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Characterizing Cellular Stress, Hippocampal Function, and Behavior in a Novel Rat Model of Alzheimer's Disease

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Abstract

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder that affects over 5 million individuals in the United States alone. While AD is primarily thought of as a disease that destroys neural networks required for memory recall and formation, AD also cause impairment in emotional regulation, cognitive flexibility, and executive function pathways. The cause of AD is unknown; however, the allele ApoE4 has been identified as a risk factor for the onset of AD. ApoE4 provides a valuable opportunity to study AD through animal models. This thesis utilized a human ApoE4 transgenic rat model (hApoE4) to investigate the biological and behavioral consequences of this AD risk allele. Cellular stress, hippocampal regulation, and behavioral tests evaluating rat cognitive function were analyzed. Cellular stress was assessed through the quantification of a novel biomarker, Growth Differentiation Factor-15 (GDF-15). Hippocampal regulation was examined in the context of neurogenesis occurrence (Doublecortin) and GABAergic neuron prevalence (Parvalbumin). It was revealed that GDF-15 serum concentrations significantly increase with age in wildtype and ApoE4 male rats, but not in ApoE4 female rats. In addition, ApoE4 males demonstrated a trend towards a reduction in PVB+ interneurons. These findings were correlated with behavioral testing data. Correlational analysis suggested the investigated biomarkers were associated with the cognitive function of hApoE4 rats, as evaluated through behavioral tests. Of most significance, this paper provides evidence suggesting the ApoE4 allele functions in a sex-dependent manner in rats, and directly alters GDF-15 levels in a potentially neurodestructive manor in female ApoE4 rats.

1. Introduction

1.1. Alzheimer's Disease

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder characterized by a rapid decline in patients' emotional and cognitive capabilities. The disease is believed to be the most common form of dementia in individuals 65+ and is the 6th leading cause of death in the United States (Mayo Clinic). Presently, over 5 million Americans suffer from Alzheimer's Disease. Unfortunately, the number of affected individuals is projected to reach 14 million by 2050 (Alzheimer's Association). The devastating symptoms of this disease, as well as its prevalence, has sparked interest in the world of biomedical research. AD research is allocated over $3.1 billion on an annual basis (Alzheimer's Association). The National Institute of Health (NIH) alone supports over 400 clinical trials dedicated to improving AD patient outcomes (NIH NIA). Despite these prolonged funding and research efforts, AD neuropathology remains poorly understood. As a result of the complexity of the disease's origins and progression, AD treatment options have been slow to advance. Thus, Alzheimer's Disease remains a critical area of study that demands further research to enhance our understanding of the AD-diseased brain. This thesis aims to expand the depth of current AD research by investigating novel questions related to neuropathology and behavior in a new rat model of AD.

AD presents in three main clinical stages: mild, moderate, and severe (Alzheimer's Association). AD is both progressive and terminal; however, the timeline of these stages can vary on an individual basis (Förstl & Kurz, 1999). As the stages suggest, the symptoms associated with AD increase in severity. In mild AD, individuals often begin to display memory and learning deficiencies, specifically regarding declarative and semantic memory. Patients might experience spatial disorientation and changes in mood. In the moderate stage of AD,

impairment in recent memory, language, logical reasoning, executive function, and visual agnosia continue to develop. In addition, patients might experience drastic changes in mood and behavior, delusions, and wandering (Förstl & Kurz, 1999). In severe AD, the symptoms observed in the moderate stage worsen. In addition, patients eventually lose the ability to communicate or interact with their environments, experience changes in physical capabilities, and require constant caretaking services (Förstl & Kurz, 1999). Thus, while AD is primarily recognized as a disease of memory and learning, it also impacts emotional and executive function-based neurological pathways. This is significant, as these behavioral symptoms of AD provide insight into the biological mechanisms underpinning disease origin and progression.

Clinically, AD has proven to be a challenging disease to treat because it is often not diagnosed until the disease has progressed to the point where a patient's memory and cognitive function are already compromised (Mantzavinos et al, 2017). However, to proceed with AD-based research, it is necessary to first recognize the pathology that is known to relate to the disease. First, AD's signature brain pathology centers around increased deposits of cortical amyloid plaques and neurofibrillary tangles (Glenner & Wong, 1984; Götz et al, 2001). The accumulation of these proteins is believed to disrupt the functioning of neuronal pathways, leading to several the symptoms observed in patients with AD. Second, AD drastically impacts functionality of the hippocampus. Individuals with AD display significantly damaged hippocampal areas and altered hippocampal regulation (Halliday, 2017). The hippocampus is an area of the brain heavily involved in memory formation, consolidation, and retrieval. Patients with AD experience a dramatic decrease in hippocampal volume, and display reduced functional connectivity within the structures that compose the hippocampus.

Interestingly, both the accumulation of problematic proteins and changes in the hippocampus are associated with the most prevalent genetic risk factor for developing AD: the ApoE ε4 allele (ApoE4). The ApoE gene is a cholesterol carrier, and involved in lipid homeostasis and transport, as well as injury repair in the brain. The ε4 allele significantly increases the risk of plaque deposits and neurofibrillary tangles in the brain, likely contributing to the cognitive and memory decline observed in AD patients (Lui et al, 2013). Thus, studying the influence of the ApoE4 allele is critical to advancing our understanding of the disease, to expand treatment options for patients diagnosed with AD.

The identification of the risk allele ApoE4 is beneficial as it provides a basis for the construction of an animal model of AD. Currently, the transgenic rat model hApoE4 offers an exciting opportunity to study both the biological and behavioral effects of this AD risk allele. Rats' shorter life span, comparatively to humans, allows potential biomarkers for AD to present themselves rapidly. In addition, rats are an excellent organism to model human behaviors (Gibbs et al, 2004; Iannaccone et al, 2009). This novel AD model organism will be used to explore the influence of the ApoE4 risk allele on biological systems. More specifically, this study aims to enhance our understanding of the impact the ApoE4 allele has on the biology and behavior of rats. Two areas of biological interest will be explored. First, ApoE4's impact of cellular stress will be investigated by examining expression of a novel inflammatory biomarker, Growth Differentiation Factor-15 (GDF-15). Second, the risk allele's influence on hippocampal regulation will be assessed through the quantification of DoubleCortin-expressing (DCX) neurons in the dentate gyrus and Parvalbumin-expressing (PVB) interneurons in the CA1 field of the hippocampus. Lastly, biology and behavior will be integrated to gain an understanding of how altered biological processes due to the ApoE4 allele might influence rat behavior.

1.2. Cellular Stress: Growth Differentiation Factor-15

As a neurodegenerative disease, AD is associated with rapid neuronal cellular death and damage. As a result, AD is associated with increases in cellular stress signals (Calabrese et al, 2006; Wen-Juan et al, 2016). Quantifying and understanding induced cellular stress due to AD is important in understanding the pathology and effects of this disease. One novel biomarker of cellular stress is GDF-15, an inflammatory protein within the TGF-beta superfamily of proteins. Under normal cellular conditions, GDF-15 expression is localized primarily to the placenta and prostate; however, it is expressed globally in low levels. During times of cellular stress, such as due to oxidative stress, hypoxia, inflammatory proteins, or general injury, GDF-15 expression is significantly increased (Wischhusen et al, 2020). Thus, GDF-15 is widely recognized as a novel biomarker for cellular stress (Appierto et al, 2009; Yang et al, 2010; Chung et al, 2017). The exact cellular impacts of GDF-15 are unclear; however, GDF-15's involvement in regulation of apoptotic, cell growth, and cell repair pathways contributes to the growing interest in GDF-15 within scientific communities (Rochette et al, 2020). Mature GDF-15 exists as a disulfide-linked homodimer and is a known ligand of receptor GFRAL (Uniprot: GDF15). The GFRAL receptor is located primarily in the brainstem; however, GFRAL receptors are expressed in neurons throughout the hippocampus (Rochette et al, 2020). In addition, GFRAL receptor activation is believed to be directly involved in neuronal survival pathways. Thus, GDF-15 and its receptor are both present and interacting with hippocampal regions within the brain.

