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The Effects of Lipopolysaccharide-induced Neuroinflammation on Learning and Forgetting in Juvenile Rats

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The Effects of Lipopolysaccharide-induced Neuroinflammation on Learning and Forgetting in Juvenile Rats.

by

Michele Barry

A Thesis Submitted In Partial Fulfillment of the Requirements for the
Master of Science in Experimental Psychology with a Concentration in Behavioral Neuroscience

In

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SETON HALL UNIVERSITY
College of Arts & Sciences

APPROVAL FOR SUCCESSFUL DEFENSE

Masters Candidate, Michele Barry, has successfully defended and made the required modifications to the text of the master's thesis for the M.S. during this Spring Semester 2016.

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Abstract

The inability to remember events experienced very early in life is referred to as Infantile Amnesia (IA) and has been observed in both humans and animals. Over the years interest in the phenomenon waned, but has recently increased with the discovery of new neurobiological methods to study brain function (e.g., Callaghan, Li & Richardson, 2014). The neurobiological mechanism behind IA has yet to be determined, but several innovative theories have been developed with these new research methods. The neurogenesis hypothesis theorizes that increased neurogenesis during early development disrupts previously established memories. The hippocampus, an area that mediates both the memory of a fearful experience and the memory of a context is an area that undergoes neurogenesis lifelong but especially early in development. The increased amount of neurogenesis in the hippocampus early in life may disrupt the memory of fearful contexts in young rats. The current study examined the effect of lipopolysaccharide (LPS), on the memory for a context paired with foot shock in developing rats using the *context preexposure facilitation effect* (CPFE) procedure. LPS is an endotoxin that activates the immune system and reduces neurogenesis in the process. Rats exposed to a context at 24 days of age and shocked, after a 22 day retention interval showed less freezing the next day than those tested after a 2 day retention interval, suggesting they forgot the context cues over time, a trait suggestive of IA. Rats injected with LPS showed significantly lower rates of freezing compared to saline-treated rats at both retention intervals, thus showing overall poorer performance rather than reduced forgetting at the longer retention interval. The results from the current study fail to support the neurogenesis hypothesis. Implications of using LPS for a test of the neurogenesis hypothesis are discussed.

Keywords: neurogenesis hypothesis, lipopolysaccharide, memory, infantile amnesia, CPFE

The Effects of Lipopolysaccharide-induced Neuroinflammation on Learning and Forgetting in Juvenile Rats

Infantile Amnesia (IA) is a term used to describe the lack of memories retained from the first few years of life in humans and the faster rate of forgetting seen in young humans and animals (Howe, 2011) even when the initial learning has been equated in the younger and older subjects (Campbell & Spear, 1972). Today researchers believe that IA persists until about 3 or 4 years of age (MacDonald, Uesiliana, & Hayne, 2000; Mullen, 1994; Usher & Neisser, 1993) and memories from before this age are not typically recalled in later childhood and adulthood. As we age, the age of our earliest memory increases. Generally adults cannot remember events before the average age of 3.5 whereas children have been shown to be able to remember events from age 2-3 over an extended period of time (Peterson & Parsons, 2005; Peterson & Whalen, 2001). A key component of IA that differentiates it from other forms of loss of memory is that forgetting during this period is higher compared to when the organism has matured past this period, over the same retention interval (Spear, 1979). Researchers have also highlighted that IA isn't a deficit in the ability of young subjects to learn. It has been argued that the initial period of infancy is actually "a period of exuberant learning" (p. 2), where associations are learned at a rapid pace (Rovee-Collier & Giles, 2010).

Early animal research demonstrated differences in memory retrieval in rats at various ages. Numerous studies (Campbell & Campbell, 1962; Campbell, Jaynes & Misanin, 1968; Campbell, Misanin, White & Lytle, 1974) trained rats of all ages on various learning tasks (e.g., fear condition, light/dark discrimination), and tested their performance on the task at different retention intervals. They found that rats were able to accurately complete the task during training, and showed good retention after a short interval. But a disparity was seen in longer

retention intervals in the different age groups. Rats that were older at the time of training were able to successfully perform the task, but younger rats did not, suggesting rapid forgetting or IA. Despite these early empirical studies demonstrating IA in rodents, interest in the phenomenon declined. However, recently some researchers have argued that a return to the study of IA in conjunction with new neurobiological methods could help better understand the neurobiology of memory in general (Callaghan, Li & Richardson, 2014; Josselyn & Frankland, 2012; Mullally & Maquire, 2014). Both animal and human research has failed to identify the neurobiological mechanisms behind IA as of yet (Callaghan, Li & Richardson, 2014) and further research is needed.

Theoretical Views of Memory

When discussing memory it is important to operationally define what memory means, this is especially important when comparing animals and humans. Traditionally memory can be broken down into short-term memory and long-term memory, the latter of which is important in IA. Short-term memory is conceptualized as a limited store of information for a temporary period of time and easily interfered with, whereas long-term memory is more stable and resistant to interference (Cowan, 2008; Santini, Huynh & Klann, 2014; Warrington, 1982). Largely as a result of adult human case studies in neurology and animal neuroscience research (see Squire, 2004) long-term memory has been conceptualized as consisting of different types, each presumed to be supported by different brain structures. Long-term memory can be divided into declarative (or explicit) and nondeclarative (or implicit). Nondeclarative memories include those that involve procedural learning (skills and habits), classical conditioning, and nonassociative learning, a main focus of animal research (Squire & Zola-Morgan, 1988). In these paradigms the animal typically learns a task over many repeated trials and then memory for this task is inferred

from the successful acquisition of the task, such as learning a discrimination task for a food reward. Animal research has also been performed with tasks involving declarative memories. In humans declarative memories involve conscious declaration of facts or events. Declarative memories may include factual information (George Washington was the first president of the USA) or memory of a single event, such as remembering where your eighth birthday party took place (also referred to as episodic memories). These declarative memories are the types of memories impacted by IA, whereas nondeclarative memories, such as learning the procedure of walking, remain intact (Mullally & Maguire, 2014). You may not remember that your first steps happened at Aunt Nancy's wedding, but you have no trouble remembering how to walk.

