EFFECTS OF CHRONIC ANTIDEPRESSANT COADMINISTRATION ON ACQUISITION,

MEMORY CONSOLIDATION, AND NEUROGENESIS AFTER REPEATED

LIPOPOLYSACCHARIDE ADMINISTRATION

by

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1. INTRODUCTION

1.1. Neural-immune Interactions

Until recently, it was assumed that the immune system and the central nervous system (CNS) operated independently to exert their effects on the body. (Cohen & Kinney, 2001; Dantzer & Kelly, 1989; Maier et al., 1994). It has recently been shown that these systems have bidirectional regulatory control over each other. Both systems play a major role in the physiological and behavioral responses to pathogen invasion. This interaction between the immune system and the CNS is thought to be highly adaptive, to keep an organism aware of its needs, such as promoting increased rest during an infected state to allow for recuperation. Given this relationship between the immune system and the CNS, it is important to focus on both systems to obtain a full understanding of the intricacies of their interaction.

Control over immune organs, such as the thymus, bone marrow, spleen, and even lymph nodes by the CNS has been shown to be largely due to autonomic nervous systems (ANS) release of catecholamines (Felten & Felten, 1991). The neuroendocrine system is also involved by regulation of activity of the hypothalamic-pituitary-adrenal (HPA) axis by cytokines, increases in corticosterone subsequently lead to increases in cytokine expression (Black, 1994; Maier et al., 1994). Not long ago, research showed that cytokines (soluble proteins that are primarily released by macrophages in the periphery and by microglia in the CNS in response to an infection), are some of the main signals that notify the CNS of immune activation in the periphery. Previous to this research, cytokines were only known to be involved in the communication between immune cells in the periphery to regulate an immune response. Moreover, the CNS helps orchestrate portions of the immune response. Certain behaviors that have been associated with illness are due to the release of cytokines,

but still not widely acknowledged by scientists are the cognitive deficits that sometimes develop during acute infection or other conditions that involve elevated cytokine levels.

1.2. Cytokines and Sickness Behavior

In addition to other cells, macrophages in the periphery release substances such as cytokines, nitric oxide, and chemokines (Maier & Watkins, 1998) following contact with a pathogen. Cytokines help coordinate and stimulate immune cells to secrete antibodies and produce memory cells to offer a faster defense in the future. They are also the major pathway of communication between immune cells in the CNS and peripheral nervous system (PNS). Cytokines enter the brain via two main pathways, 1) the neuronal route, in which vagal afferents electrically conduct inflammatory signals from the site of the infection to the CNS where microglia become stimulated and release cytokines, and 2) humoral pathways that involve production of cytokines near the circumventricular organs (CVOs) and the choroid plexus, or via active transport across the blood brain barrier (BBB) (Konsman et al., 2002). Many examples of these cytokines are named "interleukins", because of their role in the communication between leukocytes (white blood cells).

The primary cytokines that are released by macrophages after a bacterial infection are interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α) (Konsman et al., 2002). These proteins are shown to help coordinate the inflammatory response at the site of infection by attracting other immune cells that will aid in the inflammation and healing process (Maier & Watkins, 1998). Cytokines can either be classified as proinflammatory cytokines, which promote the inflammatory response or anti-inflammatory cytokines, which inhibit the inflammatory response via regulation of proinflammatory cytokines. Proinflammatory cytokines regulate a complex cascade of

changes in the organism by enlisting the help of T-cells and B-cells (producers of antibodies) which are also important in the specific defense against infection and injury (Kent et al., 1992; Maier & Watkins, 1998).

The behavioral effects of proinflammatory cytokine exposure are believed to be an adaptive response, not a symptom of bacterial infection or a sign of debilitation, and are viewed as an "evolved strategy" to combat infection and possible injury (Hart, 1988; Maier & Watkins, 1998). Some behavioral sequellae of pathogen invasion are anorexia (decreased feeding), adipsia (decreased drinking), increased slow-wave sleep, anhedonia, decreased social exploration, decreased sexual vigor, mood alterations and decreased pain threshold, that have collectively been termed "sickness behavior" (Hart, 1988; Kent et al., 1995; Konsman et al., 2002; Maier & Watkins, 1998).

One of major ways in which the body wards off infection is via the inflammatory response. With inflammation comes a fever response, marked by an increase in temperature. One reason why this is so adaptive is that microbial pathogens often cannot reproduce effectively at elevated temperatures (Kluger, 1978). Moreover, inflammation and fever also increase the intensity of the immunological response (Long et al., 1990; Maier & Watkins, 1998). Along with these adaptive effects, there are also immune-related non-adaptive events that occur in the organism, including alterations of learning and memory (Kent et al., 1995; Pugh et al., 1998; Sparkman et al., 2005). Even though there is much evidence to support the notion that cytokines (particularly IL-1β) effect behavior and cognitive abilities in organisms with infections, the physiological mechanisms that explain these decrements remain mostly unexplored.

One of the ways that researchers can stimulate the immune system is by giving lipopolysaccharide (LPS). LPS is a non-specific activator of proinflammatory cytokine release primarily from macrophages and microglia. LPS is an endotoxin that is produced from degraded cell wall of Gram-negative bacteria, and is a potent stimulator of the immune system (Borowski et al., 1998). The primary reason that researchers use LPS to model infection in animal models is that working with live bacteria and viruses represents a potential hazard to the animal and may increase experimental variability. LPS reduces this risk and allows investigators to examine effects of the immune response per se rather than the more variable effects of a live, replicating pathogen. LPS has been shown to act on Toll-like receptor-4 (TLR-4) molecules on macrophages to induce the release of cytokines, namely interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α) (Borowski et al., 1998; Larson & Dunn, 2001). As mentioned earlier, LPS-induced anorexia (decreased feeding), adipsia (decreased drinking), anhedonia, increased slow-wave sleep, decreased social exploration, decreased sexual vigor, depression, decreased locomotor activity, mood alterations and decreased pain threshold, have collectively been termed "sickness behavior"(Borowski et al., 1998; Hart 1988; Kent et al., 1995; Konsman et al., 2002; Lacosta et al., 1999; Yirmiya, 1996). These sickness behaviors are an adaptive response to acute sickness induced by a pathogen and are thought to aid in the restoration of homeostatic equilibrium (Hart, 1988; Kent et al., 1995). The behavioral changes in animals given LPS imitate the behavioral changes when animals are given cytokines (e.g., IL-1β). It is believed that these two outcomes share the same physiological mechanisms in induction of sickness behavior (Bluthé et al., 1992; Borowski et al., 1998).

