A POTENTIAL ROLE FOR VIRUS-INDUCED PERIPHERAL INFLAMMATION IN THE ONSET AND PROGRESSION OF VARIOUS ALZHEIMER'S DISEASE PATHOLOGIES

by

Whitney Summers

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Project Approved:

Michal Chumley, Ph. D.
Department of Biology
(Supervising Professor)

Gary Boehm, Ph. D.
Department of Psychology

Giri Akkaraju. Ph. D.
Department of Biology

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INTRODUCTION

At the start of twentieth century, Alois Alzheimer published literature on a 51-year-old female patient presenting with erratic behavior, disorientation, and loss of memory. During exams, objects would be shown to the patient and she would correctly name the items, but upon completion of the exam, she would forget having been examined. While reading aloud the patient would haphazardly skip lines, spell out individual words, or use enunciations that were completely unrecognizable. After four and a half years with progressively worsening symptoms, the patient died allowing a more in-depth study of her illness. Upon autopsy, Alzheimer described an evenly atrophic brain with presence of tangled fibers and abnormal protein clumps. Those irregular findings are now known as neurofibrillary tangles and senile plaques, two of the trademark biological markers of Alzheimer's disease (Alzheimer et al., 1995).

Alzheimer's disease (AD) is a growing presence in our country resulting in significant medical costs. Currently 5.4 million people are afflicted and it is estimated that one in every eight people aged 65 or older have AD (Alzheimer's Disease facts & figures). This catastrophic disease is causing great fiscal problems for our society: it is expected that the direct cost for those with AD will total $200 billion in 2012, with $140 billion coming from Medicare and Medicaid (Alzheimer's Disease facts & figures). AD is also indirectly causing increased costs across the entire health care system, making the treatment of other diseases more expensive. The fiscal effects of AD show no indication of slowing as the disease is expected to afflict upwards of 16 million people in 2050.

The root cause of AD is unknown, but the resulting effect on those inflicted is shattering. Every 69 seconds another American develops AD and many of these people

will be left to fend for themselves while living alone (Alzheimer's Disease facts & figures). Roughly 800,000 individuals with AD live alone and are subsequently exposed to risks that could easily be eliminated with the presence of a caregiver. Correspondingly, AD is extremely strenuous for the family and friends who serve as caregivers. Last year 17.4 billion hours of care went unpaid as individuals tended to their loved one afflicted with AD (Alzheimer's Disease facts & figures). These efforts often result in heartbreak as AD continues to be the sixth leading cause of death in the United States. The number of deaths from AD continues to climb while the rate of deaths from other major diseases, such as heart disease, declines with the creation of new treatments and cures.

AD is a disorder resulting in memory loss and changes in personal behaviors caused by damage and destruction of neurons in the brain. Clinically, AD is marked as a gradual decline in cognitive function associated with neuron and synapse loss and the formation of neurofibriallary tangles (NFTs) and plaques (Adams et al., 2002; Akiyama et al., 2000). NFTs are made from paired helical filaments and hyperphophorylated tau, a structural protein, important in the formation and stabilization of the microtubules in neural cells (Markesbery, 2010). Microtubules aid in proper cell development and the movement of molecules within the cell, but without the appropriate regulation of tau, the subsequent destabilization of microtubules is thought to form NFTs (Cras et al., 1995). The physical accumulation of disrupted microtubules inhibits the general functionality of neurons by interfering with crucial protein and nutrient transport. Without a functional microtubule scaffolding, cells are unable to transport proteins from the cell body out to their axon and dendrites. This prevents further communication with downstream neurons effectively halting the communication network associated with the damaged neurons.

The hallmark plaques of this diagnosis are extracellular deposits of amyloid-beta (Aβ) peptide (Murphy and LeVine, 2010). In the creation of Aβ plaques, the cell membrane-bound amyloid precursor protein is broken down and then cleaved by various enzymes, including γ-secretase. These enzymatic reactions result in release of Aβ of various lengths, some with a hydrophobic nature (Heneka and O'Banion, 2007). The early onset form of AD has recently been linked to possible genetic mutations in the amyloid precursor protein and γ-secretase genes. These mutations are thought to result in increased production of the long forms of Aβ (Seiffert et al., 2000). In contrast to the short forms of Aβ, the long forms are less soluble. This decreased solubility is especially true of the hydrophobic $A\beta_{1-42}$ forms, which are thought to have the most significant effect on the formation of the AD plaques (Heneka and O'Banion, 2007). The pathogenic forms of peptide accumulate together to create plaques at a much higher rate than the short forms and are more difficult to clear from the brain (Murphy and LeVine, 2010; Selkoe, 2001). Plaques have been shown to disrupt signal transduction of neurons leading to cognitive deficit and eventually cell death (Murphy and LeVine, 2010). Cell-cell communication between neurons is inhibited by plaques through interference at the neural synapse that results in communication network dysfunction responsible for cognitive decline (Harris et al., 2010). Similarly, plaques have a deleterious effect on neural cell survival. The accumulation of these plaques is thought to result in a deficit of neurotransmitters at the synaptic cleft and eventually death in neurons (Hooper et al., 2008). Neural disruption and cell death caused by NFTs and Aβ plaques accounts for the general brain atrophy seen in AD cases.

AD is principally classified into two forms: early onset AD and sporadic AD. As previously explained, the early onset form of AD has been linked to specific genetic features that can be transmitted to offspring. This familial form of AD represents less than 10% of all human cases of AD (Jacobsen et al., 2006). More common is the sporadic form of AD, defined as onset after the age of 65 years, which most scientists agree is not initiated by features of the amyloid precursor gene and γ-secretase gene. Unfortunately, not much is know about the onset of the sporadic form of AD.

