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Neuroinflammation and disruption in working memory in aged mice after acute stimulation of the peripheral innate immune system

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Abstract

Acute cognitive disorders are common in elderly patients with peripheral infections but it is not clear why. Here we injected old and young mice with *Escherichia coli* lipopolysaccharide (LPS) to mimic an acute peripheral infection and separated the hippocampal neuronal cell layers from the surrounding hippocampal tissue by laser capture microdissection and measured mRNA for several inflammatory cytokines (IL-1β, IL-6, and TNFα) that are known to disrupt cognition. The results showed that old mice had an increased inflammatory response in the hippocampus after LPS compared to younger cohorts. Immunohistochemistry further showed more microglial cells in the hippocampus of old mice compared to young adults, and that more IL-1β-positive cells were present in the dentate gyrus and in the CA1, CA2 and CA3 regions of LPS-treated old mice compared to young adults. In a test of cognition that required animals to effectively integrate new information with a preexisting schema to complete a spatial task, we found that hippocampal processing is more easily disrupted in old animals than in younger ones when the peripheral innate immune system is stimulated. Collectively, the results suggest that aging can facilitate neurobehavioral complications associated with peripheral infections probably by allowing the over expression of inflammatory cytokines in brain areas that mediate cognitive processing.

Keywords

aging; hippocampus; interleukin-1; laser capture microdissection; lipopolysaccharide; working memory

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Introduction

Old age is associated with increased brain inflammation (Ye and Johnson, 1999; Lee et al., 2000; Richwine et al., 2005) and stimulation of the peripheral innate immune system with lipopolysaccharide (LPS) leads to an increased inflammatory response in the brain of healthy aged mice (Godbout et al., 2005). This is noteworthy because an increased inflammatory response in the aged brain may cause cognitive deficits that are highly prevalent in elderly patients. For example, acute cognitive impairment (i.e., delirium) is common in elderly emergency department patients (Wofford et al., 1996a; Chiovenda et al., 2002) and frequently occurs in patients with infections unrelated to the CNS (Wofford et al., 1996a). Cognitive impairment complicates diagnosis and is associated with reductions in self-care, increased hospitalization and delayed recovery (Johnston et al., 1987). The severity of dementia is related to mortality of infectious disease, indicating an insidious relationship between infection and cognition whereby infection in the elderly induces cognitive impairment, and cognitive impairment exacerbates the infection.

Because of the dysregulated linkage between the peripheral immune system and brain in old mice we hypothesized that elevated levels of inflammatory cytokines in the hippocampi of aged animals would be associated with greater deficits in hippocampal-mediated learning and memory after peripheral immune activation. We focused on the hippocampus because it is sensitive to the insults of aging and is likely involved in acute cognitive disorders that are evident in elderly patients with systemic infections. Further, inflammatory cytokines can impair synaptic plasticity in the dentate gyrus (DG) and CA regions of the hippocampus (Bellinger et al., 1993; Cunningham et al., 1996; Tancredi et al., 2000; Hellstrom et al., 2005; Shaw et al., 2005) as well as disrupt hippocampal-dependent learning and memory (Palin et al., 2004; Rosi et al., 2005; Tanaka et al., 2006). A recent study reported that older rats infected with *Escherichia coli* had increased hippocampal IL-1β and deficits in hippocampal-dependent memory and learning that were not evident in similarly infected younger cohorts (Barrientos et al., 2006).

In the present study, using a mouse model and a working memory version of the water maze, we found that hippocampal processing is more easily disrupted in old animals than in younger ones after peripheral immune activation. This outcome is independent of a generalized effect on memory consolidation (Sparkman et al., 2005a; Barrientos et al., 2006), and demonstrates an important and novel aspect of infection-related cognitive impairments that is consistent with previous studies of hippocampal dysfunction (Squire, 1992; Avital et al., 2003). Furthermore, using laser capture microdissection (LCM) and immunohistochemistry (IHC), we showed the LPS-induced disruption in working memory in old mice was associated with increased expression of inflammatory cytokines in the hippocampal neuronal cell layers (DG and CA regions) and in surrounding hippocampal tissue. The results suggest that aging can influence the neurobehavioral complications associated with systemic infections probably by allowing the over expression of inflammatory cytokines in brain areas that mediate cognitive processing.

Materials and Methods

Animals

Young (3-6 mo) and old (22-24-mo) male BALB/c mice from our in-house specific-pathogen-free colony were used. Mice were housed in polypropylene cages and maintained at 23° C under a reverse phase 12 h light-dark cycle with *ad libitum* access to water and rodent chow. At the end of each study, mice were examined postmortem for gross signs of disease (e.g. splenomeglia and tumors). Data from mice determined to be unhealthy were excluded from analysis. All procedures were in accordance with the National Institutes of Health Guidelines

for the Care and Use of Laboratory Animals and were approved by the University of Illinois Institutional Animal Care and Use Committee.

Experimental Protocols

For all studies, young and old mice were injected intraperitoneally (i.p.) with sterile saline or *Escherichia coli* LPS (0.33 mg/kg; serotype 0127:B8, Sigma, St. Louis, MO). This dosage of LPS was selected because it elicits an inflammatory cytokine response in the brain resulting in transient sickness behavior in young mice (Berg et al., 2004; Godbout et al., 2005). In the first study, young (n=18) and old mice (n=18) were killed by CO_2 asphyxiation 4 h after injection of saline or LPS. Brains were quickly removed and preserved for LCM and cytokine mRNA determination or IHC staining of microglia and IL-1β. In a subsequent study, young (n=19) and old mice (n=20) were subjected to an 8-d acquisition phase in a spatial working memory test. The very next day they were injected i.p. with saline or LPS and 4 h later their performance in a hippocampal-dependent spatial working memory test was determined.

LCM

Brains were quickly removed, trimmed, embedded in Tissue-Tek® OCT Compound, chilled on dry ice and stored at -80° C until sectioning. Coronal sections 10 μm thick were cut at -20° C to prevent RNA degradation using a Leica cryocut 1800. Coronal sections containing the hippocampus were mounted on SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA) and immediately placed on dry ice and stored at -80° C. To make certain sections were collected from the same hippocampal region (approximately -2.18 mm from bregma), five 20-μm-thick sections were trimmed beginning at the anterior portion of the hippocampus before adjusting the cryostat to cut 10-μm-thick sections for LCM. Staining and LCM was performed within 7 d. Briefly, frozen sections were thawed at room temperature for 30 sec and fixed for 30 sec in 75% ethanol. After fixation, sections were rehydrated in RNase-free water for 30 sec and stained with 1% cresyl violet for 20 sec to reveal hippocampal neuronal layers. Sections were rinsed with RNase-free water for 30 sec and dehydrated in a series of ethanol baths (30 sec in 75%, 95% and 100% ethanol). Finally, sections were incubated in xylene and air dried.