1.3. Cytokines, Learning and Memory

Infections are often associated with disruptions in cognitive functions (Larson &

Dunn, 2001). Furthermore, it has been shown that disorders such as Alzheimer's disease,

Parkinson's disease, and depression have been associated with the over-expression of

cytokines (Hofman et al., 2009; Mrak & Griffin, 2001; Pollak & Yirmiya, 2002).

Proinflammatory cytokines also often contribute to learning and memory problems, both in

humans and animals, though it has not been completely determined what mechanisms are

behind the cognitive deficits (Barrientos et al., 2002; Bluthé et al., 1992). Commonalities

among the cognitive impairments indicate that cytokines, produced as a result of an

infectious pathogen, may be part of the underlying cause. However, although the behavioral

and physiological changes associated with sickness behavior have been extensively studied,

the potential learning and memory deficits have been examined to a lesser degree. In humans

it has been shown that LPS and IL-1β administration leads to learning/memory problems

(e.g., Reichenberg et al., 2001; Spath-Schwalbe et al., 1998); it has also been shown to be

detrimental in animal models (e.g., Aubert et al., 1995; Barrientos et al., 2002 Pugh et al.,

1998; Sparkman et al, 2005). For example, a number of studies showed that rodents injected

with IL-1β or LPS exhibit behavioral impairment in the Morris water maze (MWM), a test

for spatial learning/memory (Gibertini, 1996; Oitzl et al., 1993; Shaw et al., 2001; Sparkman

et al., 2005).

In a set of more compelling results, LPS and IL-1β have been shown to hinder

contextual fear conditioning. Pugh et al. (1998) showed that LPS given peripherally and

after training inhibited contextual fear conditioning, but had no effect on auditory fear

conditioning. Their methods included putting experimental subjects into a chamber in which

they received two, 2 s shocks with an intertrial interval (ITI) of 120 s. To evaluate memory,

half the rats were placed into chambers in which they were presented with an auditory

conditioned stimulus (CS) for 20 s, followed by a mild shock (US); in the contextual fear

paradigm, no auditory cue was presented. The CS was terminated upon the presentation of

the US. Immediately after this procedure, subjects were divided into four groups that were

given intraperitoneal injections of LPS at doses of 0, 0.125, 0.25 or 0.5 mg/kg then placed in

their home cage. The subjects were tested 48 h later to allow for the memory to the context

to be consolidated. Both conditions (i.e., contextual and CS fear conditioning), were then

assessed for either a fear response or an active response. Rats that were in the contextual

conditioning groups were placed into the same chambers that were used in training and

freezing responses were recorded every 10 s. Rats in the auditory fear conditioning paradigm

were given 3 min of exposure to the testing environment without CS presentation to assess

for contextual freezing behavior. After this 3 min period, the CS was presented. Freezing

was then assessed in the same manner as in the contextual-fear paradigm, every 10 s. In the

fear conditioning procedure, freezing is an indicator of memory (i.e., rats freeze because they

have learned that shock-induced pain is soon to follow the CS or exposure to the context).

LPS selectively impaired the consolidation of contextual fear conditioning but had no effect

on auditory-fear conditioning. Doses of 0.125 and 0.25 mg/kg significantly interfered with

the contextual fear conditioning while the 0.5 mg/kg dose did not relative to saline-treated

controls. The higher dose of LPS may have led to motor impairments, therefore increasing

the percent time freezing in the conditioning chamber. Furthermore, as in the tone group and

the saline control group, LPS produced no effect on freezing in an altered context or when

the auditory cue was paired with the shock. It is important to note that as it was the LPS-

treated animals that *failed* to freeze (excluding the 0.5mg/kg dose group), the effects cannot

be explained by motor impairments or altered motivation due to LPS injections. As noted

previously, injections of LPS were presumably given after a fear response was established,

and the results therefore suggest that LPS impairs some aspect of post-trial consolidation or

memory representation of the context in which the fear was established. Results also suggest

that LPS selectively impairs only certain kinds of memory processes. For example,

contextual fear conditioning (shown to be disrupted by LPS) is thought to be a hippocampus-

dependent task, whereas auditory fear conditioning (which is not disrupted by LPS) is not

believed to be hippocampus-dependent (Pugh et al., 1998).

Because contextual fear depends on a context-US association (i.e., footshock in this

case), Pugh et al. (1998) argue that if LPS interferes with contextual fear conditioning by

interfering with the acquisition of a representation of the context, then preexposure to the

context before conditioning and LPS treatment should eliminate the impairment of contextual

fear conditioning caused by LPS. On the other hand, if LPS disrupts the context-US

association, then preexposure to the context should have no effect. To examine this

hypothesis, Pugh et al. (1998) preexposed rats to the context for 2 min, 24 hrs prior to

conditioning, and the controls were just handled for the same amount of time. At this point,

the groups were not given LPS. After preexposure rats received conditioning training, and

then half the rats in both groups were given LPS doses of 0.125 mg/kg. LPS again produced

a large detrimental effect on contextual fear conditioning when LPS was given immediately

after the consolidation phase, but preexposure eliminated this effect. Therefore, the authors

argued that preexposure to the context diminished the effects of LPS on the memory

representation of the context. Coinciding with these LPS studies, Barrientos et al. (2002)

showed that IL-1β administration impedes contextual fear conditioning by interrupting the representation of the context when given directly after conditioning. They found that infusion of IL-1β bilaterally into the dorsal hippocampus reduced the effects that preexposure to the context usually has.

A study that provides additional evidence that LPS produces learning/memory decrements was reported by Sparkman et al., (2005). Four-month-old experimentally naïve C57BL/6 mice were injected intraperitoneally on day 1 of testing four hours prior to testing in a two-active avoidance conditioning procedure. Animals received 50 trials in each of 5 consecutive daily sessions. Each trial consisted of a CS or discriminative stimulus, a house light for 5 s, followed by a mild footshock (0.4mA) for up to 5 s. Each trial was separated by a 20 s ITI. Animals could learn to avoid the shock (i.e., the US), by crossing to the other side of the conditioning chamber while the CS was being presented and before the presentation of the US. This was termed an avoidance response. If the animal did not cross while the CS was presented, the US was then presented. If the animal crossed when the US was presented, the response was scored as an escape response. If the animal did not cross when either the CS or the US was being presented (total of 10 s), a null response was scored. The results of this study showed that LPS disrupted the acquisition of this task, as evidenced by the LPS-treated animals making fewer avoidance responses than did control animals. To ensure that these data were not due to motor impairments, latencies to cross to the other side of the chamber (a good indicator of motor impairment in two-way active avoidance; Sparkman et al., 2005) were recorded, and there were no significant differences between treatment groups. These findings suggest that failure to avoid by LPS-treated animals was not due to motor or motivational impairment, but rather a diminished ability to learn to avoid the footshock when

presented with the CS (optimal response). The authors argued that the LPS-treated subjects

may be less able to learn the CS-US relationship.

