EFFECTS OF EXERCISE AND AN ENRICHED ENVIRONMENT ON ALZHEIMER'S DISEASE PATHOLOGY IN CHRONICALLY STRESSED MICE

by

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disease currently affecting 5.7 million Americans, and the disease prevalence is expected to rise to 14 million by 2050. One characteristic pathological marker of AD is plaques of amyloid-beta (Aβ) peptides, which corresponds with a deterioration of memory and cognition in patients afflicted with AD. As mice do not naturally form Aβ plaques, our lab utilizes the 5xFAD transgenic mouse, a model of familial AD, wherein genetically modified human genes introduced into the mouse genome allow for the study of AD pathology. While several environmental factors impact AD pathology, stress has been found to exacerbate production of Aβ in different transgenic mouse models. Thus, the first goal of the current project was to determine if a social stressor, isolation stress, would increase levels of Aβ, number of Aβ plaques, and cognitive deficits in $5xFAD^+$ mice in comparison to group-housed controls. In addition, the second goal of this project was to determine whether the hypothesized stress-induced increase in Aβ plaques could be prevented through exposure to physical exercise alone, or to exercise and an enriched environment throughout the period of isolation. One subset of $5xFAD^+$ and $5xFAD^-$ two-month-old mice were housed in group-housed and isolated conditions. An additional subset of $5xFAD^+$ two-month-old mice were housed in isolation, housed in isolation with an exercise wheel, or housed in isolation with an exercise wheel and an enriched environment. After 3 months of group-housing or isolation, cognition was assessed through contextual fear conditioning, and brains were collected for hippocampal Aβ plaque counts and quantification of Aβ levels. Extended isolation stress significantly increased the number of hippocampal plaques and cognitive deficits in $5xFAD^+$ mice. However, these effects of social isolation were not prevented by exercise or an enriched environment in the $5xFAD^+$ mice. As AD prevalence continues to increase, understanding how stress impacts the onset and progression of AD is critical to develop therapeutic practices to alleviate disease progression.

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INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease currently affecting about 5.7 million Americans, and the number of people affected may rise as high as 14 million by 2050 (Alzheimer's Association, 2018). There is currently no treatment to prevent disease progression, and existing treatments are only partially effective in slowing the progression of disease (Rafii & Aisen, 2015). AD is the most common form of dementia, and its incidence significantly increases with age (Kukall et al., 2002). There are two main types of AD. Sporadic AD is the most common form of the disease and develops in people above the age of 65. Familial AD develops in about 5 percent of all AD patients before the age of 65, and it is primarily linked to genetic mutations (Alzheimer's Association). Characteristic AD pathology consists of intracellular neurofibrillary tangles of hyperphosphorylated tau protein and extracellular Amyloid-beta (Aβ) plaques. Additionally, AD is associated with a deterioration of memory and cognition in AD patients. Cleavage of the Amyloid Precursor Protein (APP), primarily found in the cell membranes of neurons, produces the Aβ peptide. The Aβ peptide can be cleaved at different lengths, but $A\beta_{1\text{-}42}$ is the most neurotoxic. These Aβ peptides can disrupt synaptic connections and aggregate into Aβ plaques throughout the brain, but the hippocampus is affected profoundly and early on in disease progression (Mattson, 2004).

AD pathology can be modeled in transgenic animals, which are engineered through introduction of genetic modifications. Mutations in 3 human genes in particular have been linked to familial AD: Amyloid Precursor Protein (APP), Presenilin-1 (PS1), and Presenilin-2 (PS2) genes. Forced expression of these mutations in animals produce models of AD pathology and even behavioral deficits. Mice are the most common vertebrate animals to be used as these transgenic models of AD as they are fairly inexpensive to maintain, have relatively short life spans and gestational periods, and produce large litters of offspring.

Additionally, techniques for performing genetic modifications in mice are well developed (Elder, Gama Sosa, & De Gasperi, 2010; Ertekin-Taner, 2007). While mice do not naturally form Aβ plaques, transgenic models with genetic modifications linked to familial AD do exhibit plaque pathology through production of human Aβ. In particular, our lab uses the 5xFAD transgenic mouse model, which coexpresses five familial AD mutations in human genes. These mutations correspond to increased levels of $A\beta_{1-42}$ and accelerated plaque formation (Oakley et al., 2006). However, such genetic mutations corresponding to familial AD only account for a minority of AD cases. The majority of AD cases manifest as sporadic AD (Lahiri & Maloney, 2010). The most common risk factors that contribute to the development of sporadic AD pathology are environmental factors, such as poor nutrition, head trauma, limited physical activity, alcohol abuse, and chronic stress (Fratiglioni, Ahlbom, Viitanen, & Winblad, 1993). Specifically, chronic stress plays a critical role in the development of AD (Jeong et al., 2006).