As GDF-15 is a marker of cellular stress, it is unsurprising that heightened levels of GDF-15 in humans are associated with all-cause mortality. In recent years, increased expression of GDF-15 has been linked to Alzheimer's Disease risk (Wu et al, 2021). In elderly populations, individuals with cognitive impairment and/or clinically diagnosed forms of dementia displayed

higher levels of circulating GDF-15 in comparison to neurologically healthy populations (Yuek Ling et al, 2016). In general, elevated levels of GDF-15 correlate with cognitive decline, decreased hippocampal matter, and the development of dementias (McGraph et al, 2020). As a result, GDF-15 role as a potential biomarker for worsening cognitive function or Alzheimer's risk is a present research question. In part, this area of research has developed because of AD's known impact on oxidative stress within the brain (Huang et al, 2016). More specifically, AD induces hypoxia throughout the hippocampus, leading to increased and altered oxidative stress pathways that progress from CA3 to CA1 (Cruz-Sanchez et al, 2010). As GDF-15 is a protein whose expression is induced in response to hypoxia and oxidative stress, it is not unreasonable to hypothesize that AD increases GDF-15 protein expression levels.

While evidence suggests GDF-15 increases with AD onset, GDF-15's contribution to AD is unknown. While GDF-15 levels correlate with cognitive decline, GDF-15 appears to function in a neuroprotective fashion when introduced to neurons. In AD cell cultures treated with GDF-15 secreted from human umbilical cord, a reduction in Aβ plaque levels occurred (Kim et al, 2018). This suggests GDF-15 has the capacity to function through a plaque clearing mechanism. Additionally, elevated levels of GDF-15 stimulate hippocampal neurogenesis (Kim et al, 2015). This suggests GDF-15 might impact hippocampal regulation. Lastly, in HT22- cell cultures, GDF-15 has the capacity to rescue damaged neurons through a PI3K/Akt neuronal proliferation signaling pathway (Liu et al, 2019). Thus, despite association with the onset of AD, a rise in GDF-15 might provide neurological protection during times of neurological distress, such as due to neurodegeneration.

Therefore, we hypothesize that in ApoE4 rats, GDF-15 levels will increase, as GDF-15 expression increases in human populations with AD. However, we also hypothesize that GDF-15

will function in a neuroprotective manner. In this, we predicted that rats with higher GDF-15 concentrations will exhibit lesser degrees of neurological deficiencies. Neurological deficiencies in the rats, referring to increased levels of behavioral inhibition and altered executive function, have been quantified through prior behavioral testing. Thus, GDF-15 concentrations will be quantified to determine the extent to which the ApoE4 allele influences cellular stress, and to correlate this novel biomarker's expression to AD-related behavior.

1.3. Hippocampal Regulation: Parvalbumin-Expressing Interneurons

In addition to investigating cellular stress, this thesis aimed to uncover novel factors impacting hippocampal regulation in an ApoE4 rat model of AD. As previously discussed, the hippocampus is a structure in the brain that commonly is recognized as a major participant in memory regulation. However, the hippocampus is also involved in emotional regulation, executive function, and other core pathways related cognitive flexibility (Joyce et al, 2022). In relation to AD, the CA1 field of the hippocampus presents itself as an interesting area of study. This is because the CA1 region is recognized as a key regulator of behavior, as it is a location of significant signal integration (Barrientos et al, 2016). The CA1 field of the hippocampus acts as a tripartite synapse and receives signals from both the CA3 field as well as interneurons, which contribute to the regulation of firing rates of hippocampal neurons (Shepherd & Harris, 1998; Sik et al, 1995). Of particular interest are PVB-expressing interneurons. PVB interneurons are GABAergic cells, which play an important role in maintaining functionality of the hippocampus (Nahar et al, 2021). PVB interneurons are inhibitory interneurons which synapse onto CA1 pyramidal neurons, directly influencing hippocampus neuron firing rates (Udakis et al, 2020). Thus, PVB cells play a critical role hippocampal functionality, as when active, PVB neurons inhibit neuronal communication.

PVB hippocampal interneurons are involved in both short-term and long-term memory pathways. Of note, PVB neurons support CA1 network coherence, which helps increase functional connectivity between neurons (Ognjanovski et al, 2017). This directly impacts long-term memory formation and hippocampal network plasticity. In addition to stabilizing communication between CA1 neurons, PVB interneurons have also been associated with gamma oscillations. Gamma oscillations are thought to be critical for memory formation, consolidation, and retrieval in the hippocampus (Griffiths et al, 2019; van Vugt et al, 2010). PVB interneurons are believed to be involved in both generating and maintaining gamma oscillations in the hippocampus (Nahar et al, 2021). This is significant, as changes in gamma oscillation patterns and power are observed in both humans with AD and animal models of AD (Klein et al, 2016; Stam et al, 2002). More so, PVB neurons have been studied extensively in the context of Schizophrenia, where alterations in PVB neurons are associated with the behavioral changes seen regarding this disease (Curley & Lewis, 2012). Interestingly, many of the behavioral changes seen in Schizophrenia, such as changes in executive functioning and decreases in cognitive flexibility, are similar to the behavioral changes observed in individuals with AD.

As a result, it is not of surprise that PVB interneurons have been studied within recent years in the context of Alzheimer's Disease. It has been demonstrated that PVB interneurons become hyperexcitable around 16 weeks of age in AD mice models. This hyperexcitability is cited to decrease communication within the hippocampus, through the inhibition of hippocampal pyramidal neurons (Hijazi et al, 2020). This inhibition is associated with a decrease in spatial learning and memory, directly relating PVB cells to AD symptoms. Interestingly, when PVB interneurons are regulated to function at wildtype levels within early mouse life, a dramatic

rescue in learning, memory capabilities, and plaque accumulation in AD mice is observed (Hijazi et al, 2020). This suggests that PVB neurons' function might be of clinical significance, specifically in regard to early therapeutic interventions for patients diagnosed with AD. However, PVB cells' exact role in AD is far from well understood. In part, this is because PVB neurons have been reported to increase in 15–17-week-old AD mice (Hijazi et al, 2020). This is in direct opposition to findings suggesting that human brains obtained post autopsy displayed significantly reduced number of PVB interneurons in individuals with AD. Patients with AD revealed a 60% reduction in parvalbumin cells in CA1-CA2 regions of the hippocampus (Brady et at, 1997). This is of note as it suggests specific areas within the hippocampus that might be more vulnerable to AD's influence on PVB cells (Giesers et al, 2020). Research suggests PVB interneurons' role in hippocampal regulation in AD is incredibly complex, as both increase and decrease in PVB cell activity and prevalence appear to be associated with AD. Thus, PVB interneurons and their relationship to AD warrant further study.

We hypothesize that PVB interneurons will decrease in prevalence in ApoE4 rats. We also hypothesize that rats with reduced numbers of PVB cells will display higher deficiencies in cognitive function, as research suggests PVB neurons are critical for hippocampus regulation. Assessment of cognitive function is based on behavioral testing, which was aimed to evaluate behavioral inhibition and executive function in the rats. Thus, PVB interneuron prevalence will be quantified to determine the extent to which the ApoE4 allele influences hippocampal regulation, and to evaluate this novel biomarker's role in AD-related behavior.

1.4. Hippocampal Function: Adult Hippocampal Neurogenesis

Adult hippocampal neurogenesis refers to the production and integration of new neurons into neuronal circuits within the hippocampus. Neurogenesis is believed to be a measure of

neuronal plasticity, which is thought to be involved in learning and memory pathways within the brain (Deng et al, 2010). Thus, neurogenesis is involved in the regulation and functioning of the hippocampus. As a result, within recent years, neurogenesis' influence on aging brains has been researched heavily. Specifically, research suggests that neurogenesis decreases in an age dependent manner (Boldrini et al, 2018). However, in individuals with Alzheimer's Disease, the decrease in AHN is significantly greater than observed in healthy populations (Moreno-Jiménez et al, 2019). This is speculated to be due to both the increase in amyloid plaque deposits and neuroinflammation that is associated with the disease (Sung et al, 2020). Interestingly, in AD models of AD, increasing neurogenesis drastically improves short term memory function, suggesting neurogenesis might have neuroprotective benefits in the context of AD (Kim et al, 2014). This is of particular interest, as restoring proper neurogenesis levels in AD patients presents itself as a possible method of treatment, to enhance patients' memory and cognitive function.