Immediately after dehydration LCM was performed using an Arcturus Pix Cell IIe laser capture microscope and Arcturus v2.0.0 software (Arcturus Engineering, Mountain View, CA). A laser spot size of 15 μm was used with a power setting of 35-50 mW and pulse duration of 6-10 ms. Laser power and duration were adjusted to optimize capture efficiency. To reduce binding of non-target tissue, prior to LCM Prep Strip™ membranes (Arcturus) were gently applied to compress sections and remove loose debris; CapSure™ HS LCM caps (Arcturus) were used to prevent capture film from contacting tissue; and CapSure™ cleanup pads (Arcturus) were applied to remove undesired tissue that adhered to LCM caps. The hippocampal neuronal cell layers including the CA1, CA2, CA3 regions and the DG were selectively captured on to the same LCM cap (Figure 1 D). Usually hippocampal tissue from the right and left hemispheres was captured on a single cap. Following LCM, 10 μl of extraction buffer provided in a Pico Pure™ RNA isolation kit (Arcturus) was added to the caps and incubated at 42° C for 30 min. The lysates from neuronal cell layers (Figure 1 D) obtained from 4-8 contiguous sections were combined and stored at -80° C until RNA isolation. A dissecting microscope and sterile probe were used to scrape the remaining hippocampal tissue (Figure 1 C) into an Eppendorf microcentrifuge tube with 50 μl of extraction buffer. The lysate was preserved at -80° C until RNA isolation.

Cytokine Quantification

Total RNA was isolated using the Arcturus PicoPure™ RNA isolation kit as described by the manufacturer. DNase treatment was performed on a PicoPure column with a Qiagen RNase-free DNase set (Qiagen, Valenica, CA). Total RNA was measured using the Ambion

NanoDrop® ND-1000A UV-Vis spectrophotometer (Ambion, Austin, TX), which showed each sample (i.e., pooled lysates from 8 sections) yielded about 200 ng RNA from hippocampal neuronal cell layers and 130 ng RNA from hippocampal tissue that surrounded the neuronal cell layers.

Total RNA was reverse transcribed using Sensiscript RT kit or QuantiTect RT kit as described by the manufacturer (Qiagen). When the Sensicript RT protocol was employed 50 ng of sample RNA was added to a RT master mix, which included random 9-mers (10 μM; Operon Technologies, Alameda, CA), dNTP (5mM each), RNase inhibitor RNasin (10 units; Promega, Madison, WI), 1 μl Sensiscript 20X and 2 μl 10X reaction buffer to reach a final volume of 20 μl. The RT mix was incubated at 37 °C for 60 min. The QuantiTect RT procedure used Quantiscript Reverse Transcriptase, which contained a mixture of Sensiscript and Omniscript for optimized reverse transcription of a broader range of total RNA (10 pg to 1 μg RNA). cDNAs were synthesized from 100-150 ng of total RNA at 42° C for 15 min in a final volume of 20 μl of RT mix containing Primer mix, RT buffer and Quantiscript. After RT reaction, cDNAs were stored at -20 °C.

Real-time PCR was performed using the Applied Biosystems (Foster City, CA) Taqman Assay-on Demand Gene Expression protocol as we have done previously (Berg et al., 2005). cDNA was amplified by PCR where a target sequence (IL-1β, Mm00434228_m1; IL-6, Mm00446190_m1; and TNFα, Mm00443258_m1) and a reference sequence (glucose-3 phosphate dehydrogenase, Mm99999915_g1) were amplified simultaneously using an oligonucleotide probe with a 5′ fluorescent reporter dye (6-FAM) and a 3′ quencher dye (NFQ). In our study, all PCR reactions were done in triplicate on a 384-well real-time PCR plate (Applied Biosystems) under the following conditions: 50° C for 2 min, 95° C for 10 min, followed by 45 cycles of 95° C for 15 s and 60° C for 1 min. Fluorescence was determined on an ABI PRISM 7900HT-sequence detection system (Perkin Elmer, Forest City, CA). Data were analyzed using the comparative threshold cycle (Ct) method, which for accuracy requires similar amplification efficiency between the target and the internal control gene (Livak and Schmittgen, 2001). Amplification efficiency for the target genes (IL-1β, IL-6 or TNFα) and the internal control gene (GAPDH) was validated by determining the Ct slope for serially diluted template cDNA (data not shown). Data are expressed as fold-change.

IHC Staining and Quantification

Four hours after i.p. injection of saline or LPS, mice were killed by CO_2 asphyxiation and transcardially perfused with 2mM EDTA saline and 4% paraformaldehyde and the brains were removed. Brains were postfixed over 2 d in 4% paraformaldehyde and then paraffin embedded. Coronal hippocampal sections (4 μm) were cut on a microtome and every 10^{th} section was stained for IL-1β (Peprotech, Rocky Hill, NJ) or microglia (tomato lectin, Vector Laboratories, Burlingame, CA). Sections were dewaxed and rehydrated through xylene and alcohols. For staining IL-1β, sections were incubated in citrate buffer, pH 6 and microwaved for 10 min. Endogenous peroxidase was eliminated by incubating sections in 3% H_2O_2/methanol for 15 min. Sections were washed in PBS and blocked with 20% normal goat serum before overnight incubation at 4°C with the primary antibody at 1:400 dilution in 5% serum. The sections were washed and then incubated with biotinylated goat anti-rabbit antibody (Vector Laboratories) for 40 min. For staining microglia, sections were washed in PBS and blocked with 5% normal goat serum. Sections were incubated overnight at 4°C with biotinylated tomato lectin at 1:200 dilution in 5% serum. All Staining was visualized using the ABC method and diaminobenzidine as chromagen. Sections were counterstained with hematoxylin. Isotype-matched IgG was used as a negative control for IL-1β staining. Immunostaining was visualized using an Optronix Microfire camera (model S99808, Goleta, CA) attached to a Zeiss Axio Imager A.1 microscope (Gottingen, Germany). For measurement of IL-1β-positive cells and microglia, 4 images of

each region of interest were captured from at least 4 animals per group (total of 16 images per animal). IL-1β-positive cells and microglia were counted manually at a magnification of 40× and expressed as number of cells per 100μm ^{2}.