More recent findings from our laboratory indicate that LPS administration to animals

immediately after testing day 1 in the two-way active avoidance paradigm hinders memory

consolidation, as evidenced by a reduced number of avoidance responses, and increased the

number of escape responses. This memory consolidation deficit is also increased in aged

animals (unpublished data).

Using a variation of LPS administration, in which long-term effects were examined,

Wu et al. (2007) showed that 4 bolus injections of 1 mg/kg of LPS each, one week apart,

hindered Morris water maze acquisition and hippocampal neuronal differentiation, but not

proliferation within the dentate gyrus. LPS also induced depletion of brain derived

neurotrophic factor (BDNF) and its receptor tyrosine related kinase B (TrkB). Finally,

exercise attenuated these LPS-induced deficits, by increasing neurogenesis within the dentate

gyrus, levels of BDNF, and levels of TrkB in the hippocampus (both of which are involved in

growth and survival of cells).

These data illustrate the importance of cytokines as mediators between the immune

system and the CNS (Dantzer & Kelly, 1989). Furthermore, it has been shown that IL-1β

administration inhibits long-term potentiation (LTP), an effect that may have physiological

relevance to hippocampus-dependent learning (Kelly et al., 2001). Knowing that

proinflammatory cytokines act as mediators for deficits in learning/memory in some human

populations, such as people afflicted by chronic inflammatory disease or receiving cytokine

treatment, it seems important to look more closely at the relationship between cytokines and

learning/memory. Further research may lead to therapies to alleviate some of the side effects of drug treatments for their illness that are secondary to their primary illness.

1.4. Stress, and Immune Function: a Bidirectional Relationship

Stress activates the hypothalamic pituitary adrenal (HPA) axis that, via downstream hormonal signaling, which causes the release of adrenal glucocorticoids. Glucocorticoids can bind and activate two common receptors, the high affinity mineralocorticoid receptor and the lower affinity glucocorticoid receptor (GR) that are both found at high levels within the hippocampus (de Kolet et al., 2005). Behavioral changes resulting from acute stress generally is considered an adaptive response; chronic stress, on the other hand, can cause dysregulation of the HPA axis and affect hippocampal function and structure (Joëls et al., 2004). For example, chronic stress in rodents impairs long-term potentiation (LTP; a phenomenon some believe to be a molecular model for learning) in the hippocampal CA1 subregion, and can cause dendritic retraction in the CA3 subregion of the hippocampus (Alfarez et al., 2003; Magarinos et al., 1995).

Studies have shown that psychological stressors alone can increase cytokine levels within the brain (Nguyen et al., 2000; O'Connor et al., 2003). Cytokines in turn, can cause increases in HPA activity similar to that of psychological stressors (Anisman & Merali, 2003). Along with this increase in HPA activity, a cross-sensitization may occur in which long term repeated psychological stressors or increased cytokine release may lead to increased HPA activity when an organism is challenged in the future (Johnson et al., 2004). In short, stress and immune function are tightly interwoven, and proinflammatory cytokines show effects on neuroendocrine functioning and vice versa.

Several lines of research have shown that proinflammatory cytokines (e.g., IL-1β and IL-6) are increased in patients experiencing major depression. Some studies have found an increase in the incidence of major depression in patients suffering from conditions that are linked with an increase in proinflammatory cytokines (Yirmiya, 1997). Further, patients suffering from depression have heightened cytokine levels, and this is correlated with the level of depression, the duration of the current episode, and age at the beginning of the disease (Anisman et al., 1999; Hayley et al, 2005). People receiving cytokine-based cancer therapy or Hepatitis C virus treatment, show depressive symptoms that can be alleviated by treatment with antidepressants (Musselman et al., 2001). Finally, cytokine-related mood alterations have been found in normal subjects following various immune challenges (Reichenberg et al., 2001).

In addition to human studies, animal studies have shown that when subjects are exposed to endotoxin challenges or direct stimulation with IL-1β, they show depressive-like symptoms, a portion of these can be alleviated by treatment with antidepressants (Yirmiya, 1996; Yirmiya et al., 2001). These findings from human and animal studies show that IL-1β correlates with the symptoms of depressive illness.

Along with partially orchestrating the response to immune challenges, IL-1β partially mediates the effects of stress, and it has been found that exposure to some stressors is coupled with a heightened IL-1β levels, in both the periphery and the CNS (Heinz et al., 2003; Nguyen et al., 1998; O'Connor et al., 2003). Moreover, increases of IL-1 may mediate a portion of the behavioral and neuroendocrine effects of stress (Goshen et al., 2003; Maier & Watkins, 1995). Studies have shown a heightened activation of the hypothalamic-pituitary

adrenal (HPA) axis by depressed patients (Barden et al., 1995) and that antidepressants can attenuate this effect (Reul et al., 1993).

Diminished hippocampal neurogenesis has been theorized as being a contributing factor of major depression and IL-1β may play a mediating role (Hayley et al., 2005; Kempermann & Kronenberg et al., 2003; Monje et al., 2003). Although the influence of IL-1β in this process has not been established yet, it has been shown that immune activation with LPS reduces neurogenesis (Monje et al., 2003). In a recent study, Goshen et al., (2007) found there to be a commonality between the symptoms of depression after a chronic mild stressor (CMS; a model for depression) and increased levels of IL-1β with in the hippocampus. Stressed mice showed signs of depressive-like symptoms, decreased sucrose preference, reduced social exploration, decreased neurogenesis, and adrenocortical activation. However, when using IL-1β knock out (IL-1KO) mice, these symptoms of the CMS were reduced. Further, they found that infusion of IL-1β through mini-pumps subcutaneously, could mimic the effects of CMS. They concluded that increased IL-1β is both necessary and sufficient to cause depression in patients suffering from many chronic disorders, and that procedures that reduce IL-1β can have antidepressive properties to them. They also demonstrated that the decrease in neurogenesis is dependent on IL-1 signaling, as the IL-1KO mice did not show CMS induced decrease in neurogenesis. Further, mice failed to show an increase in corticosterone induced by CMS, or that the blockade of corticosterone negated the depressive effects that CMS usually causes. The authors concluded that brain IL-1 is causally related to many depressive symptoms including behavioral symptoms, HPA activation, and reduced neurogenesis.

