SEX-MEDIATED DIFFERENCES IN THE EFFECTS OF EARLY-LIFE STRESS ON ALZHEIMER'S PATHOLOGY IN C57BL6/J MICE

by

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ABSTRACT

Alzheimer's Disease (AD) is the most common form of dementia and is currently estimated to affect over 5 million Americans. There is no treatment for AD, and the incidence is expected to increase as our population grows older. Many risk factors for AD have been identified, several of which involve stress. Our study aims to elucidate the connections between early-life stress and AD pathology in adulthood. Using a non-transgenic mouse line, maternal separation was implemented daily from post-natal day 2 or 3 (PND 2 or 3) to the time of weaning (PND 21-28) with the intention of modeling developmental stress. After weaning, the animals were allowed to age under regular conditions until adulthood. Before tissue collection, the animals were subjected to either 3 or 7 days of lipopolysaccharide (LPS) administration, modeling an acute stress event. LPS, a bacterial endotoxin, has previously been shown to exacerbate AD pathology in the form of amyloid beta and cognitive decline. After the period of LPS administration, contextual fear conditioning (CFC) was conducted to measure cognitive ability. Tissue was then collected and amyloid-β (Aβ) levels were measured. Subtle sex differences were seen but overall, results were inconsistent. Maternal separation did enhance cognitive deficits, but only in one group of males. Additionally, maternal separation and LPS interacted to increase Aβ load in females with 3 days of injections. Further studies must be completed to yield consistent results and identify a pathway of action.

1. Introduction

Pathological characteristics of the most common form of dementia, Alzheimer's disease (AD), include extracellular amyloid-beta (Aβ) plaques, intracellular neurofibrillary tangles of hyperphosphorylated tau (ptau), and general brain atrophy, including serious atrophy of the medial temporal and frontal lobes (Sanabria-Castro et al., 2017). Further, AD-related deaths increased by 71% in the United States between 2000 and 2013 (National Center for Health Statistics, 2014), a figure expected to increase as elderly populations grow. AD prevalence is projected to reach as high as 14 million by 2050 (Hebert at al., 2013), a nearly threefold increase from the prevalence reported in 2016 (Alzheimer's Association, 2016). There is no cure for AD, nor is there a precisely defined cause for 99% of AD cases (Bekris et al., 2010) which are sporadic rather than genetic in nature; this of course greatly impedes the ability of physicians to identify and treat patients before they suffer debilitating cognitive impairments. The preclinical phase of AD is marked by normal cognition with increases in Aβ, Ptau, and hippocampal atrophy that may begin more than 20 years before onset of clinical symptoms (Villemagne et al., 2013). It is unclear what triggers the development of preclinical AD, and it is difficult to diagnose without regular neuroradiological examinations and spinal taps (Villemagne et al., 2013). Identifying risk factors for AD is of critical importance to both investigating potential treatments for the disease and to slowing or reversing its prevalence in the aged American population. Indeed, if risk factors are more precisely identified and defined, the prevention of AD may prove attainable. This is in contrast to the current state of affairs, in which treatments merely assuage symptoms and do not address the core pathology of the disease.

Interestingly, AD affects the sexes differentially. Two out of every three individuals with AD are female (Hebert et al., 2013); it is unclear why this is the case. Some studies point to genetic determinants, such as *APOE* ε4, a gene that may be more commonly expressed in women (Altmann et al., 2014). Other research suggests that this is due to longer life expectancy in women, potentially because of decreased death due to heart disease. Notably, in the context of the current report, females also appear to be disproportionately affected by chronic stress and stress-related diseases such as post-traumatic stress disorder, anxiety, and depression (Olino et al., 2010; Pratchett et al., 2010). The Hispanic and African American populations are also reported to be disproportionately affected by AD (Gurland et al., 1999), although there are many other potential causes for this.

Most notably, socioeconomic factors such as poverty and discrimination may contribute to the risk for AD in minority populations(Lines, Sherif, & Wiener, 2014). Indeed, persons living with chronic psychological stressors have been shown to have over a 2-fold risk of developing AD compared to those without (Wilson et al., 2005). Furthermore, growing up in a chronically stressful environment can influence health outcomes in adulthood. For example, victims of abuse during childhood have been shown to have significant memory impairments and other general cognitive deficits (Bremner & Narayan, 1998; Bremner et al., 2000), impairments that are comorbid with decreased hippocampal volume and other neuroanatomical abnormalities (Bremner et al., 1997). Together, these studies suggest that stress, particularly when it occurs during early life, may predispose individuals to cognitive impairment and AD.