Stress is a regular aspect of both routine and significant life events, and it is mediated predominantly by the Hypothalamic-Pituitary-Adrenal (HPA) axis and the Autonomic Nervous System (ANS). In response to a stressor, the HPA axis facilitates the release of glucocorticoids, such as cortisol in humans or corticosterone in rodents, from the adrenal cortex. Glucocorticoid receptors within the brain, particularly in the hippocampus, are bound by glucocorticoids as part of a negative feedback loop to decrease the activity of the HPA axis (Besedovsky, Chrousos, & Del Ray, 2008; Jeong et al., 2006; Mcewen, 2000). However, cortisol and other stress hormones produced during acute or chronic stress can have different effects on brain and immune function. Acute, or short-term, stress, corresponds to the recruitment of stored energy and movement of immune cells from the blood into organs and tissues, which help facilitate the immune response. In addition, acute stress can facilitate and strengthen the formation of memories of events that are potentially threatening or elicit a

strong emotional response within the brain (Mcewen, 2000). Chronic, or long-term, stress, however, can lead to the suppression of the immune response and induce morphological and functional changes to the brain, particularly in the hippocampus (Mcewen, 2000). As a result, chronic stress may contribute to the development and severity of chronic illnesses including cardiovascular disease, depression, and disorders of immune function (Vanitallie, 2002). Furthermore, chronic stress exacerbates the onset and severity of cognitive dysfunction and is correlated to an increase in extracellular amyloid deposits and plaque deposition characteristic of AD (Jeong et al., 2006). This suggests chronic stress contributes to the development of AD.

In order to evaluate the effects of chronic stress, the first goal of this project is to determine whether 5xFAD mice housed in extended isolation will have an increase in hippocampal levels of Aβ, number of hippocampal Aβ plaques, and cognitive deficits, relative to group-housed controls. Secondly, this project aims to determine whether such pathological and cognitive effects from chronic isolation stress can be prevented through exposure to exercise and an enriched environment (EE). EE utilizes complex inanimate objects to provide multisensory stimulation to induce brain plasticity in study animals. Previous studies indicate EE corresponds with improved cognitive function and restoration of brain morphological changes due to chronic stress including increasing hippocampal neurogenesis and neuronal plasticity (Ashokan, Hegde, & Mitra, 2016; Bhagya, Srikumar, Veena, & Rao, 2016; Chen et al., 2010; Vega-Rivera et al., 2016). In particular, exposure to EE during a period of chronic stress may even prevent the detrimental morphological changes induced by chronic stress from occurring (Hutchinson et al., 2012). Additionally, prior studies indicate exercise helps to decrease risk of cognitive impairment and to alleviate morphological changes induced through chronic stress (Cotman, 2002; Laurin, Verreault, Lindsay, Macpherson, & Rockwood, 2001). Thus, the second goal of this project is to

determine if the hypothesized increase in hippocampal Aβ levels, hippocampal Aβ plaques, and cognitive deficits in 5xFAD mice due to isolation stress can be prevented through exposure to exercise alone or to exercise and an enriched environment throughout the period of isolation. Because the nature of EE objects and cages allows for mice to have higher levels of exercise, the effects of exercise alone will be evaluated to distinguish the effects of exercise from the effects of EE.

METHODS

Animal Subjects

The animals used in all experiments were male transgenic 5xFAD mice and wild-type controls of approximately 2 months of age, which were bred in the Texas Christian University vivarium from a stock obtained from The Jackson Laboratory (Bar Harbor, ME). All animals were treated in accordance with the protocols approved by the Institutional Animal Care and Use Committee (IACUC) of TCU, and in accordance with the guidelines described by the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). Animals were housed either in standard-sized (12.5 cm × 15 cm × 25 cm) or large-sized (30 cm × 25 cm × 30 cm) polycarbonate cages. All experimental groups were subjected to the same 12-h light/dark cycle with lights on at 0700 and off at 1900. All animals had regular access to food and water.

