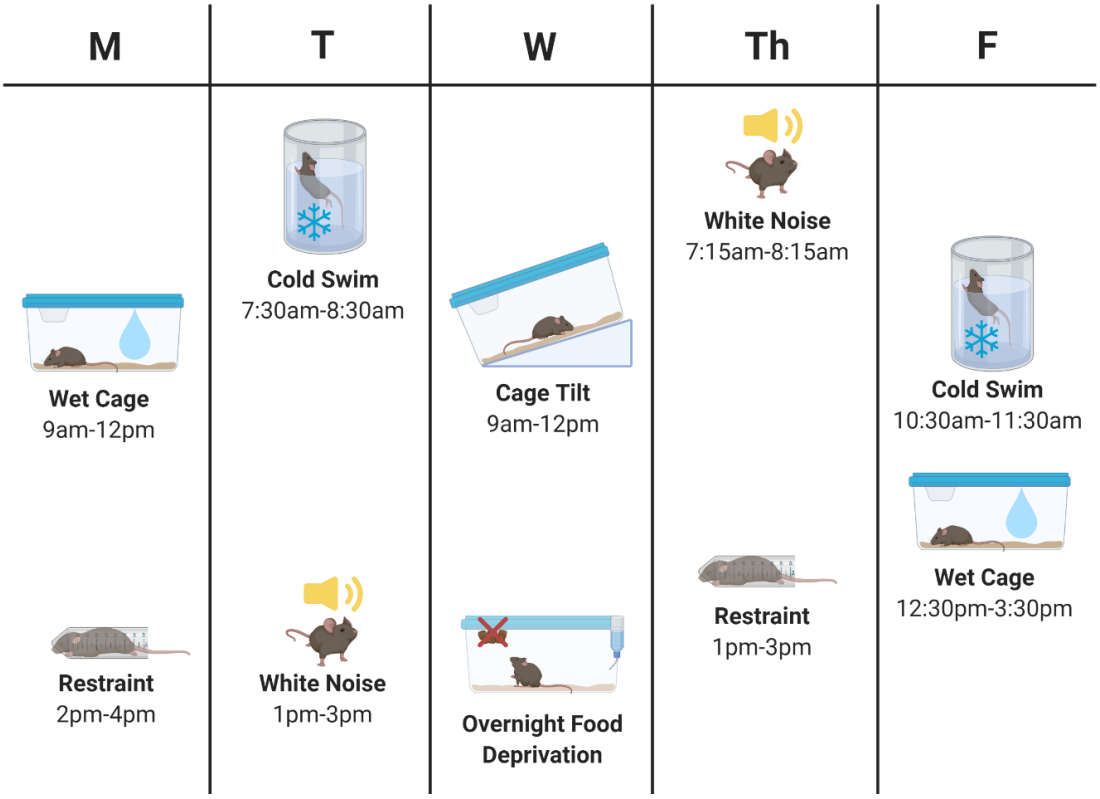
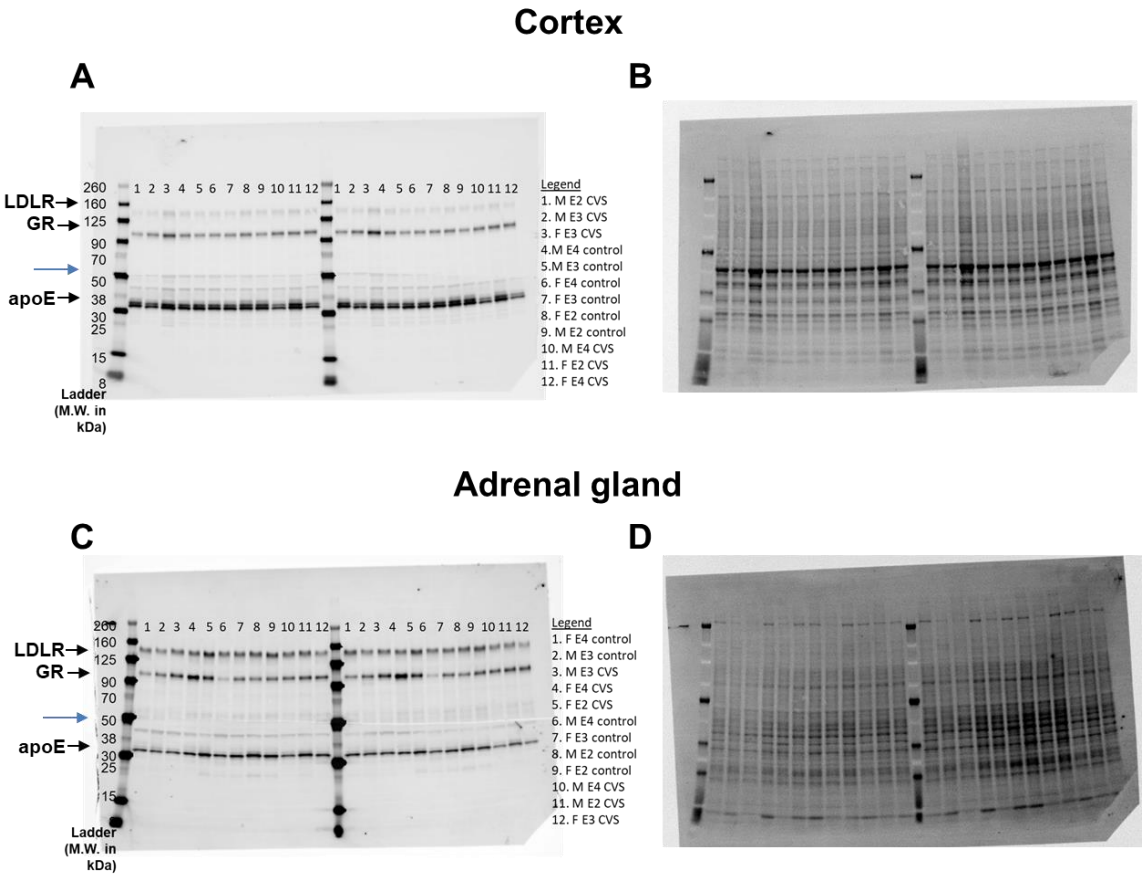
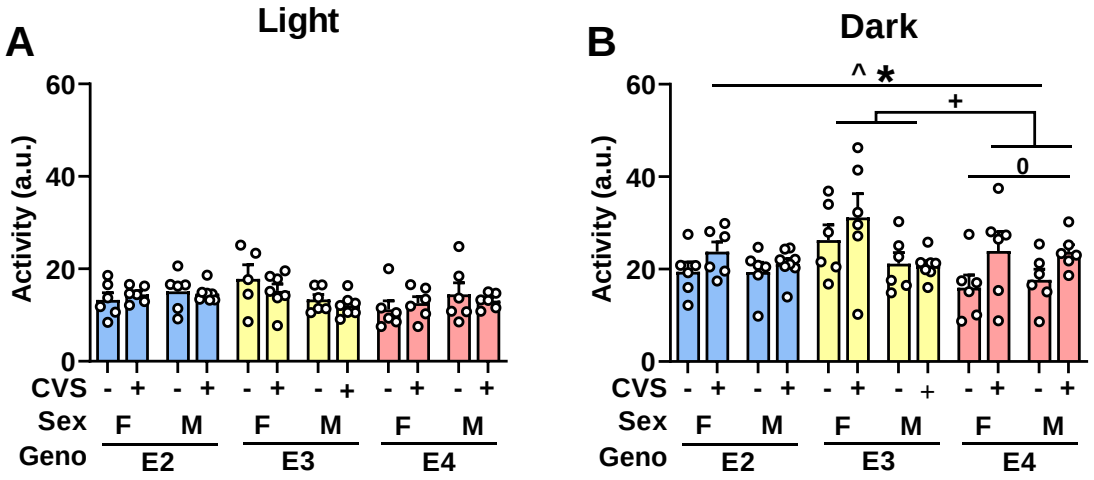
Supplementary Figures and Tables



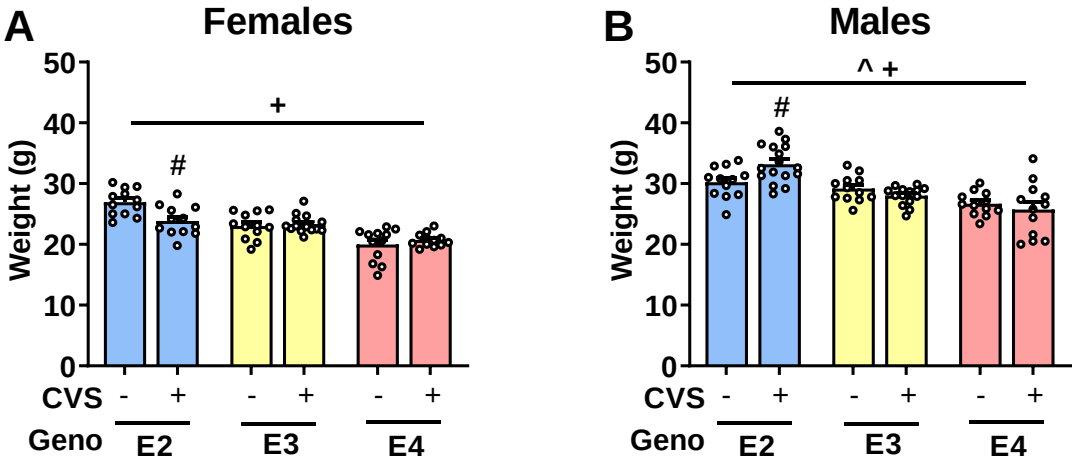
Supplementary Figure 2.1 Details of CVS schedule. Times reflect when during the day the mice were exposed to stressors; restraint stress was 15 min for each mouse and cold swim stress was 3 min for each mouse. Created with BioRender.com.



Supplementary Figure 2.2 Representative images of Western blots show in Figure 2.5. **A)** Target protein blot in cortical tissue. Black arrows show target proteins at expect bands size (LDLR – 135 kDa, GR- 95 kDa, apoE – 32-34 kDa). Samples from each group were run in duplicate on each blot. Blue arrow depicts where blot was cut. Legend shows sample group is represented in which lane. **B**) Representative total protein blot of the same blot used in (**A**) using Bio-Rad Stain Free gels. Images are taken after transfer. **C**) Target protein blots in adrenal gland tissue. **D**) Total protein image of the same blot in (**c**).



Supplementary Figure 2.3 Baseline home cage activity during A) the light cycle and B) the dark cycle. E3 mice moved more in the dark cycle than E4 mice (# $p < 0.05$) and females moved more than males (^$p < 0.05$). Although this was before CVS exposure, mice in the CVS groups moved more than control mice (* $p < 0.05$). Symbols: + refers to pairwise comparison of genotype effect, ^ refers to sex effect, * refers to CVS effects, ^{0} refers to sex x genotype interaction.



Supplementary Figure 2.4 Baseline body weights of A) female and B) male mice. Genotype was a significant factor (+$p < 0.05$). Although this was prior to CVS exposure, female E2 mice exposed to CVS weighed less than female E2 controls. Males weighed more than females and showed a similar genotype-dependent difference in baseline bodyweight. Male E2 CVS exposed mice weighed more than control counterpart. Symbols: + refers to genotype effect, ^ refers to sex effect, # refers to genotype x CVS interaction.

Supplementary Table 2.1 Statistical analyses of primary findings in home cage activity.

Measure	Model	Effect	df	Error	*F*	*p*
Activity Monitoring						
% Baseline activity						
Light Cycle	RM ANOVA	Within-subjects effects				
	Between-subject factors = Sex, Genotype,	**Week**	**1.74**	**78.133**	**10.539**	**< 0.001**
	Mauchly's test of sphericity: p = 0.043	Week*Sex:	1.74	78.133	0.078	0.902
	Greenhouse Geisser used	Week*Geno:	3.48	69.595	0.488	0.719
		Week*Group:	1.74	69.595	1.302	0.276
		Week*Sex*Geno:	**3.48**	**69.595**	**2.882**	**0.035**
		Week*Sex*Group:	1.74	69.595	1.253	0.289
		Week*Geno*Group:	3.48	69.595	1.288	0.285
		Week*Sex*Geno*Group:	3.48	69.595	0.441	0.753
		Between-subjects				
		Sex:	1	40	2.185	0.147
		Geno:	2	40	1.192	0.314
		Group:	**1**	**40**	**7.676**	**0.008**
		Sex*Geno:	2	40	0.146	0.864
		Sex*Group:	1	40	0.075	0.786
		Geno*Group:	2	40	1.478	0.240
		Sex*Geno*Group:	2	40	0.073	0.930
Dark Cycle	RM ANOVA	Within-subject effects				
	Between-subject factors = Sex, Genotype,	**Week:**	**1.497**	**59.886**	**89.235**	**< 0.001**
	Mauchly's test of sphericity: p < 0.001	Week*Sex:	1.497	59.886	0.32	0.664
	Greenhouse Geisser used	**Week* Geno:**	**2.994**	**59.886**	**3.976**	**0.012**
		Week*Group:	1.497	59.886	2.652	0.093
		Week*Sex*Geno:	2.994	59.886	1.81	0.155
		Week*Sex*Group:	2.994	59.886	0.567	0.521
		Week*Geno*Group:	2.994	59.886	1.22	0.310
		Week*Sex*Geno*Group:	2.994	59.886	1.2	0.317
		Between Subjects effects				
		Sex:	1	40	1.068	0.308
		Geno:	2	40	0.539	0.587
		Group:	1	40	0.01	0.923
		Sex*Geno:	2	40	1.121	0.336
		Sex*Group:	1	40	0.073	0.789
		Geno*Group:	2	40	0.09	0.914
		Sex*Geno*Group:	2	40	0.051	0.951
Dark/Light	RM ANOVA	Within-subject effects				
	Between-subject factors = Genotype, Group	**Week:**	**2.28**	**104.895**	**9.808**	**0.001**
	*Sex not included because only have n = 3	**Week*Geno:**	**4.561**	**104.895**	**3.372**	**0.009**
	Mauchly's test of sphericity: p < 0.001	**Week*Group:**	**2.28**	**104.895**	**3.447**	**0.03**
	Greenhouse Geisser used	Week*Geno*Group:	4.561	104.895	2.092	0.078
		Between-subjects effects				
		Geno:	**2**	**46**	**22.942**	**< 0.001**
		Group:	1	46	0.986	0.326
		Geno*Group:	2	46	1.119	0.335
		Pairwise comparisons				
		E2 vs E3:				**< 0.001**
		E2 vs E4:				**< 0.001**
		E3 vs E4:				0.991

Supplementary Table 2.2 Statistical analyses of primary findings in body weights throughout the experiment.