Stress occurs when the body senses internal or external deviations from homeostasis and releases hormonal factors in order to return to baseline (Smith & Vale, 2006). These hormonal factors include, among other biological signaling molecules, fast-acting adrenaline and slow-

acting glucocorticoids such as cortisol. Cortisol is released from the adrenal glands in response to adrenocorticotropic hormone (ACTH) being released from the anterior pituitary gland. ACTH is released in response to hypothalamic corticotropin releasing hormone (CRH), which is stimulated by neural signals originating from many brain regions including the hippocampus. Cortisol release is controlled by a negative-feedback loop in which glucocorticoid receptors (GRs) in the hippocampus, hypothalamus, and pituitary gland sense cortisol and bring down CRH and ACTH release in response to serious elevations. This system is referred to as the hypothalamic-pituitary-adrenal (HPA) axis. During acutely stressful events, cortisol release helps to prepare the body by increasing glucose availability, diverting blood flow to the muscles, and reducing unnecessary physiological processes like digestion. Unfortunately, chronic stress and extended cortisol release can result in deleterious effects such as increased fat storage, hyperglycemia, and high blood pressure. (Smith & Vale, 2006) Additionally, chronic stress can also lead to the dysregulation of the HPA axis. For example, prenatal stress has been shown to overstimulate brain GRs and trigger the downregulation of GR protein expression (Cottrell & Seckl, 2009). This, in turn, blunts the system's response to cortisol and the negative feedback loop will not function properly. This results in HPA axis hyperactivity and cortisol overload, which can negatively alter immune function (Cottrell & Seckl, 2009), among other deleterious effects (van Bodegom, 2017).

The time at which acute or chronic stress is experienced can drastically alter the outcome of these physiological events (Roque et al., 2014). Disrupting the quality of care that is provided during early life can induce significant stress in young rodents in a way that changes the trajectory of behavioral, neurological, and other physiological processes (Bath et al., 2016; Bremner et al., 1997), potentially throughout life. Many animal studies exist to support the claim

that stress during early life is associated with cognitive deficits in later life, a factor that may contribute to the development of sporadic AD (Martisova et al., 2013). In rodents, early-life stress (ELS) has been shown to dramatically alter the HPA axis (van Bodegom, Homberg, & Henckens, 2017). Cognition may also be negatively affected by ELS in rodents, also an effect apparently partly mediated by sex (Bath et al., 2017). Results are conflicting, though, on whether or not cognition is more negatively affected by stress in either sex. Male (Naninck et al., 2015) and female (Wang et al., 2016) biases have been identified, while some studies fail to identify any sex differences (Nazeri et al., 2015). Oddly, ELS may actually improve cognitive performances in some cases (Barbie-Shoshani et al., 2016). The existence of these conflicting studies demonstrates the need for more research into how ELS affects cognition. Further, there is a dearth of research into the way that ELS may affect the sporadic development of AD pathology in non-transgenic animals.

The purpose of the current study is to examine sex differences that may occur in inflammation-induced AD-associated pathology. Previous publications from our lab have shown that mice have increased cognitive deficits and Aβ loads in response to repeated exposure to the bacterial endotoxin lipopolysaccharide (LPS) (Kahn et al., 2012). These effects are also seen when mice are exposed to the viral mimetic polyinosinic:polycytidylic acid (poly I:C), which suggests that general inflammation, and not LPS specifically, may be responsible for this effect (White et al., 2016). Unpublished work from our lab has shown that psychosocial stress during adulthood (e.g. isolation stress and repeated social defeat) can exacerbate the effects of inflammation on Aβ and cognition. It is unclear what the mechanism is behind the interplay between stress, inflammation, and Alzheimer's pathology. This study exists to provide further information about what may be happening when these interactions take place. In the current

study, neonatal maternal separation (MS) is used as a model of ELS in C57BL/6 mice. We hypothesize that MS alone will be sufficient to increase cognitive deficits and Aβ load during adulthood. Work in rats has shown that MS may induce a phenotype that is very similar to that of sporadic AD, which includes increased Aβ and cognitive deficits (Martisova et al., 2013). Additionally, we hypothesize that MS will amplify the deleterious effects of repeated inflammatory insults on these pathologies. Finally, we expect these effects to present differently in male and female mice. Few studies have been conducted to show the impact of sex on MS-related stress effects or inflammation-induced AD pathology. However, the increased incidence of AD in human females leads us to hypothesize that MS will affect female mice to a greater degree than male mice.

