THE EFFECTS OF CHRONIC UNPREDICTABLE STRESS ON COGNITIVE DYSFUNCTION AND ALZHEIMER'S DISEASE PATHOLOGY

by

Amanda Key

Submitted in partial fulfillment of the
requirements for Departmental Honors in
the Department of Biology
Texas Christian University
Fort Worth, TX

May 4, 2020

THE EFFECTS OF CHRONIC UNPREDICTABLE STRESS ON COGNITIVE DYSFUNCTION AND ALZHEIMER'S DISEASE PATHOLOGY

Project Approved:

Supervising Professor: Michael Chumley, PhD.

Department of Biology

Gary Boehm, PhD.

Department of Psychology

Matthew Hale, PhD.

Department of Biology

ABSTRACT

Alzheimer's disease (AD), a form of dementia, is the 6th leading cause of death in the U.S. and its incidence continues to climb. One of the primary areas of study in this field is the role of environmental factors in disease progression. The prevalence of stress in society is ubiquitous with chronic stressors such as personal finances, work, and the economy ranking as the top causes of stress. Studies examining the role of chronic unpredictable stress (CUS) in AD progression have exhibited a deleterious effect in familial AD (FAD) models. FAD, however, only accounts for a small proportion of the AD seen in humans. The aim of this present study was to elucidate the relationship between CUS, inflammation, and AD pathology in non-transgenic mice. Both stress and inflammation have been independently shown to negatively affect AD pathology. We hypothesized that aggravating the stress response by exposing mice to CUS for 28 days while simultaneously introducing inflammation via lipopolysaccharide (LPS) injections during the final 7 days would further exacerbate the cognitive deficits and AD pathology compared to control groups.

ACKNOWLEDGMENTS

I would like to attempt to convey my gratitude to every hand that helped and every minute of time that was invested into this thesis to make its completion possible. First and foremost, I would like to thank my supervising lab professor, Dr. Chumley. I learned so much from you through your thoughtful feedback and mentorship about how to become a better scientist. Thank you for leading by example with your work ethic and for your continued patience through my time in your lab. Not only were you insightful and encouraging academically, but you were also one of my biggest cheerleaders in the pool and in life. You gave me the kind of knowledge that does not have an expiration date and your influence will stretch beyond the walls of TCU. I am truly grateful that you were at the helm of my research experience. I would also like to thank my other committee members, Dr. Hale and Dr. Boehm, for their support and the time they invested into this project.

Thank you, Kelly Brice, Paige Braden, Julia Peterman, and Chris Hagen, the graduate students in the lab who have guided me through these past two years. You all have been immensely helpful with hands-on teaching and patient oversight. I cannot begin to comprehend your impact on this lab. A special thank you to Kelly: I am honored to have been able to conduct research alongside such a wonderful scientist as yourself. Thank you for always making time to help me, gracefully juggling the innumerous demands of a student-athlete, and helping me scruff mice that were particularly snippety. Last but not least, I would like to thank the undergraduate members of the lab whose contributions make every project possible. Thank you specifically, Claire Munster, for introducing me to this lab and often accompanying me in the lab during inconvenient hours due to our restricted schedules. Research requires teamwork and it was a privilege to be a member of this team.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder affecting a substantial and ever-growing proportion of the population. It is currently the sixth leading cause of death in the United States and the only leading cause of death still on the rise (1). With the population increasing in both number and age, the burden of Alzheimer's disease is expected to double by 2060 (2). Treatments cost society approximately $100 billion each year and it has become the third most expensive disease in the United States (3). As the social, psychological, and financial burden falls not only on patients, but also their surrounding communities, questions arise regarding the cause of its pervasiveness.

Known pathological markers are useful in studying progression of the disease quantifiably. Alzheimer's disease has several characteristic pathological markers such as amyloid-beta (Aβ) plaques and neurofibrillary tangles of hyperphosphorylated tau (p-Tau) (4, 5). Aβ is a small peptide composed of 40-42 amino acids which can aggregate into insoluble, senile plaques responsible for synaptic loss and neuronal cell death (6, 7). It is presumed that Aβ deposition is the central etiological event contributing to AD, with amyloid deposition leading to neurotic plaques and neurofibrillary tangles, which then leads to neuronal damage and synaptic loss, which then leads to dementia (8, 9). However, the simplicity of this amyloid hypothesis cannot account for the cases where Aβ plaques and neurofibrillary tangles are seen in nondemented individuals postmortem (10, 11, 12) or where demented individuals show no signs of the AD pathological markers (13). This evidence suggests that this biologically complex disease does not follow a linear progression and its complete etiology is not yet fully understood. Although not as straightforward as once predicted, the amyloid hypothesis still offers a useful approach to understanding common aspects of AD and is well-studied. Aβ peptide formation

results from the processing of the amyloid precursor protein (APP), a type 1 transmembrane protein (14). APP cleavage in the brain occurs normally and can follow one of two pathways: the non-amyloidogenic pathway and the amyloidogenic pathway (15). In the non-amyloidogenic pathway, APP is cleaved by α-secretase and γ-secretase to yield protein fragments soluble alpha APP (sAPPα), the APP intracellular domain (AICD), and P3, while in the amyloidogenic pathway, APP is cleaved by β-secretase and γ-secretase to yield protein fragments soluble beta APP (sAPPβ), the APP intracellular domain (AICD), and Aβ (16, 17).