Why are some memories so prone to forgetting, while other are not affected? Since animals (and pre-verbal infants) cannot declare their factual and episodic memories declarative memory must be identified independent of conscious recollection. Mullally and Maguire (2014) suggest that there are specific characteristics of declarative memory that are easily identifiable in animals and pre-verbal infants. Declarative memories are sensitive to certain experimental manipulations that non-declarative memories are not. Some of these variables include retention interval, study duration or context. Declarative memories are also dependent on the hippocampus, and impaired when the hippocampus is damaged.

Research has supported the notion that multiple neural systems are involved in long-term memory and the hippocampus seems to be a critical area involved in declarative memory, especially episodic memory (Mullally & Maguire, 2014). The numerous systems involved in memory may develop at different rates, causing discrepancies in how memories from different systems are encoded. The neuromaturational model theorizes that infants simply lack the neural mechanisms for episodic memory storage, and that further maturation is needed for this process

to develop (Hayne, Boniface & Barr, 2000). The system involving the hippocampus (the limbic system) is seen as a slower developing system than others, making it one of the last to fully develop. The immaturity of the system that includes the hippocampus could explain why episodic memories from early in life are lost and impossible to recover (Bachevalier, 2013; Hayne, Boniface & Barr, 2000).

Not all researchers have accepted the theory of multiple brain systems and differential development of these presumed memory systems. Rovee-Collier, for example argues against a dissociation of memory systems in development and argues for a unitary, ecological model of memory (Rovee-Collier, 1997; Rovee-Collier & Giles, 2010). According to this ecological model of infant development there may not be a difference in memory processing in infants and adults, but rather as we age the information we choose to encode for learning changes. The ecological model proposes that selective attention at different ages has been mistakenly identified as two different memory systems instead of one unitary system. More rapid forgetting in infants than in adults is described as a memory deficit by the multiple brain system theory but as an adaptive survival strategy to “prune” memories by ecological theory. One neurobiological mechanism that may be incorporated by either view is neurogenesis.

Neurogenesis and Neuroinflammation

At the time of birth the brain already contains most of its neurons. As we age there is a rapid increase in the amount of connections between these neurons that are shaped by our experiences (Greenough, Black, & Wallace, 1987). Recent studies using new neurobiological techniques have suggested that neurogenesis, the formation of new neurons, continues in certain areas of the brain throughout the lifespan. These newly discovered techniques have identified

neurogenesis as a factor that may be related to alterations in memory, and has spurred a revival in IA research. Within the hippocampus the dentate gyrus undergoes lifelong neurogenesis, one of the only brain regions to continue this process through the subjects lifespan (seen in rodents, humans, and primates) (Aimone, Wiles, & Gage, 2006). Both human and nonhuman brains produce thousands to tens of thousands of new neurons each day, most of which takes place within the hippocampus (Eriksson et al., 1998; Shors, 2014). An estimated 17,000 new neurons in early adolescent rats and 9,000 in young adult rats are produced each day in the dentate gyrus in the hippocampus (Cameron & McKay, 2001; Curlik, DiFeo & Shors, 2014).

Kitamura et al. (2009) demonstrated that in adult rodents impairing the neurogenesis process within the dentate gyrus seemed to decrease the amount of contextual fear, which is a hippocampus dependent ability. The amount of hippocampal neurogenesis is believed to have a large impact on the hippocampus-dependent period of fear memory. Neurogenesis in the hippocampus could potentially facilitate extinction through new learning, rather than through unlearning of the task. In accordance to this theory a higher level of neurogenesis in the hippocampus would help clear the fear memory from the hippocampus by way of this new learning, not unlearning (Matsuoka, 2011).

In the brain there is a delicate balance between neural plasticity and making sure that the incorporation of new memories during the neurogenesis process doesn't overwrite or corrupt older neurons and their memories. If old neurons get overwritten by new neurons, it is assumed that the old memories will be lost (or be difficult to retrieve), resulting in forgetting (Abraham & Robins, 2005). Neurogenesis in the hippocampus continues through most of the lifespan in the subgranular zone of the dentate gyrus (Ming & Song, 2005; Zhao, Deng, & Gage, 2008). The new neurons bind to existing synapses in the hippocampus (Toni et al., 2008) creating the

potential for new learning to occur. Various studies have demonstrated this by promoting neurogenesis in adult mice and finding that the process facilitates new memory formation in the hippocampus (Sahay et al., 2011; Stone et al., 2011). Although neurogenesis in the hippocampus occurs across the lifespan the rate of neurogenesis in the hippocampus is highest in infancy and declines with age (Kuhn, Dickinson-Anson, & Cage, 1996; Seki & Arai, 1995). Therefore it is logical to reason that the highest amount of forgetting as a result of restructuring during neurogenesis should occur during infancy when neurogenesis is at its highest. This has led to the neurogenesis-induced-forgetting hypothesis, pointing to neurogenesis as a possible contributing factor to infantile amnesia (Josselyn & Frankland, 2012). A recent study demonstrated that high amounts of neurogenesis interrupt established hippocampal memories (Akers et al., 2014). Decreasing the amount of neurogenesis through pharmaceutical interventions during infancy may help facilitate memory of hippocampal mediated tasks and events. Akers et al (2014) used a DNA alkylating agent, temozolomide (TMZ), to reduce the amount of neurogenesis in the hippocampus pharmaceutically in infant mice. Infant mice were preexposed to a context and then treated with TMZ for four weeks. Treated mice had statistically significant improved memory for the context which was displayed through higher rates of freezing.