2. Materials and Methods

2.1. Subjects

Male and female C57BL/6J mice were bred in the Texas Christian University vivarium using breeders purchased from The Jackson Laboratory (Bar Harbor, ME). On post-natal day 2 (PND2), pups were distributed equally among dams and randomly assigned to either maternal separation or control conditions as described in section 2.2. Animals were sexed and weaned between PND21 and PND28. Animals were then housed in groups of three or four in standard polycarbonate mouse cages (30 × 20× 16 cm) (Allentown, NJ) for the remainder of the experiment. Room temperature (22˚C) and a 12-hour light/dark schedule were maintained while food and water remained available *ad libitum*. All protocols for housing, care, and experimental treatments were approved by and executed according to the standards of the Institutional Animals Care and Use Committee (IACUC) at Texas Christian University.

2.2. Experimental conditions

A 2x2 experimental design was implemented with postnatal maternal separation or control housing, and LPS or saline injections during adulthood according to the timeline shown in Fig. 1. This design was applied to groups of male and female mice who were divided into groups that would receive either 3 or 7 consecutive days of injections.

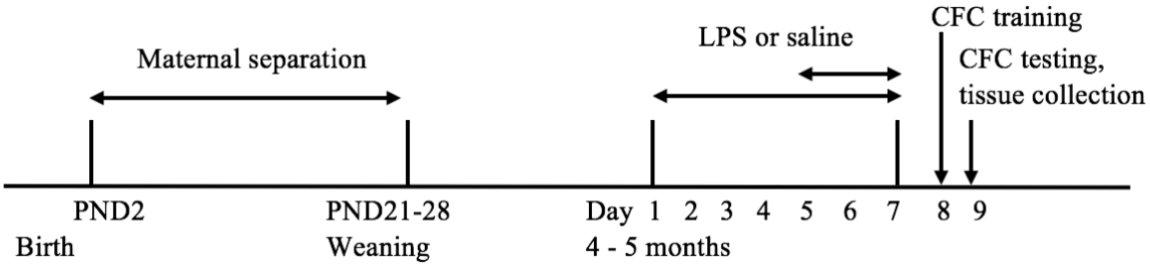

Figure 1. Experimental timeline. Beginning at 2 days after birth, maternal separation was implemented. After weaning, pups were allowed to age in normal housing conditions. At 4-5 months of age, animals were given either 3 or 7 days of i.p. LPS (250 ug/kg) or saline injections. Beginning 24 hours after the final injection, CFC training was performed. Animals were sacrificed and hippocampal tissue was isolated immediately following CFC testing.

2.2.1. Maternal separation

Beginning on PND2, maternal separation was implemented as described previously (Roque et al., 2014; Solas et al., 2010) to induce early-life stress. On the first day of separation, litters were pooled and pups were randomly distributed between the dams in order to create equal litter sizes. This was performed to eliminate baseline differences in maternal care due to differences in litter size. For 3 hours daily, pups were separated from their dam and moved to a holding cage. After this time period, pups were returned to their dams. During separation, temperature was maintained at 32˚C with a water bath and pups were supplied with a small

amount of their native nesting material. When the pups grew fur and thus were able to regulate their own temperature, they were moved to a clean, unused cage during the separation period. The cage was supplied with food and water *ad libitum*, corncob bedding, and a square of cotton nesting material. During separation, the dam was moved from the home cage to a holding cage. Pups and dam were replaced sequentially following the 3-hour interval. Separation was performed daily from 0900 to 1200 hours until pups were weaned (PND21 – PND28).