Multiple factors contribute to altered APP metabolism favoring the amyloidogenic pathway such as mutations in the APP gene, mutations in the presenilin gene (PSEN), a component of γ-secretase, mutations in the apolipoprotein E gene (APOE), partly responsible for Aβ clearance, trisomy 21, and environmental factors (8, 18, 19, 20, 21). In almost all cases, environmental and genetic factors both contribute to development of the disease (6, 7). There are two categories of AD: sporadic AD (SAD), in which environmental factors play a larger role and familial AD (FAD), in which genetic factors play a larger role. Sources estimate that FAD accounts for approximately 2-10% of the cases of AD while SAD accounts for the remaining 90-98% (22, 23, 24). Our lab studies the progression of AD in mice, which do not naturally exhibit the disease, however, there are several transgenic models for FAD in mice with mutated human copies of the APP, PSEN1, and PSEN2 genes (25, 26). These mice cleave APP and produce Aβ at an accelerated rate, leading to plaque formation that can be seen by 4-6 months of age (27, 28). Exploring the progression of FAD in transgenic mice accounts for only a small proportion of the AD seen in humans and ignores the largely important environmental factors. This study uses non-transgenic mice to highlight the environmental component of AD.

Because AD is multifactorial in nature with environmental factors contributing immensely, one area of great interest is the study of these environmental factors to determine if they play a role in development of AD, and thus could be targets for disease prevention. This includes environmental factors such as diet, exercise, sleep, and stress. The gut-brain axis (GBA) and the role gut microbiota play in neurodegenerative diseases is a recent prospect for the field, but early clinical trials have promising implications for the role of diet in AD incidence (29, 30, 31). Similarly, exercise, specifically aerobic exercise, has been shown to both minimize the risk of AD and improve cognitive function for those already diagnosed with AD (32, 33, 34). Additionally, chronic sleep restriction has been shown to increase brain vulnerability to Aβ plaques and neurofibrillary tangles (35, 36), while stress has been shown to accelerate the progression of AD in vulnerable individuals (37, 38, 39). Each of these factors offers promising implications to this field of study and this project focuses on the contribution of stress to AD.

The prevalence of stress in society is ubiquitous with chronic stressors such as personal finances, work, and the economy ranking as the top causes of stress (40). Americans on average rate their stress levels as a 4.9 out of 10 with 10 being "a great deal of stress" (41). Chronic unpredictable stress (CUS) in mice has been used as a model to study behavioral and psychiatric abnormalities such as depression and anxiety (42, 43, 44). A meta-analysis conducted by Dallé and Mabandla found that early life stress may contribute to the development of depression and later Parkinson's disease (45). Stress has also been shown to aggravate AD pathology in FAD transgenic mice (37, 38, 39). Han et al. (38) has shown that four weeks of chronic unpredictable mild stress (CUMS) increases cognitive impairment and Aβ deposition in APP/PS1 mutant mice. Ayuob et al. (46) similarly showed mice suffered memory deficits in forced swimming and elevated plus maze tests along with hippocampal neurodegenerative changes after CUMS

exposure. The aim of this study is to further elucidate the relationship between CUS and AD in non-trasngenic mice that do not naturally exhibit Aβ plaques.

Stress is defined as any state of disharmony to homeostasis and the stress response is regulated via the hypothalamic-pituitary-adrenal (HPA) axis (47). The major components of the HPA axis are the hypothalamus which releases corticotropin-releasing hormone (CRH), the anterior pituitary which releases adrenocorticotropic hormone (ACTH), and the adrenal gland which releases glucocorticoids (GC). The HPA axis shares a bidirectional relationship with the immune system. Immune cells release proinflammatory cytokines which stimulate the HPA axis to increase glucocorticoid release, which in turn negatively feeds back to the immune system to suppress further proinflammatory cytokine release (48). However, chronic exposure to stress can induce GC resistance (49, 50). The reduced inhibitory feedback caused by GC resistance triggers a feed-forward cycle between the HPA axis and inflammatory responses with each exacerbating the other (48, 51, 52).

The 28-day protocol used in this study introduced the mice to both stress and inflammation. The mice were subjected to six intermittent, randomly ordered stressors every day for 28 days. During the last 7 days of the experiment, they were also injected intraperitoneally with lipopolysaccharide (LPS), a bacterial endotoxin that induces inflammation by stimulating the innate immune system (53). LPS is a component of the outer membrane of Gram-negative bacteria which acts via toll-like receptor 4 (TLR4) to activate a cascade that results in the transcription of pro-inflammatory genes (53). Our lab has previously demonstrated that 7 consecutive days of peripheral LPS administration in mice leads to cognitive deficits in a contextual fear conditioning (CFC) paradigm (54, 55). CFC is a behavioral test that assesses the mouse's ability to associate a specific, novel context with an aversive stimulus (56). The

hippocampus and amygdala are involved in performing this task (57, 58). On the first day of CFC, the mouse is introduced to a new environment which has both visual and olfactory cues and allowed to habituate. Following the habituation period, a tone sounds, then a mild foot shock is applied. The mouse is returned to the home cage for completion of the training phase. The following day, the mouse is reintroduced into the same environment and the procedure is repeated. A computer monitors how much time the mouse spends frozen, defined as no movement other than respiration (56). Freezing time correlates to learning and memory with a higher freezing time corresponding to better learning that the specific context is associated with the aversive stimulus. Mice injected with LPS for 7 days displayed decreased freezing time compared to the control group, thus indicating that the induced inflammation leads to cognitive dysfunction (55). Our lab has additionally demonstrated that the cognitive decline associated with 7 days of intraperitoneal LPS administration is also associated with elevated hippocampal Aβ deposits (55). Seven days of LPS administration results in tolerance to the endotoxin and removal of negative inhibition on the HPA axis (55). To further expand upon these results, this project studied whether agitating the HPA axis with CUS before and during the introduction of inflammation via LPS administration would intensify the dysregulation of the HPA axis and inflammatory response, thus exacerbating Alzheimer's pathology and cognitive decline in non-transgenic mice.

METHODS

Subjects:

Experimentally naive 4–6 month-old male C57BL/6J mice, bred in the Texas Christian University vivarium from a breeding stock obtained from Jackson Laboratory (Bar Harbor, ME), were utilized for the experiment. All subjects were singly housed in standard polycarbonate

mouse cages. All subjects were on the same 12-h light/dark schedule, and both food and water were available *ad libitum*. All animals were housed and cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2010), and in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Texas Christian University.