Measure	Model	Effect	df	Error	*F*	*p*
Body weight						
% Baseline	RM ANOVA	Within-Subject				
	Between-subject factors = Sex, Genotype,	**Week:**	**2.974**	**410.428**	**23.09**	**< 0.001**
	Mauchly's test of sphericity: $p < 0.001$	**Week*Sex:**	**2.974**	**410.428**	**7.285**	**< 0.001**
	Greenhouse Geisser used	**Week*Geno:**	**5.948**	**410.428**	**10.231**	**< 0.001**
		Week*Group:	**2.974**	**410.428**	**12.395**	**< 0.001**
		Week*Sex*Geno:	**5.948**	**410.428**	**2.251**	**0.038**
		Week*Sex*Group:	2.974	410.428	2.478	0.061
		Week*Geno*Group:	**5.948**	**410.428**	**2.704**	**0.014**
		Week*Sex*Geno:	**5.948**	**410.428**	**2.679**	**0.015**
		Between-subject				
		Sex:	**1**	**138**	**6.899**	**0.01**
		Geno:	**2**	**138**	**3.824**	**0.024**
		Group:	1	138	0.531	0.467
		Sex*Geno:	2	138	1.928	0.149
		Sex*Group:	1	138	0.858	0.356
		Geno*Group:	2	138	1.381	0.255
		Sex*Geno*Group:	**2**	**138**	**5.22**	**0.007**
	Separated females and males for simplification					
	Females	Within-subject				
	RM ANOVA	**Week:**	**3.08**	**206.369**	**21.585**	**< 0.001**
	Between-subject factors = Genotype, Group	**Week*Geno:**	**6.16**	**206.369**	**8.889**	**< 0.001**
	Mauchly's test of sphericity: $p < 0.001$	Week*Group:	3.08	206.369	1.986	0.116
	Greenhouse Geisser used	Week*Geno*Group:	6.16	206.369	1.689	0.123
		Between-subject				
		Geno:	**2**	**67**	**5.851**	**0.005**
		Group:	1	67	0.021	0.884
		Geno*Group:	2	67	0.907	0.409
		Pairwise comparisons				
		E2 vs E3:				**0.004**
		E2 vs E4:				0.113
		E3 vs E4:				0.543
	Males	Within-subject				
	RM ANOVA	**Week:**	**2.589**	**183.803**	**7.075**	**< 0.001**
	Between-subject factors = Genotype, Group	**Week*Geno:**	**5.178**	**183.803**	**2.888**	**0.014**
	Mauchly's test of sphericity: $p < 0.001$	**Week*Group:**	**2.589**	**183.803**	**14.146**	**< 0.001**
	Greenhouse Geisser used	**Week*Geno*Group:**	**5.178**	**183.803**	**3.951**	**0.002**
		Between-subject				
		Geno:	2	71	0.408	0.666
		Group:	1	71	1.264	0.265
		Geno*Group:	**2**	**71**	**5.396**	**0.007**
		Pairwise comparisons				
		E2 vs E3:				0.958
		E2 vs E4:				0.749
		E3 vs E4:				0.956
		E2:	**1**	**25**	**4.878**	**0.037**
		E3:	1	24	4.145	0.053
		E4:	1	22	3.308	0.083
% Baseline during Week 2	**Females**	Between-subject				
	ANOVA	Geno:	2	67	0.442	0.645
	Between-subject factors = Genotype, Group	Group:	1	67	3.844	0.054
		Geno*Group:	2	67	0.262	0.770
		Pairwise comparisons				
		E2 vs E3:				0.727
		E2 vs E4:				0.939
		E3 vs E4:				0.970
	Males	Between-subject				
	ANOVA	Geno:	2	71	0.075	0.927
	Between-subject factors = Genotype, Group	**Group:**	**1**	**71**	**6.899**	**0.011**
		Geno*Group:	**2**	**71**	**7.386**	**0.001**
		Pairwise comparisons				
		E2 vs E3:				0.977
		E2 vs E4:				1.000
		E3 vs E4:				0.988

Supplementary Table 2.3 Statistical analyses of primary findings in water maze training.

Measure	Model	Effect	df	Error	*F*	*p*
Water maze						
Mean swim velocity	RM ANOVA	Within-subjects				
	Between-subject factors = Sex, Genotype,	**Session Type:**	**2.674**	**369.029**	**43.873**	**< 0.001**
	Mauchly's test of sphericity: p <0.001	Session Type*Sex:	2.674	369.029	2.376	0.077
	Greenhouse Geisser used	Session Type*Geno:	5.348	369.029	1.527	0.176
		Session Type*Group:	2.674	369.029	0.551	0.627
		Session Type*Sex*Geno:	5.348	369.029	0.471	0.81
		Session Type*Sex*Group:	2.674	369.029	0.526	0.644
		Session Type*Geno*Group:	5.348	369.029	1.31	0.257
		Session Type*Sex*Geno*Group:	5.348	369.029	0.207	0.966
		Between-subject				
		Sex:	1	138	1.744	0.189
		Geno:	**2**	**138**	**5.479**	**0.005**
		Group:	1	138	0.195	0.66
		Sex*Geno:	2	138	3.02	0.052
		Sex*Group:	1	138	0.003	0.953
		Geno*Group:	2	138	0.553	0.577
		Sex*Geno*Group:	2	138	0.072	0.931
		Pairwise comparisons				
		E2 vs E3:				0.684
		E2 vs E4:				0.077
		E3 vs E4:				**0.004**
Latency to platform						
Visible Sessions	RM ANOVA	Within-subjects				
	Between-subject factors = Sex, Genotype,	Session:	2.695	136.737	1.091	0.349
	Mean swim speed included as covariate	Session*Mean Velocity:	2.695	136.737	1.839	0.146
	Mauchly's test of sphericity: p < 0.001	Session*Sex:	2.695	136.737	1.076	0.355
	Greenhouse Geisser used	**Session*Geno:**	**5.39**	**136.737**	**5.061**	**< 0.001**
		Session*Group:	2.695	136.737	0.953	0.408
		Session*Sex*Geno:	5.39	136.737	0.723	0.617
		Session*Sex*Group:	2.695	136.737	1.605	0.192
		Session*Geno*Group:	5.39	136.737	1.183	0.316
		Session*Sex*Geno*Group:	5.39	136.737	1.76	0.115
		Between-subject				
		Mean Velocity:	**1**	**137**	**8.517**	**0.004**
		Sex:	1	137	1.32	0.253
		Geno:	**2**	**137**	**3.784**	**0.025**
		Group:	1	137	0.166	0.685
		Sex*Geno:	2	137	0.186	0.831
		Sex*Group:	1	137	0.315	0.575
		Geno*Group:	2	137	0.669	0.514
		Sex*Geno*Group:	2	137	0.007	0.993
Hidden Sessions	RM ANOVA	Within-subjects				
	Between-subject factors = Sex, Genotype,	Session:	6.045	221.713	1.448	0.193
	Mean swim speed included as covariate	Session*Mean Velocity:	6.045	221.713	1.922	0.074
	Mauchly's test of sphericity: p < 0.001	Session*Sex:	6.045	221.713	1.148	0.332
	Greenhouse Geisser used	Session*Geno:	12.09	221.713	1.232	0.255
		Session*Group:	6.045	221.713	0.781	0.586
		Session*Sex*Geno:	12.09	221.713	0.985	0.461
		Session*Sex*Group:	6.045	221.713	0.726	0.63
		Session*Geno*Group:	12.09	221.713	0.7	0.753
		Session*Sex*Geno*Group:	12.09	221.713	0.763	0.691
		Between-subject				
		Mean Velocity:	1	137	55.273	**< 0.001**
		Sex:	**1**	**137**	**5.166**	**0.025**
		Geno:	**2**	**137**	**5.589**	**0.005**
		Group:	1	137	3.752	0.055
		Sex*Geno:	2	137	0.672	0.512
		Sex*Group:	**1**	**137**	**4.496**	**0.036**
		Geno*Group:	2	137	0.817	0.444
		Sex*Geno*Group:	2	137	0.017	0.983

Measure	Model	Effect	df	Error	*F*	*p*
Reversal 1 Sessions	RM ANOVA	Within-subject				
	Between-subject factors = Sex, Genotype,	Session:	1	137	0.205	0.652
	Mean swim speed included as covariate	Session*Mean Velocity:	1	137	0.001	0.973
		Session*Sex:	1	137	0.614	0.435
		Session*Geno:	2	137	0.992	0.374
		Session*Group:	1	137	1.692	0.196
		Session*Sex*Geno:	2	137	1.262	0.286
		Session*Sex*Group:	**1**	**137**	**7.756**	**0.006**
		Session*Geno*Group:	2	137	2.043	0.134
		Session*Sex*Geno*Group:	2	137	0.173	0.841
		Between-subject				
		Mean Velocity:	**1**	**137**	**6.277**	**0.013**
		Sex:	1	137	0.002	0.961
		Geno:	2	137	1.151	0.319
		Group:	1	137	0.57	0.452
		Sex*Geno:	2	137	0.233	0.792
		Sex*Group:	1	137	0.568	0.452
		Geno*Group:	2	137	0.432	0.65
		Sex*Geno*Group:	2	137	0.502	0.606
Reversal 2 Sessions	RM ANOVA	Within-subject				
	Between-subject factors = Sex, Genotype,	Session:	1	137	8.346	0.004
	Mean swim speed included as covariate	Session*Mean Velocity:	1	137	2.853	0.093
	Mauchly's test of sphericity: $p < 0.001$	Session*Sex:	1	137	2.21	0.139
	Greenhouse Geisser used	Session*Geno:	2	137	0.337	0.715
		Session*Group:	1	137	0.469	0.495
		Session*Sex*Geno:	2	137	0.055	0.946
		Session*Sex*Group:	1	137	0.013	0.909
		Session*Geno*Group:	2	137	0.222	0.801
		Session*Sex*Geno*Group:	2	137	0.508	0.603
		Between-subject				
		Mean Velocity:	1	137	0.027	0.871
		Sex:	1	137	0.146	0.703
		Geno:	**2**	**137**	**5.453**	**0.005**
		Group:	1	137	0.021	0.885
		Sex*Geno:	2	137	0.967	0.383
		Sex*Group:	1	137	0.380	0.539
		Geno*Group:	2	137	0.566	0.569
		Sex*Geno*Group:	2	137	0.490	0.614
	Separated genotypes for simplification					
E2- Visible	RM ANOVA	Within-subject				
	Between-subject factors = Genotype, Group	Session:	2.479	171.118	0.925	0.417
	Mean swim speed included as covariate	Session*Mean Velocity:	2.516	171.118	3.112	0.038
	Mauchly's test of sphericity: $p = 0.015$	Session*Sex:	2.516	171.118	2.196	0.104
	Greenhouse Geisser used	Session*Group:	2.516	171.118	1.499	0.224
		Session*Sex*Group:	**2.516**	171.118	**3.62**	**0.022**
		Between-subject				
		Mean Velocity:	1	45	216	0.644
		Sex:	1	45	1.04	0.313
		Group:	1	45	0.792	0.378
		Sex*Group:	1	45	0.099	0.754
E2 Hidden	RM ANOVA	Within-subject				
	Between-subject factors = Sex, Group	Session:	7	315	0.846	0.55
	Mean swim speed included as covariate	Session*Mean Velocity:	7	315	0.844	0.551
	Mauchly's test of sphericity: $p = 0.087$	Session*Sex:	7	315	0.443	0.875
		Session*Group:	7	315	0.643	0.72
		Session*Sex*Group:	7	315	0.372	0.918
		Between-subject				
		Mean Velocity:	**1**	**45**	**4.728**	**0.035**
		Sex:	1	45	0.667	0.418
		Group:	1	45	3.957	0.053
		Sex*Group:	1	45	0.556	0.46
	After removal of mouse #21 and collapsing sex					
	RM ANOVA	Within-subject				
	Between-subject factors = Group	Session:	7	329	0.91	0.499
	Mean swim speed included as covariate	Session*Mean Velocity:	7	329	0.971	0.452
	Mauchly's test of sphericity: $p = 0.072$	Session*Group:	7	329	0.605	0.751
		Between-subject				
		Mean Velocity:	**1**	**47**	**4.802**	**0.033**
		Group:	**1**	**47**	**4.055**	**0.05**

Measure	Model	Effect	df	Error	*F*	*p*
E2 Reversal 1	RM ANOVA	Within-subject				
	Between-subject factors = Sex, Group	Session:	1	45	0.124	0.726
	Mean swim speed included as covariate	Session*Mean Velocity:	1	45	0.315	0.578
		Session*Sex:	1	45	0.875	0.355
		Session*Group:	1	45	3.782	0.058
		Session*Sex*Group:	1	45	2.893	0.096
		Between-subject				
		Mean Velocity:	1	45	1.859	0.179
		Sex:	1	45	0.328	0.57
		Group:	1	45	0.869	0.356
		Sex*Group:	1	45	0.082	0.777
E2 Reversal 2	RM ANOVA	Within-subject				
	Between-subject factors = Sex, Group	Session:	1	45	1.21	0.277
	Mean swim speed included as covariate	Session*Mean Velocity:	1	45	0.056	0.814
		Session*Sex:	1	45	0.924	0.342
		Session*Group:	1	45	1.076	0.305
		Session*Sex*Group:	1	45	0.179	0.674
		Between-subject				
		Mean Velocity:	1	45	6.01	0.018
		Sex:	1	45	0.321	0.574
		Group:	1	45	0.069	0.794
		Sex*Group:	1	45	0.766	0.386
E3 Visible	RM ANOVA	Within-subject				
	Between-subject factors = Sex, Group	Session:	2.453	115.287	4.019	0.014
	Mean swim speed included as covariate	Session*Mean Velocity:	2.453	115.287	1.507	0.222
	Mauchly's test of sphericity: p = 0.001	Session*Sex:	2.453	115.287	0.288	0.794
	Greenhouse Geisser used	Session*Group:	2.453	115.287	0.911	0.422
		Session*Sex*Group:	2.453	115.287	0.204	0.857
		Between-subject				
		Mean Velocity:	1	47	1.729	0.195
		Sex:	1	47	0.268	0.607
		Group:	1	47	0.247	0.621
		Sex*Group:	1	47	0.081	0.777
E3 Hidden	RM ANOVA	Within-subject				
	Between-subject factors = Sex, Group	Session:	7	329	0.434	0.88
	Mean swim speed included as covariate	Session*Mean Velocity:	7	329	0.623	0.737
	Mauchly's test of sphericity: p = 0.083	Session*Sex:	7	329	0.409	0.897
		Session*Group:	7	329	0.362	0.924
		Session*Sex*Group:	7	329	0.579	0.773
		Between-subject				
		Mean Velocity:	1	47	7.229	0.01
		Sex:	1	47	4.064	0.05
		Group:	1	47	0.167	0.685
		Sex*Group:	1	47	2.04	0.16
E3 Reversal 1	RM ANOVA	Within-subject				
	Between-subject factors = Sex, Group	Session:	1	47	1.26	0.267
	Mean swim speed included as covariate	Session*Mean Velocity:	1	47	0.649	0.424
		Session*Sex:	1	47	0.691	0.41
		Session*Group:	1	47	0.615	0.437
		Session*Sex*Group:	1	47	1.057	0.309
		Between-subject				
		Mean Velocity:	1	47	0.122	0.728
		Sex:	1	47	0.021	0.885
		Group:	1	47	0.153	0.697
		Sex*Group:	1	47	0.251	0.619
E3 Reversal 2	RM ANOVA	Within-subject				
	Between-subject factors = Sex, Group	Session:	1	47	4.542	0.038
	Mean swim speed included as covariate	Session*Mean Velocity:	1	47	2.404	0.128
	Mean swim speed included as covariate	Session*Sex:	1	47	0.95	0.335
		Session*Group:	1	47	0.047	0.83
		Session*Sex*Group:	1	47	0.548	0.463
		Between-subject				
		Mean Vel:	1	47	1.469	0.232
		Sex:	1	47	1.789	0.187
		Group:	1	47	0.312	0.579
		Sex*Group:	1	47	0.142	0.708