2.2.2. Injections

After animals reached adulthood (4 – 5 mo), intraperitoneal (i.p.) injections of the bacterial endotoxin lipopolysaccharide (LPS) (*Escherichia coli* serotype: 055:B5; Sigma–Aldrich, St. Louis, MO) or sterile saline (0.9%, Baxter, Deerfield IL) were administered for either 3 or 7 consecutive days. Our lab has previously optimized LPS at 250μg/kg to elicit an inflammatory response and elevate hippocampal Aβ (Kahn et al., 2012). An isovolumetric saline injection was used for the control treatment condition. Injections occurred between 0900 and 1000 hours.

2.3. Contextual fear conditioning (CFC)

A contextual fear-conditioning (CFC) paradigm was used to evaluate fear memory acquisition as a proxy for cognitive function. CFC was separated into two phases, training and testing, and all phases took place between 0800 and 1200 hours. CFC training occurred twenty-four hours after the final injection of either LPS or saline. Subjects were placed in a CFC chamber for a 120s acclimation period, followed by a single 2s 0.5 mA foot shock (aversive stimulus) delivered through an electric grid floor, followed by a final 60s interval and removal from the chamber. Animals were returned to identical chambers 24 hours later for CFC testing, which consisted of a 120s session with no foot shock or post-shock time period. Each chamber contained walls covered with a polka-dot pattern and a peppermint oil olfactory cue to increase

the novelty of the context. Freezing behavior was assessed through automated units (FreezeFrame™, Coulbourn Instruments, Whitehall, PA, USA) to indirectly assess conditioned contextual fear. Each unit held a camera that was connected to a computer operating with FreezeFrame™ Software (Coulbourn Instruments, Whitehall, PA, USA) to allow for recording and analysis of freezing behavior. Behavior was considered "freezing" if an animal's motion was measured below level 10 (the company's default setting) on a motion detection sensitivity scale of 0–1000. Percentage of time the animal engages in freezing behavior was used as a proxy to evaluate cognitive function. More freezing behavior should occur if an association between the novel context and the aversive stimulus was learned.

2.4. Tissue collection

Immediately following CFC testing, animals were sacrificed using CO2 inhalation. Hippocampal tissue was isolated, homogenized, placed in PRO-PREP (Boca Scientific, Boca Raton, FL), and snap frozen on dry ice. Tissue samples were kept at -80°C overnight, and were then centrifuged at 16,000 x *g* for 40 minutes in order to recover the tissue lysate. The lysate was then analyzed for protein quantification using a DC protein assay (Bio-Rad Laboratories, Hercules, CA) as described previously (White et al., 2016) and stored at -20°C.

2.5. Aβ ELISA

Aβ production was quantified using an $A\beta_{x-42}$ ELISA (BetaMark™, Biolegend,Catalog No: 842101), which was performed in accordance with manufacturer instructions and as detailed in our previous publications (Kahn et al., 2012; White et al., 2016). Briefly, samples were diluted with working incubation buffer (which includes the HRP-labeled detection antibody) in a 2:1 ratio, loaded into duplicate wells, and were incubated over night at 2–8 °C. The following day, all wells were washed and chemiluminescent substrate was added to each well. The plate was

then shaken briefly before luminosity was quantified (BMG LabTech FLUOstar™ Omega, Cary, NC, USA).

2.6. Statistical analysis

Behavioral and Aβ data were analyzed for significant main effects and interactions using a standard factorial 2 x 2 ANOVA, with condition (maternal separation and control group housing) and treatment (Saline and LPS) as between-subjects variables. Data from behavioral paradigms and ELISA was evaluated using SPSS™ software (IBM, Armonk, NY, USA) and were expressed in figures as the mean ± standard error of the mean (SEM). The alpha level used for all analyses was 0.05. Significant omnibus effects between treatment groups were subjected to Fisher's PLSD post hoc tests to detect differences between individual groups.

3. Results

3.1 Males

Contrary to our original hypotheses, there was no significant main effect of MS or interaction effect with injection treatment on Aβ production (Fig. 2a, c). However, as predicted, treatment with LPS significantly increased Aβ in both housing conditions and injection schedules, as expected, $F(1,29) = 28.26$, $p < 0.001$ (Fig. 2a, c). That noted, no effect of LPS on percent time freezing was seen in either group (Fig. 2b, d). Notably, there was a significant effect of MS alone on percent freezing time in animals with 7 days of injections, $F(1,29) = 5.54$, $p < 0.05$) (Fig. 2d).