1.5. Stress, Depression, Cognition, and Antidepressants

Decreased neurogenesis has been linked with diminished hippocampal volume (Bremner et al., 2000), deficits in learning and memory, and mood regulation problems (Ekdhal, et al., 2003; Shors, 2001). Despite a long-standing dogma to the contrary, neural stem cells continue to proliferate and produce neurons and glia throughout life in the subgranular zone (SGZ) of the dentate gyrus, at the border of the hilus and the granular cell layer, and subventricular zone (SVZ) of the lateral ventricle (Kuhn et al., 1996). Once the newly formed cells are born in the SGZ, they migrate to the granular cell layer where they integrate into the existing neuronal circuitry of the surrounding infrastructure (Cameron & McKay, 2001). A large number of variables modulate adult neurogenesis, including stress, age, gender, environment, and learning (Kempermann, 2002). Some investigators contend that hippocampal neurogenesis is required for memory formation in maze learning (Ambrogini, et al., 2000; Gould et al., 1999). However, this may not occur in the Morris water maze spatial learning task (Shors et al., 2001, 2002). These data suggest that there might be an underlying factor that could be addressed in therapy for learning/memory dysfunction (e.g., using a facilitator to increase neurogenesis in the hippocampus).

Depressed individuals have a decreased hippocampal volume compared to people who show no signs of mood regulation problems (Bremner et al., 2000; Sheline et al., 1996). Many researchers believe that elevated/improved neurogenesis, as the result from antidepressant treatment, may be good method to treat the symptoms of depression, if not the cure (Kempermann and Kronenberg, 2003). Chronic antidepressant pretreatment, but not acute treatment, causes an increase in hippocampal neurogenesis in mice (Kodama et al., 2004; Malberg et al., 2000), or attenuate the decrease in hippocampal neurogenesis caused by

other factors such as stress (Pechnick et al., 2007), including depression. These studies, among others, have led to what is called the "neurogenesis hypothesis of depression" (Santarelli, 2003). According to this hypothesis, depression arises from decreased neurogenesis and many antidepressants work by stimulating neurogenesis. It has been shown that pretreatment with antidepressants (e.g., imipramine) caused a decrease in the depressive-like symptoms seen after hippocampal neuronal depletion with chronic stress in mice (Pechnick et al., 2007). Furthermore, it has been shown that after olfactory bulbectomy, a well-established animal model for major depression, chronic pretreatment with imipramine (roughly 5 mg/day administered through drinking water), attenuated the decrease in neurogenesis (Keilhoff et al., 2006).

In animal models, as described previously, stress has been shown to suppress adult neurogenesis. One way that stress decreases neurogenesis is by the activation of glucocorticoid receptors in the brain. Chronic stress has been shown to reduce hippocampal proliferation and neurogenesis in rodents (Gould & Tanapat, 1999; Heine et al., 2004). After chronic stress and near-continuous HPA-axis activation, glucocorticoids may play an important role in the development of abnormal brain function (e.g., decrease in neurogenesis). For example, blockade of glucocorticoid receptors in the brain by the antagonist (mifepristone) attenuated the adverse effects of chronic stress on adulthood neurogenesis (Oomen et al., 2007).

Chronic stress is thought to play a role in major depressive disorders (for review, see Nestler et al., 2002). Over-activation of the HPA axis is found in a major portion of clinically depressed individuals, evidenced by hypertrophy of the adrenal glands, and increased levels of circulating glucocorticoids. Additionally, pretreatment with the

antidepressant clomipramine will attenuated depressive behavior induced by chronic unpredictable stress (CUS) measured in open-field and forced swim tests (Liu et al., 2008). Unpredictable chronic mild stress (UCMS) is also a way researchers model depressive disorders. Mineur et al. (2007) concluded that hippocampus-dependent tasks such as the 8-arm radial maze and contextual fear conditioning were affected by UCMS, but hippocampus-independent tasks such as cued fear conditioning was not. However, their results suggest that these effects are dependent on strain and sex differences.

Studies dealing with neurogenesis and remodeling of the brain in animal models have been expanded in the clinical literature showing that hippocampal volume is reduced in patients suffering from posttraumatic stress disorder and depression (Bremner et al., 1995). This decrease in hippocampal volume, as in nonhuman animal studies, can be attenuated or even reversed with long-term antidepressant treatment (Vermetten et al., 2003).

1.6. A Role for Adulthood Neurogenesis in Cognition and Depression?

Neurogenesis within the adult hippocampus is thought to correlate with cognitive functioning and depression, but this idea is still debated among researchers (Kempermann et al., 2004). Despite the debate, available evidence suggests that adult neurogenesis may contribute to learning and memory, and reduce the symptoms of depression. The potential contributions of adult neurogenesis seem to lie in long-term effects, rather than the acute ones. Baseline levels of neurogenesis are influenced by differing baseline levels of genetic expression of precursor proteins that lead to cell proliferation, and this correlates with the acquisition of hippocampal learning tasks (Kempermann & Gage, 2002). Researchers believe also that neurogenesis can only explain a portion (10–20% of the hippocampus-dependent behavior in the MWM. More recent studies have shown that it is the total number

of new neurons (Drapeau et al., 2003), rather than progenitor-cell proliferation (Bizon &

Gallagher, 2003), that augment hippocampal function. There are many problems that

scientists have come up with in regard to this debate (e.g., whether neurogenesis causes or

just correlates with learning and memory). What we do know is that adult neurogenesis plays

a role in cognitive functioning and mood, either directly or indirectly, but many questions

remain unanswered.

  Knowing that stress, depression, immune function, and adult neurogenesis are tightly

intertwined, the following set of experiments were conducted to further the understanding of

the relationship that they have with one another. Experiment 1 examined the effects of

chronic imipramine administration for 21 days straight and 4 bolus injections of LPS would

have on learning/memory and adulthood neurogenesis after a 3 day break. We hypothesized

that chronic imipramine would diminish the LPS reduction in both MWM learning and

BrdU-labeled cells in the dentate gyrus. The design of Experiment 2 was similar to the

design of Experiment 1, with chronic imipramine administered over 21 days and 4 bolus

injections of LPS. Following a 3 day break, neurogenesis was assessed and measured.

Experiment 1 and 2 differed in that MWM testing only occurred in Experiment 1. The

hypotheses for Experiment 2 were that chronic imipramine would ameliorate the LPS-

induced decrease in hippocampal neurogenesis alone (at both time-points assessed), and

controlled for potential effects of the learning procedure itself, which have been shown to

increase neurogenesis. Furthermore, designs of Experiment 3 and 4 were similar except that

learning/memory effects were assessed in the MWM in Experiment 3 and in contextual fear

conditioning in Experiment 4. Both experiments looked at the effects of chronic versus acute

imipramine on behavior after animals received one bolus injection of LPS immediately after

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day 1 of testing. It was hypothesized that chronic but not acute imipramine would attenuate LPS effects on memory consolidation in both MWM and contextual fear conditioning.