Given together the high amount of neurogenesis that occurs during early development and the theory that neurogenesis may increase forgetting of early memories, it is feasible that decreasing neurogenesis at the time of the experience may help decrease forgetting. One method that can be used to decrease neurogenesis is through lipopolysaccharide (LPS) administration. LPS is an outer membrane component of gram-negative bacteria that acts as a strong stimulator of immunity (Alexander & Rietcghel, 2001). LPS activates toll-like receptors on immune cells which induces cytokine release. The distinct pattern of cytokine release regulates inflammation

(Singh & Jiang, 2003) and triggers neuroinflammation. This neuroinflammation can result in cognitive deficits in adult animals (Bilbo et al., 2005) but in developing animals cytokine activation may reduce amnesia by limiting neurogenesis.

The hippocampus is especially susceptible to inflammation because of its high amounts of receptors for inflammation mediators (Green & Nolan, 2014). Inflammation increases microglia activation, which impairs basal neurogenesis and neurogenesis in the hippocampus (Ekdahl, Claasen, Bonde, Kokaia, & Lindvall, 2003). Neurogenesis could possibly be reduced by neuroinflammation, but the underlying mechanism is unidentified (Monje, Toda, & Palmer, 2003). Monje et al (2003) hypothesized that it could be the result of hypothalamic-pituitary-adrenal (HPA) axis stimulation, changes in the progenitor and neuro-vasculature cells, or as a result of activating microglia on the precursor cells.

Nevertheless, LPS injections have effects other than inhibiting neurogenesis that results in learning deficits in mice (Sparkman, Martin, Calvert, & Boehm, 2005; Shaw, Commins, & O'Mara, 2001) although the precise mechanism behind this remains unknown (Lee et al., 2008). Lee et al (2008) demonstrated that systemic injections of LPS-induced cognitive deficits, particularly in memory, that may be the result of increased amyloidogenesis. They also found that the LPS treated rats displayed a higher rate of apoptotic cell death *in vivo*. Harré et al. (2008) found that one injection of LPS to induce inflammation showed age-dependent changes in mRNA levels of *N*-methyl-d-aspartate receptors (NR), as well as produces long-lasting changes in the hippocampal and cortex NR mRNA that could last longer than two months. Also of interest, Harré et al. (2008) observed reduced NR1 mRNA from the hippocampus of rats injected with LPS on days P5, P30, and P77; moreover P50 and P30 rats displayed learning and memory deficits. NR is considered to be necessary for the hippocampus to function optimally and for

hippocampally mediated tasks to be performed successfully (Morris, Anderson, Lynch, & Baudryl, 1986). Fan et al. (2008) observed deficits in the juvenile rat hippocampus, as well as in the dopamine neurons of the substantia nigra, 16 days post LPS injection. These alterations in the brain were associated with neurological, learning, and memory deficits (Fan et al., 2005). Another study found that LPS injections resulted in an 85% decrease in new neurons in the subgranular zone and the granule cell layer, while no changes were detected in mature hilar neurons (Ekdahl et al., 2003). LPS has been shown to impact how many newly formed cells survive, even after just one ip injection (Kohman & Rhodes, 2013; Monje et al., 2003) with some studies showing an immune response in adult rats just five hours after injection (Fujioka & Akema, 2010). Injections of LPS given during a period of consolidation (immediately after a conditioning session) have been found to disrupt contextual fear conditioning in rats (Holden, Overmier, Cowan, & Matthews, 2004).

Despite these deleterious effects of LPS treatment on learning and performance Holden et al. (2004) hypothesized that low level immune system activation may improve learning, perhaps by disrupting neurogenesis. Therefore low doses of LPS may actually improve consolidation of memory in young developing learners, rather than disrupt memory performance. From the perspective of the multiple memory systems view of the ontogeny of memory it may appear counterintuitive that a defensive immune system response to a physical insult on the body results in the amelioration of a developmental deficiency. On the other hand the ecological view of the ontogeny of memory can easily incorporate LPS-induced improvement in memory within the list of the deleterious effects of LPS – that is, by improving memory LPS treatment is interfering with the adaptive value of forgetting during a time of exuberant learning (Rovee-Collier & Giles, 2010).

Contextual Fear Conditioning

Of recent research interest is understanding the mechanism for how declarative memories are consolidated. Ressler and Mayberg (2007) reviewed several animal studies that suggest that memories are not immediately consolidated and made permanent. For a few hours after the initial experience the memory exists in a more temporary state before being consolidated a few hours or days later and becoming a more lasting memory (Matsuoka, 2011). During the consolidation phase many changes are happening at once at the molecular, synaptic, and neurotransmitter level (McGaugh, 2000). When memories of fearful situations are being consolidated it is believed that the hippocampus, as well as the amygdala, and the medial prefrontal cortex are involved (Nemeroff et al., 2006). The hippocampus has been implicated in the encoding of memories of places and events (Eichenbaum, 2004). The hippocampus is also implicated in short-term memory and thought to be involved in being fearful of a context that is associated with a fearful memory. It may also serve as a storage and processing area for new memories before they are consolidated into long term memory.