Spatial Working Memory - Radial Arm Water Maze

To evaluate hippocampus-dependent memory a 5-arm radial water maze was constructed. A circular tank 100 cm in diameter and 30 cm deep and filled with water (24-26° C) to a depth of 25 cm was used. Opaque partitions were used to divide the maze into 5 arms. Each of the arms was 20 cm in width and extended 33 cm from the edge of the pool. A transparent round platform 10 cm in diameter was constructed from Plexiglas and positioned approximately 0.5 cm below the surface of the water at the end of one of 4 arms. The non-visible platform was randomly placed in one of four preset positions for each session of testing. Numerous visual cues were located outside the tank. A video camera mounted to the ceiling directly above the center of the maze was used in conjunction with the HVS VP 200 tracking system (HVS image, Ormend Cres, Hampton, England).

For all experiments, training took place during an 8 d acquisition phase with 2 sessions of 3 massed trials administered each day. Sessions were 1 h apart. A single bolus injection of saline or LPS was administered on the test day (d 9) to determine treatment effects on an animal's ability to integrate new information with existing memories to complete a hippocampus-dependent task.

To begin each trial, a mouse was pseudorandomly placed in an arm not occupied by the platform facing the wall. Mice were allowed to swim freely a maximum of 60 s or until the platform was located. After the mouse reached the platform it was required to remain there for 30 s. If the platform was not located during the 60 s, mice were guided to the platform and allowed to remain 30 s. After completion of three consecutive trials, mice were placed in their home cage under a heat lamp for approximately 10 min. Because location of the platform was changed with every new session of trials, mice were placed on the platform for 30 s preceding the start of each session of testing. Performance parameters that were determined included swim speed, latency to the platform, and distance swam.

Statistical Analysis

Cytokine data from real-time PCR and IHC staining were subjected to two-way ANOVA (Age × Treatment) using the PROC GLM procedures of SAS®. When the ANOVA revealed a significant main effect or interaction between main factors, post hoc Student's *t* test using Fisher adjustment of least squares means was employed to determine if treatment means were significantly different from one another. For spatial working memory, means for each test session were calculated and behavioral data were subjected to Repeated-Measures ANOVA as a two-way design in which test Day was a within subjects measure (i.e., repeated measure), and LPS (saline or 0.33 mg/kg) and Age (Young or Old) were between subjects measures for days 1-8. A separate ANOVA examined the effects of treatment on working memory performance for the first session of testing on day 9. When the ANOVAs revealed a significant effect of main factors or main factor interactions, differences in group means were tested using Fisher's least significant differences. Additionally, a planned comparison examined the effects of age within the group of animals that received LPS. All data are presented as means ± S.E.M.

Results

Young (3-6-month old) and old (22-24-month old) mice were injected i.p. with saline or LPS and 4 h later they were killed and mRNA encoding several inflammatory cytokines was measured in their hippocampi by real-time PCR. LCM was used to separate the neuronal cell

layers (i.e., DG and CA regions) from surrounding hippocampal tissue, thus providing a better analysis of the microenvironment of hippocampal neurons which are known to be sensitive to insults of aging and inflammatory cytokines. Figure 1 shows (A) an intact cresyl violet-stained coronal brain section that reveals the CA pyramidal and DG granular neuronal layers in an adult mouse brain; (B) the same section after the CA regions and DG were outlined using a 15 μm laser spot with a power setting of 35 mW and pulse duration of 6 ms; (C) the section after DG and the CA regions were captured on to an Arturus CapSure HS LCM cap (note the surrounding tissue remains intact); and (D) the DG and CA regions that were captured.

The goal was to obtain sufficient quantities of RNA from the captured tissue for analysis without performing *in vitro* transcription of reverse transcribed cDNA. Therefore, in an initial study increasing numbers of hippocampal neuronal cell layers (as shown in Figure 1 D) from serial sections were pooled to determine the minimum needed to measure inflammatory cytokine mRNA by real-time PCR without RNA amplification. Hippocampal tissue from young saline treated mice was used because cytokine expression was presumably lowest in this treatment group. Figure 1 E shows the relationship between the number of LCM sections pooled and the amount of RNA extracted. As anticipated the number of LCM sections pooled was positively correlated with the amount of RNA extracted (r=0.9782, $p < 0.01$). The amount of RNA extracted was not affected by age or treatment (data not shown). Total RNA in each pooled sample was reverse transcribed and the resultant cDNA served as a template for real-time PCR of GAPDH and IL-6. Figure 1 F shows the relationship between the beginning amount of RNA (logarithm) and Ct value for both GAPDH and IL-6. As the amount of starting RNA increased, the Ct values for GAPDH (r=0.9991, $p < 0.001$) and IL-6 (r=0.9956, $p < 0.001$) decreased. Total RNA isolated from two LCM hippocampal neuronal cell layer sections generated a Ct value of 36 for IL-6, which is at the limit of detection. Therefore, a minimum of four LCM sections was used to provide enough RNA for our real-time PCR protocol. To validate GAPDH as an appropriate housekeeping gene GAPDH Ct values for samples from young and aged mice treated with LPS or saline were compared. A two-way ANOVA indicated GAPDH expression was not affected by age, LPS, or an Age × LPS interaction ($p > 0.5$; data not shown). This finding is consistent with another report that found no age-related change in central GAPDH mRNA expression (Slagboom et al., 1990).

Figure 2 shows the effects of age and LPS challenge on steady-state levels of inflammatory cytokine mRNA in neuronal cell layers of the hippocampus (A) as well as in hippocampal tissue surrounding the neuronal cell layers (B). These data reveal two key points: First, they show that in hippocampal tissue surrounding the neuronal cell layers mRNA encoding IL-1β and TNFα were higher in saline-treated old mice than in similarly treated young adults ($p < 0.01$). In hippocampal neuronal cell layers IL-1β mRNA was higher in old mice than in young adults ($p < 0.01$). Neither IL-6 mRNA in the two areas sampled nor TNFα mRNA levels in neuronal layers were affected by age. These data are generally consistent with earlier reports indicating a heightened neuroinflammatory state emerges in old age (Ye and Johnson, 1999, 2001b;Godbout et al., 2005).