2. METHODS

2.1. Experimental Subjects

All four experiments utilized a total of 213 three-month-old experimentally-naïve male C57BL/6J mice that were bred in the Texas Christian University vivarium from a breeding stock obtained from The Jackson Laboratory (Bar Harbor, Maine). Experiment 1 used 64 mice to assess Morris water maze (MWM) learning, and neurogenesis within the dentate gyrus of the hippocampus. All procedures in Experiment 1 were performed after chronic imipramine (10 mg/kg for 21 days) and 4 bolus (1 mg/kg) injections of LPS on days 1, 7, 14 and 21 of imipramine treatment. Experiment 2 used a total of 63 mice to assess neurogenesis within the hippocampus directly after the 3-day break following chronic imipramine (10 mg/kg for 21 days straight), and 4 bolus injections of LPS (1 mg/kg) without the implementation of behavioral testing. Half of the animals were used for a neurogenesis cell survival experiment. Experiment 3 used 55 additional mice to assess acute verses chronic doses of imipramine with one dose of LPS (250 µg/kg) after day 1 testing in MWM (i.e., short term effects of LPS exposure). Experiment 4 utilized 31 mice to assess contextual fear conditioning, and the same procedure was used as in Experiment 3 with the exception of using contextual fear conditioning instead of MWM. All animals were housed in groups of 3–4 in standard polycarbonate mouse cages and allowed access to food and water *ad libitum*. Lights were set to an automated 0600 on and 1800 off light-dark cycle. All animals received care consistent with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996), and the experiments were conducted in accordance with a protocol

approved by the Institutional Animal Care and Use Committee (IACUC) at Texas Christian University.

2.2.1. Experiment 1

The design for Experiment 1 was a 2 x 2 factorial design, with two between-subject factors (i.e., Imipramine Pretreatment at doses of 0 and 10 mg/kg, and LPS Treatment at doses of 0 and 1 mg/kg), for a total of four treatment groups. Imipramine (Sigma, St. Louis, MO) was diluted in a small volume of pyrogen-free 0.09% saline prior to intraperitoneal (i.p.) injections. Animals received either imipramine or saline injections each of 21 days prior to a 3 day break, before MWM testing began. The LPS or saline injections were first administered intraperitoneally (i.p.) 3 weeks prior to MWM testing, at the beginning of the imipramine treatment. A total of 4 evenly spaced weekly bolus injections were given, followed by a 3 day break before behavioral testing began. Intraperitoneal injections of LPS (*Escherichia coli* serotype 0111:B4, Sigma, St. Louis, MO) was diluted in sterile 0.09% saline. For control subjects, sterile 0.09% saline (vehicle for LPS and imipramine) was used, and these volume-equivalent injections were administered at the same time(s) as those given to their experimental counterparts. Following the 3 day break, animals were tested in MWM for 6 days, the last 3 days of which a subset of animals that were subjected to neurogenesis analysis (N=32), and received consecutive 3 injections of bromodeoxyruridine (BrdU; Sigma, St. Louis, MO; 75mg/kg per injection), one on each of the last days of MWM testing (see Table 1 for Experiment 1 timeline). Animals were euthanized 24 hours after the last BrdU injection, and transcardially perfused with 0.1*M* phosphate buffer and a buffered 4% paraformaldehyde fixative. Brains were then extracted and then stored whole in 4% paraformaldehyde until subsequent immunohistochemical analysis was preformed. All mice

were visually examined for common manifestations of "sickness behavior" (e.g., hunched posture, piloerection, decreased motor activity, etc.), and weighed daily throughout the injection regimen (to determine whether or not subjects showed decreases in body weight after LPS and/or antidepressant administration), and directly prior to testing.
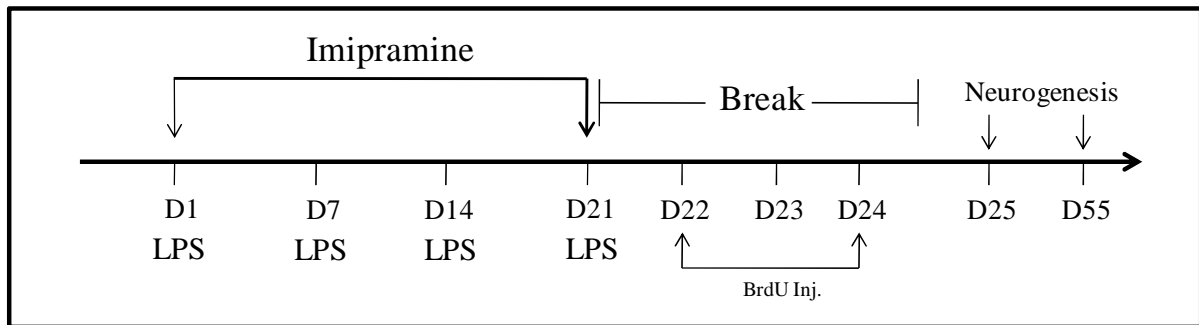
Table 1. Timeline for Experiment 1
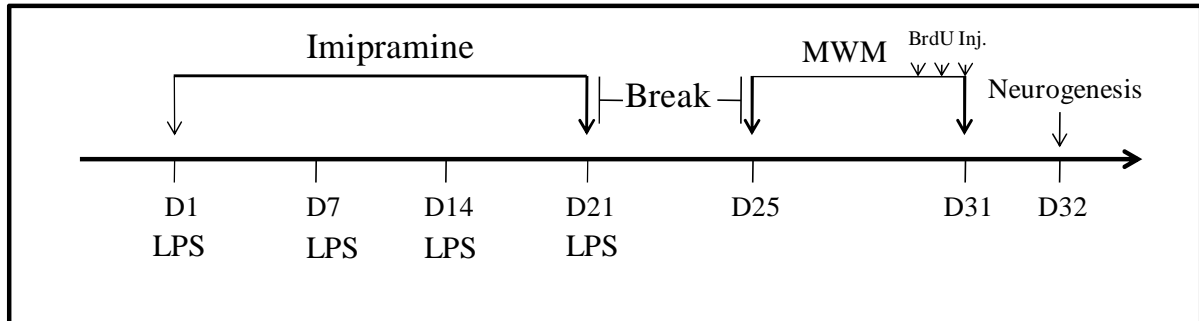
2.2.2. *Experiment 2*

Experiment 2 utilized a 2 x 2 factorial design with two between-subjects factors (i.e., Imipramine Pretreatment at doses of 0 and 10 mg/kg and LPS Treatment at doses of 0 and 1 mg/kg) for a total of four treatment groups. Following the protocol of Experiment 1, animals received imipramine or saline injections for 21 straight days, prior to a 3 day break. LPS or saline injections were administered (i.p.) 3 weeks prior to assessment of neurogenesis and co-administered with the first injection of imipramine, for a total of four evenly spaced bolus injections. Controls were treated the same way they were Experiment 1. During the three days of the break, animals that were utilized for analysis of neurogenesis (N=61) received a daily injection of BrdU (75 mg/kg per injection). Following BrdU injections, half the mice (N=31) were euthanized 24 hrs after their last injection, in preparation for immunohistochemistry (see Table 2 for Experiment 2 timeline). The other half of the mice (N=30) were housed in their home cage for 4 weeks post-BrdU injections to assess survival

of labeled cells, an indication of maturation of precursor cells. As in Experiment 1, all mice were visually examined for common manifestations of "sickness behavior", and weighed each day prior to injections. This experiment differed from Experiment 1 in that animals were assessed for neurogenesis directly after their 3 day break (prior to any effects of behavioral testing itself had on neurogenesis), and no behavioral measures were obtained.