Emerging research seeks to determine the potential contribution of peripheral inflammation on the progression of sporadic AD pathologies. Although the involvement of inflammation in neurodegenerative diseases still remains unclear, many studies have shown a positive correlation between the former and latter (Minghetti, 2005). Evidence from human population studies indicate that higher levels of C-reactive protein, synthesized by the liver in response to episodes of inflammation, correlate with increased risk of AD onset (Engelhart et al., 2004; Schmidt et al., 2002). Epidemiological studies have indicated non-steroidal anti-inflammatory drugs may reduce the risk of developing neurodegenerative disorders, like AD, later in life (McGeer et al., 1996). In contrast, human studies using the anti-inflammatory drugs Rofexcoxib and Naproxen on patients with mild to moderate forms of AD have shown no change in cognitive decline. None of the experimental groups showed significant benefit, measured in cognitive testing scores, from the various pharmaceutical interventions. Subjects in the Rofecoxib group actually showed trends indicating greater cognitive decline and increases in adverse effects including dizziness, fatigue, and hypertension not seen the placebo group (Aisen et al., 2003). One possible explanation for the adverse results of anti-inflammatory drugs on

cognitive decline in AD patients is that the drug was given too late in the neurodegenerative progression.

Prior human studies have shown a link between bacterial inflammation and increased Aβ in the brain. Nearly twenty-five years of evidence in human studies shows that inflammation and the associated processes lead to an increased production and deposition of Aβ. The Aβ can then aggregate together to create the plaques that typically appear in AD (Akiyama et al., 2000). A possible explanation for this neural progression involves the blood brain barrier (BBB). Evidence found through investigating peripheral inflammation has shown a resulting change of the dynamics in transport of the BBB that could contribute to the formation of Aβ plaques in the brain. The antigen inducing inflammation does not directly act on the BBB, but the subsequent changes in gene expression modify the Aβ transport across the BBB resulting in greater accumulation and retention of Aβ in the brain (Jaeger et al., 2009).

Much of our current understanding of AD pathology comes from experimentation using animal models. Animals can be used to study both biological and behavioral pathologies for various forms of dementia. The triple transgenic (3xTg) mouse is often considered the best model for studying AD. 3xTg means that the animal carries three mutant human genes, amyloid precursor protein, presenilin-1, and tau, allowing the animal to express both amyloid and tau pathologies similar to AD. 3xTg mice have been shown to quickly develop Aβ plagues and NFTs in the neocortex and hippocampus (Blurton-Jones et al., 2009; Bryan et al., 2009). One study using 3xTg mice found that the formation of plaques directly correlated with a decrease in neuron formation in the hippocampus. This study was evidence that the buildup of Aβ was not only damaging the

mature neurons present, but was also preventing proper neurogenesis that could further contribute to cognitive decline (Rodriguez et al., 2008). 3xTg mice are reported to first show signs of cognitive decline at 4 months of age, before plaques and tangles become clearly apparent. Immunotherapy treatments with anti-Aβ antibody were used to clear excess Aβ in the brain of affected mice. The clearance resulted in a rescuing of early memory deficits in young mice. This study strongly suggests intraneuronal Aβ as an early biological marker for cognitive dysfunction (Billings et al., 2005).

Although transgenic mouse models have been widely used in the study of AD by mimicking pathologies seen in the human population, there are noted shortcomings that make studies using wild type mice increasingly important. Not only are transgenic mice often hard to breed and house, but the genetic manipulation early in their development may provide additional variables in behavioral testing that is not fully understood and accounted for (Bryan et al., 2009). One recent study using wild type mice sought to determine if inflammation could lead to early AD markers. Non-transgenic, knock-out mice were injected with lipopolysaccharide (LPS), a common component of gram-negative bacterial cell walls, which mimics bacterial infections and induces inflammation. The authors found that repeated injections of LPS increased Aβ build-up in the brain and resulted in a deficit in hippocampus-dependent learning (Lee et al., 2008). This was one of the earliest studies attempting to connect peripheral inflammation with AD pathology and cognitive decline.

In light of this previous work, our lab has provided further evidence that repeated exposure to the bacterial endotoxin LPS results in cognitive deficit and prolonged elevation in hippocampal $A\beta_{1-42}$ in the mouse. LPS was injected in non-transgenic mice

for seven consecutive days to induce peripheral inflammation. Saline injections were used as a control to ensure that results were not secondary to the potential stress of daily injections. The previous study (Lee et al., 2008) did not account for potential sickness behaviors when assessing cognitive decline through behavioral paradigms. Thus, our lab used open field behavior protocols to determine the correct time to perform behavioral tests to assess cognitive decline. Results from the behavioral paradigms, morris water maze and contextual fear conditioning, showed cognitive decline in the mice receiving the LPS injections. Biological samples were obtained in order to measure $A\beta_{1-42}$ levels in the brain and the levels of peripheral and central pro-inflammatory cytokines. It was found that animals receiving the LPS injections had significantly greater amounts of $A\beta_{1-42}$ in their hippocampus. LPS caused significant elevation of pro-inflammatory cytokines, both centrally and peripherally, at the onset of injection with LPS, but by the end of the seventh day the mice displayed endotoxin tolerance and no longer had elevated levels of pro-inflammatory cytokines. In addition, the mice showed no sickness behaviors on the seventh day of injections (Kahn et al., 2012).