Consistent with previous studies (Laye et al., 1994; Berg et al., 2005), activation of the peripheral innate immune system with LPS increased steady-state levels of inflammatory cytokine mRNA in hippocampal tissue (Figure 2). Neurons can produce inflammatory cytokines (Molenaar et al., 1993; Ringheim et al., 1995; Renauld and Spengler, 2002; MacPherson et al., 2005) and glia are embedded within the neuronal cell layers (Lawson et al., 1990), thus it was not surprising that IL-1β, IL-6 and TNFα mRNA was increased by LPS in the DG and CA regions. The cytokine response to LPS was highest in the hippocampal tissue surrounding the neuronal layers. However, the second key point revealed in Figure 2 is that the effects of age and LPS on cytokine mRNA expression were additive so that hippocampal levels were generally higher in old mice than in young adults. In neuronal cell layers, IL-1β,

IL-6, and TNFα mRNA levels were higher in LPS-treated old mice than in similarly treated young adults (IL-1β $p < 0.01$, IL-6 and TNFα, $p < 0.05$). In hippocampal tissue surrounding the neuronal cell layers, IL-1β and TNFα mRNA were higher in old mice after injection of LPS than in younger adults ($p < 0.01$); however, LPS affected IL-6 mRNA in this region similarly in young and old mice.

To determine if changes in steady-state levels of inflammatory cytokine mRNA conferred an increase in cytokine protein, coronal brain sections that included the hippocampus from young and old mice that had been injected with saline or LPS were subjected to IHC staining to reveal IL-1β-positive cells. We focused on IL-1β because mRNA levels encoding this cytokine were consistently affected by both age and LPS in the neuronal cell layers and in hippocampal tissue surrounding the neuronal cell layers (Figure 2). Furthermore, IL-1β has been shown to inhibit LTP in both the DG and CA regions (Bellinger et al., 1993;Cunningham et al., 1996;Murray and Lynch, 1998). Shown in Figure 3 are representative photomicrographs of the DG region of old and young mice after injection of saline or LPS, and bar graphs that summarize the number of IL-1β-positive cells in all of the hippocampal areas surveyed (i.e., CA1, CA2, CA3, and DG). IL-1β-positive cells were rare in saline treated young adult and aged mice. However, LPS treatment increased the number of cells staining for IL-1β in all four areas ($p < 0.05$ in CA1 and DG; $p < 0.01$ in CA2 and CA3). A significant age × LPS interaction was observed in the CA3 region ($p < 0.01$), indicating the LPS-induced increase in IL-1β staining in this area was enhanced in the aged. A similar trend was seen in the DG (age × LPS, $P = 0.10$). Post hoc analysis indicated more IL-1β-positive cells in both the granular and polymorph layers of the CA and DG regions of LPS-treated old mice compared to LPS-treated young adults ($p < 0.05$ in CA1 and DG; $p < 0.01$ in CA2 and CA3). The staining appeared to be specific for IL-1β since no staining was evident when the primary antibody against murine IL-1β was replaced with an isotype-matched antibody control (data not shown). Furthermore, as an additional control the primary antibody was pre-hybridized to brain sections from young healthy mice that presumably had little to no IL-1β expression to eliminate antibodies with nonspecific antigen binding sites. This treatment did not reduce staining in subsequent assays with tissue from LPS challenged animals, indicating nonspecific binding was minimal (data not shown).

Microglial cells are considered the principle source for inflammatory cytokines in the CNS when the peripheral immune system is stimulated. Therefore, microglia in the DG and CA3 regions in old and young adult mice were compared by tomato lectin staining. An advantage of using lectin is that it stains microglia irrespective of their state of activation (Colton et al., 1992) and treatment with LPS does not appreciably change microglial cell morphology (Cunningham et al., 2005). Therefore, in the current study only mice given saline were examined. Figure 4 A and B are representative photomicrographs showing microglia in the DG of young and old mice. In the hippocampal regions surveyed, more cells stained with tomato lectin in old mice than in young ($p < 0.05$; Figure 4C). These data provide further evidence for an age-associated increase in neuroinflammation. Although we have not yet definitively determined the specific cell type responsible for the increased IL-1β staining shown in Figure 3, microglia are likely involved since microglial cells were in the vicinity of IL-1β-positive cells. For example, Figure 4D shows a close up (100× magnification) of a microglial cell surrounded by granular neurons in the DG; and Figure 4E shows a close up of what appears to be an IL-1β-positive microglial cell in the same hippocampal region. A role for microglia is consistent with the observation that expression of MHC class II—a marker of activated microglia—is increased in brains of old mice (Godbout et al., 2005). Furthermore, in a study with young rats IL-1β-staining in the CA1 region was co-localized with CD11b staining (a microglial cell marker) after LPS (Tanaka et al., 2006). Whatever the case, collectively these results indicate that after activation of the peripheral innate immune system with LPS both inflammatory cytokine mRNA and protein levels are higher in hippocampi of old mice than in young mice.

Inflammatory cytokines are thought to induce cognitive deficits (Wilson et al., 2002). Therefore, a study was conducted to determine if age affected hippocampal-dependent learning and memory after activation of the peripheral innate immune system with LPS. Old and young mice were subjected to an 8-d acquisition period (Figure 5 A, B and C) and effects of LPS on spatial working memory in a matching-to-place task in a 5-arm radial arm water maze were assessed 4 h after treatment on d 9 (Figure 5 D, E and F). This hippocampus-dependent assay required mice to integrate new information with existing memories to complete the task. As anticipated, during the acquisition phase there was a main effect of age ($p < 0.01$) and a day × age interaction ($p < 0.01$) in which old mice demonstrated slowed rate of acquisition of the task (i.e., displayed increased swim distances in order to locate the platform; Figure 5 A); however, old animals were equally efficient in their ability to locate the platform compared to young animals by the end of the acquisition phase (d 8; Figure 5 A). Additionally, old mice took longer to find the platform ($p < 0.05$; Figure 5 B) and there was a trend for old mice to display slower swim speeds across test sessions in the acquisition phase ($p < 0.08$; Figure 5 C). Following treatment with LPS (d 9), analysis of swim data demonstrated significant main effects of treatment ($p < 0.03$) in which LPS-treated animals swam further than saline-treated animals to locate the platform (Figure 5 D). There was also a main effect of age ($p < 0.02$) in which old animals swam further than young animals (Figure 5 D). The interaction between treatment and age was not significant ($p = 0.20$), but planned comparisons and post-hoc analysis revealed that old animals treated with LPS demonstrated a decreased efficiency to locate the platform compared to saline-injected old animals ($p < 0.02$) and young animals that received LPS ($p < 0.02$) or saline ($p < 0.01$). Latency and swim speed were not affected (Figure 5 E and C). These findings indicate that old mice are more vulnerable to deficits in spatial working memory caused by LPS. Thus, the ability to effectively integrate new information with existing memories to complete a hippocampus-dependent task may be more vulnerable to inflammation-associated disruption in old animals.