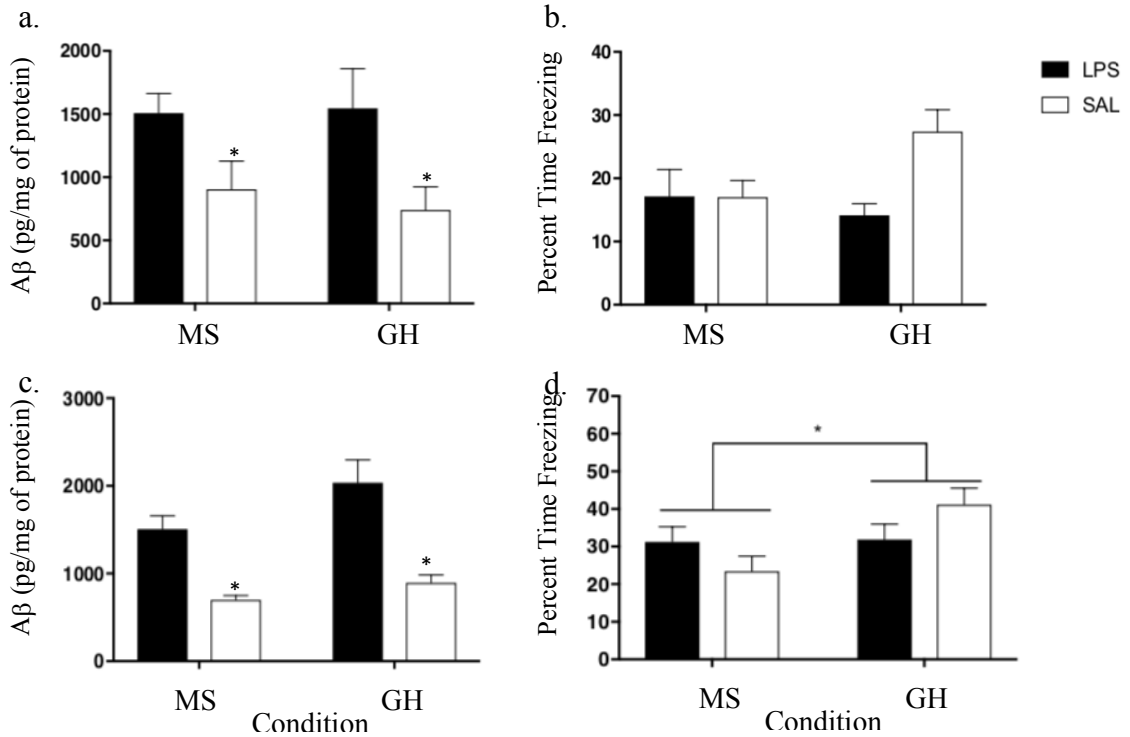

Figure 2. Amyloid-β and freezing behavior in male mice treated with 3 (a, b) or 7 (c, d) days of LPS or saline. There was no significant difference in Aβ between housing conditions; animals receiving LPS had more Aβ than those receiving saline (a, c). There was no significant difference in freezing behavior between housing conditions or treatment groups when animals received 3 days of either LPS or saline (b). Group housed animals with 7 days of either LPS or saline treatment exhibited significantly more freezing behavior than those in the maternal separation condition (d). There was not a significant effect of 7 days of LPS on freezing behavior (d). MS = maternal separation; GH = group housed; * represents $p \leq 0.05$.

3.2 Females

MS alone had no effect on Aβ load or freezing behavior in females. There was a marginally significant interaction between 3 days of LPS and MS on Aβ load, $F(1,22) = 4.197$, $p = 0.053$ (Fig. 3x). There was an effect of 7 days of LPS on Aβ load, $F(1,21) = 9.962$, $p < 0.01$ (Fig. 3b). There was no effect of housing condition on freezing behavior in the 7-day injection schedule group (Fig. 3b). Behavioral data was not collected from female animals with 3 days of injections because this was not in the original plan for this study. Behavior is typically not examined in

female animals due to the effect that the estrus cycle and its associated hormones may have on the results (Ramos-Ortolaza, 2017). This data will be collected in a future study in which these effects will be controlled for.