Measure	Model	Effect	df	Error	*F*	*p*
E4 Visible	RM ANOVA	Within-subject				
	Between-subject factors = Sex, Group	Session:	2.364	99.268	0.669	0.573
	Mauchly's test : p = 0.011	Session*Mean Velocity:	2.364	99.268	2.102	0.119
	Greenhouse Geisser used	Session*Sex:	2.364	99.268	0.47	0.658
		Session*Group:	2.364	99.268	0.678	0.534
		Session*Sex*Group:	2.364	99.268	1.431	0.243
		Between-subject				
		Mean Velocity:	**1**	**42**	**11.23**	**0.002**
		Sex:	1	42	0.017	0.897
		Group:	1	42	0.252	0.618
		Sex*Group:	1	42	0.195	0.661
E4 Hidden	RM ANOVA	Within-subject				
	Between-subject factors = Sex, Group	Session:	5.47	229.732	1.317	0.242
	Mauchly's test : p = 0.013	Session*Mean Velocity:	5.47	229.732	1.403	0.219
	Greenhouse Geisser used	Session*Sex:	5.47	229.732	2.061	0.065
		Session*Group:	5.47	229.732	1.08	0.374
		Session*Sex*Group:	5.47	229.732	1.131	0.345
		Between-subject				
		Mean Vel:	**1**	**42**	**41.577**	**< 0.001**
		Sex:	1	42	1.091	0.302
		Group:	1	42	1.483	0.23
		Sex*Group:	1	42	1.383	0.246
E4 Reversal 1	RM ANOVA	Within-subject				
	Between-subject factors = Sex, Group	Session:	1	42	0.01	0.92
		Session*Mean Velocity:	1	42	0.001	0.97
		Session*Sex:	1	42	1.877	0.178
		Session*Group:	1	42	1.191	0.281
		Session*Sex*Group:	1	42	4.275	0.045
		Between-subject				
		Mean Velocity:	**1**	**42**	**5.612**	**0.023**
		Sex:	1	42	0.071	0.792
		Group:	1	42	0.252	0.618
		Sex*Group:	1	42	1.152	0.289
E4 Reversal 2	RM ANOVA	Within-subject				
	Between-subject factors = Sex, Group	Session:	1	42	3.822	0.057
		Session*Mean Velocity:	1	42	1.792	0.188
		Session*Sex:	1	42	1.068	0.307
		Session*Group:	1	42	0.001	0.972
		Session*Sex*Group:	1	42	0.027	0.87
		Between-subject				
		Mean Velocity:	1	42	0.826	0.369
		Sex:	1	42	0.641	0.428
		Group:	1	42	0.029	0.866
		Sex*Group:	1	42	0.061	0.806

Supplementary Table 2.4 Statistical analyses of primary findings in water maze probe trials.

Measure	Model	Effect	df	Error	F	p
Probe trial 1						
% Time in Quadrant						
Female E2 Controls	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.049	**Quadrant**	**1.939**	**19.386**	**6.766**	**0.006**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				**0.017**
		Target Quadrant vs Left Quadrant :				0.632
		Target Quadrant vs Opposite Quadrant :				0.53
Female E2 CVS	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.043	Quadrant	1.699	18.69	1.167	0.325
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.221
		Target Quadrant vs Left Quadrant :				0.112
		Target Quadrant vs Opposite Quadrant :				0.997
Female E3 Controls	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.192	**Quadrant**	**3**	**33**	**18.146**	**< 0.001**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.145
		Target Quadrant vs Left Quadrant :				**< 0.001**
		Target Quadrant vs Opposite Quadrant :				**0.001**
Female E3 CVS	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.054	**Quadrant**	**3**	**39**	**7.777**	**< 0.001**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.121
		Target Quadrant vs Left Quadrant :				**0.008**
		Target Quadrant vs Opposite Quadrant :				**0.001**
Female E4 Controls	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.318	**Quadrant**	**3**	**33**	**4.311**	**0.011**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.955
		Target Quadrant vs Left Quadrant :				**0.011**
		Target Quadrant vs Opposite Quadrant :				0.056
Female E4 CVS	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.310	**Quadrant**	**3**	**30**	**4.863**	**0.007**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.169
		Target Quadrant vs Left Quadrant :				0.089
		Target Quadrant vs Opposite Quadrant :				0.17
Male E2 Controls	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.259	**Quadrant**	**3**	**30**	**9.408**	**< 0.001**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				**0.001**
		Target Quadrant vs Left Quadrant :				0.622
		Target Quadrant vs Opposite Quadrant :				**0.013**
Male E2 CVS	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.096	**Quadrant**	**3**	**45**	**5.27**	**0.003**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.854
		Target Quadrant vs Left Quadrant :				0.053
		Target Quadrant vs Opposite Quadrant :				0.062
Male E3 Controls	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.077	**Quadrant**	**3**	**33**	**24.361**	**< 0.001**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				**0.004**
		Target Quadrant vs Left Quadrant :				**< 0.001**
		Target Quadrant vs Opposite Quadrant :				**0.001**
Male E3 CVS	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.272	**Quadrant**	**3**	**39**	**12.642**	**< 0.001**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				**0.002**
		Target Quadrant vs Left Quadrant :				**0.008**
		Target Quadrant vs Opposite Quadrant :				**0.005**
Male E4 Controls	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.019	**Quadrant**	**1.705**	**18.756**	**4.597**	**0.009**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.362
		Target Quadrant vs Left Quadrant :				**0.001**
		Target Quadrant vs Opposite Quadrant :				0.243
Male E4 CVS	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.386	**Quadrant**	**3**	**33**	**17.342**	**< 0.001**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				**0.01**
		Target Quadrant vs Left Quadrant :				**< 0.001**
		Target Quadrant vs Opposite Quadrant :				**0.003**

Probe trial 2						
% Time in Quadrant						
Female E2 Controls	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.001	Quadrant	1.473	14.731	3.829	0.057
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.186
		Target Quadrant vs Left Quadrant :				0.999
		Target Quadrant vs Opposite Quadrant :				0.743
Female E2 CVS	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.114	Quadrant	3	33	0.847	0.478
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.861
		Target Quadrant vs Left Quadrant :				0.984
		Target Quadrant vs Opposite Quadrant :				0.761
Female E3 Controls	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.296	**Quadrant**	**3**	**33**	**7.685**	**< 0.001**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				**0.048**
		Target Quadrant vs Left Quadrant :				0.988
		Target Quadrant vs Opposite Quadrant :				**0.019**
Female E3 CVS	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.664	Quadrant	3	39	1.883	0.148
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.483
		Target Quadrant vs Left Quadrant :				0.991
		Target Quadrant vs Opposite Quadrant :				0.999
Female E4 Controls	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.049	Quadrant	3	33	1.539	0.223
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0..358
		Target Quadrant vs Left Quadrant :				1
		Target Quadrant vs Opposite Quadrant :				0.992
Female E4 CVS	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.063	**Quadrant**	**3**	**30**	**3.718**	**0.022**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				**0.035**
		Target Quadrant vs Left Quadrant :				0.999
		Target Quadrant vs Opposite Quadrant :				0.974
Male E2 Controls	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.158	**Quadrant**	**3**	**30**	**6.962**	**0.001**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.113
		Target Quadrant vs Left Quadrant :				0.248
		Target Quadrant vs Opposite Quadrant :				0.932
Male E2 CVS	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.106	**Quadrant**	**3**	**45**	**3.615**	**0.02**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.807
		Target Quadrant vs Left Quadrant :				1
		Target Quadrant vs Opposite Quadrant :				**0.036**
Male E3 Controls	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.307	**Quadrant**	**3**	**33**	**12.869**	**< 0.001**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				**0.003**
		Target Quadrant vs Left Quadrant :				0.518
		Target Quadrant vs Opposite Quadrant :				0.796
Male E3 CVS	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.011	**Quadrant**	**1.873**	**24.351**	**6.939**	**0.005**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.513
		Target Quadrant vs Left Quadrant :				**0.039**
		Target Quadrant vs Opposite Quadrant :				0.679
Male E4 Controls	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.102	**Quadrant**	**3**	**33**	**8.168**	**< 0.001**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				**0.007**
		Target Quadrant vs Left Quadrant :				0.26
		Target Quadrant vs Opposite Quadrant :				0.889
Male E4 CVS	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.346	Quadrant	3	33	2.844	0.053
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.57
		Target Quadrant vs Left Quadrant :				0.416
		Target Quadrant vs Opposite Quadrant :				0.982

Probe trial 3						
% Time in Quadrant						
Female E2 Controls	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.406	**Quadrant**	**3**	**30**	**5.274**	**0.045**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.997
		Target Quadrant vs Left Quadrant :				0.733
		Target Quadrant vs Opposite Quadrant :				0.171
Female E2 CVS	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.152	**Quadrant**	**3**	**33**	**6.15**	**0.002**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				1
		Target Quadrant vs Left Quadrant :				0.949
		Target Quadrant vs Opposite Quadrant :				**0.002**
Female E3 Controls	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.022	Quadrant	1.669	18.357	3.262	0.069
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				1
		Target Quadrant vs Left Quadrant :				1
		Target Quadrant vs Opposite Quadrant :				0.52
Female E3 CVS	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.074	**Quadrant**	**3**	**39**	**6.272**	**0.001**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.991
		Target Quadrant vs Left Quadrant :				0.884
		Target Quadrant vs Opposite Quadrant :				0.084
Female E4 Controls	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.166	Quadrant	3	33	0.769	0.519
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				1
		Target Quadrant vs Left Quadrant :				1
		Target Quadrant vs Opposite Quadrant :				0.916
Female E4 CVS	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.468	**Quadrant**	**3**	**30**	**3.701**	**0.022**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.901
		Target Quadrant vs Left Quadrant :				0.245
		Target Quadrant vs Opposite Quadrant :				0.066
Male E2 Controls	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.739	Quadrant	3	30	2.474	0.081
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.99
		Target Quadrant vs Left Quadrant :				0.811
		Target Quadrant vs Opposite Quadrant :				0.625
Male E2 CVS	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.299	Quadrant	2	45	1.457	0.239
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				1
		Target Quadrant vs Left Quadrant :				0.901
		Target Quadrant vs Opposite Quadrant :				0.357
Male E3 Controls	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.252	Quadrant	3	33	2.707	0.061
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.196
		Target Quadrant vs Left Quadrant :				0.813
		Target Quadrant vs Opposite Quadrant :				0.966
Male E3 CVS	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.054	**Quadrant**	**3**	**39**	**5.355**	**0.003**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.847
		Target Quadrant vs Left Quadrant :				1
		Target Quadrant vs Opposite Quadrant :				0.103
Male E4 Controls	RM ANOVA	Within-subject				
	Mauchly's test: p = 0.276	**Quadrant**	**3**	**33**	**15.771**	**< 0.001**
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				1
		Target Quadrant vs Left Quadrant :				0.984
		Target Quadrant vs Opposite Quadrant :				**0.004**
Male E4 CVS	RM ANOVA	Within-subject				
	Mauchly's test: p < 0.001	Quadrant	1.78	19.581	3.174	0.069
		Pairwise comparisons				
		Target Quadrant vs Right Quadrant :				0.426
		Target Quadrant vs Left Quadrant :				0.878
		Target Quadrant vs Opposite Quadrant :				0.051

Supplementary Table 2.5 Statistical analyses of primary findings in anxiety-related measures.

Measure	Model	Effect	df	Error	F	p
Elevated plus maze						
%Total Time in Open Arms	ANOVA	Between-subject				
	Between-subject factors = Sex, Genotype,	Sex:	1	137	1.578	0.211
		Geno:	**2**	**137**	**6.725**	**0.002**
		Group:	1	137	2.681	0.104
		Sex*Geno:	**2**	**137**	**4.011**	**0.02**
		Sex*Group:	1	137	0.202	0.654
		Geno*Group:	2	137	0.503	0.606
		Sex*Geno*Group:	1	137	0.105	0.9
		Pairwise comparisons				
		E2 vs E3:				**0.001**
		E2 vs E4:				0.226
		E3 vs E4:				0.177
Elevated zero maze						
%Total Time in Open Arms	ANOVA	Between-subject				
	Between-subject factors = Sex, Genotype,	**Sex:**	**1**	**137**	**6.225**	**0.014**
		Geno:	**2**	**137**	**5.748**	**0.004**
		Group:	1	137	2.36	0.127
		Sex*Geno:	2	137	2.892	0.059
		Sex*Group:	1	137	2.952	0.088
		Geno*Group:	2	137	0.062	0.94
		Sex*Geno*Group:	2	137	1.783	0.172
		Pairwise comparisons				
		E2 vs E3:				**0.007**
		E2 vs E4:				**0.023**
		E3 vs E4:				0.98
Plasma Corticosterone	ANOVA	Between-subject				
	Between-subject factors = Sex, Genotype,	**Sex:**	**1**	**68**	**4.82**	**0.032**
		Geno:	**2**	**68**	**8.701**	**< 0.001**
		Group:	1	68	0.263	0.61
		Sex*Geno:	**2**	**68**	**5.549**	**0.006**
		Sex*Group:	**1**	**68**	**5.253**	**0.025**
		Geno*Group:	2	68	0.431	0.652
		Sex*Geno*Group:	2	68	0.381	0.685
Adrenal Weight	ANOVA	Between-subject				
	Between-subject factors = Sex, Genotype,	**Sex:**	**1**	**66**	**23.673**	**< 0.001**
		Geno:	**2**	**66**	**8.081**	**0.001**
		Group:	1	66	0.095	0.759
		Sex*Geno:	2	66	0.321	0.726
		Sex*Group:	1	66	1.207	0.276
		Geno*Group:	**2**	**66**	**4.666**	**0.013**
		Sex*Geno*Group:	2	66	0.299	0.742
		Pairwise comparisons				
		E2 vs E3:				0.074
		E2 vs E4:				**< 0.001**
		E3 vs E4:				0.19
Females	ANOVA	Between-subject				
	Between-subject factors = Genotype, Group	**Geno:**	**2**	**30**	**6.014**	**0.006**
		Group:	1	30	0.365	0.55
		Geno*Group:	**2**	**30**	**3.679**	**0.037**
		Pairwise comparisons				
		E2 vs E3:				0.077
		E2 vs E4:				**0.006**
		E3 vs E4:				0.517
Males	ANOVA	Between-subject				
	Between-subject factors = Genotype, Group	Geno:	2	36	2.897	0.068
		Group:	1	36	0.911	0.346
		Geno*Group:	2	36	1.645	0.207
		Pairwise comparisons				
		E2 vs E3:				0.675
		E2 vs E4:				0.063
		E3 vs E4:				0.435

Supplementary Table 2.6 Statistical analyses of primary findings in Western blot assays.