Discussion

The present study demonstrates that old mice have more microglia that stain for tomato lectin and higher basal levels of several inflammatory cytokine mRNAs in the hippocampus than do young adults. Furthermore, when the peripheral innate immune system was stimulated by LPS to mimic an acute Gram-negative bacterial infection, the expression of inflammatory cytokines in the hippocampus was higher in old mice than in young ones. Using a working memory version of the water maze that required animals to effectively integrate the new information into an existing spatial schema to complete a task, we found that hippocampal processing is more easily disrupted in old animals than in younger ones after peripheral immune activation. Because inflammatory cytokines impair memory consolidation and synaptic plasticity in the DG and CA regions of the hippocampus the results suggest that aging can influence the neurobehavioral complications associated with systemic infections probably by allowing the over expression of inflammatory cytokines in brain areas that mediate cognitive processing.

In the present study we examined how activating the peripheral immune system affected inflammatory cytokines in the hippocampus and hippocampal-dependent learning and memory in old mice. Our decision to focus specifically on the hippocampus was motivated by the realization that several acute cognitive disorders that are common in elderly patients with a peripheral infection appear to involve disruptions in hippocampal processing. Our results are consistent with a recent report by Barrientos et al. (Barrientos et al., 2006). In that study, middle-aged rats (i.e., not aged) inoculated with *Escherichia coli* bacteria had elevated levels of IL-1β protein in extracts isolated from the entire hippocampal formation after manual dissection and homogenization compared to similarly treated young rats. As opposed to combining neurons, glia, and white matter from the entire hippocampus, in our study we used LCM to separate the neuronal cell layers from surrounding hippocampal tissue for analysis of

several inflammatory cytokine mRNAs. Although LCM allowed for separation of discrete regions of the hippocampus, it should be noted that the LCM samples still comprised both neurons and glia and we currently are unable to determine the contribution of the different cell types to the cytokine mRNA pool. Nonetheless, the LCM strategy allowed us to better ascertain the microenvironment that hippocampal neurons experience during acute peripheral infection.

The results showed that after injection of LPS, mRNA encoding IL-1β, IL-6, and TNFα was higher in hippocampal neuronal cell layers of old mice compared to young mice. A similar pattern was evident in hippocampal tissue surrounding the neuronal cell layers with the exception of IL-6 mRNA. With respect to cytokine mRNA expression, aging did not appear to exacerbate the effects of LPS, although levels were always higher in old animals than young ones after injection. The cytokine mRNA data were complimented by IHC staining of IL-1β-positive cells. Although the number of IL-1β-positive cells increased in all four hippocampal areas examined after peripheral injection of LPS, it is noteworthy that the greatest increase occurred in the old mice where more microglial cells stained with tomato lectin. In this respect, aging seemed to exacerbate the number of cells producing IL-1β in the CA3 region. A similar trend was observed in the DG. Thus, the expression of inflammatory cytokines in brain after peripheral infection is both discrete (region specific) and influenced by age. This may be relevant for several reasons. First, the CA region is the area of the hippocampus besieged by Alzheimer's disease and peripheral infection is a risk factor for delirium in patients with the disease (Holmes et al., 2003). Second, the DG deteriorates with age, and as already stated, infection is a risk factor for acute cognitive disorders in the elderly (Wofford et al., 1996b; Janssens and Krause, 2004). This aberrant relationship between the peripheral immune system and brain in older patients is particularly important because this segment of the population generally suffers immunosenescence and is more susceptible to infection.

We do not know what occurs in old age that might facilitate the central inflammatory response to stimulation of the peripheral immune system. It does not appear to be due to the magnitude of the signal emerging from the peripheral immune system since similar plasma cytokine levels have been reported in old and young mice, rats and human subjects after injection of LPS (Krabbe et al., 2001; Godbout et al., 2005; Barrientos et al., 2006). Furthermore, aged mice exhibited a heightened neuroinflammatory response even when LPS was injected directly into the CNS via an indwelling intracerebroventricular cannula to bypass the peripheral immune system (Huang et al., 2007). A more likely explanation is that the central cytokine compartment and microglial cells in particular, are more responsive to stimuli. A shortcoming of the current study is that we did not definitively prove that IL-1β-positive cells were microglia. Nonetheless, the fact that tomato lectin histochemistry revealed more microglial cells in the hippocampi of old mice than in young adults suggests that aging either increased the number of microglial cells or led to morphological changes that facilitated staining.

Why then, do microglial cells become more active in the aged brain? One possibility is that the effectiveness of endogenous inhibitory systems diminishes. For example, IL-10 is an anti-inflammatory cytokine that can inhibit the production of inflammatory cytokines. Both primary glial cells and coronal brain slices obtained from old mice secreted more IL-6 and less IL-10 in the absence and presence of LPS stimulation compared to glial cells or brain slices taken from young adults (Ye and Johnson, 2001a). Furthermore, the addition of recombinant IL-10 to glia or brain slices of aged animals restored IL-6 production to levels seen with glia or slices from brains of adults (Ye and Johnson, 2001a). Recently steady-state level of IL-10 mRNA was found also to be lower in the hippocampi of older rats compared to younger ones (Frank et al., 2006), and administering recombinant IL-10 to rats prior to peripheral injection of LPS prevented the LPS-induced depression in LTP (Lynch et al., 2004), suggesting IL-10 is important for maintaining hippocampal function during infection.