Table 2. Timeline for Experiment 2

2.2.3. Experiment 3

The design for Experiment 3 was a 2 X 3 factorial design, with two between-subjects factors (i.e., Chronic or Acute Imipramine Pretreatment at doses of 0 and 10mg/kg and LPS Treatment at doses of 0 and 250µg/kg), for a total of six treatment groups. Imipramine and LPS were prepared as described for Experiment 1. Animals either received chronic imipramine (10mg/kg for 21 days straight) before MWM or acute imipramine (10mg/kg; once), 24 hrs before behavioral testing in MWM, and received LPS (1mg/kg) immediately after the first session of MWM testing (see Table 3 for Experiment 3 timeline). All controls were treated as they were in prior experiments. After injections, animals were tested in MWM as they were in Experiment 1, to assess potential disruptions in memory consolidation.
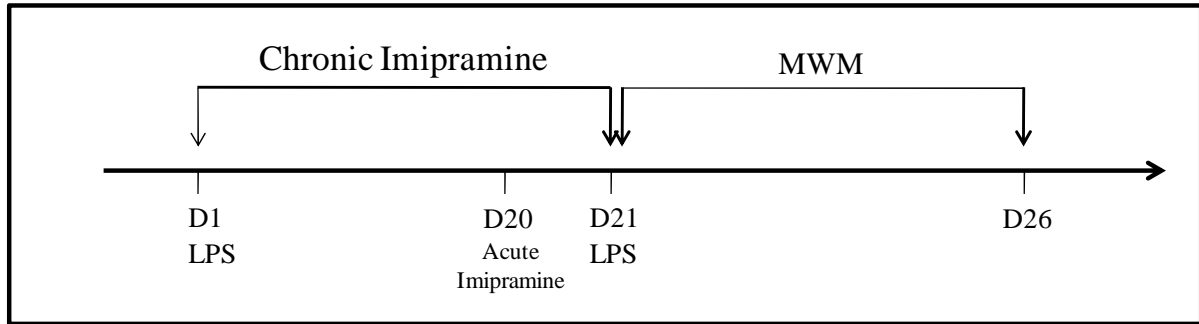
Table 3. Timeline for Experiment 3

2.2.4. Experiment 4

The design for Experiment 4 was a 2 X 3 factorial design, with two between-subjects factors (i.e., Chronic or Acute Imipramine Pretreatment at doses of 0 and 10mg/kg and LPS Treatment at doses of 0 and 250μg/kg) for a total of six treatment groups. Imipramine and LPS were prepared according to Experiment 1. Animals either received chronic imipramine (10mg/kg for 21 days straight) before contextual fear conditioning (CFC) or acute imipramine (10mg/kg; once), 24 hrs before behavioral testing in CFC, and received LPS (1mg/kg) immediately after the first session of CFC testing (see Table 4 for Experiment 4 timeline). All controls were treated as they were in prior experiments. After injections, animals were tested in CFC as they were in Experiment 3, for potential disruptions in memory consolidation.
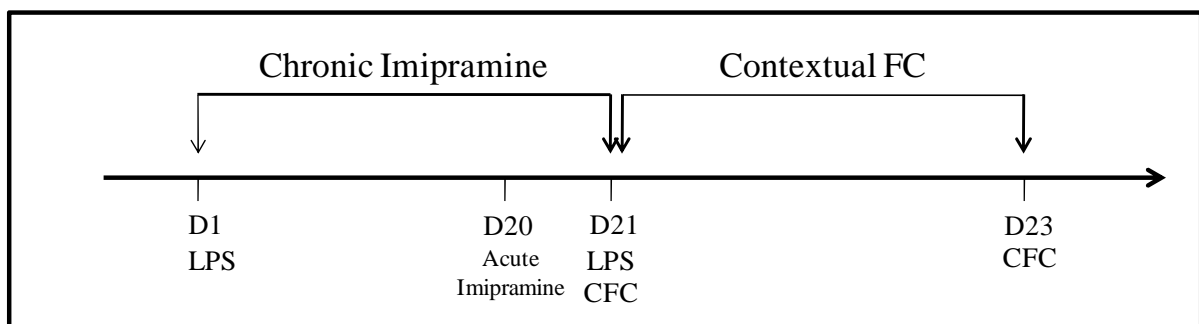
Table 4. Timeline for Experiment 4

2.3.1. Testing Procedure: Morris Water Maze

Morris water maze (MWM) testing was performed in a circular white metal tub that measures 123cm in diameter. The water in the tub was set at a constant temperature of 20°C ± 1°C and colored with white, non-toxic Crayola® paint to make the water opaque. The escape platform is made of a non-skid metal circular platform measuring 10cm in diameter and is submerged 1 cm below the surface of the opaque water. During all 5 days of spatial navigation testing, the hidden platform was held constant in the north-east quadrant of the maze. All animals received four trials per day, for 60 sec each, from each of the four quadrants of the maze (determined semi-randomly). On day 6 of testing, animals received a "probe trial", in which the escape platform was removed. Subjects received two such trials and were assessed for time spent in the quadrant where the platform was placed on the previous 5 days. All trials were recorded via an automated overhead tracking camera system (Accuscan Instruments, Columbus, OH), with which mean latency (sec) to reach the platform, swim speed (cm/sec), and total distance swam (cm) was recorded. Probe data was recorded as the mean percentage of time spent within the target quadrant.

2.3.2. Testing Procedure: Contextual Fear Conditioning

Two fully automated Freeze Monitor System units (San Diego Instruments; San Diego, CA) were used to assess contextual fear learning/memory consolidation. Each apparatus has an electrified grid floor, through which mild electric shock (0.7mA) is delivered. At floor level in each of the units (26.7cm W X 26.7cm L X 17.8cm H) are infrared photocells that sense ambulatory movements of the animals. Each of the units was connected to a computer that contained the Freeze Monitor System software that enabled

recording and analysis of freezing behavior. Movement of the animal was recorded continuously, except during the acclimation period on Day 1 (i.e., training day). All apparatus contained a peppermint smell (peppermint extract diluted 1:10 with tap H_2O), and a dotted pattern on all four walls of the chambers. A house light illuminated the testing chamber for the duration of both the acclimation day and the test day.