In order to examine the effect of LPS treatment on the consolidation of a declarative memory (i.e., increased or decreased forgetting) in animals a hippocampally-mediated task is needed that is learned quickly (episodic-like memory) and is sensitive to parameters such as retention interval and context (Mullally & Maguire, 2014). Contextual tasks that do not have an auditory component are hippocampally mediated (Rudy, Huff, & Matus-Amat, 2004). Early papers by Kim and Fanselow (1992) and Phillips and LeDoux (1992) identified that damage to the hippocampus impaired contextual fear conditioning, but did not impact the fear responses to an auditory cue. These findings highlight the theory that a functional hippocampus is needed for contextual fear conditioning and consolidation of fearful memories. This hypothesis is still of

debate as mixed results have been reported with varied timing of lesion, lesion technique, and site of lesion. Fanselow (1999) posited that the hippocampus functions to mentally join the many independent features of the context into one mental representation of the context (a conjunctive model of the context) that is then associated with the aversive unconditioned stimulus. A damaged hippocampus prevents the subject from developing a conjunctive model of the context and therefore disrupts contextual fear conditioning (Rudy, Huff, & Matus-Amat, 2004).

Fanselow (1999) introduced the *context preexposure facilitation effect* (CPFE) as evidence that is consistent with his conjunctive model view of hippocampally-mediated contextual fear conditioning. The CPFE is an extension of a phenomenon that he coined the *immediate shock deficit*. This deficit is observed when a rat is placed into a context and immediately shocked; they demonstrate no fear (freezing) when later tested in the same context. But if the rat is preexposed to the context repeatedly the day before, and then shocked in the same context the next day, at testing 24 hrs later they now demonstrate fear to the context (Kiernan & Westbrook, 1993). This context preexposure procedure ameliorates the immediate shock deficit and was coined the *context preexposure facilitation effect*. It is assumed that an immediate shock with no prior exposure fails to produce a fear response due to the rats lack of representation of the context associated with the shock (there was no opportunity to establish a conjunctive model of the context). When the rat is preexposed to the context it has time to sufficiently create a mental representation of the context to associate with the shock. Rat studies have shown evidence that the CPFE is dependent on the hippocampus (Rudy, Barrientos, & O'Reilly, 2002). Anterograde (after conditioning occurs) neurotoxic lesions in the dorsal hippocampus impaired the rat's ability to demonstrate the CPFE. A study conducted by Berrientos, O'Reilly, and Rudy (2002) demonstrated that if a rat is injected bilaterally into the dorsal hippocampus with a protein

synthesis inhibitor, anisomycin, the CPFE is abolished; presumably because memory consolidation of the context is impaired.

Robinson-Drummer and Stanton (2015) used the CPFE to examine aspects of context memory in juvenile, adolescent and adult rats as a way to study the neural basis of infantile amnesia. Rats are considered to reach sexual maturity around PND (post natal day) 50-60 (Spear, 1979). The results from Robinson-Drummer & Stanton (2015) demonstrated that rats that were preexposed to a context at PND 24 will show the CPFE at PND 26, but not 22 days later at PND 46. This effect was described as infantile amnesia and was not displayed at shorter intervals (24 hours, 8 days, or 15 days). The lack of CPFE at PND 46 supports the hypothesis that the memory of the context was forgotten, potentially due to the increased neurogenesis in the hippocampus during a key developmental stage. Based on the findings of Robinson-Drummer & Stanton (2015) it can be theorized that by following the same preexposure procedures, at PND 46 rats that show the immediate shock deficit do not remember the preexposure, suggesting infantile amnesia. If rats are treated with LPS following context preexposure and display the CPFE on PND 46, they will have remembered preexposure and will not display signs of infantile amnesia.

In the present study the CPFE will be used to test the interesting paradoxical hypothesis that emerges from the research on neurogenesis-mediated infantile amnesia and LPS-induced reduction in neurogenesis. While LPS can lead to learning and memory deficits in adult and young rats, it may have an altogether separate effect on juvenile rats under certain conditions. In developing rats a reduction of the high rate of neurogenesis by LPS treatment may ameliorate the loss of context memories established in early development. I hypothesize that 24 day-old rats who receive context preexposure and an injection of LPS will show a context preexposure effect at 22 days post injection, whereas those who receive a vehicle injection will not show the effect

because they will fail to remember the preexposed context. Preexposure and retention interval ages are in replication of Bilbo et al. (2005) and Robinson-Drummer & Stanton (2015) respectively. These results would point to a reduction in neurogenesis by LPS-induced neuroinflammation ameliorating forgetting of the preexposed context in juvenile rats. Understanding the impact neurogenesis has on developing memories would be a step towards understanding the still unknown neurobiological mechanisms behind IA.

Method

Subjects

Eight male and 8 female Sprague-Dawley rats were purchased from Harlan Co. (Indianapolis, IN) to produce 8 litters of rats. Four male pups from each litter were assigned to one of four treatment groups resulting in a total of 32 male subjects. All of the adult animals were housed in and familiarized with the vivarium for at least 2 weeks before breeding began and were given ad libitum access to food and water. Rat pups were weaned from their mother on PND 21. They were housed in groups of 4-6 in standard shoebox cages (Allentown Caging, Allentown, NJ 08501) with Harlan Teklad™ 1.8, corn-cob bedding, with ad libitum access to food and water. The vivarium was kept on a 12 hour light/dark cycle, and within temperature ($22^{\circ} \pm 5^{\circ}$ C) conditions. All procedures were approved by the Seton Hall University Institutional Animal Care and Use Committee.