Chronic Unpredictable Stress Paradigm:

When mice were 4-5 months old, they were exposed to either the CUS or control protocol for 28 days. The CUS group consisted of 21 mice while the control group consisted of 20 mice. In the CUS paradigm, subjects were intermittently exposed to 4-6 various stressors throughout the day in a random order. The six aversive stressors included: restraint – mice were placed in a 50mL plastic tube (CELLTREAT Scientific, Pepperell, MA) with openings in both sides for air flow for 30 minutes; forced swim – mice were placed in a 1000mL beaker filled with 800mL of lukewarm water for 5 minutes; wet bedding – 100-200mL of water was poured into the home cage to dampen bedding for 2 hours, then mice were transported to new home cages with dry bedding; cage tilt – home cages were placed on a 45 degree incline for 2 hours; empty cage – mice were placed in an empty study cage without bedding but with access to food and water for 1 hour; and nesting removal overnight. In the control paradigm, mice were housed in groups of 3-4 in identical home cages in the same study room and not exposed to any of the stressors. During the last 7 days of the study, mice were injected with either LPS or saline. During injection week, the mice were weighed daily to monitor a proper LPS response. On the 29th day, mice were subjected to CFC training. On the 30th day, mice were subjected to CFC testing followed by hippocampal tissue collection.

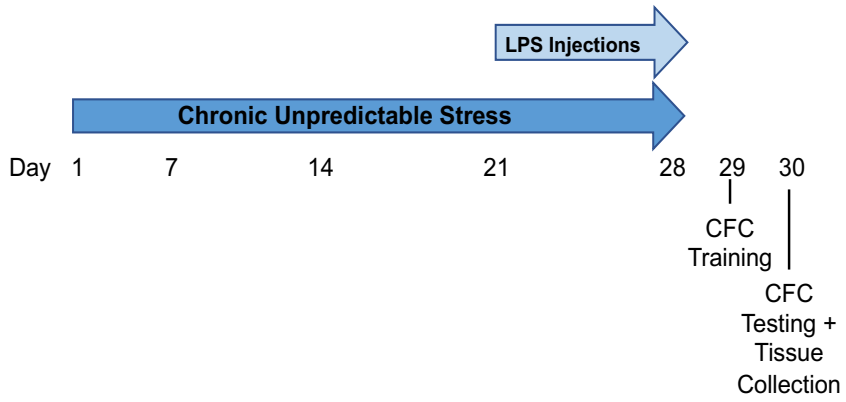

Figure 1. 28-day CUS Timeline: The mice underwent CUS for 28 consecutive days. During the last 7 days, mice were also injected with LPS or saline. The mice were then subjected to CFC training on the 29th day and CFC testing on the 30th day which was followed by tissue collection.

Intraperitoneal LPS Injections:

LPS or saline injections occurred during the last 7 days of CUS. Following the first 21 days of CUS, mice were intraperitoneally injected once-daily for 7 consecutive days with either LPS (*Escherichia coli* serotype 026: B6; Sigma Aldrich, St. Louis, MO) or sterile, phosphate-buffered saline (Dulbecco's PBS; Caisson Laboratories). LPS was administered to CUS subjects at a concentration of 250 µg/kg of body weight while 200µL of sterile phosphate-buffered saline was administered to all control subjects.

Contextual Fear Conditioning:

Twenty-four hours after the conclusion of CUS and injections, mice underwent contextual fear conditioning (CFC). CFC is a behavioral test which evaluates learning after exposure to an aversive stimulus. CFC is performed using a chamber (7W x 7D x 12H) with an electrified grid floor that delivers a 0.7 mA shock to the feet for two seconds (Coulbourn Instruments, Whitehall, PA). Additionally, animals are provided with both visual and olfactory cues: black and white polka dot walls and peppermint oil (peppermint oil in a 1:10 dilution of distilled water). The process for CFC consists of two days: one day of training followed by one day of testing. During training, mice were placed in the CFC apparatus and allowed to acclimate to the new surrounding for 120 seconds, followed by a brief, mild foot shock. Mice remained in

the apparatus 60 seconds after the foot shock before being returned to their home cages. Testing occurred twenty-four hours after training. During testing, mice were placed back in the CFC apparatus for 120 seconds, this time with no shock. Freezing behavior was monitored and computed by FreezeFrame™ software (ActiMetrics Software, Wilmette, IL) during both the training and testing trials. Since freezing is a mouse's natural fear response, freezing is measured to assess the strength of the learned association between the context of the CFC chamber and aversive foot shock. As previously explained, higher percentage of time freezing during testing correlates to better learning.

Tissue Collection and Protein Quantification:

Immediately following CFC testing, mice were euthanized via rapid decapitation. The hippocampus was removed from each animal and both hemispheres of the hippocampus were collected and placed in 250 μL of Proprep (PRO-PREP, Bulldog Bio, Portsmouth, NH) and disrupted using a pestel. Tissue lysates were then rapidly frozen on dry-ice following the collection process and stored at -80º C. For later assays, tissue samples were thawed on ice and then centrifuged at 15,000 rpm for 40 minutes to collect clear lysates. Protein assays (DC Protein Assay; Bio-Rad Laboratories, Hercules, CA) were performed to determine the protein concentrations in all hippocampal lysates before conducting enzyme-linked immunosorbent assays (ELISAs).