Measure	Model	Effect	df	Error	*F*	*p*
Target Protein Analyses						
Cortex						
GR/apoE	ANOVA	Between-subject				
	Between-subject factors = Sex, Genotype,	**Blot:**	**1**	**47**	**91.416**	**< 0.001**
	Blot included as covariate	**Sex:**	1	47	1.787	0.188
		Geno:	**2**	**47**	**18.256**	**< 0.001**
		Group:	**1**	**47**	**19.702**	**< 0.001**
		Sex*Geno:	2	47	2.28	0.114
		Sex*Group:	1	47	0.491	0.487
		Geno*Group:	**2**	**47**	**4.331**	**0.019**
		Sex*Geno*Group:	2	47	0.247	0.782
		Pairwise comparisons				
		E2 vs E3:				**0.003**
		E2 vs E4:				**< 0.001**
		E3 vs E4:				0.052
LDLR/apoE	ANOVA	Between-subject				
	Between-subject factors = Sex, Genotype,	**Blot:**	**1**	**47**	**55.47**	**< 0.001**
	Blot included as covariate	Sex:	1	47	0.422	0.519
		Geno:	**2**	**47**	**4.852**	**0.012**
		Group:	**1**	**47**	**13.391**	**0.001**
		Sex*Geno:	2	47	0.785	0.462
		Sex*Group:	1	47	0.37	0.546
		Geno*Group:	**2**	**47**	**3.846**	**0.028**
		Sex*Geno*Group:	2	47	0.854	0.432
		Pairwise comparisons				
		E2 vs E4 p = 0.009				
Adrenal gland						
apoE/total protein	ANOVA	Between-subject				
	Between-subject factors = Sex, Genotype,	**Blot**	**1**	**46**	**16.498**	**< 0.001**
	Blot included as covariate	Sex	1	46	1.499	0.227
		Geno	**2**	**46**	**9.568**	**< 0.001**
		Group	1	46	0.008	0.93
		Sex * Geno	2	46	0.079	0.924
		Sex * Group	1	46	0.18	0.673
		Geno * Group	2	46	0.442	0.645
		Sex * Geno * Group	2	46	0.055	0.946
GR/total protein	ANOVA	Between-subject				
	Between-subject factors = Sex, Genotype,	Blot	1	46	0.886	0.352
	Blot included as covariate	Sex	1	46	0.319	0.575
		Geno	2	46	0.157	0.855
		Group	**1**	46	**6.365**	**0.015**
		Sex * Geno	2	46	1.52	0.229
		Sex * Group	1	46	0.239	0.627
		Geno * Group	2	46	0.346	0.71
		Sex * Geno * Group	2	46	1.261	0.293
GR/apoE	ANOVA	Between-subject				
	Between-subject factors = Sex, Genotype,	**Blot**	**1**	**46**	**7.143**	**0.01**
	Blot included as covariate	Sex	1	46	0.05	0.824
		Geno	**2**	**46**	**8.167**	**0.001**
		Group	**1**	**46**	**13.738**	**0.001**
		Sex * Geno	**2**	**46**	**4.3**	**0.019**
		Sex * Group	1	46	0.923	0.342
		Geno * Group	2	46	2.759	0.074
		Sex * Geno * Group	2	46	2.474	0.095
LDLR/apoE	ANOVA	Between-subject				
	Between-subject factors = Sex, Genotype,	Blot	1	46	28.258	**< 0.001**
	Blot included as covariate	Sex	1	46	0.213	0.647
		Geno	**2**	**46**	**8.286**	**0.001**
		Group	**1**	46	0.416	0.522
		Sex * Geno	2	46	0.428	0.654
		Sex * Group	1	46	0.001	0.982
		Geno * Group	2	46	0.408	0.668
		Sex * Geno * Group	2	46	2.014	0.145

Supplementary Table 2.7 Statistical analyses of primary findings in target protein and 7-ketocholesterol levels.

Measure	Model	Effect	df	Error	*F*	*p*
Plasma apoE	ANOVA	Between-subject				
	Between-subject factors = Sex, Genotype,	**Sex**	**1**	**52**	**4.587**	**0.037**
		Geno	**2**	**52**	**81.78**	**< 0.001**
		Group	**1**	**52**	**6.892**	**0.011**
		Sex * Geno	**2**	**52**	**3.699**	**0.031**
		Sex * Group	1	52	0.003	0.957
		Geno * Group	**2**	**52**	**6.161**	**0.004**
		Sex * Geno * Group	2	52	0.061	0.941
Cortical apoE	ANOVA	Between-subject				
	Between-subject factors = Sex, Genotype,	Sex	1	58	0.158	0.692
		Geno	2	58	0.935	0.399
		Group	**1**	**58**	**48.91**	**< 0.001**
		Sex * Geno	2	58	2.916	0.062
		Sex * Group	1	58	0.331	0.567
		Geno * Group	2	58	1.785	0.177
		Sex * Geno * Group	2	58	1.99	0.146
Cortical MAP-2	ANOVA	Between-subject				
	Between-subject factors = Sex, Genotype,	Sex	1	59	0.116	0.735
		Geno	2	59	0.386	0.681
		Group	**1**	**59**	**74.256**	**< 0.001**
		Sex * Geno	**2**	**59**	**6.801**	**0.002**
		Sex * Group	1	59	0.995	0.323
		Geno * Group	**2**	**59**	**4.019**	**0.023**
		Sex * Geno * Group	**2**	**59**	**3.792**	**0.028**
	ANOVA	Between-subject				
	Between subject factors = Genotype, Group	Geno	2	65	0.448	0.641
		Group	**1**	**65**	**58.565**	**< 0.001**
		Geno * Group	**2**	**65**	**3.344**	**0.041**
7-ketocholesterol						
Cortex	ANOVA	Between-subject				
	Between-subject factors = Genotype, Group	Genotype	2	26	0.214	0.809
		Group	**1**	**26**	**6.53**	**0.017**
		Genotype * Group	**2**	**26**	**4.715**	**0.018**
		Sidak's multiple comparisons				
		E2 control vs E2 CVS:				0.716
		E3 control vs E3 CVS				0.11
		E4 control vs E4 CVS				**0.013**
Liver	ANOVA	Between-subject				
	Between-subject factors = Sex, Genotype,	Sex	1	36	1.472	0.233
		Geno	2	36	0.318	0.729
		Group	**1**	**36**	**18.856**	**< 0.001**
		Sex * Geno	2	36	0.668	0.519
		Sex * Group	1	36	0.01	0.92
		Geno * Group	2	36	0.295	0.746
		Sex * Geno * Group	2	36	0.552	0.58
Plasma	ANOVA	Between-subject				
	Between-subject factors = Sex, Genotype,	Sex	1	34	0.083	0.775
		Geno	**2**	**34**	**24.421**	**< 0.001**
		Group	1	34	0.005	0.943
		Sex * Geno	2	34	0.799	0.458
		Sex * Group	1	34	0.036	0.851
		Geno * Group	2	34	0.133	0.876
		Sex * Geno * Group	2	34	0.285	0.754

Supplementary Table 2.8 Nonsignificant Western blot results of apoE, LDLR, and GR for main effects of sex, genotype, and CVS. Values are shown as intensity normalized to total protein or apoE and hence have no units listed. *p* values refer to effects of genotype and CVS exposure; significance was $p < 0.05$.

Tissue	Measure	E2		E3		E4		*p*
		Control	CVS	Control	CVS	Control	CVS	
Cortex	apoE/Tot Prot	1.26 ± 0.27	1.15 ± 0.23	1.18 ± 0.28	1.01 ± 0.22	1.04 ± 0.23	0.81 ± 0.16	n.s.
	LDLR/Tot Prot	0.056 ± 0.013	0.052 ± 0.011	0.050 ± 0.011	0.062 ± 0.016	0.051 ± 0.013	0.061 ± 0.012	n.s.
	GR/Tot Prot	0.74 ± 0.11	0.71 ± 0.093	0.79 ± 0.11	1.08 ± 0.15	0.82 ± 0.11	0.98 ± 0.15	n.s.
Adrenal Gland	LDLR/Tot Prot	1.125 ± 0.12	1.21 ± 0.16	1.00 ± 0.055	1.094 ± 0.12	1.14 ± 0.13	0.98 ± 0.090	n.s.
Hippocampus	apoE/Tot Prot	1.31 ± 0.093	1.33 ± 0.10	1.24 ± 0.10	1.23 ± 0.10	1.20 ± 0.11	1.16 ± 0.081	n.s.
	LDLR/Tot Prot	0.056 ± 0.0071	0.059 ± 0.0071	0.052 ± 0.0072	0.048 ± 0.0067	0.050 ± 0.0070	0.043 ± 0.0061	n.s.
	GR/Tot Prot	1.32 ± 0.20	1.37 ± 0.19	1.28 ± 0.20	1.29 ± 0.20	1.37 ± 0.22	1.32 ± 0.21	n.s.
	LDLR/apoE	0.042 ± 0.0043	0.044 ± 0.0040	0.41 ± 0.0037	0.038 ± 0.0032	0.041 ± 0.0036	0.036 ± 0.0039	n.s.
	GR/apoE	1.02 ± 0.13	1.05 ± 0.11	1.05 ± 0.13	1.07 ± 0.13	1.14 ± 0.13	1.16 ± 0.16	n.s.
mPFC	apoE/Tot Prot	0.65 ± 0.15	0.65 ± 0.13	0.67 ± 0.13	0.76 ± 0.18	0.57 ± 0.13	0.60 ± 0.13	n.s.
	LDLR/Tot Prot	0.040 ± 0.0054	0.048 ± 0.0051	0.036 ± 0.0039	0.036 ± 0.0051	0.043 ± 0.0066	0.042 ± 0.0054	n.s.
	GR/Tot Prot	0.21 ± 0.023	0.22 ± 0.019	0.22 ± 0.023	0.22 ± 0.025	0.23 ± 0.022	0.25 ± 0.029	n.s.
	LDLR/apoE	0.12 ± 0.042	0.13 ± 0.045	0.10 ± 0.036	0.12 ± 0.052	0.15 ± 0.061	0.13 ± 0.049	n.s.
	GR/apoE	0.60 ± 0.23	0.55 ± 0.16	0.53 ± 0.16	0.63 ± 0.24	0.66 ± 0.20	0.66 ± 0.17	n.s.
Liver	apoE/Tot Prot	0.40 ± 0.068	0.42 ± 0.081	0.31 ± 0.055	0.36 ± 0.066	0.36 ± 0.067	0.36 ± 0.061	n.s.
	LDLR/Tot Prot	0.11 ± 0.020	0.12 ± 0.025	0.083 ± 0.016	0.10 ± 0.019	0.10 ± 0.017	0.10 ± 0.018	n.s.
	LDLR/apoE	0.32 ± 0.046	0.35 ± 0.043	0.31 ± 0.036	0.30 ± 0.038	0.30 ± 0.029	0.34 ± 0.058	n.s.

Supplementary Table 2.9 Additional sterol and oxysterol measures. Values are in ng/mg tissue, except cholesterol which is reported as ug/mg tissue. *p* values refer to effects of genotype and CVS exposure. Significance was set to $p < 0.05$.

Measure	E2		E3		E4		*p*
	Control	CVS	Control	CVS	Control	CVS	
Hippocampus							
Cholesterol	6.79 ± 0.57	6.59 ± 0.85	7.40 ± 0.46	6.74 ± 0.34	6.90 ± 0.32	6.28 ± 0.96	n.s.
Cholestanol	22.34 ± 2.60	24.93 ± 1.59	26.40 ± 1.39	31.37 ± 1.06	24.38 ± 3.72	22.35 ± 2.91	n.s.
Desmosterol	126.50 ± 16.60	114.37 ± 25.07	122.27 ± 11.17	132.04 ± 11.17	108.36 ± 28.51	145.18 ± 38.66	n.s.
Lathosterol	14.46 ± 1.51	11.62 ± 2.77	13.10 ± 0.85	10.23 ± 0.75	9.55 ± 1.13	10.20 ± 1.20	n.s.
Cortex							
24S-hydroxycholesterol	62.18 ± 25.46	71.81 ± 21.81	77.66 ± 24.50	101.07 ± 22.27	64.29 ± 15.98	88.35 ± 13.47	n.s.
25 hydroxycholesterol	0.78 ± 0.14	0.60 ± 0.11	0.63 ± 0.11	0.68 ± 0.12	0.64 ± 0.047	0.61 ± 0.046	n.s.
27-hydroxycholesterol	1.08 ± 0.089	0.99 ± 0.11	0.92 ± 0.12	0.97 ± 0.19	0.74 ± 0.12	0.90 ± 0.091	n.s.

Chapter 3: Conclusions and future directions

General Discussion

As stated in Chapters 1 and 2, from these dissertation studies, I wanted to address 3 points: 1) Is there additional evidence that apoE isoform influences the behavioral and cognitive changes associated with stress exposure?, 2) How does sex modulate these effects, and 3) Do these apoE isoform-specific changes relate to downstream function of LDLR?