Interestingly, GDF-15, while associated with the onset of AD, has been shown to directly impact levels of neurogenesis. GDF-15 released from a human umbilical cord increased neurogenesis in both in vivo and in vitro trials (Kim et al, 2015). This raises the question as to the extent GDF-15 influences neurogenesis in individuals diagnosed with AD. Doublecortin (DCX) has emerged as a prominent marker for adult neurogenesis, due to the protein's role in new neuron migration (Ayanlaja et al, 2017).

We hypothesize that DCX neurons will decrease in prevalence in ApoE4 rats, as neurogenesis decreases in humans affected by AD. We also hypothesize that DCX neuron prevalence will be correlated with GDF-15 concentrations, as GDF-15 is believed to increase neurogenesis within the hippocampus. Thus, neurogenesis will be quantified using the DCX

biomarker, to determine the extent to which the ApoE4 allele influences hippocampal regulation, and to evaluate this novel biomarker's role in AD-related behavior.

2. Methods

2.1. Rat Colony Conditions

Subjects were Sprague-Dawley rats (n=20 female, n=20 male), which arrived in the colony on postnatal day 25 (Horizon Discovery Lab). Half of both the male (n=10) and female (n=10) rats were hApoE4 knock-in (hAPoE4 KI), while the remaining rats were wildtype. All rats were housed in same-sex pairs in clear polycarbonate cages (30.5 x 30.5 x 18.5 cm), with a thin layer of corncob bedding and a wire bar lid. Cages were individually ventilated (Thoren Caging Systems, Inc., Hazleton, PA). At 14 months of age, male rats outgrew their cages and were put into larger cages made of the same material (Thoren #8 Expanded Rat Cage: 30.8 cm x 40.60 cm 22.23 cm). Once rats reached 18 months of age, the corncob bedding was replaced with shredded paper bedding (Carefresh paper fiber bedding; Petsmart).

The colony room was kept at 21 +/-1°C with 10-55% humidity and the rats were kept on a 12-hour light/12-hour dark cycle. Lights turned on at 08:00 daily. All procedures were carried out within the light phase of the cycle. Rats had access to ad libitum food (Harlan Rat Chow) and water (tap). Rat food and water was refilled every 3 days. Once every week, rat body weights were recorded, rat cages were cleaned, and rats participated in enrichment. Enrichment consisted of same-sex rats from multiple cages interacting and being handled by research assistants for approximately 30 minutes.

Extensive behavioral testing was conducted when the rats were between the ages of 2-4 months, and then again when rats were between the ages of 16-18 months. At 4 months of age, blood serum samples were collected from each rat and centrifuged at 4°C for 10 minutes at

14,000g. The supernatant collected from each sample was stored in 5mL microcentrifuge tubes at -80°C.

At 18 months, rats were deeply anesthetized using isoflurane in 1.5% oxygen, decapitated, and brains were removed rapidly. Brains were hemisected along the midsagittal plane and post-fixed in 4% paraformaldehyde in PBS at 4°C, then transferred to 0.1% sodium azide at 4°C prior to sectioning. Within the first 30 seconds following sacrifice, approximately 500 µl of trunk blood was extracted from each rat and centrifuged at 4°C for 10 minutes at 14,000g. The supernatant collected from each sample was stored in 5mL microcentrifuge tubes at -80°C.

In this thesis, rat cohorts will be identified in the following manner: Female Wildtype (FWT), Male Wildtype (MWT), Female ApoE4 (FA4), and Male ApoE4 (MA4).

2.2. Rodent Behavioral Testing

This thesis builds upon prior research conducted with these rats. Specifically, behavioral assessments were performed to gauge rat behavioral inhibition and cognitive function. Regarding behavioral inhibition, rat behavior was analyzed in an Open Field (OF) experiment (Appendix, Additional Figure 4). In this experiment, rats were placed in a novel box. Behavioral inhibition, which is used interchangeably with anxiety-like behavior in this paper, was measured through two metrics. First, the amount of time it took the rat to enter the center of the box was recorded. This metric is referred to as latency to enter. The greater the latency to enter, the higher the demonstration of behavioral inhibition. Second, the number of times the rat entered the center of the box was recorded. Number of entrances also alludes to behavioral inhibition, in which the higher the number of entrances, the lower the demonstrated anxiety-like behavior. The second

behavioral test was a set-shifting task (Appendix, Additional Figure 5). The set-shifting task aimed to gauge rat cognitive flexibility. Rats were incentivized to learn a set of behavioral based rules. Then the rules were altered. The ability for the rats to learn and unlearn rule sets was measured through a trial to criterion (TTC) metric. TTC refers to the number of trials a rat had to attempt before showing mastery of the rule set (or completing the task correctly). The TTC was set to 80%. Higher TTC scores indicate a greater number of trials were necessary for the rat to be correct 80% of the time. Thus, higher TTC scores suggest greater cognitive impairment, while lower TTC scores suggest greater degrees of cognitive flexibility.

2.3. GDF-15 ELISA

Two separate immunoassays were performed to quantify concentrations of GDF-15 in rat serum. The first ELISA was performed with the serum collected from the rats at 4 months of age. This provided a baseline level of GDF-15 concentrations in the young, adult rats. A second ELISA was performed with the serum collected from the rats at 18-months, at the time of sacrifice. This was done to assess GDF-15 concentrations in the rats at an older age. The Quantikine™ ELISA Mouse/Rat GDF-15 Immunoassay (R&D Systems, Bio-Techne) was used to assay the GDF-15 concentrations. The Quantikine™ ELISA Mouse/Rat GDF-15 Immunoassay (R&D Systems, Bio-Techne) was used to assay the GDF-15 concentrations. GDF-15 levels were measured in accordance with the manufacturer's instructions and are shown in pg/mL.

When performing the ELISA at 4-months of age, several of the rats lacked large enough samples of serum. For rats that did not have the required amount of serum, the amount of available serum was diluted. Buffer was used to bring the smaller sample sizes up to the volume

required in the ELISA protocol. The results produced by the ELISA readings were multiplied by the dilution factors during analysis to account for the original dilutions.

2.4. Brain Sectioning

The right and left-brain hemispheres of the rats were randomly selected for blocking. Brains were blocked to isolate the hippocampal region. Brains were sectioned with the use of a cryostat. Coronal sections of the hippocampus were taken at 40 μm thickness. Every section was retained into serial wells, to produce a total of 6 sets of tissue through the rostral-caudal extent of the hippocampus. Each serial well contained every 6th section, for approximately 30 sections. Sections were stored in 0.1% sodium azide at 4°C prior to immunohistochemical staining.

2.5. Immunohistochemistry Staining: DCX

Staining:

Protocols were adapted from previously described methods (Rao & Shetty, 2004; Glenn et al., 2007). Free-floating hippocampal sections were rinsed in PBS (pH 7.4), incubated in 0.6% hydrogen peroxide for 30 minutes at room temperature, rinsed in PBS, incubated in 3% normal horse serum (NHS; Vector Laboratories, Burlingame, CA) in PBS and 0.1% triton-X-100 (TTX; Sigma) in PBS solution for 30 minutes at room temperature, and then incubated with the DCX primary antibody (DCX in rabbit, 1:200) for 24 hours at 4°C on a shaker . Next, sections were rinsed in PBS, incubated with secondary antibody for one hour at room temperature (biotinylated horse anti-rabbit; 1:200; Vector Laboratories), rinsed again, then subsequently incubated in to an avidin-biotin complex (ABC; Vector Laboratories) for one hour at room temperature. The sections were rinsed again PBS and stained with Vector ImmPact SG solution for neuron visualization (Vector Laboratories). Tissue was rinsed and stored in 4°C until mounting. Sections

were mounted on 1% gelatin-coated slides and counterstained using an ethanol series. Coverslips were applied.

Quantification of DCX+ Neurons:

For each brain, each section containing the dentate gyrus was used in analysis. Each section with the dentate gyrus was imaged at 10x. ImageJ was used to count the number of DCX+ cells in each section (see Additional Figures for a representative image). As the number of sections varied per brain, the average number of DCX+ neurons per brain was calculated to compare between rats.