Experimental Design

In the first experiment, $5xFAD^+$ and $5xFAD^-$ animals were housed in standard-sized polycarbonate cages in isolated or group-housed conditions of 3-4 animals per cage for 3 months (Table 1). In the second experiment, $5xFAD^+$ animals were housed in three separate isolated conditions for 3 months: a) in a large-sized (30 cm × 25 cm × 30 cm) polycarbonate cage with an enriched environment (EE), b) in a standard-sized (12.5 cm × 15 cm × 25 cm) polycarbonate cage with an exercise wheel (EX) (Med Associates Inc., Albans, VT), and c) in

a standard-sized polycarbonate cage alone (Table 2). Aside from a larger cage, EE cages contained cognitively stimulating objects including wire mesh climbing walls, PVC pipe tunnels, plastic and paper huts, steel climbing chains, wooden sticks, extra nesting material, and exercise wheels (Bhagya et al., 2016; Chen et al., 2010; Marianno, Abrahao, & Camarini, 2017; Vega-Rivera et al., 2016; Image 1). To maintain environmental novelty, EE objects were rearranged every 3-4 days, and one existing object was exchanged for a new object. EX and isolated animals were also briefly handled to control for routine stress (Ashokan et al., 2016; Bhagya et al., 2016; Marianno et al., 2017; Vega-Rivera et al., 2016). EE and EX animals always had access to the exercise wheel.

Genotype	Housing Condition	Number of animals (n)
$5xFAD^+$	Group housed	6
	Isolated	6
$5xFAD^-$	Group housed	12
	Isolated	11

Figure 1. Experiment 1 design

Housing Condition	Number of animals (n)
Isolated	10
Exercise (EX)	9
Enriched Environment (EE)	8

Figure 2. Experiment 2 design

Figure 3. Conditions for the enriched environment

Contextual Fear-Conditioning

After 3 months of isolated or group-housed conditions, animal learning for both experiments was assessed in a contextual fear-conditioning (CFC) paradigm. The behavior testing apparatuses used were fully automated fear-conditioning units (Coulbourn Instruments, Whitehall, PA, 7W × 7D × 12H) and FreezeFrameTM software (ActiMetrics Software, Wilmette, IL). This paradigm paired a mild aversive stimulus (two 0.5 mA shocks) delivered by an electrified grid floor with the context of chambers containing polka-dotted wall patterns and a peppermint olfactory cue (Kranjac et al., 2012). Movement of the animal was monitored and recorded continuously. Animals were placed in the chambers and allowed a 180 s acclimation period followed by a single 2 s, 0.5 mA shock. Another 2 s 0.5 mA shock was administered 60 s later. Animals remained in the chamber an additional 60 s and were then transported back to their home cages in clear polycarbonate transport boxes. Animals were returned to the chambers 24 h later, and freezing behavior was monitored for 180 s. Freezing behavior is considered an innate fear response in rodents, and, in this context, the percent of time spent freezing was used as a dependent variable to indicate whether an association between the context and aversive stimulus was learned.

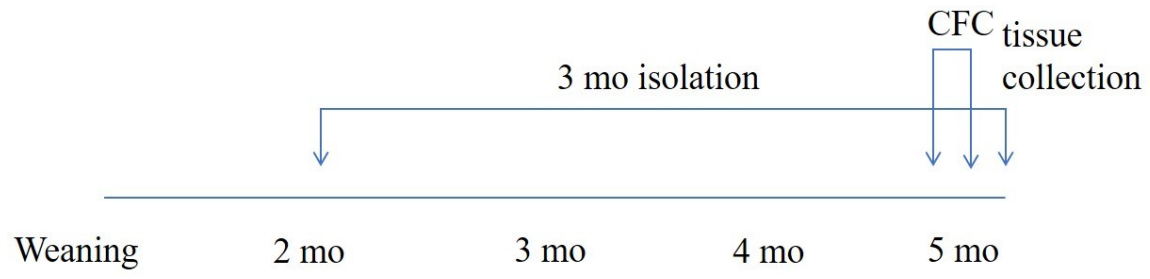

Figure 4. Experimental timeline. All animals underwent CFC training after three months of isolation or group-housing, followed by testing 24 hours later. Tissue collection occurred 24 hrs after testing in all animals.