E2-specific changes in response to stress

The findings from my dissertation studies, summarized in **Figure 3.1**, support previous studies that have found that apoE genotype influences behavioral and cognitive changes at baseline and after stress exposure. Not only did I find evidence suggesting that cognitive performance in the water maze is specifically impaired in E2 mice exposed to CVS, but E2-specific changes were also found in adrenal measures and in body weight changes. The impairments in spatial cognition and peripheral changes in adrenal gland weights and HPA axis-associated protein markers seen highlight the range of symptoms seen in those with PTSD and argue that CVS exposure recapitulates some aspects of this disorder. These data thus emphasize the need to consider apoE genotype to most appropriately treat stress- and trauma-related disorders, such as PTSD. It is especially important for future human subject studies to include and report E2 carriers within their datasets as this study suggests that apoE genotype may modulate several factors that influence PTSD prognosis, including cholesterol metabolism and markers within the HPA axis, such as corticosterone and GR levels.

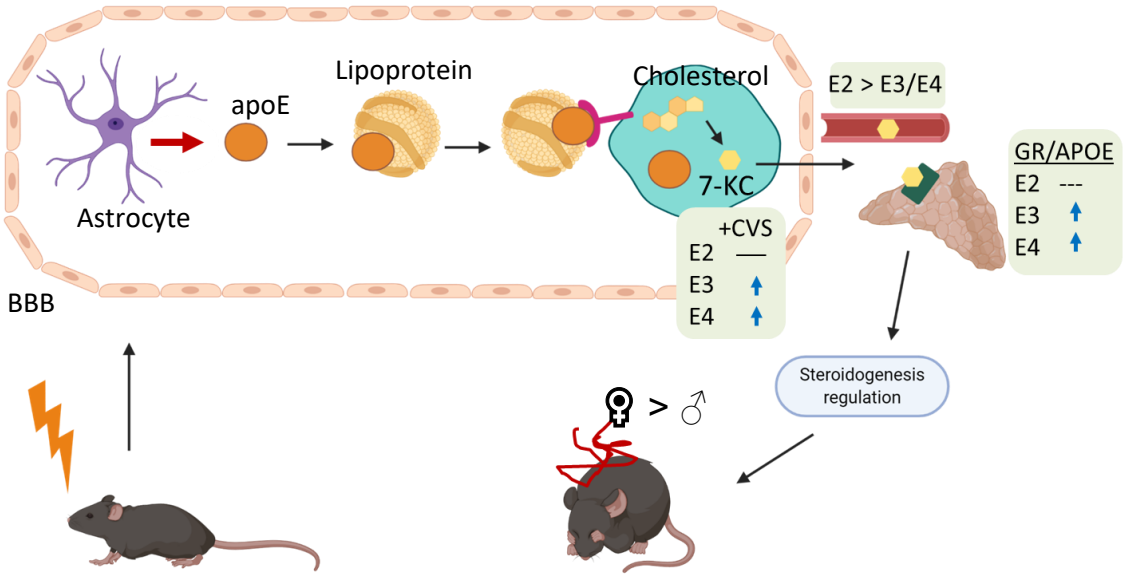


Figure 3.1 Summary of major findings. I found that after CVS there was a decrease in cortical levels of apoE. E2 female mice exposed to CVS lacked increases in cortical 7-ketocholesterol levels that were seen in E3 and E4 mice, despite no changes in LDLR levels. This change is proposed to lead to dysregulation of glucocorticoid synthesis in the adrenal gland, where I found similar apoE-isoform differences in GR normalized to apoE levels, likely due to transcriptional regulation. Dysregulation then corresponded to changes in behavior and cognition, most prominently spatial impairment in E2 mice that was worse in females compared to males.

Sex-differences in apoE isoform specific changes

Sex differences have been of rising interest in understanding PTSD (see Chapter 1 for overview). Behaviorally, E2 females demonstrated impairment in the first water maze probe trial that was not seen in E2 males. However, E2 males appeared more sensitive to changes in weight gain. Previous results suggest that 7-ketocholesterol activity in adipocytes may contribute to obesity (Wamil et al., 2008), yet levels in the plasma and periphery suggest this is unlikely to be the cause of the male-specific change in weight gain since CVS-related differences in 7-ketocholesterol in the liver were found regardless of sex. Differences in protein measures were not dependent on sex suggesting that these sex-dependent differences in cognitive impairments are likely influenced by additional biological aspects like sex hormones that should be considered in future studies. Thus, these sex x genotype specific responses are intriguing and require

additional studies. The role of estradiol, for example, mentioned in Chapter 1 may be critical in this association.

Biological mechanism for apoE isoform-specific changes in behavior and cognitive performance

While it is unlikely that binding affinity or expression of LDLR is altered, my results from 7-ketocholesterol support my hypothesis that the E2-specific changes are related to dysregulation of cholesterol metabolism. As mentioned in the discussion of Chapter 2, LDLR protein levels were not altered at the time point assessed after CVS, suggesting that amounts of LDLR are not necessarily involved in the E2-specific changes after CVS compared to E3 and E4 but that the binding affinity in E2 compared to E3 and E4 may be problematic when there is less apoE available after CVS exposure. Thus, because there is less apoE, cholesterol metabolism as an output of apoE-LDLR cholesterol intake is most affected in E2 mice. Remarkably, 7-ketocholesterol was the only oxysterol or sterol measured for this study that was significant different due to greater levels in E2 or CVS exposure (see **Suppl. Table 2.9** for additional sterols and oxysterols assessed). Future studies would certainly need to be done to parse out the exact mechanisms that result in specifically altered 7-ketocholesterol levels.

Neither ApoE nor cholesterol crosses through the BBB, but certain oxysterols, including 7-ketocholesterol, can enter and exit the CNS via the BBB. Thus, the apoE-isoform interaction with CVS exposure in 7-ketocholesterol may be one top-down mechanism that apoE influences the stress response by signaling to the periphery although it is likely the changes within the brain that result in behavioral differences. This is supported by the striking differences in protein levels in the periphery, including plasma, liver, and adrenal gland measures. There is very likely bottom-up feedback from the adrenal gland via glucocorticoids, as is true in the normal function of the HPA-axis, although further studies will need to determine apoE's role in the periphery in this response. Conditional KO animals could be used with respect to determining the importance

of CNS vs peripheral apoE, *i.e.* by downregulating expression in the adrenal gland or liver. Also, apoE in the periphery is secreted by macrophages and can further activate other macrophages, highlighting the potential role of immunoregulation in the peripheral response (Mahley, 1988).

Evaluating the apoE TR CVS model

As an overall model, the apoE TR mice CVS paradigm has demonstrated further evidence that apoE is an important genetic factor in stress response, especially in the context of severe or chronic stress, such as that seen in PTSD. While the behavioral measures included in these experiments covered a broad range—arousal, avoidance, cognitive impairments, it is critical to note that mouse models, including the one used here, can only inform researchers of particular aspects of the disorder. While animal studies allow researchers to decrease the potential influence of confounding variables, environmental changes, behavioral studies in mice are still subject to be easily perturbed by seemingly innocuous differences, such as different experimenters. While the experimental design of this project was created so that as many variables could be accounted for, some of potential confounding factors, such as the effects of pair-housing, are discussed in greater detail under Methodological Considerations and Appendix: Additional Methodological Notes.

Previously in Chapter 1, I reviewed the current literature on apoE isoforms in populations with PTSD. These results from human subject studies showed inconsistent findings on the influence of apoE isoforms. My own studies in apoE TR mice suggests that there are unique changes in E2 mice, but not E4 mice, in response to stress that supports that E2 carriers are more susceptible to changes in response to stress. This is in addition to the effects of CVS across all genotypes that support the use of CVS as a model of PTSD-like changes.

Taken together, my data suggest the importance of more fully understanding the role of lipid metabolism both in the brain and the periphery to better understand the biological changes associated with mental health disorders. As mentioned in Chapter 1, current treatments primarily

focus on symptoms but do not completely lead to remission in all individuals, thus highlighting strong need for additional targeted therapies. Genotyping for apoE in PTSD patients may thus allow treatments to target cholesterol dysregulation in E2 carriers. This dissertation thus provides a promising avenue for future studies to address more individualized therapies by suggesting novel targets like cholesterol metabolism for E2 carriers..

Methodological Considerations

Regional specificity

The lack of significant differences within the hippocampus in any of the protein and sterol analyses is perplexing considering the role it has in the tasks employed (spatial learning, contextual processing, etc.) as well as its involvement in fear learning and the stress response (see introduction). This argues, that at least in the case of sterols, cholesterol synthesis is not affected by CVS or apoE isoform. Analyses within the mPFC present a similar puzzle considering its extensive role in decision making, contextual processing, and salience detection (See Chapter 1). Methodological considerations (discussed further below) may contribute to this; for example, dissections of these small regions may have introduced variability. In addition, as these tissues were collected 12 days after the final stressor, I may have missed earlier changes, which is discussed further below.

However, while future studies should further explore the regional specificity seen here, the findings in the cortex and adrenal gland are certainly well-defined. The similarity between the genotype x CVS exposure interaction found in both tissues further support the specificity of these regions in the association between E2 and CVS. However, both tissues were not changed in all measures equally suggesting that while there is some tissue specificity, there are additional factors that need to be understand.

Human apoE mice

The human apoE TR mice have provided enormous information about the role of apoE in human disorders (Balu et al., 2019). However, there are certain caveats about the use of these mouse lines that should be kept in mind. For example, the E2 mice have demonstrated both in my data (**Figure 3.2A)** and others (Sullivan et al., 2004) far higher plasma cholesterol levels than E3 and E4 mice. It should be noted that while my data correspond with other data, plasma cholesterol was measured using samples from the first cohort of animals during which both CVS and control mice were housed concurrently in the same room (see Appendix: Additional Methodological Notes). These differences in cholesterol are paradoxical to what is seen in humans. In fact, human E2 carriers show lower plasma cholesterol levels and lowered risk to developing atherosclerosis unless they have an additional health condition such as diabetes or obesity resulting in Type III hyperlipidemia (Mahley & Rall, 2000), suggesting that another apolipoprotein or metabolic agent is dissimilar enough in the mice to cause the discrepancy. Besides the fact that mice alone metabolize cholesterol at different rates than humans in general, this isoform-specificity may tie in to how E2 displays poorer binding affinity to LDLR. Thus, perhaps mouse LDLR differs from human LDLR in a critical manner. Although not feasible for this dissertation, future studies could assess if human LDLR mice crossed with the apoE TR mice circumvents this potential confound. Previous work using hemizygous human LDLR (hLDLR) mice crossed with E2, E3, or E4 mice showed that hLDLR lowered plasma cholesterol levels in general. Furthermore, hLDLR/E2 plasma cholesterol levels were similar to hLDLR/E3 and hLDLR/E4 (Johnson et al., 2014). Such plasma cholesterol levels are more similar to what is seen in humans. Taken with the profound effects seen in tissues outside the CNS in the current study, the human apoE/hLDLR cross may further aid in understanding the translational relevance of the changes in lipid metabolism such as 7-ketocholesterol in relation to the stress response.

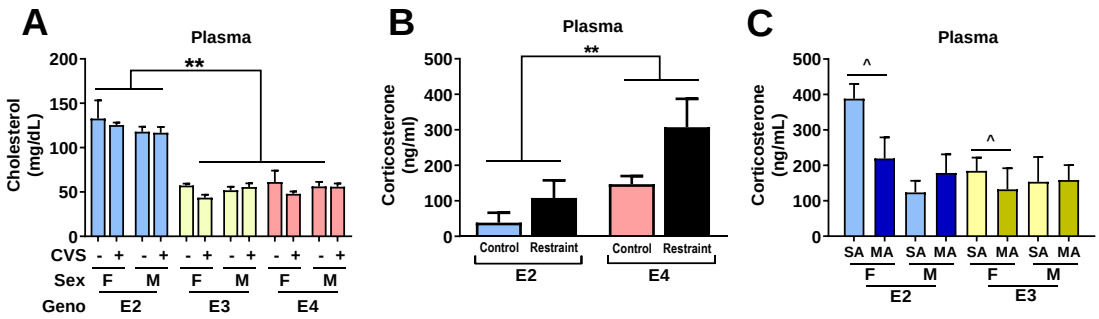


Figure 3.2 Plasma corticosterone levels in apoE TR mice A) at baseline or after 30 min of restraint stress (+$p < 0.05$) and B) after a single injection of either saline (SA) or 1mg/kg methamphetamine (MA) (^$p < 0.05$). C) Plasma cholesterol levels are higher in E2 mice compared to E3 and E4 mice.

Similar to cholesterol levels, corticosterone levels were elevated in E2 mice regardless of CVS exposure compared to E3 and E4 mice. While it would be informative to know if plasma corticosterone levels correlated with plasma cholesterol levels, this was not possible in this study because the samples were not taken from the same animals for the two measures. This isoform-specific difference has not been seen at baseline in past work (Johnson et al., 2015) nor in preliminary studies (**Figure 3.2**). In fact, in one small pilot study from our lab (unpublished data) looking at the effects of 30 min restraint stress in E2 and E4 mice, E4 mice showed significantly higher corticosterone levels compared to E2 mice (**Figure 3.2 B** $F_{1, 13} = 6.58$, $p = 0.024$). Restraint stress showed a trend for an increase in corticosterone levels ($p = 0.077$). This is particularly interesting as it further suggests that E2 mice lack a typical HPA axis response. Furthermore, in mice injected with either saline or a low dose (1mg/kg) of methamphetamine and tested for behavioral and cognitive changes associated with methamphetamine exposure differences, only females showed higher levels of corticosterone **(**unpublished data, **Figure 3.2 C**, $F_{1, 27} = 4.98$, $p = 0.034$). These data support that the effects in E2 mice described in Chapter 2 are due to increased overall stress levels in the control group rather than an inherent difference in these mouse lines. It should be noted that genotype differences between studies in plasma corticosterone levels may be due in part to the nature of the blood collection. The data in Chapter

2 involved blood collection from the mandibular vein in awake mice, whereas the other studies mentioned above collected blood at euthanasia via cervical dislocation and decapitation or lethal ketamine cocktail injection. Thus, collection procedure may have influenced the genotype differences seen.

Potential effects of pair-housing

As noted above, pair-housing the mice may have resulted in the inconsistent results between the current study and Johnson et al. 2015 we have found in the apoE TR mice. Furthermore, it precluded the assessment of nest scores, a measure of self-care which we have previously reported genotype differences after CVS exposure (Johnson et al., 2015). Social housing was chosen to avoid potentially worsening the effects of CVS. Ros-Simó and Valverde have shown that social isolation in young (3 weeks old) CD1 male mice results in greater anxiety-like behavior as well as hyperactivity and greater increases in plasma corticosterone after a stressor compared to mice that were group housed (Ros-Simó & Valverde, 2012). Thus, pair housing after CVS may have acted as "community support" and mitigated the behavioral effects of CVS. Previous studies show that social buffering can occur when social support lessens the effects of stressors (Davitz & Mason, 1955). More recent work shows evidence that if the conspecific spectator is fearful, there will be increased responses in the naïve-exposed animal to the stressor, but if both animals are exposed to the same stressor, then the shared experience results in attenuated fear-related behaviors (Lee & Noh, 2016). In humans, strong social support can help decrease the development of PTSD in adults with chronic illness (Dinenberg et al., 2014) as well as those in the military (Pietrzak et al., 2011).