We are now seeking to determine if prolonged viral inflammation produces similar results as bacterial inflammation in the scope of AD pathology progression. Although an infection by both bacteria and viruses results in inflammation, the signal transduction pathway leading to the onset of inflammation is slightly different. Viral inflammation often proceeds through Toll-Like Receptor 3 (TLR3) in contrast to bacterial infections that activate the TLR4 pathways. Both pathways lead to a slightly different cytokine, or messenger molecule, production that would support the idea of varied downstream effects. To induce virus-like inflammation we used Polyinosinic:polycytidylic acid (Poly I:C)

injected daily for seven consecutive days. Poly I:C is a synthetic double stranded RNA-like molecule that mimics the structure of viral double stranded RNA and subsequently activates immune cells to induce inflammation without using a live pathogen. Previous studies using rodents have shown Poly I:C stimulates the immune system through TLR3 and subsequent NF-κB activation resulting in inflammation (Alexopoulou et al., 2001). One such study used Poly I:C to induce inflammation late in gestation to assess the lasting effects on the compromised fetus. The authors found that immune system challenge to the female during late gestation resulted in increased tendency toward AD-like neurodegeneration and cognitive decline in the offspring when they reached adulthood (Krstic et al., 2012). In order to determine cognitive decline in rodents many studies have used contextual fear conditioning behavioral paradigms.

Numerous behavioral paradigms have been developed to assess changes in memory and cognition in rodents. Many of these experimental models often rely on spatial recognition learning and memory to assess changes in hippocampus-dependent functions. The hippocampus has been found to play a major role in the conversion of short-term memory to long-term memory and spatial recognition. One of these behavioral paradigms is contextual fear conditioning (CFC) and it has been shown previously to detect changes in learning and memory in mice caused by genetic mutation or pharmaceutical intervention. CFC employs a previously discovered innate behavior of mice to freeze when faced with an inescapable stressor or fear (Pugh et al., 1998). This paradigm works by introducing the animals to a neutral context, often termed the conditioning apparatus, and then pairing this context with a negative stimulus. One such negative stimulus commonly used is a short mild foot shock (Anagnostaras et al., 2000).

After multiple pairings of the neutral context with the negative stimuli, the rodent will begin to view the previously neutral context as aversive and subsequently freeze (Anagnostaras et al., 1995). Mice with damage or decreased functioning of the hippocampus have been found to show decreased freezing behaviors (Rudy and Pugh, 1998). CFC has been used in many AD models, including our LPS study (Kahn et al., 2012), to show AD-like cognitive impairment. Animals that have been found to display AD-like physiological markers, either through genetic alterations or pharmaceutical intervention, also show impairment in CFC testing (Corcoran et al., 2002; Gerlai et al., 2002; Jacobsen et al., 2006; Steele et al., 2012).

Induction of inflammation in mice through Poly I:C had been previously shown to induce sickness behaviors, including decreased locomotion and decreased tendency toward burrowing, which could lead to skewed results in CFC (Kranjac et al., 2012b). To ensure that sickness behaviors are not falsely interpreted as better memory in CFC, burrowing studies will be used to determine proper testing time following Poly I:C injections. The burrowing protocol is an extremely sensitive hippocampus-dependent task that was developed to detect cytokine-induced disruption (McLinden et al., 2012). Our labs previous work with LPS did not require the employment of burrowing studies to determine proper time to test behavior because the rodents developed an endotoxin tolerance to LPS. Specifically, the animals did not display sickness behaviors after seven consecutive days of a single injection of LPS. The mechanism for this tolerance is not completely known, but it is hypothesized that this physical adaption works to prevent excessive inflammation that could lead to endotoxin shock (Kahn et al., 2012). By using

burrowing we will be able to rule out sickness behaviors caused by Poly I:C injections as a confounding factor in memory testing.

We hypothesize that repeated injections of Poly I:C will result in increased $A\beta_{1-42}$ production, cognitive deficits, and AD-like pathology in non-transgenic mice similar to results of consecutive LPS injections (Kahn et al., 2012; Lee et al., 2008). CFC will be used to determine changes in hippocampus-dependent learning between the mice receiving Poly I:C and those receiving saline. We will distinguish cognitive decline from the effects of sickness behaviors through the employment of burrowing studies. Burrowing studies will allow us to determine the earliest time that CFC can be performed after injections such that sickness behaviors will not significantly affect the results. Biological assays, including ELISA and proteins assays, will be used to determine increases in $A\beta_{1-42}$ and pro-inflammatory cytokines. Increased levels of pro-inflammatory cytokines in the periphery would indicate that inflammation occurred, which could be a factor in the potential progression of an AD-like pathology.

MATERIAL AND METHODS

Subjects and housing

All experiments utilized male C57BL/6J mice from the Texas Christian University vivarium. Animals were treated and housed following the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and in agreement with standards approved by the Institutional Animal Care and Use Committee (IACUC) of Texas Christian University.

Testing subjects were housed in standard cages (12.5cm x 15cm x 25cm) and in groups of three and four animals. Both control groups and experimental groups were

housed in the same conditions, including light schedules and food availability. Lights were on at 0600 and off at 1800 daily. Food was readily available for the mice to consume as they desire.

Biological assays

Poly I:C injections and tissue preparation

Poly I:C (Midland Certified Reagent Co., Midland, TX) injections were administered for seven consecutive days prior to behavioral testing and biological assays in order to assess the potential of Poly I:C to increase $A\beta_{1-42}$ and alter behavior. A control group of mice was administered saline following the same regimen as Poly I:C to ensure that behavior alterations do not come from the stress of repeated injections. Injections were administered intraperitoneally at a weight dependent dose of 12 mg/kg for Poly I:C groups. In a pilot study to compare Poly I:C with LPS, we used our standard 250 mg/kg dose of LPS (Kahn et al., 2012).

After the completion of injections and at the designated times, the mice were euthanized following IACUC-approved protocols. The hippocampal tissue samples were obtained for study by protein assay and ELISA procedures. The extracted tissues were homogenized with protein extraction solution (PRO-PREP, Boca Scientific, Boca Raton, FL.) including protease inhibitors. The tissue samples were allowed to further lyse on ice for 30 minutes. The lysates were centrifuged at 15,000 rpm for 30 minutes. The clear lysate was then removed for DC Protein Assay (Bio-Rad Laboratories, Hercules, CA.).