Tissue Collection/Extraction

Following behavioral testing, animals were anesthetized with a mixture of ketamine (100mg/kg) and xylazine (5mg/kg). Once properly sedated, animals were given heparin through an intraperitoneal injection and then transcardially perfused with 1X phosphate-buffered saline for 7 minutes (PBS; pH 7.4). One perfused hemisphere was harvested and placed in a 4% paraformaldehyde solution (PFA; pH 7.4), and it was stored at 2−8 °C until it was sectioned using a vibratome (Leica Biosystems, Buffalo Grove, IL). The hippocampus was isolated from the remaining hemisphere and homogenized in a solution containing protease inhibitors for protein extraction (PRO-PREP, Boca Scientific, Boca Raton, FL), snap frozen in dry ice, and stored at −80 °C until it was further processed. The homogenized tissue samples were centrifuged at 16,000 × *g* for 40 min, after which the clear lysate was removed and a DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) was conducted according to manufacturer instructions.

DC protein assay

DC Protein Assays utilize a working reagent that is used with detergent-based buffers. The protein standard curve consisted of dilutions from 0.2 – 1.5 mg/ml of γ-globulin, made in the same buffer as the lysates. 5 µl of standards and 5 µl of sample were pipetted in duplicates into a 96 well plate with 25 µl of reagent A' (consisting of a mixture of reagent A and reagent S) and 200 µl of reagent B in each well. After sitting for 15 min in the dark at room temperature, the plate was placed into the plate reader (BMG LabTech FLUOstar Omega, Cary, NC) and the optical density of the samples were read at 750 nm. The results were then used to standardize protein content.

Aβ ELISA

Following general quantification of proteins through the DC Protein Assay, the BetaMark Aβ x-42 ELISA (BioLegend, San Diego, CA) was performed according to manufacturer

instructions. Samples were diluted in a 2:1 ratio using the incubation buffer, plated in equal volumes in duplicate with the Horseradish Peroxidase (HRP)-labeled detection antibody, and incubated overnight at 2–8 °C. The following day after incubation, the wells were washed five times with wash buffer, and the 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added to each well. The plate was then incubated for 45 min in the dark at room temperature, and the optical density was read at 620 nm (BMG LabTech FLUOstar Omega, Cary, NC).

Histochemistry for Aβ Plaque Analysis

Forty micrometer brain tissue sections were obtained and placed in a 24-well plate containing 0.03% sodium azide and 1% PFA mixture in each well. Before staining began, all sections were washed in deionized water (DI water) 3x for 10 minutes each to remove traces of sodium azide and PFA. Following the third wash, 500 µl of Thioflavin T was added to each well to wash for 5 min. The Thioflavin T was discarded, and each sample was washed with 70% ethanol for 2 min. The 70% ethanol was discarded, and each sample was washed with 50% ethanol 2x for 2 min. Finally, the 50% ethanol was discarded, and each sample was washed with DI water 2x for 2 min. Each of the samples were washed in 1X PBS and mounted on slides. Early, middle, and late sections were selected from each animal sample. From the prepared slides, Aβ plaques in the hippocampus were visualized with a Zeiss LSM 710 confocal microscope (Carl Zeiss Microscopy, Thornwood, NY). Images were processed through the ImageJ program (U.S. National Institutes of Health, Bethesda, MD), and the number of Aβ plaques in the hippocampus was counted for each animal sample.

RESULTS

Experiment 1

Isolation stress leads to decreased cognitive function regardless of genotype

In order to determine the effects of three months of isolation on cognitive function, freezing behavior in the CFC paradigm was evaluated. An increase in freezing behavior is interpreted as an increase in learning of the association between the context and aversive stimulus. A two-way analysis of variance (ANOVA) was performed and indicated there was a significant effect of housing condition (isolated or group housed; $F(1, 31) = 9.968$, $p = 0.04$). However, there was no significant effect of genotype ($5xFAD^+$ or $5xFAD^-$; $F(1, 31) = 0.277$, *NS*) or interaction between housing condition and genotype ($F(1, 31) = 0.074$, *NS;* Figure 5). Thus, isolated animals froze significantly less than group housed animals regardless of genotype. This decrease in freezing time indicates decreased learning in isolated animals compared to the group housed animals.