Apparatus



Figure 1. Picture of the actual conditioning chamber used to administer footshock, taken from the author. The small circular light on top of the box is a pacing light signaling when the experimenter was to score the rats behavior. An identical chamber (not shown) was to the right of the pictured chamber.

Two identical conditioning chambers (see Figure 1) containing two levers on one wall and a centrally placed food tray were used throughout the study (26.7 cm x 23.9 cm x 26.7 cm; Ralph Gebrands Instruments, Arlington, MA). The chambers were used to expose the rats to electric footshock in a unique context. The floors of each chamber contained 17-18 stainless steel rods grids (0.23 cm diameter), spaced 1.3 cm apart. The rods are wired to a generator and scrambler (ENV-416s Standalone Grid Shocker/Scrambler; Med Associates Inc., Albans, VT) that were controlled by MED PC computer software. The experimenter sent a shock activation signal to the shock generator by pushing up on one of the metal levers from the outside of the chamber. One white light bulb (6 watt, 120 volts) illuminated each chamber. On top of each chamber was a small white light that was out of the animals' view. These two lights served as

spacing lights during the tests for freezing to signal the experimenter when to make an observation of freezing in each chamber (see Procedure). A video camera was used to record each testing session and the video feed was inputted into a laptop in an adjoining room. The experimenter monitored the rats from this laptop and ANY-maze software (Stoelting Co.) ran in the background to analyze freezing behavior. Seventy percent ethanol alcohol was used to sanitize the chambers before and after each subject.

Procedure

Context preexposure. Preexposure to the test-chamber context began on PND 24 for all rats. The rats were transported from the housing cages to the testing chamber, in separate transportation cages, two at a time for preexposure to the testing chambers. The transportation cages were two identical standard shoebox cages with corncob bedding. The outsides of the plastic walls and floors of the cages were covered with black contact paper so the rats could not see out and the tops of the cages were obscured with cardboard tops with black contact paper facing the inside of the cage. Each subject was placed in the testing chamber and allowed to explore for five minutes. They then were transported back to their home cages in the same transportation cage and in the same method in which they were moved to the testing room. Once back in their home cages, they remained there for about 40 seconds before being removed and transported back to the testing room for further preexposure, replicating the preexposure procedure used by Bilbo et al. (2005). This context preexposure procedure was completed a total of 6 times per subject and was utilized because research suggests that the transportation cues are an important component of the context cues in a fear conditioning task (Bevins et al., 1997; Bilbo et al., 2005). After the final context preexposure each subject received an injection intraperitoneally (ip) of either 0.1 mg/kg LPS ($n = 16$) in a volume of 1 ml/kg, a dose found to be

effective at inducing neuroinflammation in previous research (Harré et al., 2008), or the same volume of physiological saline ($n = 16$).

Context fear conditioning training and testing. All rats were exposed to test chamber-footshock pairings after a retention interval of 2 or 22 days post ip injection (See Figure 2 for design summary). On PND 26 half of the LPS treated rats ($n = 8$) and half of the saline treated rats ($n = 8$) were transported to the testing room in the same manner as in the preexposure procedure and received a two second 1.5 mA shock 2 seconds after being placed in the chamber. The subject was then immediately removed and returned to their home cage. A 2 day retention interval rather than the typical 1 day retention interval (Robinson-Drummer & Stanton, 2015), was used to minimize the possibility that LPS-induced malaise would interfere with learning during exposure to the single context-shock pairing. Any LPS-induced illness effects (e.g., fever, malaise) were expected to have dissipated by the second day after LPS injection. The remaining rats received context fear conditioning training after a 22 day retention interval ($n = 8$ LPS treated, $n = 8$ saline treated). On PND 46 these subjects were transported to the testing room and received the same shock (1.5 mA for 2 seconds) as the other groups and were returned to their home cages. Twenty-four hours after each group received training (PND 27 for the 2-day retention group and PND 47 for the 22-day retention group) all rats were returned to the testing room. Subjects were immediately removed from the transportation cages upon entering the room and placed in the testing chamber. Freezing, defined as no movement, except necessary for respiration (Fanselow & Bolles, 1979) was observed for a period of 6 minutes. The experimenter was blind to subject's treatment condition, but not retention interval condition, during testing.

Freezing was measured in two ways: 1) experimenter time sampling ratings of freezing and 2) total freezing by the ANY-maze software. For the time sampling procedure the

experimenter used the pacing lights above each chamber to measure freezing every 3 seconds, alternating between boxes. In this way freezing was measured in each rat every 6 seconds throughout the 6 minute session. The experimenter scored for freezing live, from an adjacent room through a laptop video feed. All sessions were recorded. ANY-maze software (Stoelting, Co.) was used on a laptop computer to track freezing rates, in conjunction with freezing rates recorded by the experimenter. Two measures of freezing behavior were used to compare the two methods when measuring freezing behavior of young rats, and to serve as a reliability check.