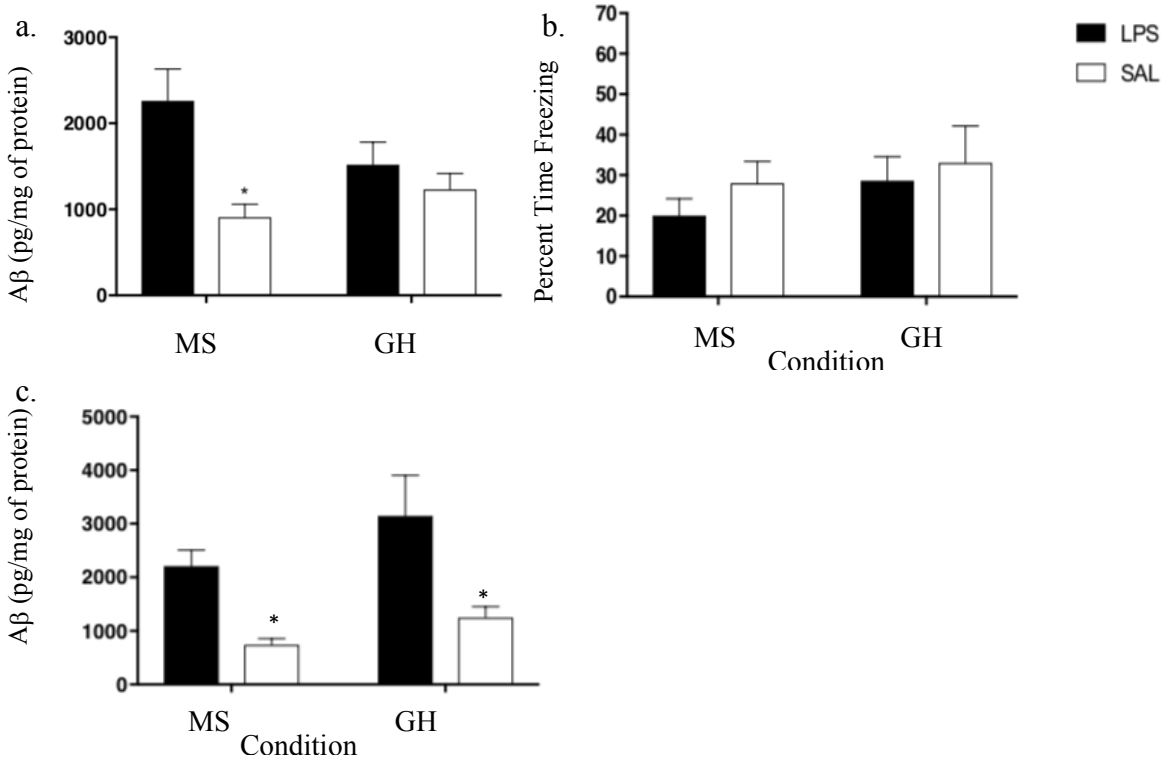

Figure 3. Amyloid-β and freezing behavior in female mice treated with 3 (a) or 7 (b, c) days of LPS or saline. There was an interaction between MS and 3 days of LPS on Aβ load (a). No significant differences in freezing behavior were seen in animals with 7 days of injections (b). There was an effect of treatment, but not condition, on animals with 7 days of injections (c) MS = maternal separation; GH = group housed; * represents $p \leq 0.05$.

4. Discussion

Although subtle differences were noted, the hypotheses were not fully supported, as no consistent sex differences were observed. An interesting effect was seen in males exposed to 7 injections of LPS. There was no enhancement of cognitive dysfunction in response to LPS. However, there was a significant difference between the housing conditions, which means that

maternal separation alone was responsible for increasing cognitive deficits (Fig. 2d). This group did not exhibit changes in Aβ in response to housing condition (Fig. 2c). In females with only 3 days of injections, the animals exposed to early-life stress exhibited higher Aβ load than the other groups (Fig. 2a). It is unknown whether they had comorbid cognitive pathology. Overall, MS was not sufficient to increase Aβ load or cognitive impairment. Inflammation and MS did not consistently interact, either. Further, LPS did not consistently produce the expected effect on Aβ load and cognition.