To determine a behavioral consequence of the inflammatory cytokine response in the hippocampus after activation of the peripheral innate immune system, we compared the effects of LPS on cognitive deficits in young and old mice. A previous study showed that middle-aged rats had impaired memory in the Morris water maze and impaired contextual fear conditioning after inoculation with *Escherichia coli* (Barrientos et al., 2006). The important results suggested that bacterial infection impeded hippocampal functioning presumably through a generalized effect on memory consolidation. However, our study illustrates that inflammatory stimuli can have deleterious effects on cognitive processing in old animals that are independent of generalized effects on memory consolidation.

In the current study peripheral LPS injection produced impairment of working memory in old mice compared young ones. We do not expect that LPS disrupted the retrieval of information learned beforehand, as LPS has not been shown to disrupt behavior in a previously learned task (Aubert et al., 1995; Sparkman et al., 2005b). Therefore, we postulate that LPS disrupted cognitive processing in the old animals and impeded their ability to effectively integrate the new platform position into existing schema. The results suggest that the hippocampus in old animals is vulnerable to cytokine-induced disruption of cognitive processing when the peripheral immune system is activated as would occur during an acute infection. Furthermore, this effect appears to be independent of generalized amnesia or effects upon memory consolidation, as animals' behavior was assessed *after* acquisition of the task. These behavioral results are consistent with hippocampal dysfunction, as the hippocampus seems to be a critical component for forming relational representations and strategic flexibility in the Morris water maze (Squire, 1992). Additionally, the effect of LPS on cognitive processing is consistent with previous findings (Gibertini, 1996; Sparkman et al., 2006), although the effects of age may be dependent upon the parameters utilized during testing that may alter task difficulty. In the current study, both young and old animals had been trained to the same performance criteria prior to the test day. This is important in that both age groups demonstrated the same capacity to effectively learn the task prior to treatment. However, old animals were less effective in locating the platform when challenged with LPS. Therefore, this may be a useful model of acute cognitive confusion associated with peripheral infection in the elderly. In any case, these results suggest that to treat acute cognitive disorders it might be useful to minimize the brain inflammatory response that is associated with peripheral infections.

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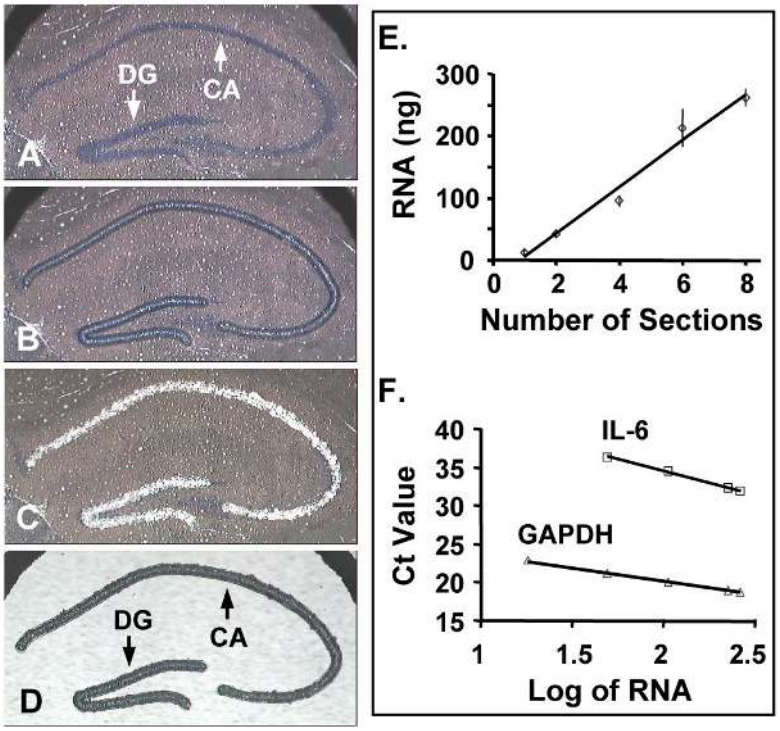

Figure 1.

Laser capture microdissection (LCM) of hippocampal tissue. Prior to determination of steady-state levels of inflammatory cytokine mRNA the DG and CA regions were separated from surrounding hippocampal tissue by LCM. (A) An intact cresyl violet-stained coronal brain section that reveals the CA neuronal cell layer and DG in a young adult mouse brain; (B) the same section after the CA regions and DG were outlined using a 15 μm laser spot with a power setting of 35 mW and pulse duration of 6 ms; (C) the section after the CA regions and DG were captured on to an Arturus CapSure HS LCM cap (note the surrounding tissue remains intact); and (D) the CA regions and DG that were captured. Increasing numbers of hippocampal neuronal cell layers (D) were pooled to determine the minimum needed to measure inflammatory cytokine mRNA by real-time PCR without RNA amplification. (E) The number of LCM sections pooled was positively correlated with the amount of RNA extracted ($r=0.9782$, $p < 0.01$). (F) In real-time PCR, as the amount of starting RNA (logarithm) increased, the Ct values for GAPDH ($r=0.9991$, $p < 0.001$) and IL-6 ($r=0.9956$, $p < 0.001$) decreased. As described in the results it was determined that a minimum of four LCM sections were needed to provide enough RNA for our real-time PCR protocol.