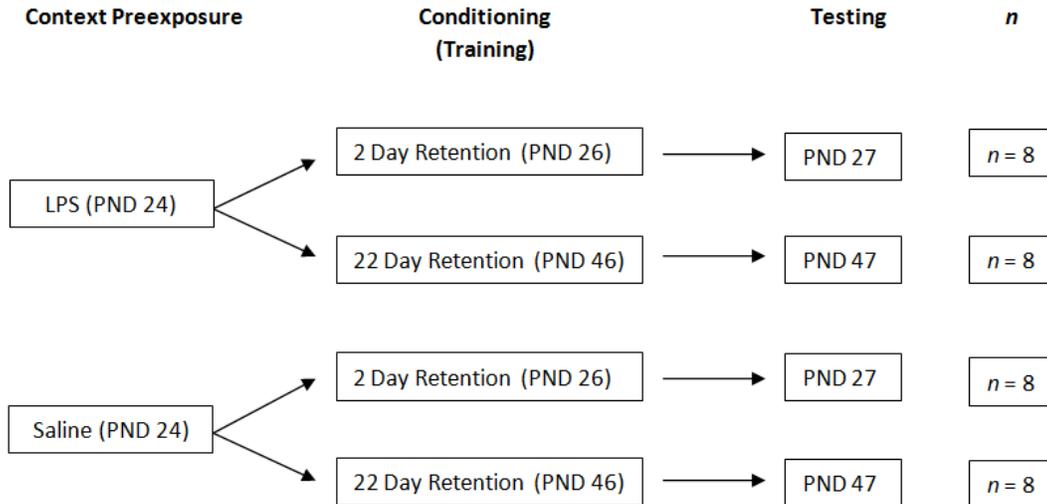


Figure 2. Study design (*n* = 32). Subjects received a LPS or saline injection on PND 24 after context preexposure. Training occurred after either a 2 or 22 day retention period (PND 26 or PND 46) after preexposure and testing for conditioned fear occurred 1 day after training.

Data Analysis

A 2 (treatment: LPS, Saline) x 2 (Retention interval: 2, 22 days) between groups analysis of variance (ANOVA) was performed to analyze the data. Statistical significance was set at $p <$

0.05. Effect sizes were interpreted using Cohen's (1992) guidelines for partial eta squared: small (.01), medium (.06) and large (.14).

Results

Experimenter Ratings of Freezing

The observational data was converted to percent freezing with the formula: number of observations with freezing behavior / total number of observations x 100. Figure 3 shows the mean percent freezing scores observed by the experimenter in the 4 groups tested. Greater freezing suggests a stronger association of the preexposed context conditioned stimulus (CS) with the footshock unconditioned stimulus (UCS). Reduced freezing suggests poorer associative learning. Overall LPS disrupted learning in treated rats over rats that received saline. The 2 day and 22 day retention interval groups that were injected with LPS showed lower rates of freezing (2 day $M = 51.49$, $SD = 33.07$; 22 day $M = 42.21$, $SD = 32.23$) than the corresponding saline-treated groups (2 day $M = 80.24$, $SD = 14.94$; 22 day $M = 56.98$, $SD = 30.81$). Moreover, both LPS and saline treated rats tested at the 22 day retention interval showed a lower rate of freezing than those tested at 2 days, suggesting forgetting of the context cues over time. A 2 (treatment: LPS, Saline) x 2 (Retention interval: 2, 22 days) between groups analysis of variance (ANOVA) was performed on freezing rates. Statistical significance was set at the traditional $p < 0.05$. The ANOVA revealed a statistically significant main effect of treatment, $F(1,28) = 4.583$, $p = .041$, $\eta^2_p = .141$, with a large effect size supporting the impression that LPS disrupted the acquisition of conditioned freezing overall. However, there was no Treatment x Retention interaction, $F(1,28) = .474$, $p = .497$, $\eta^2_p = .017$, or a main effect of retention interval, $F(1,28) = 2.563$, $p = .121$, $\eta^2_p = .084$, although a medium effect size was found. My hypothesis was based on

replicating a decrease in freezing behavior over the 22 day retention interval as reported by Robinson-Drummer and Stanton (2015). Because ANOVA does not test directional hypotheses, to better test my a priori directional hypothesis I used a half-tailed test based on a generalization of chi-square as suggested by Karl Wuensch (2006). The half-tailed t-test was done by taking the p value from the main effect of retention interval and dividing by 2, which resulted in a marginally significant difference ($p = .06$) between the 2 day and 22 day retention intervals.