In the current study, Aβ load did not predict cognitive impairment. In humans, Aβ may not be sufficient to produce clinical cognitive impairment (Jack et al., 2009). For example, some individuals with high brain Aβ exhibit no other dementia-associated signs or symptoms (Hedden et al., 2012). Additionally, recent AD clinical trials involving immune-mediated clearance of Aβ showed no difference in cognitive functioning after treatment (van Dyck, 2018). Evidence exists for the toxicity of Aβ at the synapse (Niu et al., 2018; Jonson et al., 2018), but this is clearly not the only factor that leads to the general neurodegeneration seen in AD. The degree of synaptic loss typically parallels cognitive decline (Poirel et al., 2018), and so the next phase of this study should examine how synaptic markers (e.g. PS95) are altered in response to ELS. The mechanisms behind synaptic loss in AD are debated. One line of research suggests that microglia are aberrantly active during the early stages of AD, potentially due to up-regulation of the classical complement cascade (Hong et al., 2016). Other studies show that lack of brain derived neurotrophic factor (BDNF) stimulation may lead to synaptic loss and cognitive deficits (Stimpson et al., 2018). Some have shown that BDNF expression may be downregulated by psychological stress (O'Keefe et al., 2014). It is possible that these pathways are affected by MS,

and if these effects are different in male and female animals then cognition would be differentially affected as well.

Sex hormones within the brain may have affected the outcomes of the female mice in this study. It has been shown that in humans, females who have had their ovaries removed may be protected against AD (Rocca et al., 2007; Bove et al., 2014). *APOE* ε4, which is one of the most reliable genetic determinants of sporadic AD, may interact with estrogens to increase AD risk (Yaffe et al., 2012; Kang & Grostein, 2012). Sex hormones are responsible for the development of sexual dimorphisms in the fetal and pubescent brain (Arnold & Breedlove, 1985), and sex hormones modulate behavior during post-pubescent life (Berenbaum & Beltz, 2011) which confers a high density of sex hormone receptors throughout the brain. Estrogens also play a role in maintaining synaptic plasticity in the adult hippocampus and amygdala (Hyer et al., 2017; Frankiensztajn et al., 2018). It is possible that sex differences in ELS studies are mediated by hormonal factors, which may partially explain the results of the current study. Future studies should investigate aromatase activity, estrogen receptor density, and estrogen concentration within the hippocampus.

To this end, maternal separation may not be sufficient to reliably produce levels of stress in the offspring that are of sufficient severity to trigger increases in AD-like pathology. The effects of ELS are only seen when maternal behavior is significantly altered. MS may inhibit maternal care during separation, but increases in maternal behaviors such as arch-backed nursing and licking have been shown after reuniting the dam with her pups (van Bodegom, 2017). This compensatory mechanism may ameliorate any MS-mediated effects. In the current study, maternal behavior was not quantitatively monitored. Thus, future studies must include this monitoring in order to ensure that MS is sufficiently altering maternal behavior. Additional

studies will include the ELS paradigm of nesting material restriction, which has been demonstrated to be a more reliable form of stress for both the dam and her pups (Fuentes, et al., 2108). Neonatal or prenatal bedding restriction may induce more obvious changes in offspring phenotype so that AD-like pathology and sex differences can be studied more thoroughly and reliably.

The current research in our lab focuses on neuropathological and behavioral changes in pups, but not in dams. A search of the current literature has revealed no studies on MS dams. During the current study, it was noted that the second litter from MS dams typically did not survive past PND2. One would expect the stress of MS to be much greater in dams, especially after undergoing multiple rounds of MS with multiple litters. This may potentially induce epigenetic changes in the dams that could be passed on to her offspring. It may be worthwhile to investigate how the pups of an MS-experienced dam are affected compared to those of an MS-naïve dam. Additionally, if epigenetic changes are induced by MS, it would be interesting to both examine multiple generations of MS pups as well as investigate the specific epigenetic changes that are taking place. This may potentially serve as a model for human poverty, which typically manifests across multiple generations and is suspected to do so in large part due to epigenetic modifications (Dolinoy, Weidman, & Jirtle, 2007; Rothstein, Harrell, & Marchant, 2017). Learning how to prevent AD beginning in the earliest stages of life will greatly decrease the fiscal, physical, and emotional loads of the aging population in the United States and around the world.

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