Social dominance structures in mice may have also affected behavioral and biochemical measures throughout the experiment and is something to consider for future studies. However, when mice were placed back into pair housing after CVS, there were no signs of fighting noted

suggesting that social dominance may not have shifted throughout the housing changes. Group housing could also be considered more relevant to humans, considering that social isolation is generally not associated with trauma. Future studies assessing behaviors (*e.g.* social interactions as well as grooming) would be informative to learn if post-trauma coping behaviors indicate which animal is more likely to develop greater CVS-related behavioral changes. The important of group housing vs single housing is explored in the Appendix: Avoidance behavior in apoE mice. The potential effects of housing also emphasize the importance of husbandry details in behavioral testing, especially in the context of complex health disorders.

Time course of changes in apoE and 7-ketocholesterol

This work shows molecular changes that are measurable at 12 days after the final stressor. However, due to the infrequent sampling (*i.e.* only at the end of behavioral testing), this study cannot inform us when the changes in apoE and 7-ketocholesterol arise. We also have tissue from a small cohort of mice that underwent an acute stressor, 15-min restraint, which will be important to assess for possible changes in apoE and 7-ketocholesterol levels; however, I hypothesize that the differences described here will be found to a lesser degree in these tissues because of the acute nature of the stress exposure. Considering the preliminary findings mentioned above showing lower levels of plasma corticosterone in E2 mice compared to E3 mice, there may be a subset of changes that occur quickly, such as corticosterone signaling that precedes longer term changes like apoE expression levels.

Future directions

There are certainly several lines of study to pursue to fully understand the role of 7-ketocholesterol and apoE isoform in response to chronic stress. While a mouse model was chosen in order to assess apoE isoforms because E2 and E4 are found at lower rates in the human

population, it is important that E2-specific changes, such as that seen in 7-ketocholesterol, be explored in human cohorts although samples would be challenging to obtain considering the ethical concerns.

Considering the brain regions involved in PTSD-related changes (discussed in the introduction, namely fear learning, threat detection, executive function and emotion regulation, and contextual processing), future work should expand on the brain regions that were assessed in this dissertation work. Regions such as the amygdala are critical to look at; however, as immunohistochemistry did not work well with methodology employed, it was not included in this dissertation (Appendix: Additional Methodology Notes).

Resiliency to trauma exposure in PTSD has now come to the forefront of research. This is perhaps due to the acknowledgment that many animal models currently assess PTSD-related symptoms with the assumption that all animals within the PTSD group will display phenotypes rather than a subset, as is seen in human populations (Cohen & Zohar, 2004). Since interventions after traumatic experiences generally poorly mitigate PTSD symptoms (Chapter 1), resiliency research redirects researchers' attentions to understanding why some individuals are resistance to the effects of traumatic exposure instead of why some individuals are more susceptible. This shift in focus explores factors that may be critical in helping an individual adapt after trauma in a healthy manner. Horn et al. reviews recent work on resiliency in PTSD and highlights the importance of social factors, such as support systems or children bonding with their caregiver, as well as personal agency, such as the ability to actively cope with the traumatic situation vs avoiding (Horn et al., 2016). In terms of biological factors, studies provide evidence that it is not just an absence of vulnerability that results in resiliency. For example, mice resilient to the effects of chronic social defeat stress show increased levels of molecular plasticity and adaptive responses in gene expression (Jung et al., 2015; Krishnan et al., 2007). This highlights the strong social nature of mice and supports the importance of social housing in mouse behavior.

While previous research using the human apoE TR mice have included an expansive range of behavioral and cognitive tests, future studies assessing apoE isoforms in the context of stress/trauma should incorporate these other behaviors such as fear extinction and acoustic startle to better understand the impact of the different apoE isoforms. Additional experiments have started to attempt to parse out the effects of apoE isoform on different behaviors, specifically avoidance behavior (see Appendix: Avoidance Behavior in apoE Mice).

The ultimate goal for the animal work included in this dissertation is of course the implications for translational findings. As mentioned in Chapter 1, studies in human populations presents challenges because of the low allele frequencies of E2 and E4. Furthermore, in particular for apoE studies in PTSD patient populations, most studies have been limited to male combat veterans. Both sex and ethnic background modulate the influence of apoE isoform on Alzheimer's disease (Farrer et al., 1997). Thus, more research is warranted to understand how these factors interact to influence PTSD. In a small collaborative study with the OHSU Intercultural Psychiatry Program, we have genotyped Cambodian and Vietnamese patients receiving therapy for a variety of mental health disorders to assess if E2 carriers are more prevalent than expected based on known allele frequencies (see Appendix: Translational Implications of apoE Genotype on PTSD Susceptibility).

Considering the complex heterogeneity seen in the biological underpinnings of PTSD, one hope for future work would be for apoE genotype to be used in conjunction with multiple biomarkers to improve the "PTSD biological profile." Public health for PTSD needs to be addressed on the preventative as well therapeutic level (Watson, 2019). Understanding who is at increased risk of developing PTSD as well as the biological correlates associated with this susceptibility will hopefully inform public health how to create opportunities not just for treatment but also prevention.

PTSD has a truly complex biological basis—highlighted by the number of findings tying in genetics—resulting in the conclusion that no single factor should be a biomarker (Shalev et al., 2017). This heterogeneity underscores that there is much to be understood about the biological changes associated with the disorder and that for better treatment, research needs to address this heterogeneity using a more granular approach. Further experiments exploring how to attenuate the cognitive impairments associated with E2 in the context of chronic stress will be beneficial to developing targeted therapies even if it only applies to a subset of those afflicted with PTSD. Hence, this dissertation has contributed further understanding of the role of apoE isoforms and their functional outcomes in the stress response and emphasizes novel factors to consider in the PTSD biological profile.

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Appendix

Additional Methodology Notes

Testing of control and CVS groups simultaneously

The first cohort of this study included mice exposed to CVS, as well as controls, in an attempt to avoid confounds associated with the different cohorts. When mice were exposed to CVS, they were housed in a separate room than controls to avoid indirect effects and then placed back in the same room as controls after. In addition, plasma samples were taken from all animals prior to the elevated plus and zero mazes, as well as after the tests using mandibular vein bleeds (one on each side according to OHSU IACUC approval). However, behavioral data from the water maze suggested that the mice were overly stressed. Due to a concern that scents and sounds transmitted more freely between activity monitoring cages compared to the typical home cages on Thoren ventilation racks, the simultaneous testing of groups was removed in favor of testing controls and CVS mice separately. The additional blood samplings were also removed as a precaution of inducing stress in the control mice. For this dissertation, the effects of multiple sampling were not controlled for since this procedural detail was removed for the cohorts included.

Western blot troubleshooting

Original western blot protocols for this dissertation work used chemiluminescent staining to visualize antibody binding. While trying to determine concentrations for the GR antibody, it became apparent that LDLR KO animal tissues (from different colonies from the Tavori and Raber labs) showed nonspecific binding signaling at the same size expected for LDLR (**Figure A.1 A-B**). Other backgrounds bands appeared, although these were not at sizes of interest. As a

result, my methodologies switched to incorporate near infrared fluorescent staining, which did not result in background bands (**Figure A.1 C**).

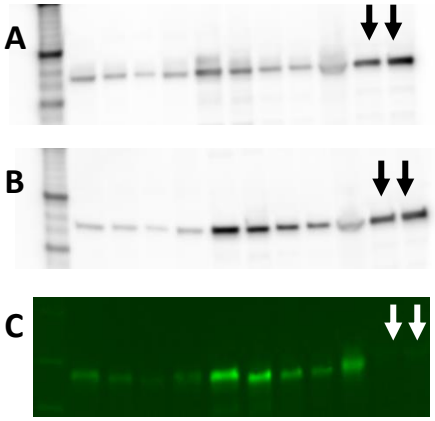

Figure A.1 Western blot confirmation of LDLR antibodies. A) Primary antibody treatment using chemiluminescence. B) No primary control using chemiluminescence shows that the secondary antibody has false positives. C) NIR protocol results in clean bands. Arrows point to LDLR KO samples.

Immunohistochemistry

Earlier proposals of my dissertation work included the use of immunohistochemistry to better address regional specificity of LDLR, apoE, and c-Fos. Specifically, I had exposed E2, E3, and E4 male and female mice to either CVS or control conditions and then euthanized these mice 3 hours after the final stressor to assess for early immediate gene expression, *i.e.* c-Fos, and potential colocalization with markers of interest, including LDLR, apoE and GR. Regions of interest included the central and basolateral nuclei of the amygdala, subregions of the hippocampus, and medial prefrontal cortex. While this would have undoubtedly been neuroanatomically informative, the lack of success in a series of extensive pilot studies to optimize this protocol led to the decision to exclude immunohistochemistry from my dissertation and focus on using Western blot analysis of protein quantification.

Avoidance Behavior in apoE Mice

[Note: The following study is being completed for submission. I would like to acknowledge Sarah J. Holden with her help acquiring this data, as well as Payel Kundu and Destine Krenik for their help with dissections.]

Introduction

Relationship of avoidance to PTSD

Avoidance behavior is one of the primary PTSD diagnostic criteria and may be especially important to assess during early stages considering their predictive power for later PTSD symptom severity (Perkonigg et al., 2005; Shin et al., 2015). Unlike fear conditioning, in which an animal is forced to experience shocks, avoidance assays allow for the animal to either actively or passively escape the negative stimulus. This "choice" activates separate circuits from those in fear conditioning (LeDoux et al., 2017).

As discussed in Chapter 3: General Discussion, the apoE TR mice CVS paradigm has been greatly informative in understanding behavioral and cognitive symptoms associated with PTSD and potential underlying biological changes. However, as also previously mentioned, the CVS paradigm does have caveats. Although the CVS model and subsequent behavioral testing assesses anxiety-like behavior using the elevated plus and zero mazes in which rodents can choose to avoid the more anxiogenic open areas, there are other tests that can address this. One example is the passive avoidance test in which mice are placed into one of two chambers that are connected by a gate that begins in the closed position. The chambers are both dark at first and after a brief habituation period, a cue light turns on in the first chamber at which point the gate is opened. Mice are then free to escape the aversive light and enter the second, still-dark chamber. Upon entering, however, they are given a mild foot shock in this novel environment. Time to

cross into the dark chamber is recorded. To test memory after a 24-hour period, mice are then placed back into the first chamber, and the trial repeats, except without a foot shock if the mouse does cross into the second chamber. Latency to cross is again recorded and expected to be higher than the first day. A recent model of mild traumatic brain injury has shown that after a mild physical trauma, mice expressing amyloid precursor protein showed increased latencies to cross when mice were re-exposed to the test environment for numerous subsequent days, similar to extinction training in fear conditioning (Cheng et al., 2019). I used a behavioral paradigm similar to this previous study to address whether avoidance behavior is differentially affected by apoE isoform. Data from the current study suggests that E2 mice show poorer passive avoidance memory compared to E3, E4, and wildtype (WT) mice.

Methods

Animals

Male and female mice were included in this study and used according to IACUC guidelines at OHSU. Mice were either WT (C57BL/6J) or human apoE TR E2, E3, or E4 mice bred in house. Animals were 2-4 months of age and singly housed at the start of the study. On the 11th day after passive avoidance training, mice were group housed (2-5 mice/cage) with their original littermates for the rest of the study. Throughout the entire study, mice had access to food and water *ad libitum* and were maintained on a 12 : 12 hr light cycle. Group sizes involved are shown in Table A.1.

Table A.1 Group sizes

Groups				
	E2	**E3**	**E4**	**WT**
♀	13	14	14	15
♂	13	14	15	15

Behavioral Testing

Similar testing parameters, including a 0.3 mA foot shock, were used as described previously (Cheng et al., 2019) to assess passive avoidance memory over 49 days after the initial training session. On the 50th day after passive avoidance training, mice were also assessed for locomotion and exploration in the open field during a 5 min trial. Ethovision 14 was used to record and measure distance moved and time within the center of the arena (Noldus, Netherlands). The next day mice were assessed for depressive-like behavior in the forced swim task in which mice are allowed to swim in a container filled with room temperature water for 6 min. Videos were recording and time spent immobile in the later 5 min of the trial was rated by an experimenter blind to the genotype of the mice. All behavioral testing occurred during the light phase. The timeline is shown below in **Figure A.2**. Mice were euthanized via cervical dislocation after the forced swim test and tissues were collected for future analyses.

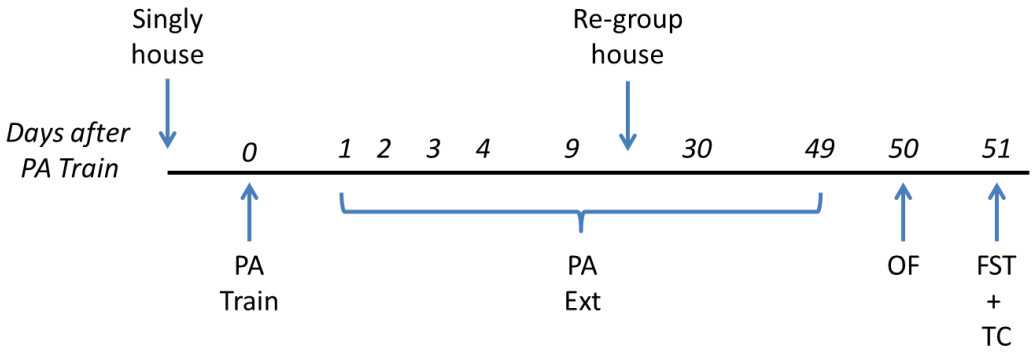

Figure A.2 Experimental design. PA = Passive Avoidance, Ext = Extinction, OF = Open Field, FST = Forced Swim Test, TC = Tissue Collection.

Statistical Analysis

Data are reported as mean ± SEM. Analysis was conducted using SPSS v25 (IBM, Armonk, NY, USA) and visualized with GraphPad v.8.2.0 (Prism, La Jolla, CA, USA). Multi-factorial ANOVA were used and included genotype and sex as factors. Sidak's correction for multiple comparison's was used and pairwise comparisons using the estimated marginal means

are noted when appropriate. For passive avoidance testing, data collected when mice were singly housed (Days 1-9 post-training) were analyzed separately from data collected when mice were returned to their original group housing (Days 30-49 post training). Repeated measures were applied and Mauchly's test of sphericity was used to assess if sphericity corrections (*i.e.* Greenhouse-Geisser corrections) were needed. Significance was set to $p < 0.05$.