2.6. Immunohistochemistry Staining: PVB

Staining:

Protocols were adapted from previously described methods (Rao & Shetty, 2004; Glenn et al., 2007). Free-floating hippocampal sections were rinsed in TBS (pH 7.4), incubated in 0.6% hydrogen peroxide for 30 minutes at room temperature, rinsed in TBS, incubated in 3% normal horse serum (NHS; Vector Laboratories, Burlingame, CA) in TBS and 0.1% triton-X-100 (TTX; Sigma) in TBS solution for 30 minutes at room temperature, and then incubated with the PVB primary antibody (PVB in rabbit, 1:2000) for 24 hours at 4°C on a shaker . Next, sections were rinsed in TBS, incubated with secondary antibody for one hour at room temperature (biotinylated horse anti-rabbit; 1:200; Vector Laboratories), rinsed again, then subsequently incubated in to an avidin-biotin complex (ABC; Vector Laboratories) for one hour at room temperature. The sections were rinsed again TBS and stained with Vector ImmPact SG solution for neuron visualization (Vector Laboratories). Tissue was rinsed and stored in 4°C until mounting. Sections

were mounted on 1% gelatin-coated slides and counterstained using an ethanol series. Coverslips were applied.

Quantification of PVB+ Neurons:

PVB+ cells in the CA1 field of the hippocampus were counted using the optical fractionator method. Three sections of brain tissue were selected per brain for cell counting. Unbiased stereological methods were used to count the numbers of PVB+ cells in 3 sections through the dorsal hippocampus in each rat. StereoInvestigator (Microbrightfield Inc, Williston, VT) was used to systematically sample within the free-hand outlined CA1 region and count numbers of labeled cells (see Additional Figures for a representative image). A 200x200 counting frame was used, and approximately 30-40 sites per sample were selected. Cells were counted at 20x. The average number of cells within the three selected samples was used to compare the number of PVB+ neurons within a given rat brain to other brains.

2.7. Statistical Analysis

GraphPad Prism was used to perform statistical analysis of the results of this study and generate figures. For each set of data, normality was assessed and taken into consideration when performing statistical tests. Data sets that passed normality testing were assessed using Gaussian parameters, while data sets which failed normality were assessed using non-Gaussian parameters. In addition, planned comparisons were one-tailed t-tests used to test a priori hypothesis. Figured display error bars in terms of standard error, SEM. All biological measures were analyzed using a 2x2 completely between subjects ANOVA with the factors Sex (female and male) and Genotype (WT and ApoE4).

A Spearman correlation matrix was used to determine correlation between the biological and behavioral results. A correlation matrix was generated for each rat cohort, for GDF-15 concentrations, difference in GDF-15 concentrations from 4-months to 18-months, number of PVB+ cells, and the results from two behavioral tests. The behavioral test results were obtained from preexisting behavioral data from an open-field behavioral test and a set-shifting task behavioral test.

3. Results

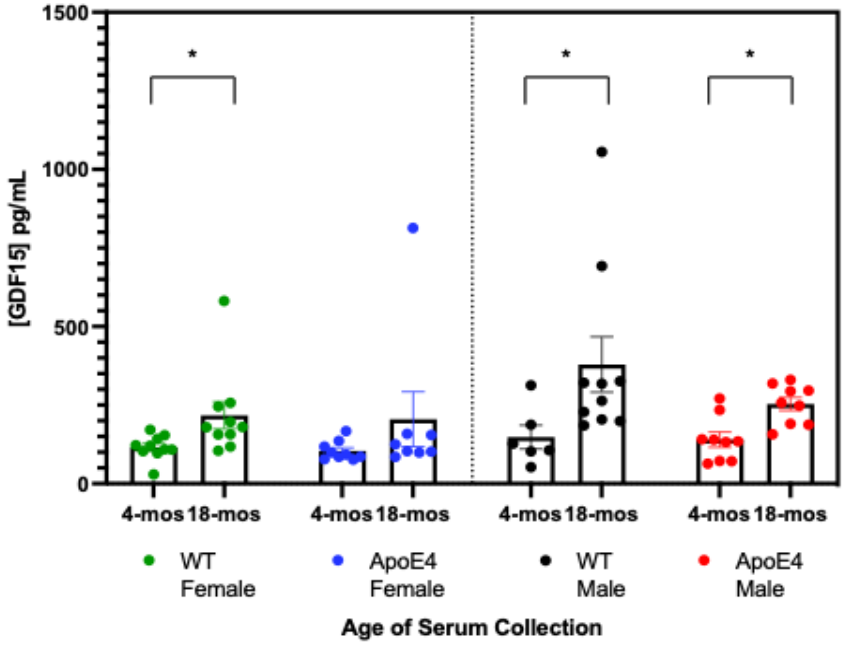
GDF-15 Concentrations in Rat Serum Samples at 4- and 18-months of Age

Two ELISAs were performed to quantify GDF-15 concentrations in rat blood serum, at 4-months and 18-months of age. At 4-months of age, the ANOVA test revealed no significant difference in GDF-15 concentrations between FA4 (n=9), FWT (n=10), MA4 (n=9), and MWT rats (n=6) (all $p > 0.05$). At 18-months of age, again the ANOVA test showed no significant difference in GDF-15 serum concentration between FA4 (n=8), FWT (n=10), MA4 (n=9), and MWT (n=10) rats (all $p>0.05$). Within rat cohorts, there were several statistically significant findings. Between 4-months and 18-months of age, paired comparison revealed a significant increase in GDF-15 concentrations within FWT, MWT, and MA4 rat cohorts ($p = 0.018$, 0.036, 0.001 respectfully) (Figure 1A). The paired comparison for FA4 rats between 4-months and 18-months of age did not reveal a significant change in GDF-15 blood serum concentrations ($p>0.05$).

GDF-15 blood serum concentration in rat cohorts at 18-months was also evaluated by percent increase from baseline. Baseline was defined as the average GDF-15 concentration in wildtype rats at 4-months (FWT= 115.85 pg/mL, MWT= 148.24 pg/mL) (Figure 1B). The

percent difference from baseline was significantly greater in the FWT cohort than the FA4 cohort (t-test, p=0.027). There was no significant difference in percent difference between the MA4 and MWT cohorts (t-test, p =0.20).

(A)



(B)

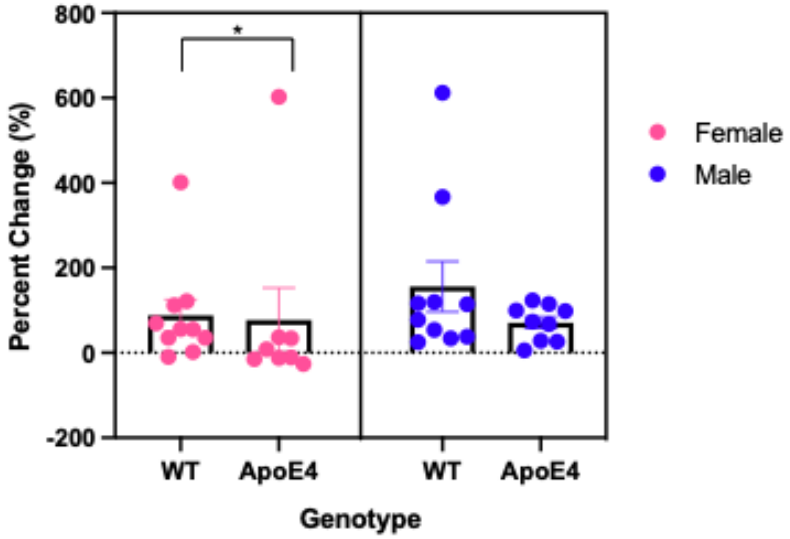


Figure 1. Mean (± SEM) of (A) concentration of GDF-15 (pg/mL) in serum of WT and ApoE4 rats at 4-months and 18-months of age. WT female, WT male, and ApoE4 male rats showed significant increase in GDF-15 concentrations ($p < 0.05$). And, (B) percent change in GDF-15 concentrations in rat serum between 4-months and 18-months. Female WT and female ApoE4 rats showed a significant difference in percent change from baseline, where baseline was defined as the average concentration of GDF-15 in WT rats at 4 months of age.

Parvalbumin Cells in CA1 Field of Hippocampus

The number of PVB+ neurons represent the average number of PVB+ cells located in the CA1 field of the hippocampus, within a singular 40 μm section of brain tissue (Appendix, Additional Figure 1). Paired comparison revealed no significant difference in the number of PVB+ cells in FA4 and FWT brains ($p>0.05$) (Figure 2). A non-parametric t-test was used to gauge significance within the male cohort, as the male cell counts failed the test for normality. The t-test revealed the number of PVB+ cells in MA4 and MWT brains approached significance ($p=0.065$). While this value fails to show a difference at the 5% significance threshold, it is significant at a 10% significance threshold.