$A\beta_{42}$ ELISA:

Following tissue collection and protein quantification, a Mouse $A\beta_{42}$ ELISA (Invitrogen, ThermoFisher Scientific) was completed to quantify soluble $A\beta_{42}$ from the hippocampal region. First, lysates were diluted in incubation buffer (1:2 dilution). The $A\beta_{42}$ standard was reconstituted with standard reconstitution buffer and diluted sequentially. 100 μL of standards

and lysates were plated into the antibody-coated wells and incubated at room temperature for two hours, after which the plates were aspirated and washed four times with 1X wash buffer. The plate was then treated with $A\beta_{42}$ Detection Antibody solution and incubated at room temperature for one hour, after which the plates were washed and treated with 100 μL of HRP-tagged detection antibody (Anti-Rabbit IgG). Following incubation at room temperature for 30 minutes, the plates were washed and treated with 100 μL stabilized chromogen (tetramethylbenzidine) and incubated at room temperature in the dark for 30 minutes. Subsequently, 100 μL of stop solution was added to each well and the plate was read at an absorbance of 450nm (BMG LabTech FLUOstar Omega, Cary, North Carolina).

Statistical Analyses:

All data was analyzed using Statistical Package for Social Sciences (SPSS; Version 23.0, IBM, Armonk, NY). The experimental design of the study consisted of two, two-way analyses of variance (ANOVAs): stress condition – CUS vs. control and treatment condition – LPS or saline. Multiple ANOVAs were conducted to examine the dependent variables of both stress conditions and hippocampal Aβ lysates. In all statistical analyses, an alpha level ≤ 0.05 was considered significant.

RESULTS

A 2x2 analysis of variance (ANOVA) was performed to assess the behavioral effect of 28 days of CUS and 7 days of LPS injections on CFC performance. Evaluation by the SPSS interquartile range rule established two mice in the study as statistical outliers because they demonstrated increased freezing behavior before the presentation of the foot shock during the training session. These two mice were excluded from further statistical analysis. The results revealed a significant main effect of treatment condition such that the mice receiving LPS

injections froze more than the mice receiving saline injections (LPS or saline; $F(1,35) = 6.172$, $p = 0.018$) (Figure 2). No significant main effect was found in stress condition (CUS or control; $F(1,35) = 0.087$, NS) or interaction between treatment condition and stress condition ($F(1,35) = 1.988$, NS).

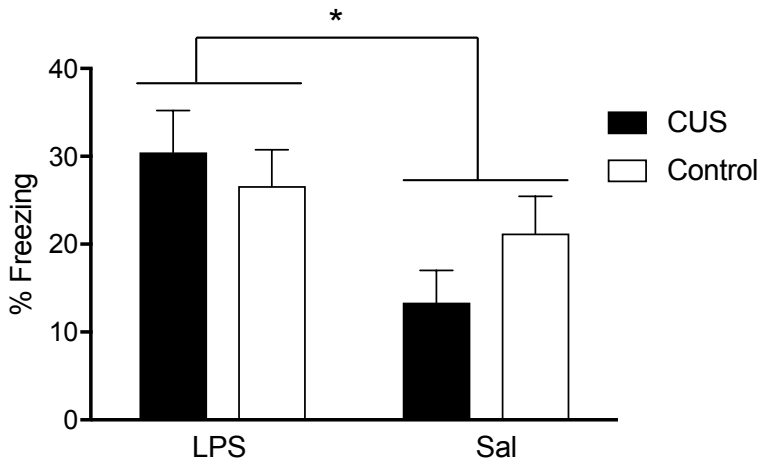

Figure 2. Contextual Fear Conditioning: A 2x2 ANOVA indicated a significant effect in treatment condition such that mice receiving LPS injections froze significantly more than mice receiving saline injections (p= 0.018). There was no statistical significance found in the freezing time between stress conditions.

A second 2x2 ANOVA was performed to assess the pathological effect of 28 days of CUS and 7 days of LPS injections on hippocampal soluble $A\beta_{42}$ peptide. The results revealed no main effect of treatment condition ($F(1,37) = 0.647$, NS) or stress condition ($F(1,37) = 0.430$, NS) (Figure 3). An interaction between treatment condition and stress condition was found to be approaching significance ($F(1,37) = 3.105$, $p = 0.086$) such that the mice subjected to CUS and LPS injections had the highest level of soluble Aβ in the hippocampus.

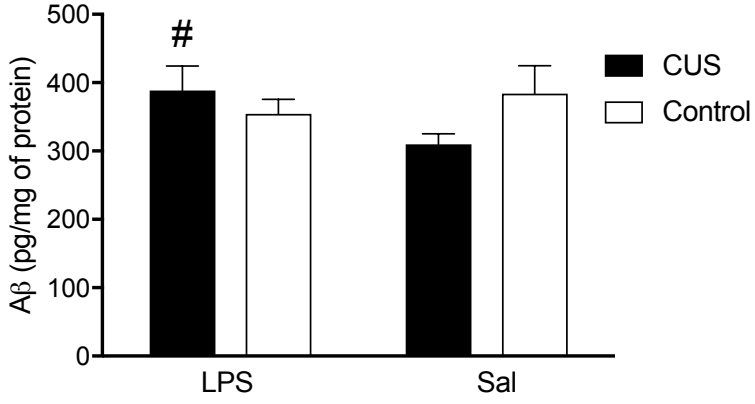

Figure 3. Hippocampal $A\beta_{42}$: A 2x2 ANOVA indicated an interaction between LPS and CUS that was approaching significance, such that mice in the CUS stress condition receiving LPS injections had the most hippocampal Aβ (p= 0.086). There was no main effect found in treatment condition or stress condition.

Mice were weighed daily during injection week to ensure that the mice receiving LPS injections had an appropriate inflammatory response to the endotoxin. Weight loss is expected during the first few days after injections as the mice will be exhibiting sickness behavior. A repeated-measures ANOVA revealed a significant main effect of treatment such that the mice receiving LPS injections weighed less than the mice receiving saline injections ($F(1,37) = 11.865$, $p = 0.001$) (Figure 4). There was also a significant Treatment X Day interaction ($Fs(1,37) > 5.923$, $ps < 0.020$), such that animals receiving LPS injections weighed less than the mice receiving saline injections on days 2, 3, 4, 5, and 6 of injection week ($ps < 0.020$) (Figure 4).