DC protein assay

The DC protein assay used both a working reagent and detergent-based buffers. Dilutions ranging from 0.2 mg/ml – 1.5 mg/ml were used to prepare the protein standard

curve. Dilutions were made in the same buffer as the lysates. 5µl of the dilution standards and the samples were pipetted into a 96 well plate along with 25µl of regent A' and 200µl of reagent B. Following a 15 minute reaction time, the plate was placed into the plate reader (BMG LabTech FLUOstar Omega, Cary, NC). The plate reader then determined the optical density of the wells at 750nm. These results were used to normalize the protein content.

$A\beta_{1-42}$ ELISA procedure

The BetaMark $A\beta_{1-42}$ ELISA (Covance Research Products, Dedham, Massachusetts) utilizes a 96-well plate and follows a 48-hour procedure. The wells were coated with antibody and filled by either sample or standards of known concentration. Prior to the start of the assay, the working incubation buffer and standard intermediates were made. The Standard Diluent was utilized to create the standard curve and the Aβ standard. The samples were then diluted with the working incubation buffer, including the HRP-labeled detection antibody, at a 2:1 ratio. 100µl, in duplicate, of each dilution of the standard curve and 100µl, in triplicate, of each unknown was added to the plate. The plates were then allowed to incubate at 2-8 ° Celsius over night. The next day the wells were washed five times each with the 1X wash buffer. Following the washes, 200µl of the substrate for HRP enzyme, TMB, was added to the wells. The plates were allowed to incubate for 45 minutes in the dark at room temperature and then the plate was read at an optical density of 620nm.

Statistics

For this group of experiments, we used analysis of variance (ANOVA) statistics to discover if there were significant differences between experimental groups. If a

significant omnibus F was found, Fisher's PLSD post-hoc tests were used to determine which experimental groups were significantly different.

Behavioral Paradigms

Contextual fear conditioning

Contextual fear conditioning relies on mouse freezing behaviors that were measured using a Freeze Monitor System and software (7 in x 7 in x 12 in). The unit (Coulbourn Instruments, Whitehall, PA) floor is an electric grid that administers an aversive stimulus to the mouse. The units ceiling has a small light bulb and camera that is used to monitor the animal and detect movements. The CFC protocol used designates the first day as training and the second day as testing. Forty-eight hours after the final injection of Poly I:C or saline, the mice were placed in the CFC unit for training. Two time specific training protocols were used in these experiments.

(1) Two-shock experiment: The training session began with a 120-second acclimation phase, immediately followed by a 2-second 0.7mA mild foot shock. Mice remained in the unit for a 60-second phase before another 2-second 0.7mA shock was administered. After another 60-second phase the mice were removed from the unit with training completed. The total training time for the two-shock protocol is 244 seconds.

(2) Single-shock experiment: The training session started with a 120-second acclimation phase, immediately followed by a 2-second 0.7mA mild foot shock, and a 60-second phase to remain in the unit. In total, training for the single-shock protocol is 182 seconds.

The day following training, mice were once again placed in the CFC unit for testing. During testing no shocks were delivered and the movements of the animals were

continuously recorded for 90 seconds. Freezing behaviors observed were recorded as total freezing time (seconds) and are indications of hippocampus-dependent contextual learning. Previous work done in our lab has shown that the incorporation of an olfactory contextual cue and wall design (black polka dots) increases freezing times. Increased freezing times indicate an increase in learning of the context-shock pairing. Olfactory contextual learning is thought to be a hippocampus-dependent task. Thus, in this study peppermint oil (Adams Extract, Gonzales, TX) mixed with water at ratio of 1:10 was used as an olfactory cue. The oil-water mixture was placed in a dish below the grid floor.

Burrowing

Burrowing is a hippocampus-dependent and species-specific task that is extremely sensitive in assessing sickness behaviors. During testing time, mice were single-housed in a cage containing a burrowing tube (a plastic gray cylindrical tube that is closed at one end, measuring 20.3cm in length, 5.7cm in diameter, and raised by screws to a height of 2.5cm). The mice were injected with a single Poly I:C or saline injection for seven consecutive days. Immediately following the last injection, the tubes were filled with 200 grams of mouse chow. After 4 hours had elapsed, the amount of mouse chow remaining in the tube that had not been removed by the burrowing of the mouse was measured using a digital scale and recorded. This protocol was repeated and burrowing data was collected at the 24-, 48-, and 72-hour mark after the final injection.

RESULTS

Appearance of animals and weight loss from Poly I:C

Animals were weighed and visually inspected daily during the seven days of injections, for both Poly I:C- and saline-injected groups (data not shown). Animals that

received seven injections of Poly I:C displayed classical sickness-related symptoms such as weight loss, lethargy, and decreased grooming, that continued through day seven. On day one, both groups showed no significant difference in starting weight ($t_{(75)}$ =0.977, ns). On day seven, a weight loss measurement was taken by subtracting the starting weight on day one from the ending weight on day seven. This difference score yielded a significant difference in weight loss ($t_{(75)}$ =5.854, p<0.0001). These results reveal a significant decrease in weight for animals that had received seven consecutive injection of Poly I:C.

Experiment 1: Poly I:C-induced Aβ$_{1-42}$ production

Two experimental groups, Poly I:C- or saline-injected mice, were established to determine if repeated injections with Poly I:C would lead to increased Aβ $_{1-42}$ in the hippocampus of the C57BL/6 mouse. Animals received one injection for seven consecutive days. Twenty-four hours after the final injection, animals were euthanized following IACUC approved protocols, hippocampal tissue was immediately obtained and Aβ $_{1-42}$ ELISA procedure was conducted. As seen in Figure 1, the results from the biological assay shows a significant increase in the amount of Aβ $_{1-42}$ in the hippocampus of a mouse that received seven consecutive days of Poly I:C injections as compared to the saline-injected group ($F_{(1,18)}$ =5.17, p<0.05).