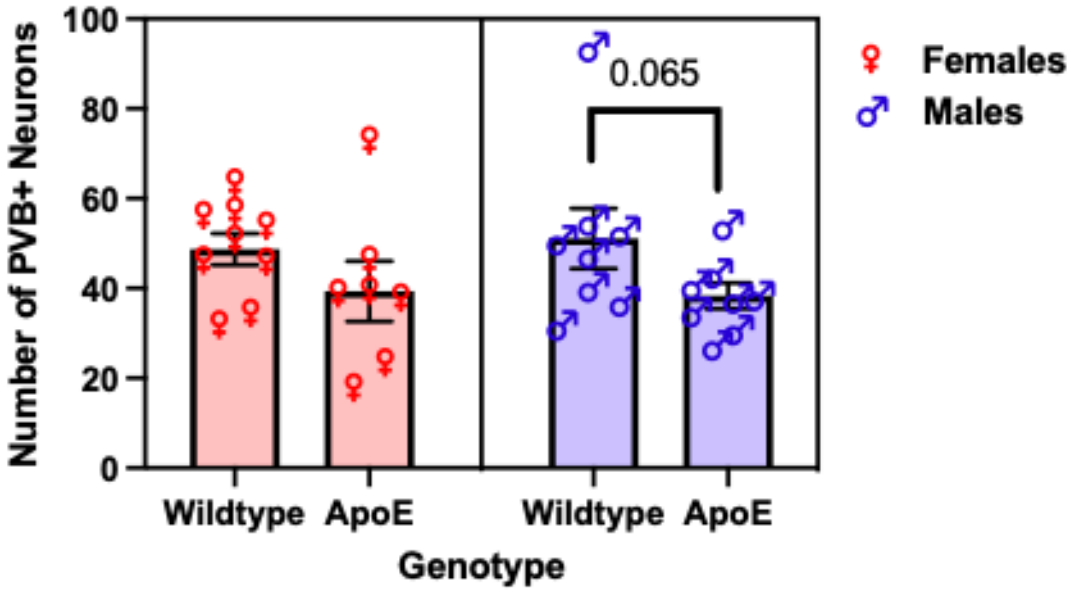


Figure 2. Mean (± SEM) numbers of PVB+ labeled cells in the CA1 field of the hippocampus. In male WT and ApoE4 rats, there was a trend towards fewer PVB+ cells in the ApoE4 cohort. This decrease in PVB+ cells in male ApoE4 was significant at a 10% significance threshold, but not at a 5% threshold ($p= 0.065$).

Doublecortin Cells in Dentate Gyrus of Hippocampus

The number of DCX+ positive neurons represent the average number of DCX+ cells in the dentate gyrus within a singular 40 μm section of brain tissue (Appendix, Additional Figure

2). Due to difficulties with the antibody stain, a smaller subsect of each rat cohort was used to investigate DCX+ cells. The ANOVA test revealed no significant differences between FA4 (n=3), FWT (n=2), MA4 (n=3), and MWT (n=2) rat cohorts (all $p>0.05$) (Figure 3). Paired comparisons between FA4 and FWT and between MA4 and MWT revealed cell counts were approaching significant differences; however, the small n value is a limiting factor ($p=0.08$ and 0.06, respectively).

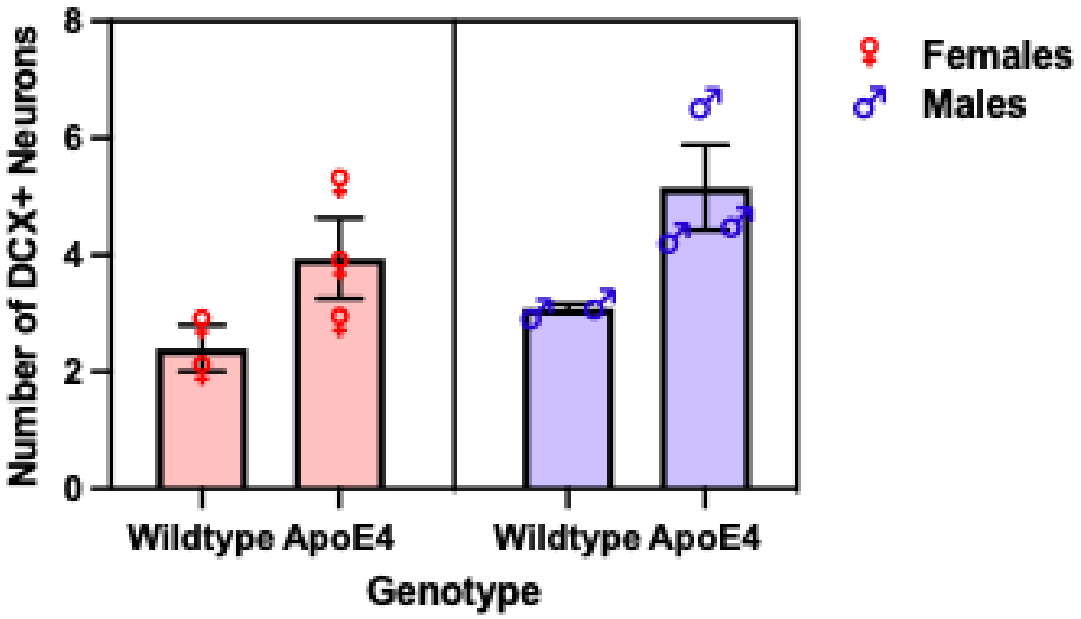


Figure 3. Mean (± SEM) numbers of DCX+ labeled cells in the dentate gyrus of the hippocampus. No significant difference in DCX+ cells was observed between the rat cohorts.

Integrating Biological Markers and Behavioral Testing Results

A spearman nonparametric correlation test was performed to evaluate the biological and behavioral findings. A separate correlation test was performed for each rat cohort FA4 (n=10), FWT (n=10), MA4 (n=10), and MWT (n=10) (significant results in Table 1; see Appendix Additional Figure 3 for complete correlation results).

In the female wildtype cohorts, a significant negative correlation was revealed between rats' levels of GDF-15 at 4-months of age and reversal TTC ($p = 0.034$). Lower levels of GDF-15 at 4-months of age were correlated to higher degrees of cognitive impairment, or greater TTC scores ($r = -0.644$). Correlation analysis also revealed a significant positive correlation among PVB+ neurons and FWT rats change in serum concentrations of GDF-15 from 4-months to 18-months of age. The higher the increase in GDF-15 concentrations from 4 to 18-months, the higher the number of PVB+ neurons within the CA1 field of the hippocampus at 18-months of age ($r = 0.6$, $p = 0.048$) (Table 1A).

In the male wildtype cohort, a significant positive correlation was revealed between changes in serum concentration of GDF-15 from 4 to 18-months and reversal TTC ($p=0.042$). The greater the increase in GDF-15 within 4 to 18 months, the greater the assumed impairment in cognitive flexibility ($r=0.76$). Additional correlation analysis revealed a negative correlation between PVB+ neurons and open field latency to enter ($r = -0.68$, $p = 0.038$). MWT rats with lower prevalence of PVB+ neurons displayed greater behavioral inhibition and heightened anxiety-like behaviors, as latency to enter the OF increased with decreased PVB+ neurons (Table 1B).

In the female ApoE4 cohort, a significant negative correlation was revealed between both GDF-15 serum concentrations at 18-months and changes in serum concentrations of GDF-15 from 4- to 18-months in relation to open field latency to enter ($r = -0.85, -0.756$ respectfully, $p = 0.007, 0.029$ respectfully). This indicates a negative relationship between GDF-15 levels and behavioral inhibition, in which higher levels of GDF-15 at 18-months or higher levels of increased GDF-15 between 4 to 18-months are associated with lower behavioral inhibition and anxiety-like behaviors. This finding is strengthened by an additional correlation that revealed a

positive relationship between GDF-15 concentrations at 18-months of age and number of open field entrances (r=0.693, p = 0.0439). The higher the concentration of GDF-15, the higher the number of OF entrances, suggesting lower behavioral inhibition and anxiety-like behaviors in FA4 rats with higher GDF-15 concentrations (Table 1C).