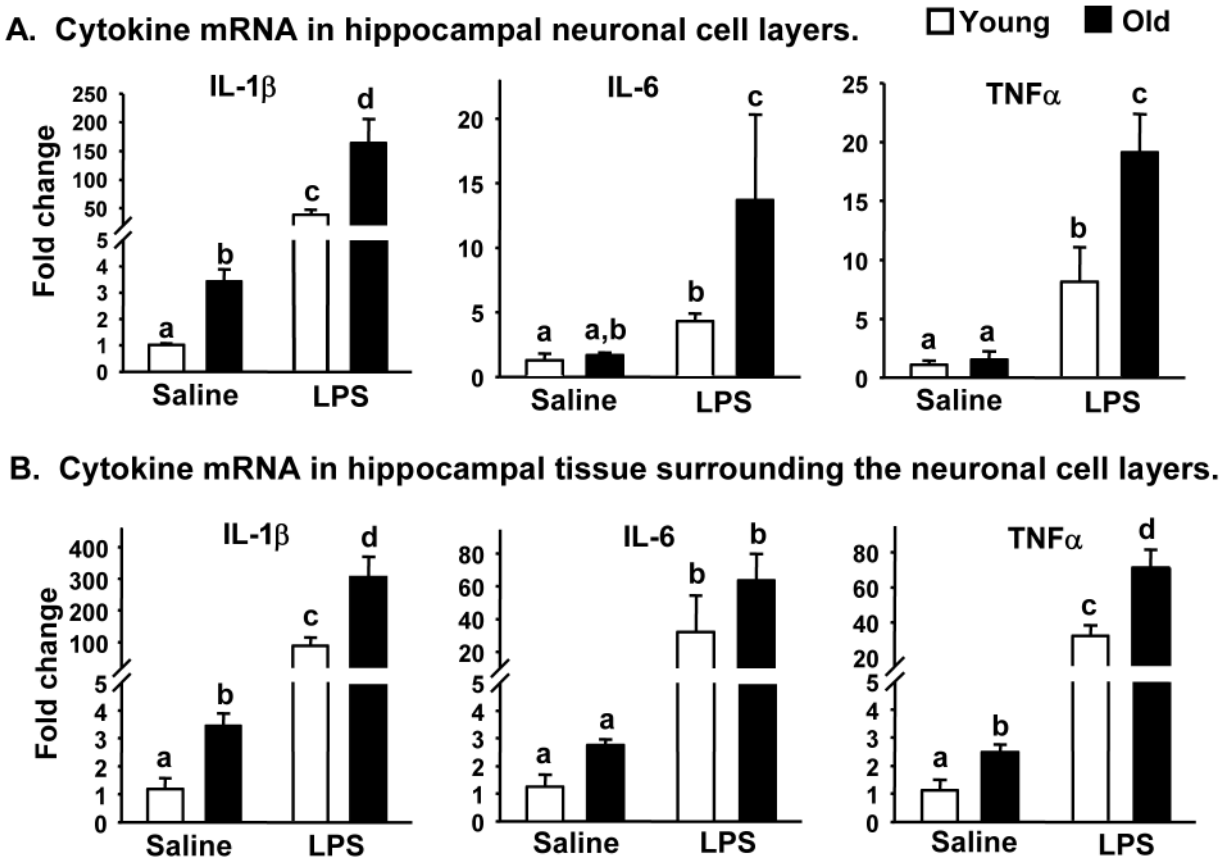

Figure 2.

Inflammatory cytokine mRNA in hippocampal tissue that was separated by laser capture microdissection. Young and old mice were injected i.p. with LPS or saline and killed 4 h later for tissue collection. Inflammatory cytokine mRNA levels in hippocampal neuronal cell layers (DG and CA regions) (A) and surrounding hippocampal tissue (B) were determined by real-time PCR. Bars represent mean ±S.E.M. Means with different letters are significantly different from each other, $p < 0.05$.

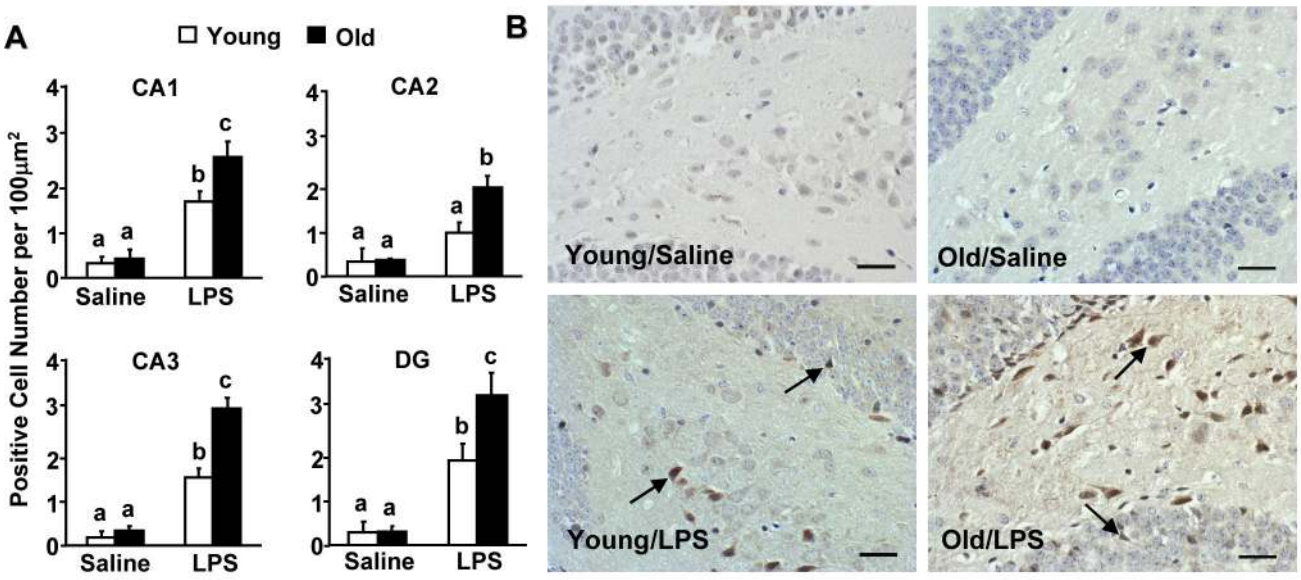

Figure 3.

Immunohistochemical staining of IL-1β-positive cells in hippocampus. Young and old mice were injected i.p. with LPS or saline and 4 h later their brains were collected. Bar graphs summarize the number of IL-1β-positive cells per 100μm^2 (A). Bars represent mean ±S.E.M. Means with different letters are significantly different from each other, $p < 0.05$. Pictures are representative photomicrographs of the DG for an old and young mouse given LPS or saline at a magnification of 40×. (B) Scale bar = 25 μm.