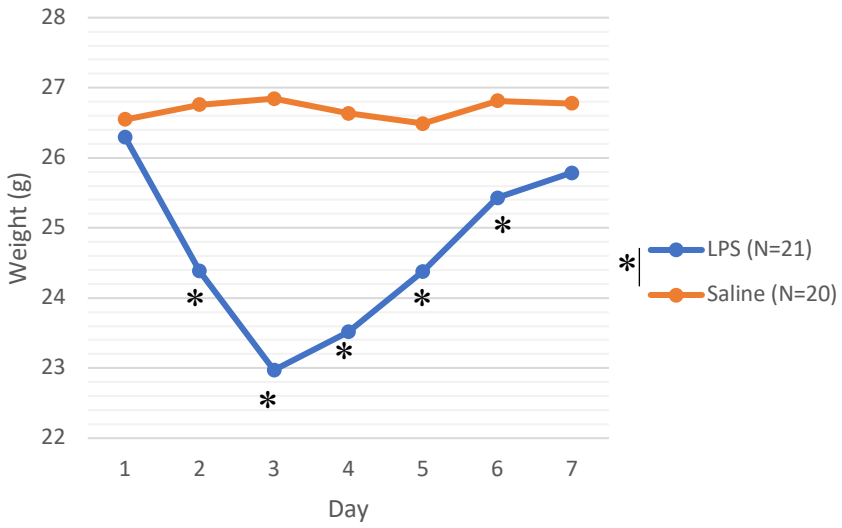

Figure 4. Mouse Weight Loss During Injection Week: The mice receiving LPS injections weighed significantly less than the mice receiving saline injections during days 2,3,4,5, and 6 ($ps < 0.020$) and overall during the injection week ($p = 0.001$).

DISCUSSION

This experiment tested the hypothesis that 28 days of exposure to chronic unpredictable stress and 7 days of LPS injections would cause cognitive deficits and increased Aβ accumulation in non-transgenic mice. The results did not support this hypothesis. In the behavioral experiment conducted using CFC, the mice injected with LPS froze significantly more than the mice injected with saline (Figure 2), indicating increased cognitive performance,

the opposite of the expected effect. Exposure to CUS had no effect on cognitive performance in the CFC test. There was also no significant interaction between the stress condition and treatment condition in the behavioral experiment (Figure 2). The pathological analysis revealed that mice exposed to CUS and receiving LPS injections had the highest levels of hippocampal Aβ (Figure 3). The interaction between stress condition and treatment condition was approaching significance, but not yet there. Neither CUS nor LPS injections significantly increased Aβ as expected.

Though our lab has previously demonstrated that 7 days of LPS administration increases cognitive dysfunction and Aβ accumulation (54, 55), the results from this study did not support similar findings. The LPS injections in this study decreased cognitive dysfunction and had no effect on Aβ accumulation (Figure 2, Figure 3). To ensure that the LPS was being administered properly and accurately inducing sickness behavior, the mice's weights were monitored during injection week. As expected, the mice receiving LPS injections lost weight during the first several days after injections and exhibited sickness behavior while the mice receiving saline injections maintained their weight and did not exhibit sickness behavior (Figure 4). Sigma Aldrich, the company responsible for making the LPS used in these experiments, produces serotypes of varying potency, measured by their endotoxin units. The endotoxin units of the serotype used in this experiment were much lower than the endotoxin units used in the previous trials exhibiting cognitive effects. Perhaps a higher potency of LPS is needed to cause effects in cognitive performance and Aβ deposition which this serotype did not achieve. Another possible confounding variable was the original health of the mice. After the experiment, it was discovered that the mice tested positive for various pathogens including pinworms, murine norovirus, and *Helicobacter pylori.* If they were infected with pathogens and already displaying sickness

behavior, this could have confounded the effect of the LPS, though the weight data revealed no sickness behavior in the control mice when compared to the mice exposed to LPS (Figure 4).

Exposure to CUS had no significant effect on either CFC performance or levels of hippocampal Aβ (Figure 2, Figure 3). In this experimental design, the mice undergoing CUS were removed from their home room and taken to a separate room in which the stressors were conducted. After the stressors were completed, the mice were returned to their home room. This resulted in the mice in the CUS stress condition being removed and replaced into the home room approximately eight times per day. Since the control mice were housed in the same room as the CUS mice, the additional noise and disruption from sliding the cages in and out of the rack could have disturbed and stressed the control mice. Exposing the control mice to stress would have confounded the effect of CUS so as to see no difference in CFC performance or Aβ accumulation between the two groups. To address this concern, two further CUS experiments were similarly conducted in which the CUS paradigm lasted only 21 days and the control and CUS groups were housed in different rooms to decrease unnecessary disruption of the control mice. The results of the CFC test have been analyzed and both of the 21-day CUS experiments revealed a significant main effect of stress so that the mice who underwent CUS froze significantly less than the control mice, revealing cognitive dysfunction as predicted. The $A\beta_{42}$ ELISAs have yet to be conducted for these experiments. These results have been delayed due to the unforeseen circumstances surrounding the COVID-19 pandemic. When the ELISAs are able to be completed, we hypothesize we will see a main effect of stress so that the CUS mice display significantly higher levels of hippocampal Aβ compared to control mice. We also hypothesize we will see a significant interaction between stress condition and treatment condition so that the

CUS mice that received LPS injections have the highest levels of Aβ compared to any other group.

Future studies involving the impact of CUS on AD pathology would help expand the knowledge we have about this disease and its contributing factors. Further directions include modifying the stressors, modifying the length of the CUS timeline, including female mice, including mice of different ages, and studying the effects of CUS independent of inflammatory stress. Our lab is currently conducting studies regarding shorter CUS timelines and older mice in which the preliminary results look promising.