There were no significant correlations between biological markers and behavior in male ApoE4 rats.

A.

WT Females	[GDF-15] pg/mL 4-mos	Change in [GDF-15] from 4- to 18-mos
Reversal TTC	-0.644 (p-value= 0.034)	
PVB+ Cell Count		0.6 (p-value= 0.048)

B.

WT Males	Change in [GDF-15] from 4- to 18-mos	Open Field Latency to Enter
Reversal TTC	0.76 (p-value=0.042)	
PVB+ Cell Count		-0.68 (p-value=0.038)

C.

ApoE4 Females	[GDF-15] pg/mL at 18-mos	Change in [GDF-15] from 4- to 18-mos
Open Field Latency to Enter	-0.85 (p-value=0.007)	-0.756 (p-value=0.029)
Open Field Number of Entries	0.693 (p-value=0.0439)	

Table 1. Selected results extracted from Spearman nonparametric correlation testing, performed between biological and behavioral results. A separate correlation was run for each rat cohort, to examine correlation trends within each rat population. Selected results refer to results that were significant at a 5% significance threshold in a) WT female b) WT male and c) ApoE4 female rat cohorts.

4. Discussion

The objective of this thesis was to characterize cellular stress, hippocampal function, and behavior in a rat model of AD. The hApoE4 transgenic rat provided a novel opportunity to gain insight into both potential biological and behavioral impacts of the ApoE4 allele. Cellular stress was successfully characterized through the quantification of GDF-15 levels in the blood serum of rats. Hippocampal function was then evaluated through PVB and DCX immunohistochemical staining. Finally, with the biological data generated through these experiments, a correlational analysis was performed to contextualize biological results with behavioral findings. The results

of this investigation support prior research suggesting the ApoE4 allele alters rat biology and behavior. In addition, findings of this study suggest the ApoE4 allele might operate in a sex-dependent manner, which warrants further study.

The first major objective of this study was the characterization of cellular stress. Characterization of cellular stress was analyzed through the use of the novel biomarker for cellular distress, GDF-15. In FWT and MWT rat cohorts, a significant increase in GDF-15 was observed from 4 to 18-months of age. This was expected, as increases in GDF-15 are seen to positively correlate with age in healthy human populations, due to normal aging processes (Liu et al, 2021). Thus, the significance in the increase in GDF-15 in the wildtype rats was not remarkable. MA4 rats also displayed an increase in GDF-15 concentrations from 4 to 18-months of age. However, the increase in GDF-15 was no more significant in ApoE4 rats than WT rats. This finding deviates from the initial hypothesis which predicted ApoE4 rat models would experience heightened levels of GDF-15 expression in comparison to WT controls. In addition, this result goes against prior research which demonstrates an increase in GDF-15 in AD patients compared to health controls (Wu et al, 2021). This deviation from the hypothesis is only furthered by the FA4 results. FA4 rats displayed no increase in GDF-15 between 4 to 18-months of age. In this, FA4 rats not only deviate from the expected increase in GDF-15 in AD models comparison to health controls, they also deviate from the expected age-related increase in GDF-15 that is not dependent on disease. This finding is of particular interest as females are at a heightened risk for the onset of AD. This extreme derivation from the expected increase in GDF-15 suggests the ApoE4 allele might be impacting rats in a sex-based manner.

The GDF-15 biological results can also be contextualized in relation to behavioral findings. Most interestingly, a significant correlation was demonstrated between GDF-15

concentrations and cognitive performance in FA4 rats. FA4 rats with higher levels of GDF-15 revealed lower levels of behavioral inhibition and anxiety-like behaviors. This finding is in direct support of the initial hypothesis that GDF-15 might function in a neuroprotective manner within diseased brains. This is because, despite being the one rat population to not demonstrate a significant increase in GDF-15 with age, the FA4 rats were the only rats to display this significant correlation between GDF-15 levels and OF behavioral testing. Females are believed to be more suspectable than males to the impact of the ApoE4 allele. Thus, these findings support the prediction that female ApoE4 rats might be at a neurological disadvantage due to a reduction in GDF-15 levels, as GDF-15 might function in a neuroprotective manner.

While this study was correlational in nature, thus limiting the conclusions that can be drawn, these findings warrant further research. This is because GDF-15 is thought to play a role in stimulating neuronal growth, enhancing neuronal survival, and regulating neuronal firing patterns (Liu et al, 2016; Schoder et al, 2003; Subramaniam et al, 2003; Lu et al, 2016). These finding suggests that ApoE4 female rats might not benefit from the same degree of neuronal protection that is awarded through increased GDF-15 serum concentrations in wildtype rats. This is supported by the behavioral findings, which suggest a greater degree of cognitive impairment in the ApoE4 females, which allude to neuronal deficiencies. Additionally, while the ApoE4 male rats did demonstrate a significant increase in GDF-15, this increase was not as drastic as expected. Thus, the effect of the ApoE4 allele and GDF-15 in male rats also requires further evaluation.

The correlation between GDF-15 concentrations and WT rat behavioral results are also important to consider. In FWT rats, lower GDF-15 levels correlated with higher degrees of cognitive impairment. This opposes the results seen in MWT, in which greater increases in GDF-

15 were correlated to higher decreases of cognitive impairment. While, again, this study cannot make causal claims, these results suggest the complexity of GDF-15 expression. This thesis suggests GDF-15 might not be functioning in a consistent manner across all ages and sexes (Doerstling et al, 2018). This is important to consider when evaluating findings suggesting GDF-15 levels correlate with all-cause mortality in otherwise healthy populations.

The second major objective of this study was to examine hippocampal regulation through the quantification of PVB+ neurons in the CA1 field of the hippocampus. While no significant findings presented themselves, ApoE4 rats overall expressed fewer PVB+ cells and this effect was more pronounced in males. Therefore, the findings do somewhat support the original hypothesis predicting a decrease in PVB+ cells in ApoE4 rats. This trend is supported by prior studies that demonstrate a decrease in PVB+ cells in animal models of AD (Leung et al, 2021). Interestingly, here, male ApoE4 rats experienced the more significant trend. Male ApoE4 rats' difference in PVB+ cells in comparison to the control group was very close to approaching statistical significance. This is interesting as it further suggests that the ApoE4 allele might function in distinct ways within male and female ApoE4 KI rats. In female KIs a more significant alteration was seen in relation to GDF-15 serum concentrations, whereas in male KIs a more significant alteration was seen in the trend towards a reduction in PVB+ interneurons.

The PVB cell results can be contextualized in regard to behavioral findings. In relation to behavior, PVB+ interneurons are relevant as they function to inhibit neuronal firing, as PVB+ cells are GABAergic. In WT males, lower levels of PVB+ neurons correlated with higher levels of behavioral inhibition and anxiety-like behavior in rats. This is significant when considering the male ApoE4 rats, which showed no significant correlation with this behavioral metric, suggesting potential alterations in the PVB cell's activity. This is of importance when

considering the role PVB+ neurons play in hippocampal regulation, as a decrease in the number or activity of PVB+ cells can cause hyperexcitability in neuronal circuits. This is critical when considering AD, as dysregulation in neuronal circuits due to hyperexcitability because of alterations in GABAergic neurons is predicted to relate to deficiencies in memory and cognitive function (Ramsey et al, 2019). Thus, while this study cannot provide causal conclusions, it does provide evidence suggesting PVB-expressing neurons' role in AD requires future research to fully understand the implications of ApoE4 on hippocampal regulation. More specifically, in order to gain a better appreciation for these behavioral results, the activity of PVB+ cells should be examined in addition to cell prevalence in future studies.

Unfortunately, this study was not able to meaningfully evaluate DCX+ cells in this novel AD rat model. Neurogenesis is known to decline in aging rat populations. As a result of this feature of neurogenesis, there were very few DCX+ cells in the dentate gyrus of the 18-month-old rats. The preliminary data that was collected suggests a trend towards ApoE4 rats expressing higher levels of DCX+ cells. However, because of the limited sample size, the results of this section of the study are unable to be used in meaningful analysis and were not evaluated in the context of behavior. With that being said, the preliminary results found in this study suggest that neurogenesis in the dentate gyrus warrants further study in relation to the ApoE4 allele, as a clear trend in an increase in DCX+ cells was present in both male and female ApoE4 rats.