Results

Passive avoidance

There were no significant differences in the latency to cross to the dark chamber during the training day of passive avoidance (**Figure A.3 A-B**). During the first 5 testing days after training, WT mice overall showed faster latency to cross, which was largely driven by the male WT mice (genotype: $F_{3,105} = 5.03$, $p = 0.003$; sex x genotype interaction: $F_{3,105} = 3.00$, $p = 0.034$). During group housing test days, males showed higher latencies compared to females $F_{1, 105}= 5.13$, $p = 0.026$) and WT mice again had the lowest average latency to cross compared to the human apoE mice ($F_{3,105} = 8.21$, $p < 0.001$).

Latency times were also normalized to the first test day after training (**Figure A.3 C-D)**. During the test days during singly housing, mice overall tended to increase over days ($F_{2.10, 220.64} = 3.48$, $p = 0.001$) that was largely affected by genotype ($F_{6.30, 220.64} = 2.63$, $p = 0.016$). E2 mice were the highest, indicating they tended to increase in their latency score of this extinction period ($F_{3,105} = 3.07$, $p = 0.031$). This genotype difference was not seen during the final days of testing when the animals were group housed.

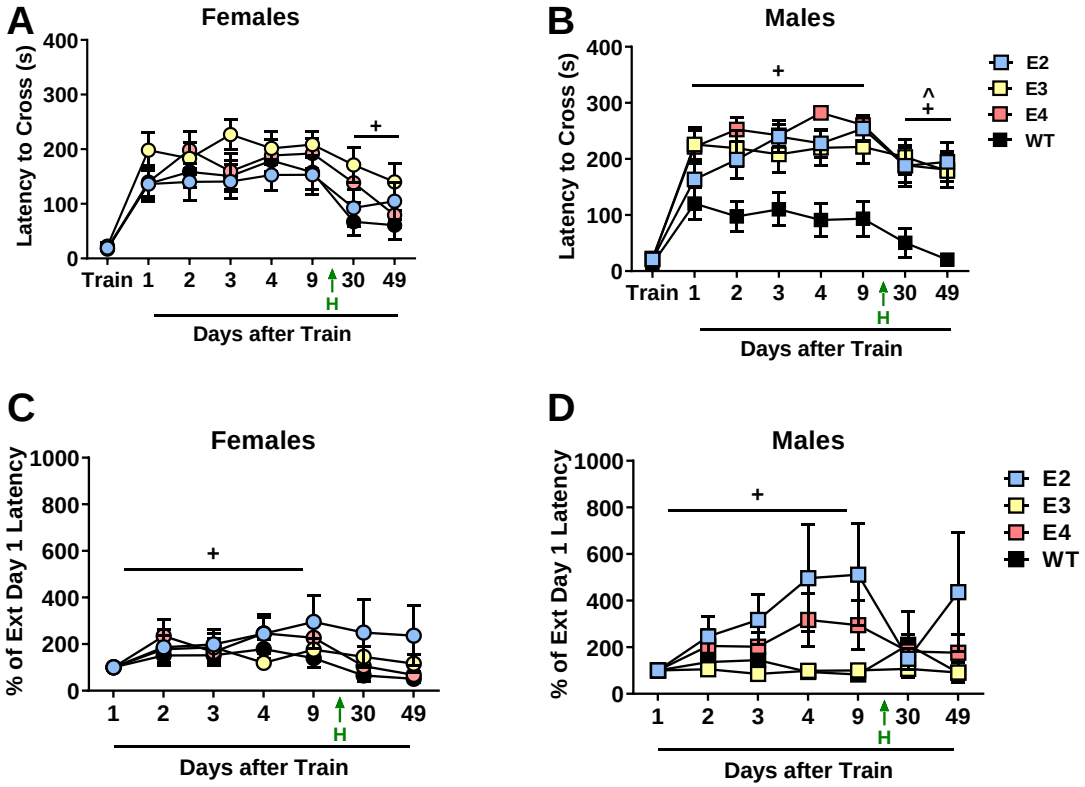

Figure A.3 Passive avoidance extinction in hapoE and WT mice. WT mice crossed to the other chamber in less time than both **A**) female and **B**) male hapoE mice during single housing days (+ $p < 0.05$). After mice were placed back into group housing (denoted with H on the x axis), there was a significant effect due to sex with males crossing slower than females (^ $p < 0.05$) in addition to a genotype difference again driven by WT mice. **C**-**D**) When latency to cross was normalized to the first day after training, there was a main effect of genotype that was driven by overall higher % of Extinction Day 1 Latency in E2 mice vs E3, E4, and WT mice.

Open field

Total distance moved within the open field arena showed that males explored the arena less than females ($F_{1,105} = 12.87$, p = 0.001) and that E2 mice explored less than E3 mice (p = 0.013) and WT explored most ($F_{3,105} = 16.65$, $p < 0.001$, E2 vs WT $p < 0.001$, E3 vs WT p = 0.003, E4 vs WT $p < 0.001$). On the other hand, E3 mice spent the most time in the center of the arena ($F_{3,105} = 8.033$, $p < 0.001$).

Forced swim test

Analyses of the time spent immobile during the forced swim test showed a trend for genotype differences ($F_{3, 105} = 2.68$, $p = 0.051$).

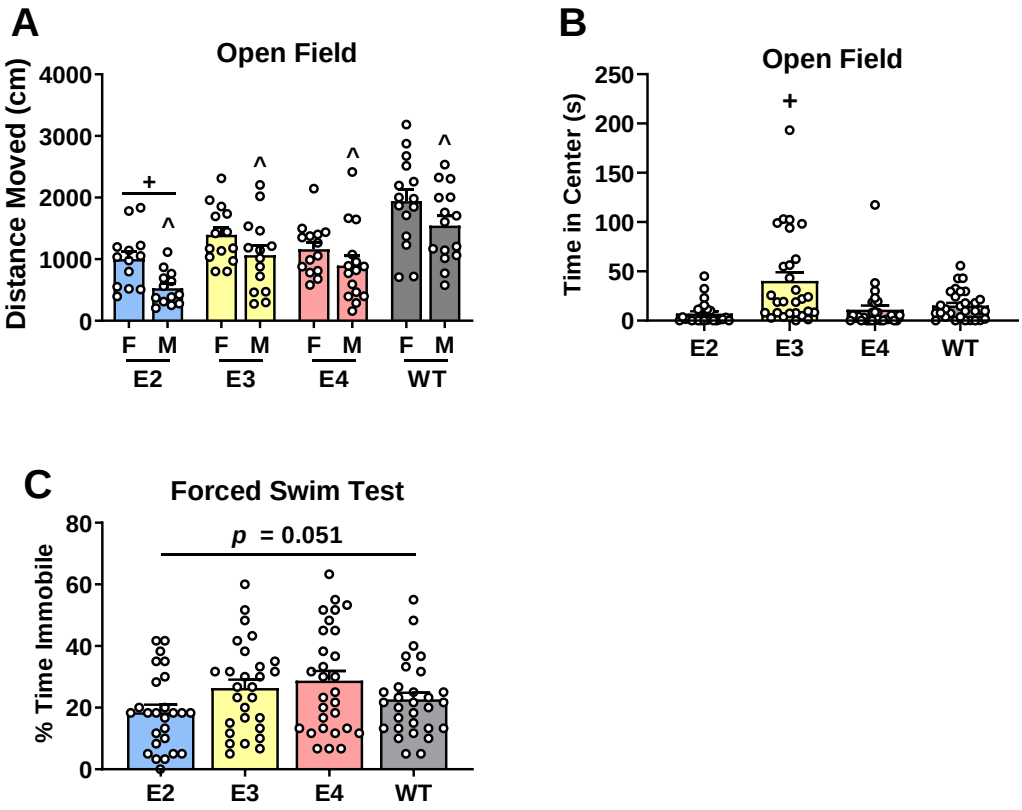

Figure A.4 Behavioral tests after passive avoidance extinction. A) Males explored the open field arena less than females (^$p < 0.05$). E2 mice explored the least compared to E3, E4, and WT mice. B) E3 mice spent the most time exploring the center of the open field (sexes shown collapsed, +$p < 0.05$). C) There was a trend in genotype ($p = 0.051$).

Discussion

These data show evidence that the E2 isoform leads to unique behavioral changes in passive avoidance behavior that further support E2 as a genetic risk factor for behaviors associated with PTSD. E2 mice longer latency to cross to the dark platform across extinction learning suggesting that avoidance behavior grew stronger over time although this does appear

driven by the males. Excitingly, this genotype effect disappeared when mice were regroup-housed emphasizing the importance of social support systems.

The purpose of this study was not to create a model of PTSD, but to assess the diagnostically critical symptom of avoidance in the apoE TR mice. The addition of open field and the forced swim test allow additional understanding of potential confounding factors (such as hyperactivity or depressive-like behavior). Findings in the open field show that E2 and E4 mice may have increased levels of anxiety compared to E3 mice.

However, behavior in E3 mice were markedly different from WT mice, highlighting differences between mouse and human E3 apoE that should not be ignored. Previous studies using the apoE TR E3 and E4 mice and strain-matched WT mice (C57BL/6J) showed similar levels of exploration compared to E3 mice but lower percent time freezing, *i.e.* fear behavior, compared to E3 mice during both fear acquisition and memory tests (Villasana et al., 2016). Indeed, homology between mouse and human apoE amino acid sequences has been reported to be only 70% (Rajavashisth et al., 1985). Furthermore, murine WT apoE contains the arginine at the equivalent 112 and 158 residues resulting in a sequence more similar to E4. Murine WT apoE as a result only has a single-domain whereas human apoE has 2 (Nguyen et al., 2014). The results presented here further support that expression of human apoE instead of murine apoE leads to significant behavioral differences, presumably due to these structural differences.

It should be noted that the single foot shock used in this passive avoidance paradigm was not intended to model PTSD induction (although it is presumably a significant experience for a rodent), but rather to highlight a separate behavioral pathway important in PTSD. Thus, the additional behavioral tests (open field and forced swim) may more closely reflect baseline measures and in fact are similar to previous studies in these mice (Siegel et al., 2012).

The forced swim test has long been used as a measure of depressive-like behavior due to its predictive validity for the therapeutic effects of selective serotonin reuptake inhibitors as well

as other drugs. However, there is growing acknowledgment within the behavioral neuroscience field that that it is a poor behavioral assay for depressive-like behavior. Instead, the behaviors during this test may represent active coping strategies during inescapable stressors. In addition to other tests that measure specific facets of depression, such as sucrose preference to assess anhedonia, an "emotionality score" may be more informative when it comes to complex psychiatric disorders (Gururajan et al., 2019). While we recognize that the field is moving away from it and will include more informative behavioral assays in the future, we want to be fully transparent and acknowledge the entirety of the behavioral testing the mice underwent.

Although it did not reach significance, male and female E2 mice appeared to respond differently after group housing in the final extinction trials. Social buffering, or the effect of the presence of a conspecific to decrease the response to stressors, has been shown to have sex differences. For example, males that were group housed showed exacerbated decreases in proliferating cells within the dentate gyrus of the hippocampus, while in females the decrease in proliferating cells was ameliorated with group housing. There were no sex differences in behavior or cognitive performance however (Tzeng et al., 2016).

Future work will address how the behaviors seen in this passive avoidance paradigm differs from a similar fear conditioning paradigm. Such work can help elucidate the differences between fear and avoidance circuitry and provide a broader view of the changes in disorders such as PTSD.

Translational Implications of apoE Genotype on PTSD Susceptibility

Note: The following work was done in collaboration with David J. Kinzie, Daniel Towns, and James Boehnlein at the OHSU Intercultural Psychiatry program and is being prepared for submission. The work has been adapted for inclusion here.

I wish to sincerely thank Kim Truong-Pham, Kanya Pou, and Loan Huynh for their services as counselors at the clinic and aiding with translating and collecting the saliva samples. We also thank Dr. Clive Woffendin and his staff at Oregon Clinical and Translational Research Institute for genotyping. Thank you to Reed Hall for his help with sorting the data.

Introduction

Post-Traumatic Stress Disorder is characterized by four symptom subsets including re-experiencing, negative cognition and mood, altered arousal and reactivity, and avoidance behaviors (DSM-V). Yet, despite knowing the traumatic event leading to the diagnosis, treatment options have remained suboptimal. The difficulty associated with treating PTSD is in part due to the high variability seen within the disorder, ranging from the types of symptoms to the severity (Shalev et al., 2017).

Genome-wide association and twin studies suggest a strong heritable component emphasizing the need to understand genetic influences on both susceptibility and prognosis of the disorder. Previous studies have assessed the influence of apolipoprotein E in PTSD (see Chapter 1: ApoE and its relationship to stress response for summary). However, apoE allele prevalence rates vary widely among different ethnic populations (Singh et al., 2009; Tan et al., 2003), adding additional complexity to studying apoE genotypes in an already highly heterogenous disorder such as PTSD. Furthermore, even disorders highly correlated to apoE genotype, such as the E4

allele and its associated risk for Alzheimer's disease and cardiovascular disease are influenced by race and ethnicity (Farrer et al., 1997; Osuntokun et al., 1995; Rajan et al., 2017; Tzourio et al., 2008) highlighting the need to better understand apoE and its suggested associated altered risk and symptom severity for PTSD in a different population.

The OHSU Intercultural Psychiatry Program (IPP) has been a long-standing, successful teaching clinic for cross-cultural psychiatry (Boehnlein et al., 2008). At the OHSU IPP, PTSD rates were surprisingly high in this population of Southeast Asian refugees after a secondary, more structured interview to assess for PTSD (Kinzie et al., 1990). The cross-cultural nature of treating refugees, especially from countries with vastly different cultures and belief systems, perhaps contributed to this high diagnosis rate during the second interview. Civilian patients with PTSD, such as refugees typically have high rates of mental health concerns (Goodkind et al., 2014). Thus, we sought to better understand if apoE genotype influences the prevalence and severity of PTSD symptoms in Cambodian and Vietnamese refugees. High prevalence rates of diabetes and hypertension have been previously seen in this population and were also assessed as comorbidities (Kinzie et al., 2008).

Methods

Subject enrollment, saliva sample collection and genotyping

Subjects of Cambodian or Vietnamese background were recruited at the Oregon Health & Sciences University (OHSU) Intercultural Psychiatry Program. Accredited counselors provided translation for the study as the majority of the subjects do not speak English. Upon enrolling in the study, subjects signed consent forms, which were made available in Vietnamese or Khmer. Saliva samples were collected for apoE genotyping, coded with a study ID, and frozen in a secure -20°C freezer. Staff at the Oregon Clinical and Translational Research Institute determined apoE genotype. Information regarding sex, age, ethnicity, PTSD diagnosis (none, current, in remission,

etc.), primary PTSD symptoms, PTSD severity at time of diagnosis, presence of other health conditions (specifically, diabetes, cardiovascular disease, hypertension, sleep problems, substance use disorders, and smoking status), presence of comorbid psychiatric disorders, and current medication were also retrieved from patient health records. All documents and protocols were approved of by the OHSU Institutional Review Board.