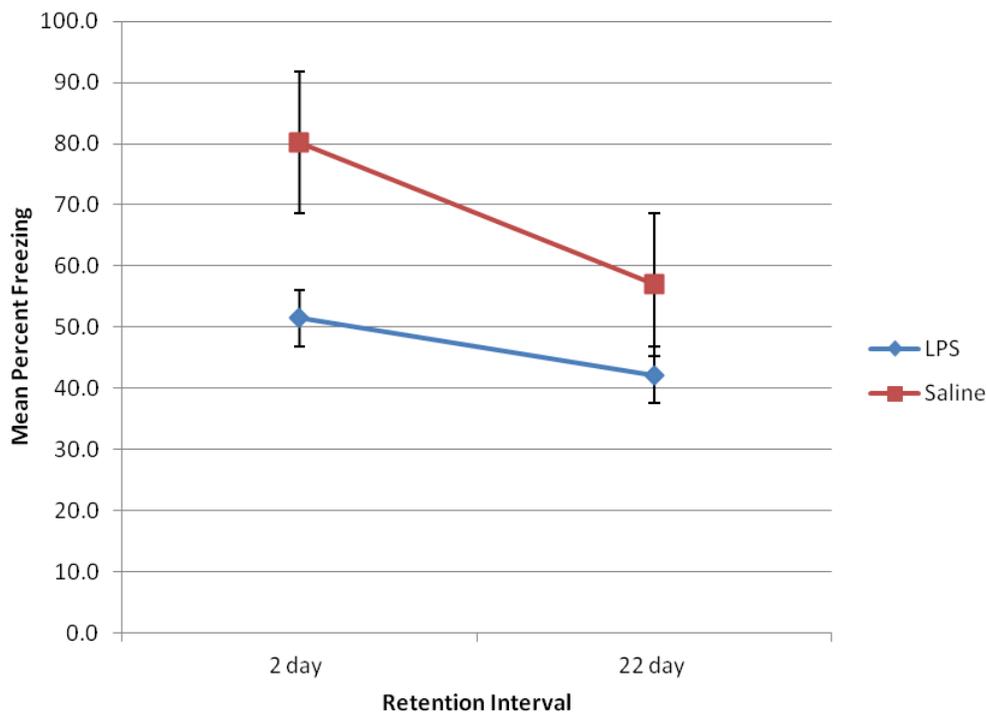


Figure 3. Mean percent freezing of the 4 treatment groups (LPS-2, LPS-22, Sal-2, Sal-22) as observed by the experimenter. The error bars are standard errors of the mean.

ANY-maze Measure of Freezing

For the ANY-maze data percent freezing was calculated as: total minutes freezing / session length in minutes x 100. As before, a 2 (treatment: LPS, Saline) x 2 (Retention interval:

2, 22 days) between groups analysis of variance (ANOVA) was performed on the data. Figure 4 shows the percentage of total freezing during the 6 minute test session as calculated by the ANY-maze software. The results are very similar to the observation data described previously. The main effect of LPS-treatment was again statistically significant with a large effect size, $F(1,28) = 9.126, p = .005, \eta^2_p = .246$, but the main effect of retention interval was again not statistically significant with a small effect size, $F(1,28) = .797, p = .379, \eta^2_p = .028$. The treatment by retention interval interaction was also not significant, $F(1,28) = .219, p = .644, \eta^2_p = .008$. A half-tailed test of retention interval did not yield a significant value ($p = .19$) for this data, however.

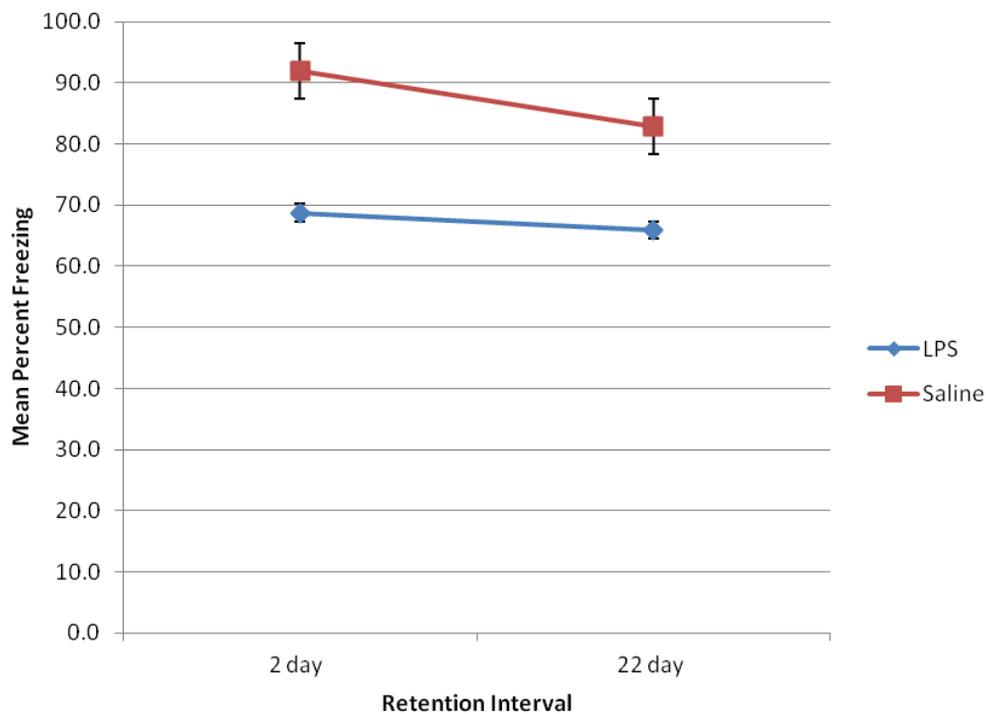


Figure 4. Mean percent freezing of the 4 treatment groups (LPS-2, LPS-22, Sal-2, Sal-22) as measured by ANY-maze. The error bars are standard errors of the mean.

Discussion

The current study aimed to examine the effect of LPS treatment on the learning of a context-shock association in juvenile (24 day old) rats after a 2 or 22 day retention interval between context preexposure and context-shock pairings. LPS (or saline) treatment occurred immediately after context preexposure. By triggering immune system activation LPS may interfere with learning by disrupting consolidation of the memory of the context and preventing the acquisition of condition fear to the context (Holden et al., 2004; Kitamura, et al., 2009; Matsuoka, 2011). However the neurogenesis hypothesis of IA suggests that because LPS disrupts neurogenesis, the forgetting typically seen at 22 days (Robinson-Drummer and Stanton, 2015) may be ameliorated by preventing new neurons from disrupting previously established memories of a context, a hippocampally mediated task. LPS resulted in significantly lower rates of freezing compared to saline rats at both retention intervals failing to provide any support to the neurogenesis hypotheses.