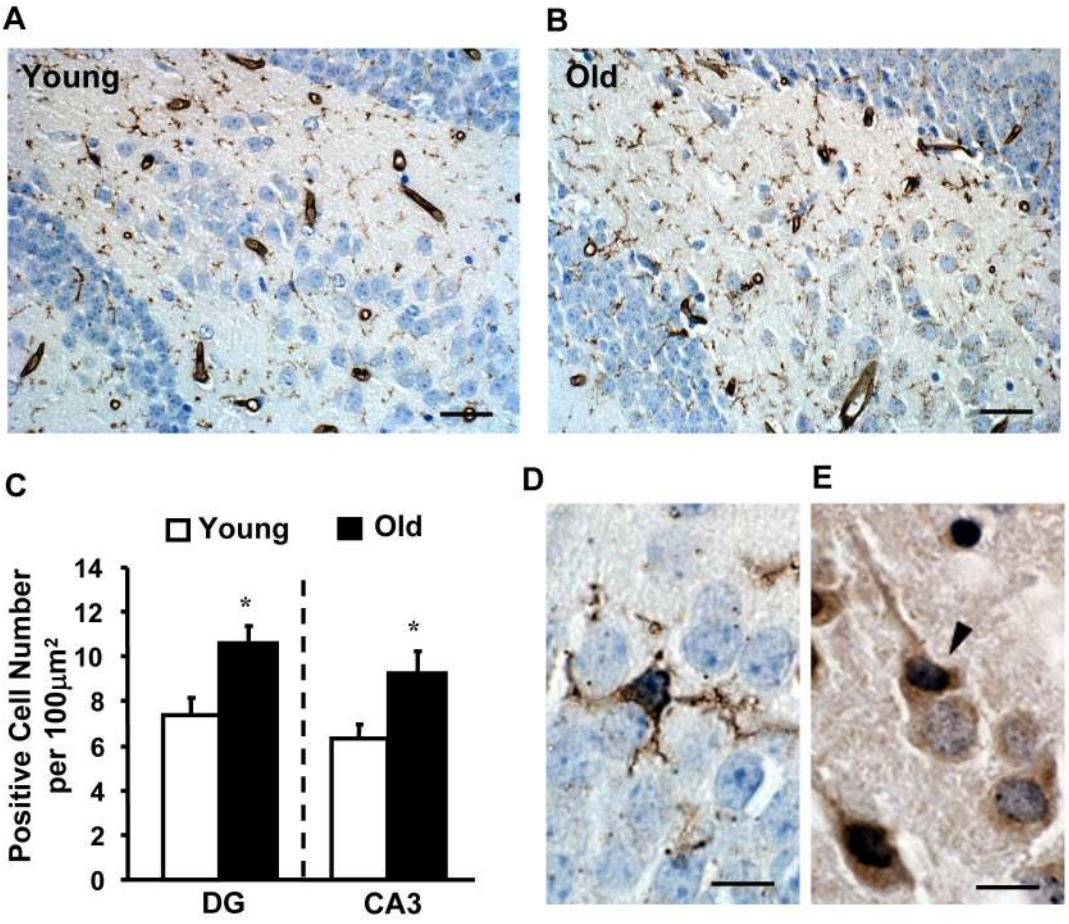

Figure 4.

Tomato lectin staining of microglia in hippocampi of young adult and old mice. Representative photomicrographs showing at 40×magnification microglia stained with tomato lectin in the DG of young adult (A) and old (B) mice. Scale bar = 25 μm. The bar graph (C) summarizes the number of microglia that stained with tomato lectin per 100μm^2 for both the DG and CA3 regions in young and old mice. Data are presented as mean ± S.E.M. Means with different letters are significantly different from each other, $p < 0.05$. The photomicrographs to the lower right show a tomato lectin stained microglial cell (D) and an IL-1β-positive cell (E) at 100× magnification within the DG. Scale bar = 10 μm.

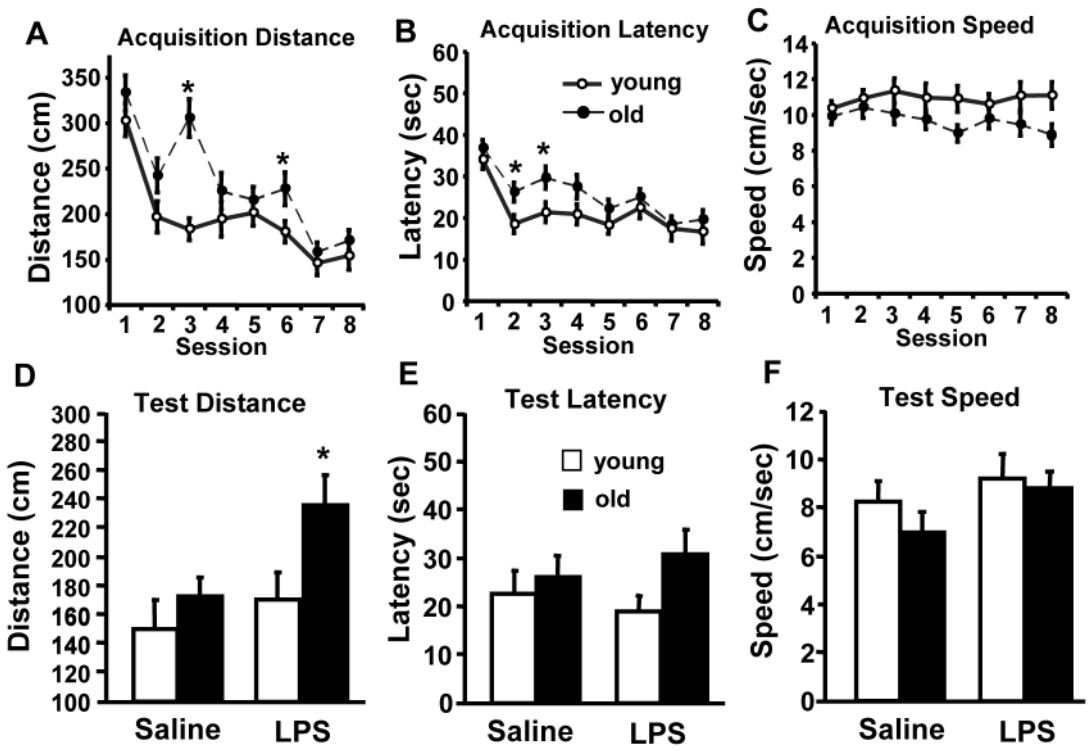

Figure 5.

Effects of LPS on performance of young and old mice in the matching-to-place test. Young and old mice were trained in the radial arm water maze for 8 d. After the 8 d acquisition training mice were injected with saline or LPS and performance in the matching-to-place test was evaluated 4 h later. Performance of acquisition and test phase in young (white circles) and old (black circles) mice was shown in mean distance (A and D), latency to find the platform (B and E) and swim speed (C and F). Data are presented as mean±S.E.M. * - denotes significant difference between groups as determined by Fisher's PLSD ($p < 0.05$).