Experiment 2: Burrowing behavior

To determine if 7 consecutive days of Poly I:C injections would result in sickness behaviors that could distort our assessment of cognitive differences, we utilized the burrowing paradigm. The burrowing paradigm relies on the innate behavior of mice to burrow or tunnel. When an animal is experiencing sickness behaviors they exhibit decreased locomotion and burrowing. Animals received either Poly I:C or saline for

seven consecutive days. Immediately following the seventh injection animals were placed in single-housed testing cages. Burrowing data was obtained at the 4-, 24-, 48-, and 72-hour mark following the final injection (Figure 2). 4 hours after the last injection, burrowing data indicated that Poly I:C was significantly suppressing burrowing behaviors ($t_{(13)}$ =4.33, p<0.001). The 24-hour burrowing data still showed a slight difference between the Poly I:C and saline groups, with Poly I:C-injected mice burrowing less than saline-injected mice ($t_{(13)}$ =2.08, p=0.058, ns). The 48-hour burrowing data showed no remarkable difference between the two groups ($t_{(13)}$ =0.644, p=0.530, ns). Similarly, the 72-hour burrowing data showed no difference between the groups ($t_{(13)}$ =0.77, p=0.445, ns). Although the difference was not statistically significant 24 hours after the final injection, we decided in light of the slight difference it would be most appropriate to allow a larger time gap before testing cognitive differences. Results indicated that sickness behaviors are resolved fully by 48 hours after the final injection with Poly I:C. Thus, through the burrowing paradigm we were able to the determine that the appropriate time to test cognitive difference between the two groups, saline and Poly I:C, was 48 hours after the final injection.

Experiment 3: Contextual fear conditioning

We utilized contextual fear conditioning to determine any potential differences in learning between Poly I:C and saline-injected animals. Animals received seven consecutive daily injections that were completed 48 hours prior to testing. With previously obtained burrowing data we determined that sickness behaviors that could potentially confound our CFC results would be resolved by 48 hours after the last Poly I:C injection. Initial testing using the two-shock CFC protocol showed no significant

difference in freezing time between the groups during training ($F_{(1,14)}$ =1.170, ns) and testing ($F_{(1,14)}$ =0.023, ns). We were concerned that the two-shock protocol was too easy for the mouse to learn, thus not sensitive enough to detect small cognitive differences. To combat this concern we then tested a different set of Poly I:C- and saline-injected animal groups using the single-shock CFC protocol. We hypothesized that the single-shock CFC protocol should be more difficult to learn and more sensitive to potential differences between the two groups. Single-shock CFC results again showed no significant difference between the groups in freezing time during training ($F_{(1,15)}$ =2.268, ns) and testing ($F_{(1,15)}$ =0.013, ns). As seen in Figure 3, these results indicate that following seven consecutive injections with Poly I:C there is no significance cognitive deficits in learning.

Experiment 4: Could clearance of Aβ $_{1-42}$ explain lack of cognitive deficits?

One possible hypothesis regarding the lack of cognitive differences observed in Poly I:C- versus saline-injected mice is that Poly I:C treated animals had a longer recovery after the seventh injection before testing began, which could allow for Aβ clearance from the hippocampus prior to testing. An ELISA study was completed on hippocampal tissue obtained immediately after CFC testing in order to assess the potential hippocampal clearance of Aβ. Following seven consecutive days of Poly I:C or saline treatment, a 48-hour recovery period, and CFC training and testing days, animals were euthanized following IACUC approved protocols and tissue was obtained for further study. The results from the biological assay (Figure 4) shows that there remained a significant increase in the amount of Aβ $_{1-42}$ in the hippocampus of the animals that received seven consecutive days of Poly I:C injections as compared to the saline-injected group ($F_{(1,10)}$ =19.52, p<0.01).

Experiment 5: Poly I:C- verse LPS-induced $A\beta_{1-42}$ production

Our lab had previously shown that 7 consecutive days of LPS injections resulted in increased $A\beta_{1-42}$ in hippocampal tissue and cognitive deficits (Kahn et al., 2012). Although 7 consecutive daily injections with Poly I:C also resulted in a significant increase in $A\beta_{1-42}$ in hippocampal tissue, there was no remarkable cognitive changes. A pilot study was performed to further evaluate potential reasons why Poly I:C injections did not induce cognitive deficits similar to LPS injections in the C57BL/6 mouse. The pilot utilized ELISA procedure to assess the levels of $A\beta_{1-42}$ in the hippocampus of animals from three study groups: saline-, Poly I:C, and LPS-injected animals. Animals again received seven consecutive days of injections. Twenty-four hours after the final injection, animals were euthanized following IACUC approved protocols and hippocampal tissue was obtained. As seen in Figure 5, there was a significant difference in $A\beta_{1-42}$ in hippocampal tissue among the different groups ($F_{(2,18)}$ =8.593, p<0.01). Fisher's PLSD post-hoc analysis revealed that the only significant difference in hippocampal $A\beta_{1-42}$ was between LPS- and saline-injected animals ($F_{(2,18)}$ =0.0025, p<0.0025). There was no significant difference noted between LPS- and Poly I:C-injected animals or Poly I:C- and saline-injected animals.