On day 1 of contextual fear conditioning (CFC), all groups were placed into the chambers, and then the trial began with a 90 sec acclimation period. After the initial 90 seconds, a 2-second 0.7 mA shock was delivered. Following the delivery of the footshock a 90 second inter-trial interval (ITI) period began, during which the amount freezing was measured. In addition, the shock was delivered for the second time at 182 seconds after the initiation of the experiment, and for the third time at 274 seconds. At all times, freezing was measured during the ITI of 90 sec after the shock was administered. The total time spent in the chamber for one animal per session on Day 1 was 366 seconds, and freezing was measured for a cumulative 270 seconds. Immediately after the training session, animals were given their respective treatment (i.e., LPS or saline). Following the training day, animals were given a 48 hr break before the testing phase started. On Day 3 (testing day) the shock was not administered at any time during the session. The day 3 session was a total of 720 seconds long, during which time freezing was measured and the light remained on during the whole experiment, as it was during the training day.

2.3.3. Assay Procedure: Bromodeoxyruridine Staining (IHC)

Once finished with Morris water maze testing in Experiment 1, or after their 3 day break in Experiment 2, mice were deeply anesthetized with sodium pentobarbital and then transcardially perfused through the ascending aorta with 0.1M phosphate buffer (pH 7.4),

followed by 4% paraformaldehyde in 0.1*M* phosphate buffer (pH 7.4). For analysis of

adulthood neurogenesis (i.e., newly generated cells), 5-bromo-2'-deoxyuridine (BrdU) which

incorporates into newly synthesized DNA during the S-phase of cell replicating replacing

thymidine during DNA synthesis, was used and dissolved in 0.9% NaCl, and sterile-filtered

at 0.2μm. Mice were injected 3 times (75mg/ml; 24 hrs apart) with BrdU, beginning 4 days

prior to tissue processing. After perfusion, brains were post-fixed in 4% paraformaldehyde at

4°C until immunohistochemical analysis was performed. Fresh sections were cut in the

coronal plane (40 μm) using a Leica VT1000 S vibratome (Leica, Wetzlar, Germany), and

placed into 48-well tissue culture plates containing 1% paraformaldehyde until staining

procedures were conducted. For the staining procedure, six evenly-spaced (i.e., rostrally to

caudally) free-floating sections were selected then rinsed 3 times for 30 min per wash in

diH$_2$O. To allow tissue to be penetrated by antibodies, free-floating sections were incubated

in 2N HCL, for a total of 30 min at 37°C. Sections were then washed 3 times for 2 min each

in 1X PBS, and put immediately into 0.1*M* Sodium Borate (pH=8.5) and allowed to incubate

at room temperature for 10 min. Following incubation in Sodium Borate, sections were

washed for an additional 3 times for 2 min each in 1X PBS. Once washed, sections were put

in 1° monoclonal rat anti-BrdU antibody (1:500; Abcam, Cambridge, MA) in blocking buffer

(5% normal donkey serum) in PBST (0.3% TritonX-100 in 1X PBS) overnight at 4°C. The

next day, sections were washed at room temperature for 6 times 10 min each in PBST. Once

washed, sections were incubated overnight at 4°C in the secondary antibody (donkey anti-rat

IgG- HRP; 1:500; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) in blocking

buffer described previously. Once again, the following day, sections were washed at room

temperature for 6 more times, 5 times 10 min each in PBST followed by 1 time in MilliQ

water for 10 additional min. Once the final wash was completed, tissues were incubated in a

3, 3'-Diaminobenzidine stain (DAB Plus Substrate Kit; Invitrogen, Carlsbad, CA) for 30 min

at room temperature, per manufacturer's instructions. When enough stain penetrated the

tissue, the sections were washed in MilliQ water again till they were mounted on gelatinized

histological slides. Sections were allowed to dry overnight, and then were subjected to a

ethanol dehydration process, followed by xylene, until coverslipped using Cytoseal 60

mounting media.

For quantification of BrdU labeled cells in the dentate gyrus, a total of six

representative coronal sections were used. DAB/BrdU labeled positive cells in the granular

cell layer and subgranular zone (within two cell bodies lengths from the granular cell layer)

was visualized using a light microscope (Zeiss Axio Imager. Z1, Carl Zeiss, Jena, Germany)

with a camera (AxioCam MRc, Carl Zeiss, Jena, Germany) attached. Granular cell

layers/dentates were first visualized using a 5X objective to trace around the area of interest,

and all cell counts were performed using a 40X oil immersion objective. Once the cell

numbers per dentate was recorded, a mean was obtained (i.e., average of BrdU positive cells

across all 12 dentates) for the total number of BrdU-positive cells per animal.

2.4. Statistical Analyses

The data for the MWM was analyzed using standard repeated-measures analysis of

variance (ANOVA) procedures (Statview 5.0, SAS Institute Inc., Cary, NC), with

Imipramine (0 and 10mg/kg) and LPS Treatment (0 and 1mg/kg) as the between-subjects

variables, and Test Day (days 1–6 for MWM) as the within-subjects (i.e., repeated-measures)

variable in Experiment 1 and Acute versus Chronic Imipramine (0 and 10mg/kg) and LPS

Treatment (0 and 1mg/kg) as the between-subjects variables, and Test Day (days 1–5 for

MWM) as the within-subjects (i.e., repeated-measures) variable in Experiment 3. Standard

factorial ANOVAs were used to determine main effects and interactions among treatment

conditions (Acute versus Chronic Imipramine [0 and 10mg/kg] and LPS Treatment [0 and

1mg/kg]) in CFC. The criterion for rejecting the null hypothesis was set at an alpha level of

$p < 0.05$. Standard factorial ANOVAs were also used to compare differences in the means of

BrdU labeled cells across all treatment conditions when applicable. Significant main effects

and interaction effects were subjected to Fisher's PLSD post-hoc analysis to determine

significant differences between treatment groups.

3. RESULTS

3.1. Experiment 1

3.1.1. Experiment 1: Weight difference scores

Weight difference data showed that there was a main effect of Condition (($F(1,60)=$

210.86, $p<0.0001$; see Figure 1)), in which LPS-treated animals had a higher difference score

(i.e., weight before injection subtracted by weight 24 hrs post-injection) in weight compared

with saline-treated animals. However, there was no main effect of Imipramine or a

Condition X Imipramine interaction for difference scores in weight (($F(1,60)= 0.18$;

$F(1,60)= 1.47$; see Figure 1)). Moreover, there were LPS X Timepoint, and Imipramine X

Timepoint interactions (($F(2,120)= 19.83$, $p<0.0001$; $F(2,120)= 4.11$, $p<0.05$, respectively;

see Figure 1)), but no Condition X Imipramine X Timepoint interaction (($F(2,120)= 1.56$; see

Figure 1)). Fisher's PLSD revealed that, for the LPS X Timepoint interaction, at all three

timepoints LPS-treated animals had a higher difference between the pre- and post-injection

than did their saline-treated counterparts ($p's<0.0001$). As for the Imipramine X Timepoint

effect, Fisher's PLSD showed that there were no differences among the groups, but the overall F-value is significant across all timepoints.