Though the results of this study do not support the hypothesis that CUS leads to AD pathology and behavioral effects in non-transgenic mice, results from the modified 21-day studies suggest further research is needed in this area to draw conclusions regarding CUS's possible causative role in AD. Broader studies involving the development of AD in non-transgenic mice are important to understand the progression of the sporadic form of AD which effects the majority of AD patients. By better understanding the environmental factors that contribute to the onset of SAD, people can attempt to minimize their exposure to harmful influences or seek treatment to mitigate disease progression during its earlier stages if already exposed.

REFERENCES

1. Heron, Melonie. “Deaths: Leading Causes for 2017.” *Center for Disease Control and Prevention: National Vital Statistics Report*, vol. 68, no. 6, 24 June 2019, www.cdc.gov/nchs/data/nvsr/nvsr68/nvsr68_06-508.pdf.
2. Matthews, Kevin, et al. “Racial and Ethnic Estimates of Alzheimer's Disease and Related Dementias in the United States (2015–2060) in Adults Aged ≥65 Years.” *Alzheimer's & Dementia*, vol. 15, no. 1, 19 Sept. 2018, pp. 17–24., doi.org/10.1016/j.jalz.2018.06.3063.
3. Meek, Patrick, et al. “Economic Considerations in Alzheimer's Disease.” *Pharmacotherapy*, vol. 18, no. 2P2, 17 Jan. 2012, pp. 68–73., https://doi.org/10.1002/j.1875-9114.1998.tb03880.x.
4. Andreasen, Niels, et al. “Cerebrospinal Fluid Levels of Total-Tau, Phospho-Tau and Aβ42 Predicts Development of Alzheimer's Disease in Patients with Mild Cognitive Impairment.” *Acta Neurologica Scandinavia*, vol. 107, no. s179, 21 Feb. 2003, pp. 47–51., https://doi.org/10.1034/j.1600-0404.107.s179.9.x.
5. Weller, Jason, and Andrew Budson. “Current Understanding of Alzheimer’s Disease Diagnosis and Treatment.” *F1000Research*, vol. 7, 2018, p. 1161., doi:10.12688/f1000research.14506.1.
6. Checler, Frederic. “Processing of the β-Amyloid Precursor Protein and Its Regulation in Alzheimer's Disease.” *The Official Journal of the International Society for Neurology*, vol. 65, no. 4, Oct. 1995, pp. 1431–1444., https://doi.org/10.1046/j.1471-4159.1995.65041431.x.

7. Lu, Daniel C., et al. “Amyloid Beta Protein Toxicity Mediated by the Formation of Amyloid-? Protein Precursor Complexes.” *Annals of Neurology*, vol. 54, no. 6, 2003, pp. 781–789., doi:10.1002/ana.10761.
8. Hardy, John, and David Allsop. “Amyloid Deposition as the Central Event in the Aetiology of Alzheimer's Disease.” *Trends in Pharmacological Sciences*, vol. 12, 12 Oct. 1991, pp. 383–388., doi:10.1016/0165-6147(91)90609-v.
9. Hamos, J. E., et al. “Synaptic Loss in Alzheimer's Disease and Other Dementias.” *Neurology*, vol. 39, no. 3, 1989, pp. 355–355., doi:10.1212/wnl.39.3.355.
10. Price, Joseph, and John Morris. “Tangles and Plaques in Nondemented Aging and ‘Preclinical’ Alzheimer's Disease.” *Annals of Neurology*, vol. 45, no. 3, Mar. 1999, pp. 358–368., doi.org/10.1002/1531-8249(199903)45:33.0.CO;2-X.
11. Dominy, Stephen S., et al. “Porphyromonas Gingivalisin Alzheimer’s Disease Brains: Evidence for Disease Causation and Treatment with Small-Molecule Inhibitors.” *Science Advances*, vol. 5, no. 1, 23 Jan. 2019, doi:10.1126/sciadv.aau3333.
12. Herrup, Karl. “The Case for Rejecting the Amyloid Cascade Hypothesis.” *Nature Neuroscience*, vol. 18, no. 6, 2015, pp. 794–799., doi:10.1038/nn.4017.
13. Thomas, Kelsey R., et al. “Objective Subtle Cognitive Difficulties Predict Future Amyloid Accumulation and Neurodegeneration.” *Neurology*, vol. 94, no. 4, 2019, doi:10.1212/wnl.0000000000008838.
14. Lu, Daniel C., et al. “Amyloid Beta Protein Toxicity Mediated by the Formation of Amyloid-? Protein Precursor Complexes.” *Annals of Neurology*, vol. 54, no. 6, 2003, pp. 781–789., doi:10.1002/ana.10761.

15. Chow, Vivian W., et al. “An Overview of APP Processing Enzymes and Products.” *NeuroMolecular Medicine*, vol. 12, no. 1, 2009, pp. 1–12., doi:10.1007/s12017-009-8104-z.

16. Wilkins, Heather M., and Russell H. Swerdlow. “Amyloid Precursor Protein Processing and Bioenergetics.” *Brain Research Bulletin*, vol. 133, July 2017, pp. 71–79., doi:10.1016/j.brainresbull.2016.08.009.

17. Haas, C, et al. “Beta-Amyloid, Protein Processing and Alzheimer's Disease.” *Arzneimittel-Forschung*, vol. 45, no. 3A, Mar. 1995, pp. 398–402., www.ncbi.nlm.nih.gov/pubmed/7763333.

18. Laws, Simon M., et al. “Expanding the Association between TheAPOEgene and the Risk of Alzheimer's Disease: Possible Roles ForAPOEpromoter Polymorphisms and Alterations InAPOEtranscription.” *Journal of Neurochemistry*, vol. 84, no. 6, 2003, pp. 1215–1236., doi:10.1046/j.1471-4159.2003.01615.x.