DISCUSSION

In the present study we sought to determine if Poly I:C-induced peripheral inflammation could produce elevated levels of $A\beta_{1-42}$ and if Poly I:C-induced Aβ elevations could result in changes in behavior and cognition. Our lab has previously shown that LPS-induced inflammation results in elevated Aβ and associated cognitive deficits in the hippocampus-dependent task morris water maze and CFC (Kahn et al.,

2012). Although LPS mimics a bacterial inflammatory process and Poly I:C mimics a viral inflammatory process, we expected to see similar physiological changes from the peripheral inflammation. Therefore, the project attempted to compare behavioral, cognitive, and physiological findings for Poly I:C-induced inflammation versus previously studied LPS-induced inflammation in C57BL/6J mice. With these findings we hope to better understand the potential role of peripheral inflammation in the onset of AD-like pathologies, specifically elevations of Aβ in the hippocampus.

Our findings demonstrated that 7 consecutive days of injections of Poly I:C resulted in significantly elevated levels of Aβ in the hippocampus. Hippocampal-Aβ levels were initially assessed in tissue obtained 24 hours after the final Poly I:C injection. These findings are consistent with previous studies using LPS-induced peripheral inflammation (Kahn et al., 2012; Lee et al., 2008). Similar to studies that displayed a correlation between increases in Aβ and cognitive decline using LPS as an inflammatory mediator, we predicted that Poly I:C-injected animals would display cognitive deficits.

Prior to cognitive testing, it was necessary to assess if seven consecutive injections of Poly I:C would lead to sickness behaviors that could confound behavioral results. Sickness behaviors are the physiological manifestation of the cellular immune response to an antigen. Sickness behaviors observed in mice include, but are not limited to, decreased locomotion, changes in motivation, and weight loss (McLinden et al., 2012). These sickness behaviors have been previously shown in mice after both Poly I:C and LPS injections utilizing a burrowing protocol sensitive to sickness (Kranjac et al., 2012a; McLinden et al., 2012). These sickness behaviors could alter the animals' performance on learning tasks and therefore interfere with the analysis of behavioral data. As

expected, our study found that following 7 consecutive injections of Poly I:C, animals had significant weight loss compared to animals that received 7 consecutive injections of saline, consistent with the onset of sickness behaviors. However, unlike LPS-injected mice that show endotoxin tolerance by the seventh daily injection (Kahn et al., 2012), Poly I:C-injected mice were found to display sickness behaviors, as assessed using burrowing, after seven days of Poly I:C treatment. In a previous study to determine when tolerance would take effect using Poly I:C injections, mice were injected daily for 21 days, and immediately after each injection animals were placed in burrowing cages overnight. This study showed that at no point did the mice cease to display sickness behavior (Kranjac, D., unpublished data). These results indicate an absence of tolerance development to Poly I:C. Due to the potential risk if sickness behaviors confounding our cognitive tests, we needed to establish a timeframe when Poly I:C-induced sickness behavior would no longer be evident after the final injection. To this end burrowing studies were employed to assess sickness behaviors following 7 consecutive injections of Poly I:C. Our results indicated that although there was no significant difference between the saline-injected group and the Poly I:C-injected group ($p=0.058$) 24 hours after the final injection, we determined that waiting 48 hours after the final injection would lead to a decreased chance of having sickness behavior confound our behavior data.

Contextual fear conditioning (CFC) was utilized to assess possible changes in learning and memory following Poly I:C injections. Initial experiments followed the two-shock paradigm, however results showed no significant differences between the groups. We were concerned that the two-shock protocol was too easy for the mouse to learn, thus not sensitive enough to detect small cognitive differences. To address this

issue we tested additional groups of animals using a single-shock CFC paradigm. We hypothesized that the single-shock CFC protocol should be more difficult to learn and more sensitive to potentially small cognitive differences between the two groups. Our results from this CFC protocol again indicated no significant difference between the groups. Thus, our results indicated that following seven consecutive daily injections with Poly I:C there are no significant cognitive deficits.

One possible explanation for the lack of cognitive deficits observed in Poly I:C-injected mice could be that Aβ produced during the inflammation had been cleared from the hippocampus prior to CFC testing, as there was a delay of 48 hours. To evaluate this possibility an ELISA was performed on hippocampal tissue obtained immediately after CFC testing. The results revealed that the amount of Aβ in Poly I:C-injected animals was still significantly elevated at the time of CFC testing.

In an attempt to provide an explanation for the lack of cognitive deficits in the presence of Poly IC-induced Aβ, a pilot study was performed to compare LPS- and Poly I:C- induced peripheral inflammation. The pilot study utilized an ELISA to assess the levels of Aβ in the hippocampus of animals in one of three experimental groups: saline-, Poly I:C-, and LPS-injections. We found that LPS treatment resulted in greater Aβ than Poly I:C. Although the difference between Poly I:C and LPS was not statistically significant, there was strong indication that LPS resulted in greater Aβ elevations. Interestingly, the only significant difference found was between LPS and saline, with the difference between Poly I:C and saline being statistically non-significant.

The next step for our lab will be to perform additional studies with a larger sample size to try and replicate the results of the three-group pilot. Additionally, further

work needs to be done to try and normalize potency and Aβ elevations of Poly I:C and LPS in order to establish a more accurate comparison. In these studies we plan to reduce the dose of LPS, leading to a reduced inflammatory response, and thus attempt to produce the same levels of Aβ in the LPS and Poly I:C groups. We hypothesize that there is a basal level of Aβ that does not interfere with cognition, and anything above this level would lead to cognitive deficits. We could also chose to increase Poly I:C doses to produce the same level of Aβ as produced in our LPS studies, but doing so may prolong the sickness behaviors following the final injection. Not only would this prolong the time required for the experiment, but there are also substantiated concerns that excess Aβ may dissipate or be cleared from the brain during the time required for resolution of sickness behaviors. If LPS doses are reduced to a level that produces $A\beta_{1\text{-}42}$ elevation equal to that of Poly I:C and cognitive deficits are absent, this would further suggest the importance of $A\beta_{1\text{-}42}$ levels on disruptions in learning and memory.