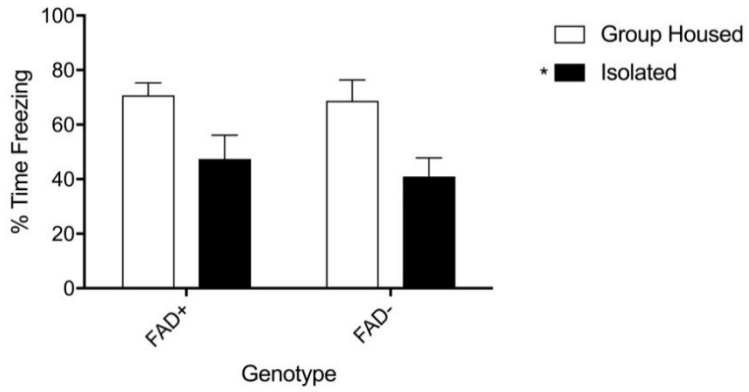

Figure 5. Isolation stress leads to decreased cognitive function regardless of genotype. Both $5xFAD^+$ and $5xFAD^-$ isolated animals froze significantly less than group housed animals on testing day. Bars represent means ± SE; n=6-12 animals per condition. Significance differences ($p<0.05$) are designated by *.

Isolation stress leads to increased plaques in 5xFAD$^+$ mice

In order to determine the effect of three months of isolation on Aβ plaque number in 5xFAD$^+$ mice, plaques were counted in the hippocampus and subiculum of a late tissue section from each animal. A Student's t-test was performed and indicated there was a significant increase in plaque number in isolated animals relative to group-housed animals (t (1, 9) = 2.582, p = 0.030; Figure 6).

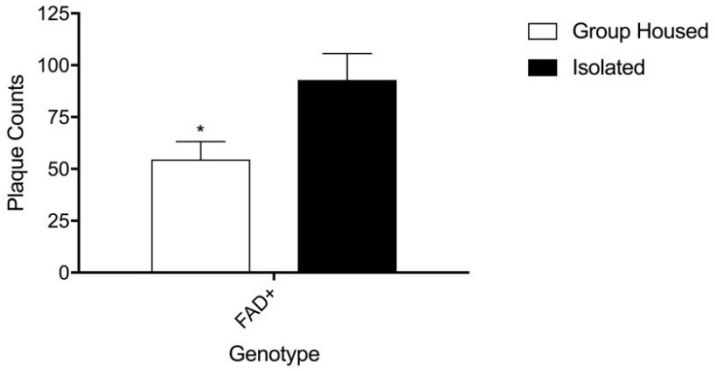

Figure 6. Isolation stress leads to increased plaque number in 5xFAD$^+$ mice. Plaque counts were significantly higher in isolated animals compared to group housed animals. Bars represent means ± SE; n=8-10 animals per condition. Significance differences ($p<0.05$) are designated by *.

Isolation stress does not impact levels of hippocampal Aβ

In order to determine the effect of three months of isolation stress on levels of Aβ in the hippocampus, Aβ was quantified with an Aβx-42 ELISA. A two-way ANOVA was performed and indicated a significant effect of genotype (5xFAD$^+$ or 5xFAD$^-$; F(1, 32) = 95.377, $p < 0.001$). However, there was no significant effect of housing condition (isolated or group housed; F = 0.433, *NS*) or interaction between genotype and housing condition (F(1, 32) = 0.244, *NS*; Figure 7). Overall, 5xFAD$^+$ mice had significantly more hippocampal Aβ than 5xFAD$^-$ mice regardless of housing condition.

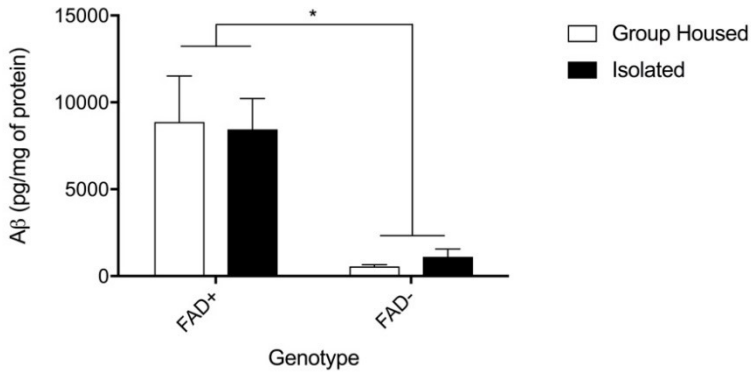

Figure 7. Isolation stress does not impact levels of Aβ. Concentration of Aβ in the hippocampus was significantly higher in 5xFAD^{+} mice compared to 5xFAD^{-} mice irrespective of housing condition. Bars represent means ± SE; n=6-12 animals per condition. Significance differences ($p<0.05$) are designated by *.