19. Bird, Thomas D. “Genetic Aspects of Alzheimer Disease.” *Genetics in Medicine*, vol. 10, no. 4, 2008, pp. 231–239., doi:10.1097/gim.0b013e31816b64dc.

20. Chen, Yi-Gang. “Research Progress in the Pathogenesis of Alzheimer’s Disease.” *Chinese Medical Journal*, vol. 131, no. 13, 5 July 2018, pp. 1618–1624., doi:10.4103/0366-6999.235112.

21. Hartley, Dean, et al. “Down Syndrome and Alzheimer's Disease: Common Pathways, Common Goals.” *Alzheimer's & Dementia*, vol. 11, no. 6, 2014, pp. 700–709., doi:10.1016/j.jalz.2014.10.007.

22. Robakis, Nikolaos K. “Mechanisms of AD Neurodegeneration May Be Independent of Aβ and Its Derivatives.” *Neurobiology of Aging*, vol. 32, no. 3, Mar. 2011, pp. 372–379., doi:10.1016/j.neurobiolaging.2010.05.022.

23. Alzheimers. “Risk Factors.” *Alzheimer's Research & Prevention Foundation*, Alzheimer's Research & Prevention Foundation, 5 Feb. 2017, alzheimersprevention.org/alzheimers-info/risk-factors/.

24. Shea, Yat-Fung, et al. “A Systematic Review of Familial Alzheimer's Disease: Differences in Presentation of Clinical Features among Three Mutated Genes and Potential Ethnic Differences.” *Journal of the Formosan Medical Association*, vol. 115, no. 2, Feb. 2016, pp. 67–75., doi:10.1016/j.jfma.2015.08.004.

25. Eratne, Dhamidhu, et al. “Alzheimer’s Disease: Clinical Update on Epidemiology, Pathophysiology and Diagnosis.” *Australasian Psychiatry*, vol. 26, no. 4, 2018, pp. 347–357., doi:10.1177/1039856218762308.

26. Drummond, Eleanor, and Thomas Wisniewski. “Alzheimer’s Disease: Experimental Models and Reality.” *Acta Neuropathologica*, vol. 133, no. 2, 2016, pp. 155–175., doi:10.1007/s00401-016-1662-x.

27. Garcia-Alloza, Monica, et al. “Characterization of Amyloid Deposition in the APPswe/PS1dE9 Mouse Model of Alzheimer Disease.” *Neurobiology of Disease*, vol. 24, no. 3, Dec. 2006, pp. 516–524., doi:10.1016/j.nbd.2006.08.017.

28. Jankowsky, Joanna L., et al. “Mutant Presenilins Specifically Elevate the Levels of the 42 Residue β-Amyloid Peptide in Vivo: Evidence for Augmentation of a 42-Specific γ Secretase.” *Human Molecular Genetics*, vol. 13, no. 2, 25 Jan. 2003, pp. 159–170., doi:10.1093/hmg/ddh019.

29. Yusufov, Miryam, et al. “Alzheimer's Disease and Diet: a Systematic Review.” *International Journal of Neuroscience*, vol. 127, no. 2, 9 Mar. 2016, pp. 161–175., doi:10.3109/00207454.2016.1155572.

30. Sureda, Antoni, et al. “Oral Microbiota and Alzheimer’s Disease: Do All Roads Lead to Rome?” *Pharmacological Research*, vol. 151, Jan. 2020, p. 104582., doi:10.1016/j.phrs.2019.104582.

31. Rosa, Francesca La, et al. “The Gut-Brain Axis in Alzheimer’s Disease and Omega-3. A Critical Overview of Clinical Trials.” *Nutrients*, vol. 10, no. 9, 2018, p. 1267., doi:10.3390/nu10091267.

32. Cass, Shane P. “Alzheimer’s Disease and Exercise.” *Current Sports Medicine Reports*, vol. 16, no. 1, 2017, pp. 19–22., doi:10.1249/jsr.0000000000000332.

33. Morris, Jill K., et al. “Aerobic Exercise for Alzheimer's Disease: A Randomized Controlled Pilot Trial.” *Plos One*, vol. 12, no. 2, 10 Feb. 2017, doi:10.1371/journal.pone.0170547.

34. Bernardo, T.c., et al. “Physical Exercise and Brain Mitochondrial Fitness: The Possible Role Against Alzheimer's Disease.” *Brain Pathology*, vol. 26, no. 5, Sept. 2016, pp. 648–663., doi:10.1111/bpa.12403.

35. Kincheski, Grasielle C., et al. “Chronic Sleep Restriction Promotes Brain Inflammation and Synapse Loss, and Potentiates Memory Impairment Induced by Amyloid-β Oligomers in Mice.” *Brain, Behavior, and Immunity*, vol. 64, Aug. 2017, pp. 140–151., doi:10.1016/j.bbi.2017.04.007.

36. Ahmadian, Nahid, et al. “Review Paper: Tau Pathology of Alzheimer Disease: Possible Role of Sleep Deprivation.” *Basic and Clinical Neuroscience Journal*, vol. 9, no. 5, 2018, pp. 307–316., doi:10.32598/bcn.9.5.307.

37. Cortese, Aurelio, et al. “The Arctic/Swedish APP Mutation Alters the Impact of Chronic Stress on Cognition in Mice.” *European Journal of Neuroscience*, vol. 50, no. 5, 13 July 2019, pp. 2773–2785., doi:10.1111/ejn.14500.

38. Han, Bing, et al. “Chronic Stress Contributes to Cognitive Dysfunction and Hippocampal Metabolic Abnormalities in APP/PS1 Mice.” *Cellular Physiology and Biochemistry*, vol. 41, no. 5, 2017, pp. 1766–1776., doi:10.1159/000471869.