Our findings build upon previous data that indicate potentially detrimental effects of peripheral inflammation. Evidence suggests that peripheral inflammation leads to elevated levels of $A\beta_{1\text{-}42}$ in the brain that may have a role in the progression of AD-like pathologies, including deficits in learning and memory. Those afflicted with chronic inflammatory conditions, such as Crohn's disease, Diabetes Mellitus, and Lupus, need to be aware of this potential increased risk. These findings will be useful as we seek to target inflammation or Aβ production in an attempt to combat the associated cognitive decline. Understanding the link between inflammation and neurodegeneration is imperative for further research.

FIGURE 1

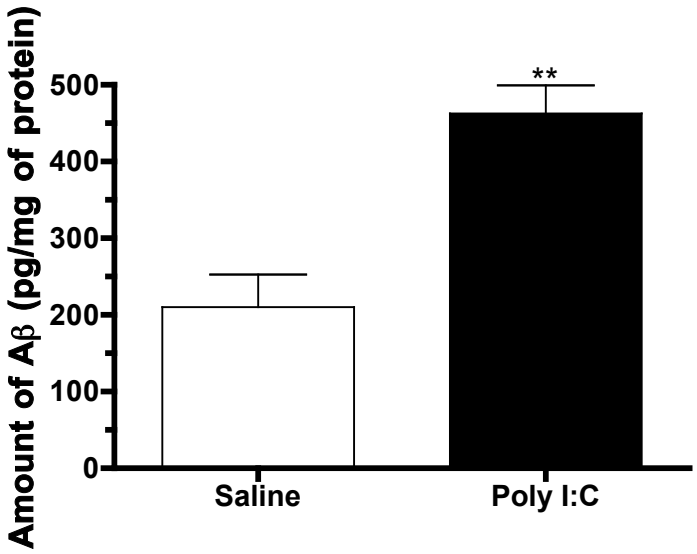

Figure 1. Peripheral Poly I:C-induced Aβ production. 24 hours after the 7th injection, Aβ was significantly elevated in the hippocampus of Poly I:C-treated animals (12mg/kg) as compared to saline treated controls. ** $p<0.05$, bars represent mean +/-SEM

FIGURE 2

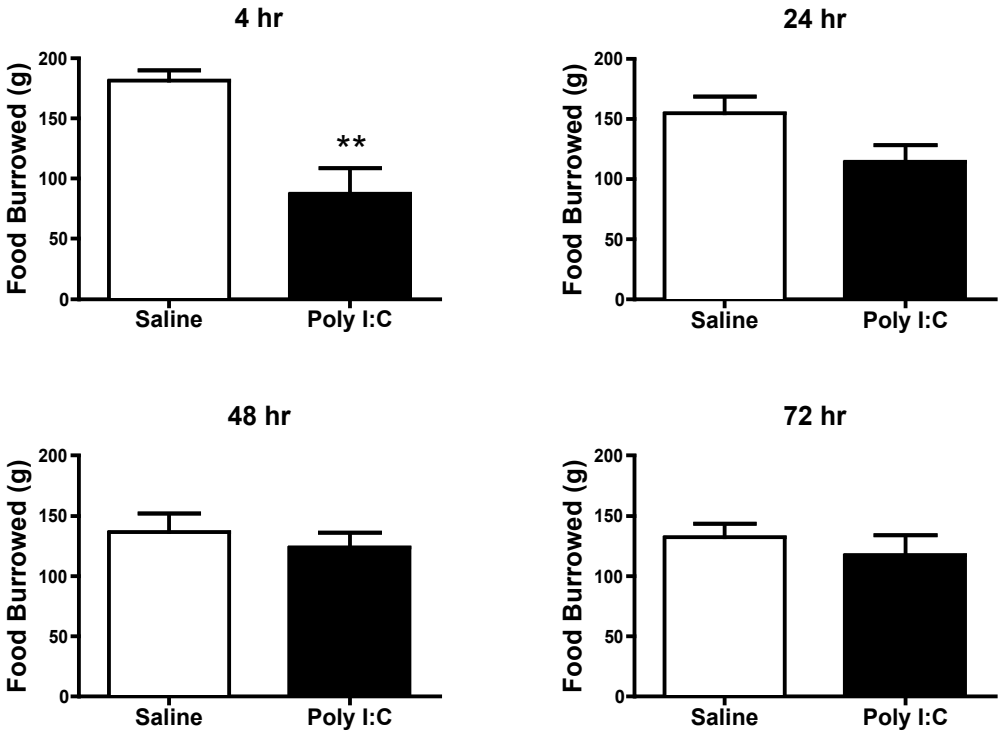

Figure 2. Burrowing behavior after 7 consecutive days of i.p. Poly I:C injection (12mg/kg). 4 hours following the 7th injection, Poly I:C suppressed burrowing behavior. 24 hours following the 7th injection, Poly I:C continued to decrease burrowing (p=0.058). 48 and 72 hours following the 7th injection, Poly I:C no longer suppressed burrowing activity. **p<0.01, bars represent mean +/-SEM