Thus, the findings of this thesis successfully allowed for the characterization of cellular stress, hippocampal regulation, and behavior in a novel rat model of AD. Most significantly, this study suggests evidence for novel biological impacts of the ApoE4 allele on rats. Female ApoE4 rats do not experience an increase in GDF-15, which is predicted to operate in a neuroprotective manner. Male ApoE4 rats, and female ApoE4 rats to a less extent, demonstrate a strong trend

towards a reduction in expression of PVB+ interneurons in the CA1 field of the hippocampus. Additionally, these biological alterations do correlate both directly and indirectly to behavioral metrics evaluating rat cognitive impairment, providing a strong argument for future research in relation to both PVB+ cell expression and serum GDF-15 concentration. In addition, this study provides a strong case for the importance of considering sex-based differences in neurological function and neuropathology. It is important to study AD in both male and female organisms. This study supports this conclusion as ApoE4 serves as just one example of a potentially sex-dependent protein associated with the onset of AD. More so, it is also important when studying AD to consider changes in biological markers at an individual level. Between cohorts, no significant difference in GDF-15 levels were noticeable at either 4-months or 18-months of age. Significance in changes of GDF-15 were only present when looking within a specific rat population. Taken together, the results from this experiment suggest that future AD and ApoE4 research must be conducted in a manner that can reflect both sex-based and individual-based analysis. Thus, the ApoE4 knock-in rat offers a strong model system to investigate alterations in cellular stress, hippocampal regulation, and behavior, in order to expand our understanding of AD.

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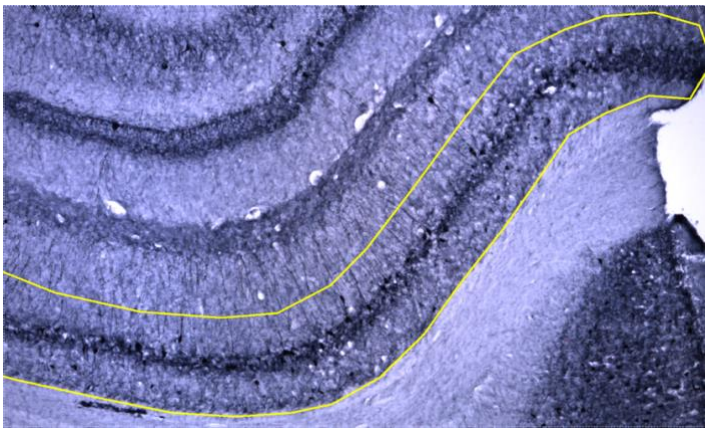
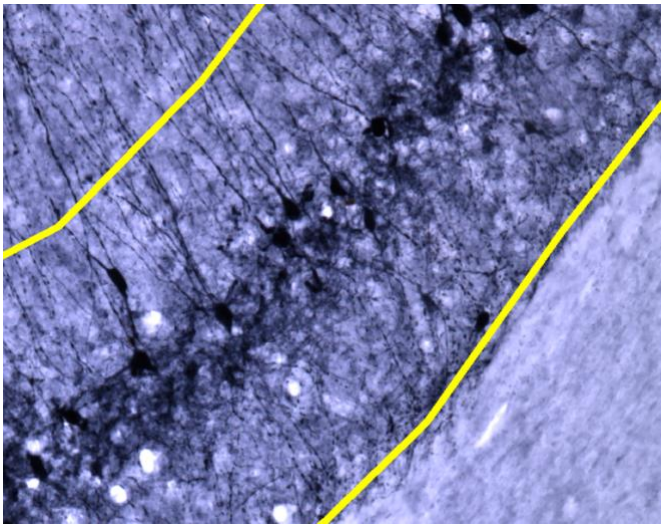
Additional Figures

(A)

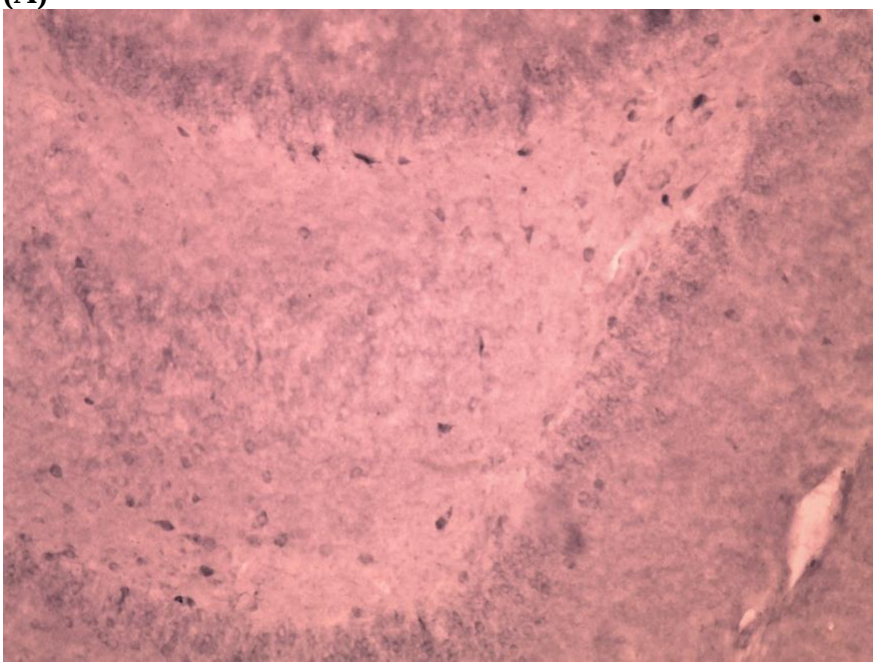


(B)



Additional Figure 1. Representative PVB+ staining at (a) 10x magnification and (b) 20x magnification. Yellow line encapsulates a part of the CA1 field of the hippocampus.

(A)



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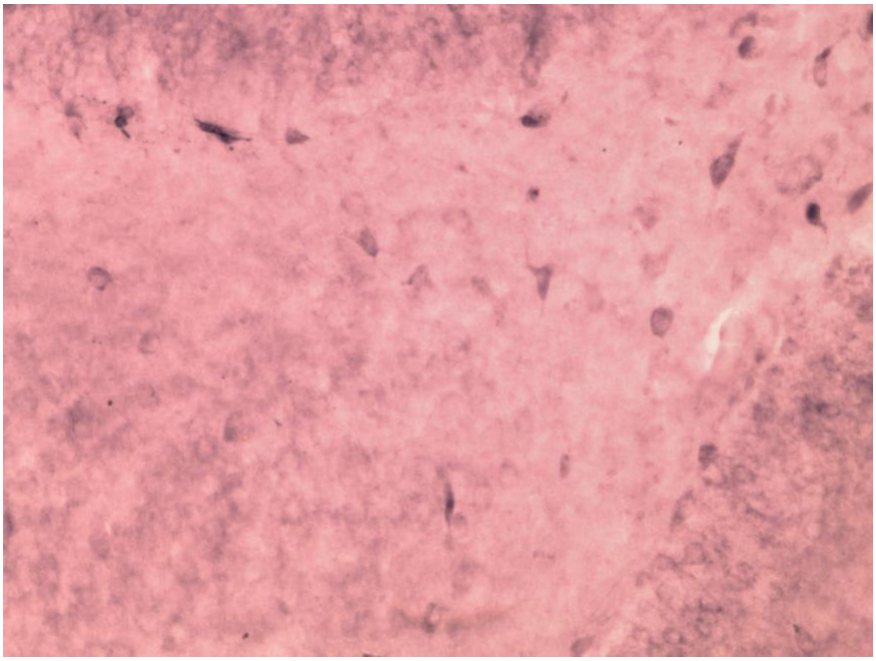


Additional Figure 2. Representative DCX+ staining at (a) 10x magnification and (b) 20x magnification in the dentate gyrus of the hippocampus.