Experiment 2

Exercise and Enriched Environment did not ameliorate cognitive deficits

In order to determine whether exercise or an enriched environment would restore cognitive function, freezing behavior in the CFC paradigm was analyzed. All animals exhibited running activity, thus no animals were excluded from analyses. A one-way ANOVA was performed and indicated significant differences between the three groups ($F(2,24) = 8.167$, $p = 0.02$). In contrast to the hypothesis, the post hoc LSD test indicated that the percent freezing time for exercise animals was significantly lower compared to isolated animals ($p = 0.010$) and to the enriched environment animals ($p = 0.001$) on testing day. There was no significant difference between the percent freezing time for the enriched environment and isolated animals (*NS*; Figure 8) on testing day.

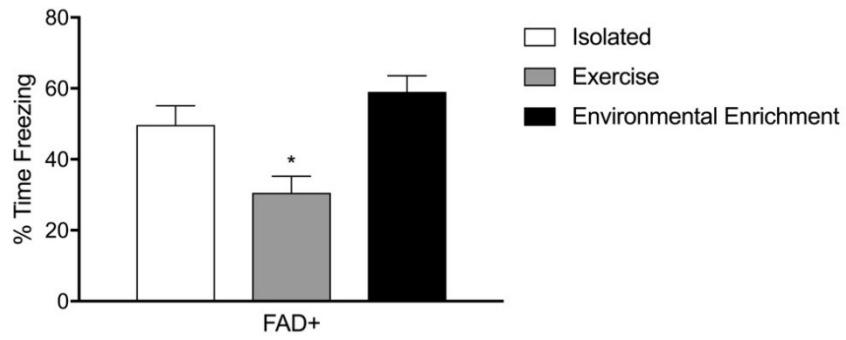

Figure 8. Exercise and enriched environment did not ameliorate cognitive deficits. The percent freezing time for exercise animals was significantly lower than the percent freezing time for both enriched environment and isolated animals. There was no significant difference in the percent freezing time between enriched environment animals and isolated animals. Bars represent means ± SE; n=8-10 animals per condition. Significance differences ($p<0.05$) are designated by *.

Enriched Environment and Exercise did not prevent buildup of Aβ Plaques

In order to determine whether enriched environment or exercise would prevent Aβ plaque buildup seen in isolated animals, plaques were counted in the hippocampus and subiculum of a late tissue section from each animal. A one-way ANOVA was performed and indicated there were no significant differences between the three groups ($F(2,22) = 0.472$, *NS*; Figure 9).

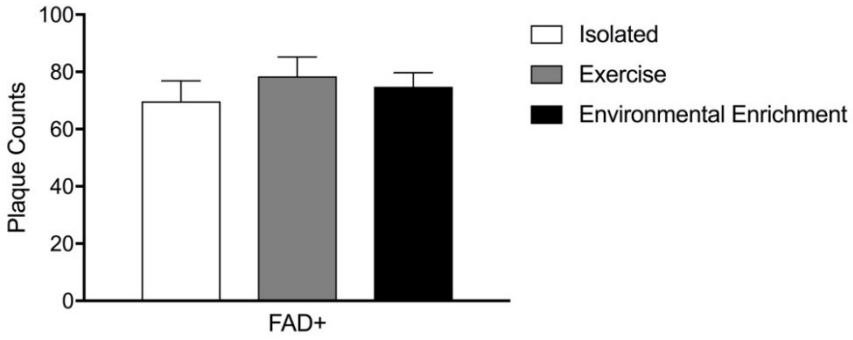

Figure 9. Enriched environment and exercise did not prevent buildup of Aβ Plaques. There was no significant difference between plaque number in enriched environment and

exercise animals relative to isolated animals. Bars represent means ± SE; n=8-10 animals per condition. Significance differences ($p<0.05$) are designated by *.