Saline rats froze significantly more than LPS rats, suggesting LPS disrupted overall learning. One possible explanation for this may be the dose of 0.1 mg/kg LPS used could have been high enough to produce a neuroinflammatory response, thereby causing a learning deficit, but not sufficient to decrease neurogenesis. No direct measure of neurogenesis was included in the current experiment, so it cannot be determined with certainty if neurogenesis was impacted by LPS. Another possibility is that the timing of LPS treatment was not ideal and should have been administered after 24 hours to allow for consolidation of the context memory. The LPS treated groups may have never learned the association between the shock and the context, even after context preexposure. By administering LPS immediately after context preexposure there may have been no opportunity for a memory consolidation and therefore no “engram” to be

covered up by more neurogenesis. One concern with interpreting these results is identifying the difference between learning issues and performance issues, particularly in the LPS-2 day retention interval group. As previously mentioned LPS may have disrupted learning of the contextual task, thereby decreasing fear during testing. It is also possible that 2 days after LPS injection the rats were feeling negative side effects of the drug, interfering with their performance. Anecdotally, rats injected with LPS showed no symptoms of LPS-induced sickness in the days following injection. Observations showed no difference in eating and drinking behavior between LPS and saline rats, and no noted behavioral differences. Moreover any undetected illness is unlikely to have lingered over 22 days in the 22 day retention group who also showed a deficit. LPS appears to have disrupted the consolidation (as seen in Holden et al., 2004) or eventual retrieval of the memory of the context (as seen in adult rats in Czerniawski & Guzowski, 2014 and Czerniawski, Miyashita, Lewandowski, & Guzowski, 2015).

Based upon the results of Robinson-Drummer and Stanton (2015) I predicted that rats tested at the 22-day retention interval would display less freezing than the rats tested after a 2 day retention interval. This effect of the retention interval on conditioned freezing is an important characteristic necessary to define the CPFE task as a measure of IA. In addition because the CPFE is a hippocampally-dependent task this decline in performance is consistent with the argument that there is rapid forgetting of a declarative memory. The results showed a decline in freezing across the retention interval, and the half-tailed test of experimenter data just missed the traditional cutoff for statistical significance. Interestingly the decrease in percent freezing in the Robinson-Drummer and Stanton's (2015) study when comparing their 1 day retention and 22 day retention groups was only by 10% (see their Figure 1). In the current study saline-treated groups decreased by 23.26% (looking at experimenter freezing data, implications

of different freezing measures are discussed later). One weakness in the design of the present experiment is that additional control groups were not included to compare freezing in groups that did not experience preexposure to the context. Thus it is difficult to know what level of “baseline” freezing occurred in the present experiment. For example, the relative novelty of the chamber alone elicits some freezing independent of the context-shock association. Robinson-Drummer and Stanton (2015) did include control groups that were preexposed to an alternate unfamiliar context for comparison and obtained a statistically significant effect of retention interval compared to the control groups (i.e., a statistically significant preexposure condition x retention interval interaction). Because of the lack of controls to assess baseline freezing in the present experiment confirmation of forgetting over the 22 retention interval may have been made more difficult. Nevertheless, the half-tailed test based on my *a priori* directional hypothesis resulted in a marginally statistically significant difference in retention interval in saline-treated rats. Thus, the medium-sized effect size from the ANOVA results and the directional t-test analysis provides some support for an effect of the retention interval on conditioned freezing.

Inspection of Figures 3 and 4 reveals a disparity between freezing scores for the two methods of measurement used. The difference in mean freezing scores for each of the four groups seen between the experimenter recorded data and ANY-maze data, can be explained multiple ways. Freezing is a behavior that is hard to detect and accurately measure as it can be hard to distinguish between freezing behavior and immobility produced by a lack of exploratory behavior. A discrepancy between what is defined as actual freezing may exist between the human experimenter and ANY-maze software. The experimenter reported a lower rate of freezing than ANY-maze, potentially due to a more stringent definition of freezing behavior. Other possible contributing factors include the method in which freezing was observed. The

experimenter measured freezing at an interval of every six seconds for six minutes, a total of 61 interval measurements. The number of intervals spent freezing was then converted into a percentage. ANY-maze recorded total freezing time during the session. This difference in how frequently freezing was measured within the six minute time frame may contribute to the inconsistencies of mean freezing between the two methods used. Other contributory factors are anecdotal in nature and could not be measured at the time of testing. When the experimenter viewed the live feed of the animal in the testing chamber to determine freezing rates, the feed displayed an indicator of the subject being tracked by ANY-maze. On numerous occasions the experimenter observed inaccuracies in this tracking location, where the tracking location did not match the animal's movement. Possible contributory factors that could have caused the ANY-maze software to not accurately track the subject include an insufficient amount of light in the chambers, not enough of a contrast between the color of the animal and the background, the small size of the animals, and having to track animals from a side view, rather than a view from above (which has a higher accuracy rate). Despite the limitations of the two tracking methods used both approaches showed a statistically significant main effect of LPS treatment on conditioned freezing with a large effect size.

The present study used the CPFE to measure the effects of LPS on contextual fear conditioning in developing rats but further research needs to identify if this procedure is a reliable and valid measure of IA. Future research using the CPFE should ensure to include the proper control groups to help shed light on the theoretical issues regarding the ontogeny of memory and whether a multiple memory systems approach or an ecological unitary system approach is most useful. The effects of LPS are complex, with the present study giving evidence of a disruptive effect of LPS on learning, possibly during consolidation or contextual memory

retrieval. To better understand the effects of immune system activation on CPFE and retention interval a detailed parametric study is needed with the appropriate manipulations of LPS dose and time of injection. Because of the complex effects of LPS, to test the neurogenesis hypothesis of IA it may be beneficial for future researchers to use other methods to reduce neurogenesis.

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