39. Han, Bing, et al. “Chronic Stress Aggravates Cognitive Impairment and Suppresses Insulin Associated Signaling Pathway in APP/PS1 Mice.” *Journal of Alzheimer's Disease*, vol. 53, no. 4, 8 July 2016, pp. 1539–1552., doi:10.3233/jad-160189.

40. Clay, R A. “Stressed in America.” *Monitor on Psychology*, vol. 42, no. 1, Jan. 2011, pp. 60–60., www.apa.org/monitor/2011/01/stressed-america.

41. “Health Care, Mass Shootings, 2020 Presidential Election Causing Americans Significant Stress, New Stress in America™ Survey Finds.” *American Psychological Association*, American Psychological Association, 5 Nov. 2019, www.apa.org/news/press/releases/2019/11/americans-significant-stress.

42. Herrup, Karl. “The Case for Rejecting the Amyloid Cascade Hypothesis.” *Nature Neuroscience*, vol. 18, no. 6, 26 June 2015, pp. 794–799., doi:10.1038/nn.4017.

43. Monteiro, Susana, et al. “An Efficient Chronic Unpredictable Stress Protocol to Induce Stress-Related Responses in C57BL/6 Mice.” *Frontiers in Psychiatry*, vol. 6, 2 Feb. 2015, doi:10.3389/fpsyt.2015.00006.

44. Campos, Alline C., et al. “Animal Models of Anxiety Disorders and Stress.” *Revista Brasileira De Psiquiatria*, vol. 35, no. suppl 2, 2013, doi:10.1590/1516-4446-2013-1139.

45. Dallé, Ernest, and Musa V. Mabandla. “Early Life Stress, Depression And Parkinson’s Disease: A New Approach.” *Molecular Brain*, vol. 11, no. 1, 19 Mar. 2018, doi:10.1186/s13041-018-0356-9.

46. Ayuob, Nasra Naeim, et al. “Ocimum Basilicum Improve Chronic Stress-Induced Neurodegenerative Changes in Mice Hippocampus.” *Metabolic Brain Disease*, vol. 33, no. 3, 22 June 2018, pp. 795–804., doi:10.1007/s11011-017-0173-3.

47. Tsigos, Constantine, and George P Chrousos. “Hypothalamic–Pituitary–Adrenal Axis, Neuroendocrine Factors and Stress.” *Journal of Psychosomatic Research*, vol. 53, no. 4, 2002, pp. 865–871., doi:10.1016/s0022-3999(02)00429-4.

48. Silverman, Marni N., and Esther M. Sternberg. “Glucocorticoid Regulation of Inflammation and Its Functional Correlates: from HPA Axis to Glucocorticoid Receptor Dysfunction.” *Annals of the New York Academy of Sciences*, vol. 1261, no. 1, July 2012, pp. 55–63., doi:10.1111/j.1749-6632.2012.06633.x.

49. Miller, Gregory E., et al. “A Functional Genomic Fingerprint of Chronic Stress in Humans: Blunted Glucocorticoid and Increased NF-KB Signaling.” *Biological Psychiatry*, vol. 64, no. 4, 15 Aug. 2008, pp. 266–272., doi:10.1016/j.biopsych.2008.03.017.

50. Rohleder, Nicolas. “Acute and Chronic Stress Induced Changes in Sensitivity of Peripheral Inflammatory Pathways to the Signals of Multiple Stress Systems – 2011 Curt Richter Award Winner.” *Psychoneuroendocrinology*, vol. 37, no. 3, Mar. 2012, pp. 307–316., doi:10.1016/j.psyneuen.2011.12.015.

51. Sternberg, Esther M. “Neural Regulation of Innate Immunity: a Coordinated Nonspecific Host Response to Pathogens.” *Nature Reviews Immunology*, vol. 6, no. 4, Apr. 2006, pp. 318–328., doi:10.1038/nri1810.

52. Sternberg, Esther M. “Neural Regulation of Innate Immunity: a Coordinated Nonspecific Host Response to Pathogens.” *Nature Reviews Immunology*, vol. 6, no. 4, Apr. 2006, pp. 318–328., doi:10.1038/nri1810.

53. Batista, Carla Ribeiro Alvares, et al. “Lipopolysaccharide-Induced Neuroinflammation as a Bridge to Understand Neurodegeneration.” *International Journal of Molecular Sciences*, vol. 20, no. 9, 9 May 2019, p. 2293., doi:10.3390/ijms20092293.

54. Kahn, Marielle S., et al. “Prolonged Elevation in Hippocampal Aβ and Cognitive Deficits Following Repeated Endotoxin Exposure in the Mouse.” *Behavioural Brain Research*, vol. 229, no. 1, 1 Apr. 2012, pp. 176–184., doi:10.1016/j.bbr.2012.01.010.

55. Kranjac, Dinko, et al. “Peripheral Administration of d-Cycloserine Rescues Memory Consolidation Following Bacterial Endotoxin Exposure.” *Behavioural Brain Research*, vol. 243, 15 Apr. 2013, pp. 38–43., doi:10.1016/j.bbr.2012.12.053.

56. Curzon, Peter, et al. “Cued and Contextual Fear Conditioning for Rodents.” *Methods of Behavior Analysis in Neuroscience*, 2nd ed., CRC Press/Taylor & Francis, 2009, www.ncbi.nlm.nih.gov/books/NBK5223/.

57. Goosens, K. A. “Contextual and Auditory Fear Conditioning Are Mediated by the Lateral, Basal, and Central Amygdaloid Nuclei in Rats.” *Learning & Memory*, vol. 8, no. 3, 1 May 2001, pp. 148–155., doi:10.1101/lm.37601.

58. Phillips, R G, and J E LeDoux. “Differential Contribution of Amygdala and Hippocampus to Cued and Contextual Fear Conditioning.” *Behavioral Neuroscience*, vol. 106, no. 2, Apr. 1992, pp. 274–285., doi:10.1037//0735-7044.106.2.274.