DISCUSSION

The aim of our experiments was to test the hypotheses that 1) chronic isolation stress in 5xFAD mice would result in an increase in Aβ levels, Aβ plaque deposition, and cognitive deficits and 2) exposure to exercise alone (EX) or to exercise and an enriched environment (EE) would prevent the hypothesized exacerbation of AD pathology and cognitive function due to chronic isolation stress. These hypotheses were partially supported. Results from Experiment 1 demonstrated that extended isolation in 5xFAD mice resulted in an increase in Aβ plaques and cognitive deficits, but it did not affect levels of Aβ. Both $5xFAD^+$ and $5xFAD^-$ isolated mice froze significantly less than group-housed animals, indicating an increase in cognitive deficits. Additionally, while $5xFAD^+$ mice in both housing conditions, group-housed and isolated, did have a significant increase in Aβ levels relative to the $5xFAD^-$ mice, this was due to the established increase in levels of $A\beta_{1-42}$ in the 5xFAD transgenic model of familial AD (Oakley et al., 2006). However, there was no significant difference in Aβ levels between group-housed and isolated conditions, regardless of genotype. Previous studies show mixed results on the effects of stress on levels of Aβ and number of Aβ plaques (Sayer, Robertson, Balfour, Breen, & Stewart, 2008; Stuart, King, Fernandez-Martos, Summers, & Vickers, 2017). However, some evidence indicates the effects of the stress response may have a greater influence on plaque deposition than on Aβ production (Dinkins et al., 2015). This is consistent with our results. If plaque deposition occurs at a higher rate in the presence of potentially increased levels of Aβ, the plaque number may increase with no significant difference in Aβ levels. Further evidence suggests that an increase in metabolic oxidative stress and in production of stress hormones involved in the HPA axis such as

corticotropin releasing factor (CRF) or glucocorticoids, during an extended stress response, may play a role in Aβ plaque formation (Dong & Csernansky, 2009; Lee et al., 2009), which is consistent with our results. Alternatively, evidence suggests chronic stress exacerbates impairment of phagocytic activity in microglial cells in AD, which further prevents microglial cells from removing Aβ plaques and allows for increased accumulation (Ghosh & Geahlen, 2015). This is also consistent with our results.

Experiment 2 expanded on the results of Experiment 1 in an attempt to see if exercise and an enriched environment would prevent the effects of chronic isolation stress on AD pathology and cognitive function in 5xFAD mice. Results from Experiment 2 demonstrated exercise and an enriched environment did not prevent the increase in Aβ plaque number and cognitive deficits in 5xFAD mice induced by chronic isolation stress. This implies the effects of social isolation stress may be so detrimental that certain therapeutic measures cannot prevent them (Cotel et al., 2012). Furthermore, the 5xFAD transgenic mouse model is considered a very progressive model of AD due to accelerated production of Aβ and formation of Aβ plaques, as well as severe cognitive deficits. These mice start producing Aβ around 1.5 months of age and begin displaying Aβ plaques around 2 months of age (Oakley et al., 2006). Previous studies have shown that extended exposure of 5xFAD mice to exercise and an enriched environment does not affect number of Aβ plaques and cognitive deficits in the absence of a chronic stressor (Cotel et al., 2012; Huttenrauch et al., 2017). Thus, use of such an aggressive model of AD may also contribute to the inability of exercise and enriched environment to alleviate the extreme effects of social isolation stress on AD pathology. Future studies may see some prevention of the effects of social isolation through implementing pharmaceutical interventions, such as antioxidants, or other therapeutic practices (Hsiao, Kuo, Chen, & Gean, 2012; Menachem-Zidon et al., 2008).

In addition, the results of Experiment 2 indicated an increase in cognitive deficits in the exercise animals due to the decrease in time spent freezing during CFC. However, previous studies indicate that higher levels of physical activity in mice due to voluntary access to a running wheel leads to hyperactivity (Rhodes, 2001; Rhodes, 2003). Thus, the hyperactivity in the mice may have led to decreased freezing during CFC that may not necessarily corresponded with cognitive deficits.

In conclusion, social isolation stress in 5xFAD mice results in an increase in Aβ plaques and cognitive deficits, and these effects cannot be prevented through exercise and an enriched environment. With regards to humans with AD, this suggests the importance of maintaining social interaction both prior to and throughout progression of AD in order to prevent exacerbation of disease progression through chronic social isolation. Since AD is the sixth leading cause of death in the U.S. and currently affects about 5.7 million Americans, research regarding therapeutic practices to alleviate or prevent disease progression is becoming increasingly important.

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