FIGURE 3

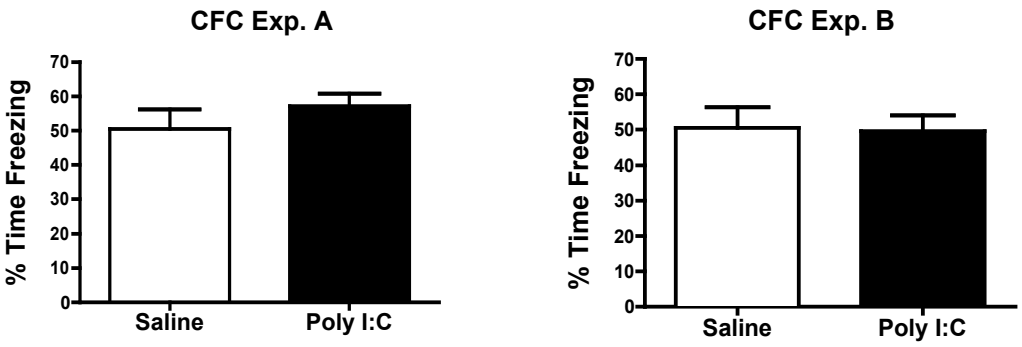

Figure 3. No cognitive deficits displayed in CFC. Experiment A: 48 hours after 7 consecutive days of Poly I:C treatment (12mg/kg), animals were trained using two foot shocks. Pre-shock motor activity was not different between groups, supporting the absence of sickness behaviors (data not shown). 24 hours after training, there was no difference in freezing behavior. Experiment B: to increase task difficulty, a separate Poly I:C treatment experiment was conducted in which only one presentation of the shock stimulus was administered during training. Again, 24 hours after training there was no difference in learning between the groups. Bars represent mean +/-SEM

FIGURE 4

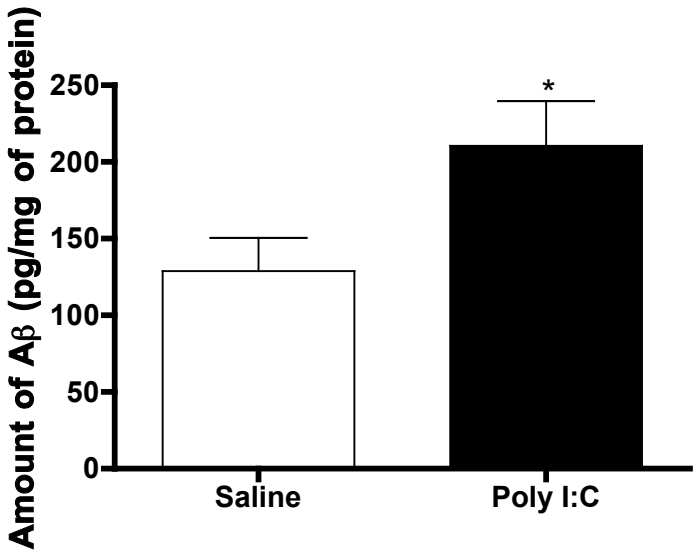

Figure 4. Peripheral Poly I:C-induced Aβ production. Immediately following CFC testing, Aβ remains significantly elevated in the hippocampus of Poly I:C-treated animals (12mg/kg) as compared to saline treated controls. *p<0.05, bars represent mean +/-SEM

FIGURE 5

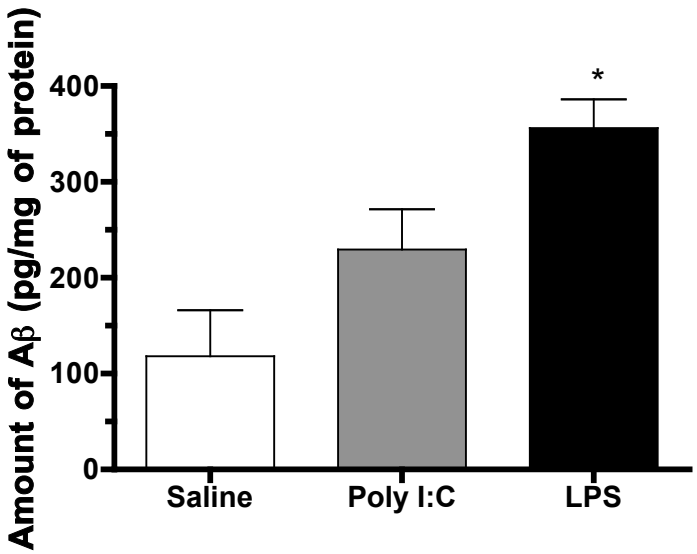

Figure 5. Aβ production following LPS or Poly I:C administration. Aβ is significantly elevated in the mouse hippocampus after 7 consecutive i.p. injections of LPS (250μg/kg), but not Poly I:C (12mg/kg), as compared to saline treated controls. *p<0.05, bars represent mean +/-SEM

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ABSTRACT

Alzheimer's disease (AD) is a progressive disorder characterized by neuronal cell death and atrophy in regions of the adult brain, including the hippocampus and cortex. Two hallmark pathologies of AD are extracellular amyloid-beta (Aβ) plaques and intracellular neurofibrillary tangles. Presence of these pathologies can limit normal cell signaling properties leading to learning and memory deficits and, ultimately, cell death. Chronic inflammation has been implicated in the onset and progression of these AD pathologies. Our lab has previously shown that peripheral injections of a bacterial mimetic leads to increased Aβ levels in the mouse hippocampus, as well deficits in hippocampus-dependent learning. The current study was designed to further our understanding of peripheral inflammation-induced AD-like pathology by using polyinosinic:polycytidylic acid (Poly I:C) which produces an inflammation similar to that caused by double-stranded viral RNA. Mice were given intraperitoneal (i.p.) injections of Poly I:C or saline for 7 consecutive days. Similar to our findings using the bacterial mimetic LPS, hippocampal tissue from animals receiving peripheral Poly I:C contained significantly higher levels of Aβ peptide over that found in saline injected control animals. However, unlike LPS, 7 consecutive injections of Poly I:C leads to sickness behavior that does not disappear until 48 hours after the final injection. Cognitive testing at that time revealed no deficit in hippocampus-dependent learning. Our studies implicate that both bacterial and viral inflammation can produce elevated levels of Aβ in the hippocampus, but pilot data suggests a minimum level of Aβ may be necessary to produce cognitive deficits.