Statistical analyses

Due to the low expected frequencies of E2 and E4, genotypes were assessed by grouping all E2+ (E2/E3, E2/E4) were compared to E2- and subsequently all E4+ (E3/E4, E2/E4, and E4/E4) to E4- subjects. Binomial tests were used.

Results

Results are being finalized. Preliminary analyses suggest that within our cohort of individuals, there is a greater percentage of E2 carriers in our PTSD+ group than would be expected based on Hardy-Weinberg equilibrium.

Conclusion

This study will provide additional evidence of the influence of apoE genotype on PTSD, especially when it comes to non-traditionally assessed populations. This population has been shown to deal with trauma and may react to treatment significantly differently due in part to cultural norms. For example, patient-doctor interactions are confounded by feelings of shame and discomfort at sharing significant, traumatic events with American doctors that presumably do not share such experiences (Kinzie et al., 1990).

It should be noted that due to the nature of the population that the clinic serves, many of these subjects have been patients of the IPP for decades, since they relocated to Portland, Oregon.

Furthermore, due to the severe nature of the traumas experienced, PTSD severity was not assessed by DSM-V criteria and instead determined by a clinician assigning a category (*i.e.*, "mild," "moderate," or "severe". While these factors do limit the generalizability of the study, our findings suggest that E2+ genotype may lead to longer-lasting and perhaps even more severe cases of PTSD. However, apoE genotype of previous patients at the IPP are unavailable and highlights the need to replicate this study elsewhere to confirm the findings. Understanding factors influencing PTSD is especially important as research shows children of Vietnamese refugees are more susceptible to developing mental health disorders (Vaage et al., 2011).

Additional publications

During my dissertation work, I have had tremendous opportunities to expand my research experience beyond what I proposed for my experiment. These have led to the following publications listed below.

1. **Torres ERS**, Boutros SW, Meshul CK, and Raber J. ApoE isoform specific differences in behavior and cognition associated with sub-chronic MPTP exposure. *Learn. Mem.* Accepted July 7, 2020.

Most patients with Parkinson's disease will also develop dementia. Increased rates of dementia in those with Parkinson's disease has been associated with E4. To better understand prodromal changes, such as circadian dysfunction, we used young female and male E3 and E4. Mice were either treated with saline or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that induces parkinsonism-like changes. We found that E4 mice were more affected by MPTP treatment in activity monitoring, indicative of circadian rhythm dysfunction, as well as in cognitive performance in the water maze. This study highlights the use of prodromal timepoints in Parkinson's disease research and support the influence of E4 in this disease. I performed behavioral and cognitive testing, assessed protein levels, analyzed the data, and wrote the manuscript.

2. Raber J, Anaya AF, **Torres ERS**, Lee J, Boutros S, Grygoryev D, Hammer A, Kasschau K, Sharpton TJ, Turker MS, Kronenberg A. Effects of six sequential charged particle beams on behavioral and cognitive performance in B6D2F1 female and male mice. *Front. Physiol*. Accepted July 15, 2020.

As a follow up to previous work in understanding the effects of space irradiation, our group assessed the behavioral and cognitive effects in C57BL/6J × DBA2/J F1 male and female mice after exposure to sham or sequential six-beam irradiation (protons, ^{4}He ions, ^{16}O ions, ^{28}Si ions, ^{48}Ti ions, and ^{56}Fe ions) at 0, 25, 50, or 200 cGy at 4-6 months of age. We found female mice treated with 50 or 200 cGy showed cognitive impairments in the novel object recognition test. Both males and females showed deficit in avoidance learning in the passive avoidance test. To assess if the gut-brain axis may have a role in these behavioral changes, we assessed the gut microbiome which primarily corresponded to differences between males and females. Interestingly, several taxa were identified that responded to radiation in a dose-dependent manner. These data suggest that the gut-microbiome is sensitive to radiation and that radiation results in cognitive impairment. For this study, I mentored interns on the behavioral tests and protein analyses. I analyzed the data and wrote the manuscript along with the other co-authors.

3. Burfeind KG, Zhu X, Norgard MA, Levasseur PR, Huisman C, Buenafe AC, Olson B, Michaelis KA, **Torres ERS**, Jeng S, McWeeney S, Raber J, and Marks DL. (2020). Circulating myeloid cells invade the central nervous system to mediate cachexia during pancreatic cancer. eLife, 9, e54095. https://doi-org.liboff.ohsu.edu/10.7554/eLife.54095

Inflammation has been considered important in the mechanisms underlying weight loss and anorexia seen in cancer patients. However, the source of inflammation is unknown during malignancy. Using a mouse model pancreatic ductal adenocarcinoma. Our findings suggest that the chemokine receptor type 2/ C-C motif chemokine ligand 2 axis recruits neutrophils into the brain and drives anorexia and muscle wasting. I helped analyze behavioral data and reviewed the manuscript.

4. Rhea EM, **Torres ERS**, Raber J, and Banks WA. Insulin BBB pharmacokinetics in young apoE male and female transgenic mice. *PLOS ONE. 2020 Jan 31;15(1):e0228455. doi: 10.1371/journal.pone.0228455. eCollection 2020.*

Female sex and E4 synergistically increased the risk of developing AD; however, it is still unknown why. Since insulin resistance is associated with cognitive impairment and AD, blood brain barrier transport of insulin was assessed. Young E3 and E4 male and female mice were used to assess differences in early life. We found insulin binding in the BBB vasculature are genotype- and sex-dependent. These data may help explain changes in CNS insulin signaling associated with E4 and female sex later in life. I helped plan the study, analyze the data, and reviewed the manuscript.

5. **Torres ERS**, Hall R, Bobe G, Choi J, Impey S, Pelz C, Lindner JR, Stevens JF and Raber J. Integrated Metabolomics-DNA Methylation Analysis Reveals Significant Long-Term Tissue-Dependent Directional Alterations in Aminoacyl-tRNA Biosynthesis in the Left Ventricle of the Heart and Hippocampus Following Proton Irradiation. *Front. Mol. Biosci.* 6:77 2019. doi: 10.3389/fmolb.2019.00077

Proton irradiation can induce long-term changes in the brain and behavioral performance. To better understand the underlying mechanism, we assessed the metabolome and DNA methylation patterns in hippocampal and left ventricle of the heart tissues in mice 22 weeks after proton irradiation. We found tissue-dependent changes in aminoacyl-tRNA biosynthesis using integrated analysis between metabolomics and DNA methylation. I trained and supervised interns that helped identify metabolites in the dataset, analyzed the data, and wrote the manuscript.

6. Bading-Taika B, Akinyeke T, Magana AA, Choi J, Ouanesisouk M, **Torres ERS**, Lione LA, Maier CS, Bobe G, Raber J, Miranda CL, & Stevens, JF. (2018). Phytochemical characterization of Tabernanthe iboga root bark and its effects on dysfunctional metabolism and cognitive performance in high-fat-fed C57BL/6J mice. *Journal of Food Bioactives*, 3, 111–123. https://doi.org/10.31665/JFB.2018.3154

This study explored the components of iboga root bark as a insulinotropic agent. C57Bl6J male mice were fed a high-fat diet to induce hyperglycemia. Water with ibogaine, a component of boga extract, was given to part of the mice along with the high-fat diet. Effects of the high-fat diet compared to low-fat diet include increased body weight, increased plasma glucose, triacylglyerols, total cholesterol, LDL-cholesterol, insulin, leptin, and pro-inflammatory mediator. Only MCP-1, a proinflammatory marker was reduced in the low dose of ibogaine group. Spatial learning and memory in the water maze was impaired in groups receiving iboga extract, suggesting that iboga extract should be taken with caution. My role in this study was to help analyze the water maze data and review the manuscript.

7. Raber J, Yamazaki J, **Torres ERS**, Kirchoff N, Stagaman K, Sharpton T, Turker MS and Kronenberg A. Combined effects of three high energy charged particle beams important for space flight on brain, behavioral and cognitive endpoints in B6D2F1 female and male mice. *Front. Physiol.* 12 March 2019. doi.org/10.3389/fphys.2019.00179

Space irradiation can have long-term effects on cognition, but many studies only address one type of particle. To address this and broaden understanding of its effects, male and female C57BL/6J × DBA2/J F1 were exposed to sequential irradiation consisting of protons, ^{16}O, and ^{28}Si. Higher doses of 3-beam irradiation lead to object recognition impairment in the mice. Males treated with a mid-level dose showed greater home cage activity as well as increased depressive-like behavior in the forced swim test. In cortical

tissue, irradiated males showed decreased in neurotrophic marker BDNF compared to sham-irradiated males while only irradiated females showed increased microglial activation in CD68 levels. There were also dose-dependent changes in the diversity and composition of the gut microbiome. I analyzed data and performed protein measurements as well as reviewed the manuscript.

8. **Torres ERS**, Akinyeke T, Stagaman K, Duvoisin RM, Meshul CK, Sharpton TJ, Raber J. Effects of Sub-Chronic MPTP Exposure on Behavioral and Cognitive Performance and the Microbiome of Wild-Type and mGlu8 Knockout Female and Male Mice. *Front. Behav. Neurosci*. 2018 Jul 18;12:140. doi: 10.3389/fnbeh.2018.00140. eCollection 2018.

Although motor dysfunction is the primary symptom associated with Parkinson's disease, patients often complain of gastrointestinal complications long before motor problems arise. Glutamatergic neurotransmission has been linked to both Parkinson's disease as well as gastrointestinal dysfunction. To model this, we used subchronic treatment of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin used to model Parkinsonism in WT and metabotropic glutamate receptor 8 (mGlu8) KO mice. Motor and non-motor behavioral performance was assessed. Using male and female mGlu8 KO mice and controls, we found that mGlu8 KO mice were protected from motor and cognitive deficits seen in WT mice. In addition, gut microbiome analyses showed significant association between microbiome alpha-diversity and locomotor performance in addition to microbiome composition and fear learning. These data suggest that MPTP's effects may be mediated by the gut microbiome. I helped perform behavioral testing and MPTP treatment in addition to analyzing the data and writing the manuscript.

9. Raber J, **Torres ERS**, Akinyeke T, Lee J, Weber Boutros SJ, Turker MS, Kronenberg A. Detrimental Effects of Helium Ion Irradiation on Cognitive Performance and Cortical Levels of MAP-2 in B6D2F1 Mice. *Int. J. Mol. Sci*. 2018 Apr 20;19(4). pii: E1247. doi: 10.3390/ijms19041247.

How space irradiation affects the brain is largely unclear. The long-term effects of helium irradiation were assessed using male and females from the F1 generation of C57Bl/6J x DBA2/J (B6D2F1). Irradiated B6D2F1 mice demonstrated cognitive impairments ~~in~~ object recognition and passive avoidance tests, suggesting task-dependent effects. In addition, protein levels of apoE and MAP-2 in brain tissues suggest that dose-dependent changes occur in a non-linear manner and that low-doses of helium irradiation may be more harmful. I assisted with behavioral and cognitive testing, performed protein measurements, and analyzed data as well as reviewed the manuscript.

10. Johnson LA, **Torres ERS**, Impey S, Weber S, Patel E, Akinyeke T, Alkayed NJ, and Raber J. Apolipoprotein E4 Mediates Insulin Resistance-Associated Cerebrovascular Dysfunction and the Post-Prandial Response. *J. Cereb. Blood Flow Metab.* 2017; doi: 10.1177/0271678X17746186

Metabolic dysfunction is associated with increased risk for age-related cognitive decline and AD. E4 has higher risk of these conditions compared to E3. ApoE TR E3 and E4 female mice were fed a high-fat diet to induce obesity and insulin resistance. E4 mice showed greater cognitive deficits compared to E3 mice reduced cerebral blood volume, and lowered glucose uptake. E4 mice also acutely benefitted from a glucose dose given prior to cognitive testing. I assisted with cognitive testing, tissue collection, analyses, and reviewed the manuscript.

11. Johnson LA, **Torres ERS**, Impey S, Stevens JF, and Raber J. Apolipoprotein E4 and Insulin Resistance Interact to Impair Cognition and Alter the Epigenome and Metabolome. *Sci. Rep.* 2017 Mar 8;7:43701. doi: 10.1038/srep43701.

This study sought to determine shared mechanisms behind E4 genotype and insulin resistance in their influence on cognitive dysfunction. High-fat diet was fed to female E3 and E4 mice to decrease insulin sensitivity. Mice were tested for cognitive impairment on the water maze; E4 mice on the high-fat diet showed greater impairments compared to E3. Hippocampal tissues were analyzed using genome-wide measures of DNA hydroxymethylation as well as untargeted metabolomics. This information was then integrated to determine pathways most affected by genotype and diet. Pathways highlighted were purine metabolism, glutamate metabolism, and the pentose phosphate pathway. I went through the raw data of metabolomics to identify metabolites and helped analyze these data along with integrating it with the methylation analysis. I also helped interpret the data and reviewed the manuscript.

12. McGinnis GJ, Friedman D, Young KH, **Torres ERS,** Thomas CR Jr, Gough MJ, Raber J. Neuroinflammatory and cognitive consequences of combined radiation and immunotherapy in a novel preclinical model. *Oncotarget.* 2017; 8(6):9155-9173.

Cancer patients report cognitive impairment after cancer treatments. We assessed behavioral and cognitive changes in BALB/c mice treated with radiotherapy alone or with immunotherapy. Our findings argue that while combined treatment controls tumor growth, it affects the brain and induces changes in anxiety, cognitive performance, and neuroinflammation. I assisted with data collection (activity monitors) and analysis and reviewed the manuscript.

13. Weiss JB, Weber SJ, **Torres ERS**, Marzulla T, Raber J. Genetic inhibition of Anaplastic Lymphoma Kinase rescues cognitive impairments in Neurofibromatosis 1 mutant mice. *Behav. Brain Res.* 2017; 321:148-156.

The majority of patients with neurofibromatosis are heterozygous for NF1 loss of function mutations. These mutations have been linked to anaplastic lymphoma kinase (Alk). To assess whether genetic inhibition of Alk ameliorates symptoms associated with NF1 mutations, NF1 heterozygous mice were crossed with Alk KO mice and test for cognitive performance and circadian activity. NF1/Alk KO mice improved water maze and contextual fear extinction learning compared to NF1 mice. NF1/Alk KO mice also demonstrated normal circadian activity levels compared to NF1 heterozygous mice. This study demonstrates that inhibiting Alk may be therapeutically beneficial for neurofibromatosis patients. I analyzed circadian activity and reviewed the manuscript.