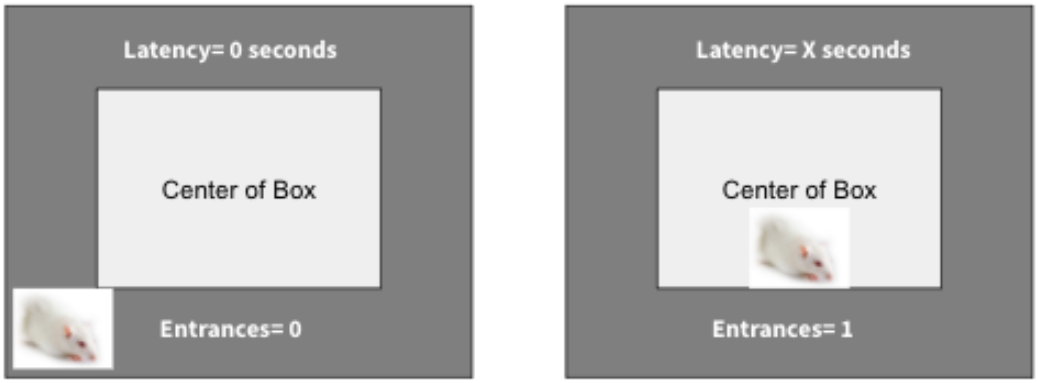
ApoE4 Females	[GDF-15] at 4-months	[GDF-15] at 18-months	Difference in [GDF-15] between 4- and 18-months	Visual TTC	Reversal TTC	Open Field Latency to Enter	Open Field Number of Entries	Number of PVB+ Neurons
[GDF-15] at 4-months	1.000	0.071	-0.036	-0.866	0.800	0.203	-0.294	0.143
[GDF-15] at 18-months	0.071	1.000	0.964	-0.258	-0.100	-0.854	0.693	-0.100
Difference in [GDF-15] between 4- and 18-months	-0.036	0.964	1.000	0.000	-0.200	-0.757	0.586	0.000
Visual TTC	-0.866	-0.258	0.000	1.000	-0.258	0.272	-0.577	
Reversal TTC	0.800	-0.100	-0.200	-0.258	1.000	-0.335	0.289	-0.500
Open Field Latency to Enter	0.203	-0.854	-0.757	0.272	-0.335	1.000	-0.763	0.296
Open Field Number of Entries	-0.294	0.693	0.586	-0.577	0.289	-0.763	1.000	0.000
Number of PVB+ Neurons	0.143	-0.100	0.000		-0.500	0.296	0.000	1.000

WT Males	[GDF-15] at 4-months	[GDF-15] at 18-months	Difference in [GDF-15] between 4- and 18-months	Visual TTC	Reversal TTC	Open Field Latency to Enter	Open Field Number of Entries	Number of PVB+ Neurons
[GDF-15] at 4-months	1.000	0.029	-0.486	-0.152	-0.152	0.213	0.034	-0.600
[GDF-15] at 18-months	0.029	1.000	0.714	0.051	0.244	0.019	0.382	-0.452
Difference in [GDF-15] between 4- and 18-months	-0.486	0.714	1.000	0.516	0.759	-0.273	0.338	-0.400
Visual TTC	-0.152	0.051	0.516	1.000	0.275	-0.690	-0.020	0.000
Reversal TTC	-0.152	0.244	0.759	0.275	1.000	-0.400	-0.127	0.232
Open Field Latency to Enter	0.213	0.019	-0.273	-0.690	-0.400	1.000	-0.325	-0.683
Open Field Number of Entries	0.034	0.382	0.338	-0.020	-0.127	-0.325	1.000	0.041
Number of PVB+ Neurons	-0.600	-0.452	-0.400	0.000	0.232	-0.683	0.041	1.000

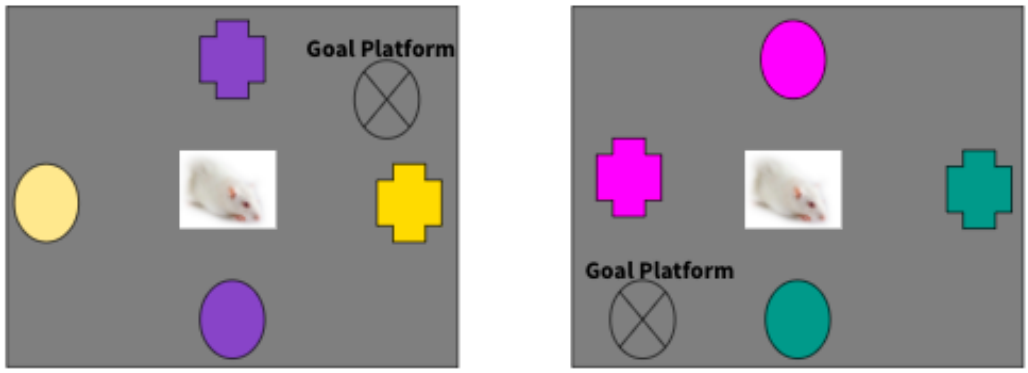
ApoE4 Males	[GDF-15] at 4-months	[GDF-15] at 18-months	Difference in [GDF-15] between 4- and 18-months	Visual TTC	Reversal TTC	Open Field Latency to Enter	Open Field Number of Entries	Number of PVB+ Neurons
[GDF-15] at 4-months	1.000	-0.405	-0.833	0.335	-0.220	-0.044	-0.109	-0.143
[GDF-15] at 18-months	-0.405	1.000	0.810	-0.372	0.153	-0.244	-0.055	-0.464
Difference in [GDF-15] between 4- and 18-months	-0.833	0.810	1.000	-0.671	0.317	-0.114	-0.027	-0.257
Visual TTC	0.335	-0.372	-0.671	1.000	0.549	0.500	-0.500	-0.335
Reversal TTC	-0.220	0.153	0.317	0.549	1.000	-0.257	-0.159	-0.108
Open Field Latency to Enter	-0.044	-0.244	-0.114	0.500	-0.257	1.000	-0.304	-0.342
Open Field Number of Entries	-0.109	-0.055	-0.027	-0.500	-0.159	-0.304	1.000	0.069
Number of PVB+ Neurons	-0.143	-0.464	-0.257	-0.335	-0.108	-0.342	0.069	1.000

WT Females	[GDF-15] at 4-months	[GDF-15] at 18-months	Difference in [GDF-15] between 4- and 18-months	Visual TTC	Reversal TTC	Open Field Latency to Enter	Open Field Number of Entries	Number of PVB+ Neurons
[GDF-15] at 4-months	1.000	0.782	0.273	0.546	-0.644	-0.091	-0.208	0.100
[GDF-15] at 18-months	0.782	1.000	0.745	0.464	-0.293	0.079	-0.283	0.400
Difference in [GDF-15] between 4- and 18-months	0.273	0.745	1.000	0.464	0.293	0.128	-0.151	0.600
Visual TTC	0.546	0.464	0.464	1.000	-0.151	0.621	0.170	0.089
Reversal TTC	-0.644	-0.293	0.293	-0.151	1.000	0.312	-0.119	0.647
Open Field Latency to Enter	-0.091	0.079	0.128	0.621	0.312	1.000	-0.196	0.201
Open Field Number of Entries	-0.208	-0.283	-0.151	0.170	-0.119	-0.196	1.000	-0.111
Number of PVB+ Neurons	0.100	0.400	0.600	0.089	0.647	0.201	-0.111	1.000

Additional Figure 3. Complete spearman correlation matrix values for integrating biological markers and behavioral testing results. Separate correlations were run for each of the rat cohorts, ApoE4 female, WT male, ApoE4 male, and WT female rats.



Additional Figure 4. Example set-up of Open-Field behavioral test. A rat is placed in a box. The time it takes the rat to enter the center of the box is recorded. This metric is referred to "latency to enter". The number of times a rat enters the center of a box is also recorded. Both metrics aim to evaluate the behavioral inhibition and anxiety-like behaviors of rats.



Addition Figure 5. Example set-up of Set-Shifting behavioral test. A rat is placed in a water-maze, to incentivize the rat to locate a platform. The rat must learn the visual cue of the shapes and colors, to know where the platform is. Once the rat has learned this rule, the rule is changed. The colors and shapes are shifted on the rat, and the rat must relearn how to locate the platform. The number of times it takes for the rat to successful reach the platform and complete the task is evaluated to gauge cognitive function and cognitive flexibility. The metric for this behavioral test is called trials to criterion (TTC), in which the number of trials before a rat is correct 80% of the time is recorded. Higher TTC indicate higher levels of cognitive impairment.

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