In examining raw weights more closely, the data for Timepoint 1 indicated there were no main effects of Condition, Imipramine, or a Condition X Imipramine interaction before LPS administration (($F(1,60)= 1.28$; $F(1,60)= 0.004$; $F(1,60)= 0.204$, respectively; data not shown)). However, 24 hours after LPS administration on Timepoint 1, there was a Condition effect (($F(1,60)= 7.85$, $p<0.01$; data not shown)) demonstrating that LPS-treated animals had a significant reduced body weight compared to their saline-treated counterparts. No significant Imipramine or Condition X Imipramine interactions were found (($F(1,60)= 0.46$; $F(1,60)= 0.02$, respectively; data not shown)). As for Timepoint 2 weights before LPS administration, and same as in Timepoint 1's before LPS administration weights, the findings revealed were no main effects of Condition, Imipramine, or Condition X Imipramine interaction before injection LPS (($F(1,60)= 0.03$; $F(1,60)= 0.41$; $F(1,60)= 0.17$, respectively; data not shown)). Once again, however, the findings showed that after LPS administration on Timepoint 2, there was an effect of Condition (($F(1,60)= 4.18$, $p<0.05$; data not shown)), revealing that LPS-treated animals had a significantly reduced body weight compared to their saline-treated counterparts. Looking at raw weights 24 hrs after LPS injection at Timepoint 2, the data once again indicated no significant Imipramine or Condition X Imipramine interactions (($F(1,60)= 0.47$; $F(1,60)= 0.82$, respectively; data not shown)), as was the case with Timepoint 1, after LPS administration. Finally, when examining weights before LPS injection at Timepoint 3, the findings indicated, with Timepoint 1 and Timepoint 2 before LPS administration weights, that there were no main effects of Condition, Imipramine, or Condition X Imipramine interaction before LPS administration (($F(1,60)= 0.37$; $F(1,60)=$

0.11; $F(1,60)= 0.11$, respectively; data not shown)). Interestingly, and differing from

Timepoint 1 and 2, prior to LPS administration there was a main effect for Condition on

weight difference. The data for weights 24 hrs after LPS administration on Timepoint 3

showed no Condition effect (($F(1,60)= 0.13$; data not shown)), indicating that LPS-treated

animals did not have a significantly reduced body weight compared to their saline-treated

counterparts. Furthermore, there were no significant Imipramine or Condition X Imipramine

interactions (($F(1,60)= 0.003$; $F(1,60)= 0.74$, respectively; data not shown)). Note, that for

the fourth injection timepoint, weights after LPS administration were not taken, preventing

data analysis. This is the case for the fourth timepoint in Experiment 2 as well.

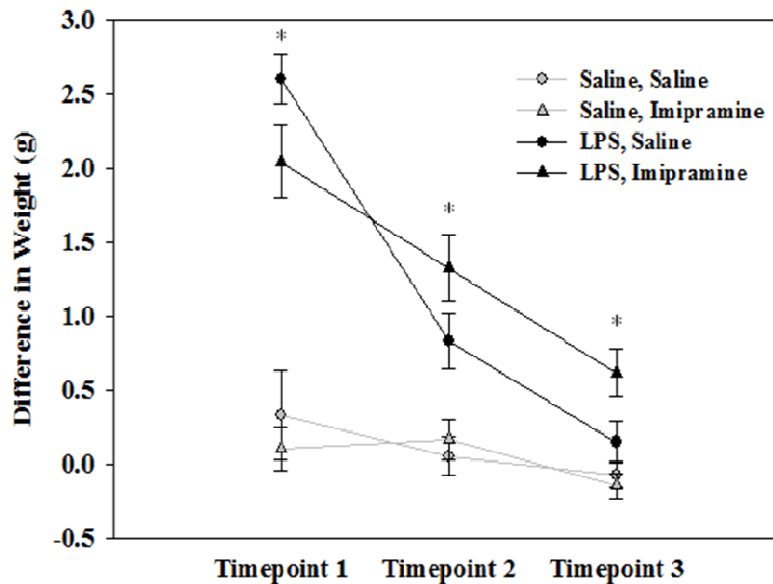Figure 1. Administration of chronic imipramine and 4 bolus injections of LPS (data shown for 3 timepoints) significantly decreased body weight (before compared to 24 hrs after LPS administration) relative to saline-treated subjects, in 3-month-old animals across all three timepoints ($p<0.0001$). * indicates a significant difference from saline-treated subjects. Symbols represent mean ± SEM.

3.1.2. Experiment 1: Morris Water Maze

     For performance in the MWM analyses no main effects of Condition, were obtained for the latency to find the platform, distance traveled, speed, or percent time in the correct quadrant (($F(1,60)$=0.02; $F(1,60)$=0.05; $F(1,60)$= 0.13; $F(1,60)$= 0.60)), respectively; see Figures 2A–D). Moreover, there were no main effects of Imipramine for latency to find the platform, distance traveled, speed, or percent time in the correct quadrant (($F(1,60)$=0.03; $F(1,60)$=0.03; $F(1,60)$= 0.09; $F(1,60)$= 0.62, respectively; see Figures 2A–D)), or any Condition X Imipramine interactions (($F(1,60)$=0.09; $F(1,60)$=0.05; $F(1,60)$= 0.01; $F(1,60)$= 0.05, respectively; see Figures 2A–D)). Furthermore, there were no Condition X Day interactions for latency to find the platform, distance traveled, speed, or percent time in the correct quadrant (($F(4,240)$=0.02; $F(4,240)$=0.18; $F(4,240)$= 0.61; $F(4,240)$= 0.21, respectively; see Figures 2A–D)), Day X Imipramine interactions (($F(4,240)$=0.41; $F(4,240)$=0.71; $F(4,240)$= 0.93; $F(4,240)$= 0.26, respectively; see Figures 2A–D)), or Condition X Imipramine X Day interactions (($F(4,240)$=0.48; $F(4,240)$=0.28; $F(4,240)$= 0.33; $F(4,240)$= 1.76, respectively; see Figures 2A–D)). Neither weekly bolus injections of LPS nor chronic imipramine administration had any effect on test measures.
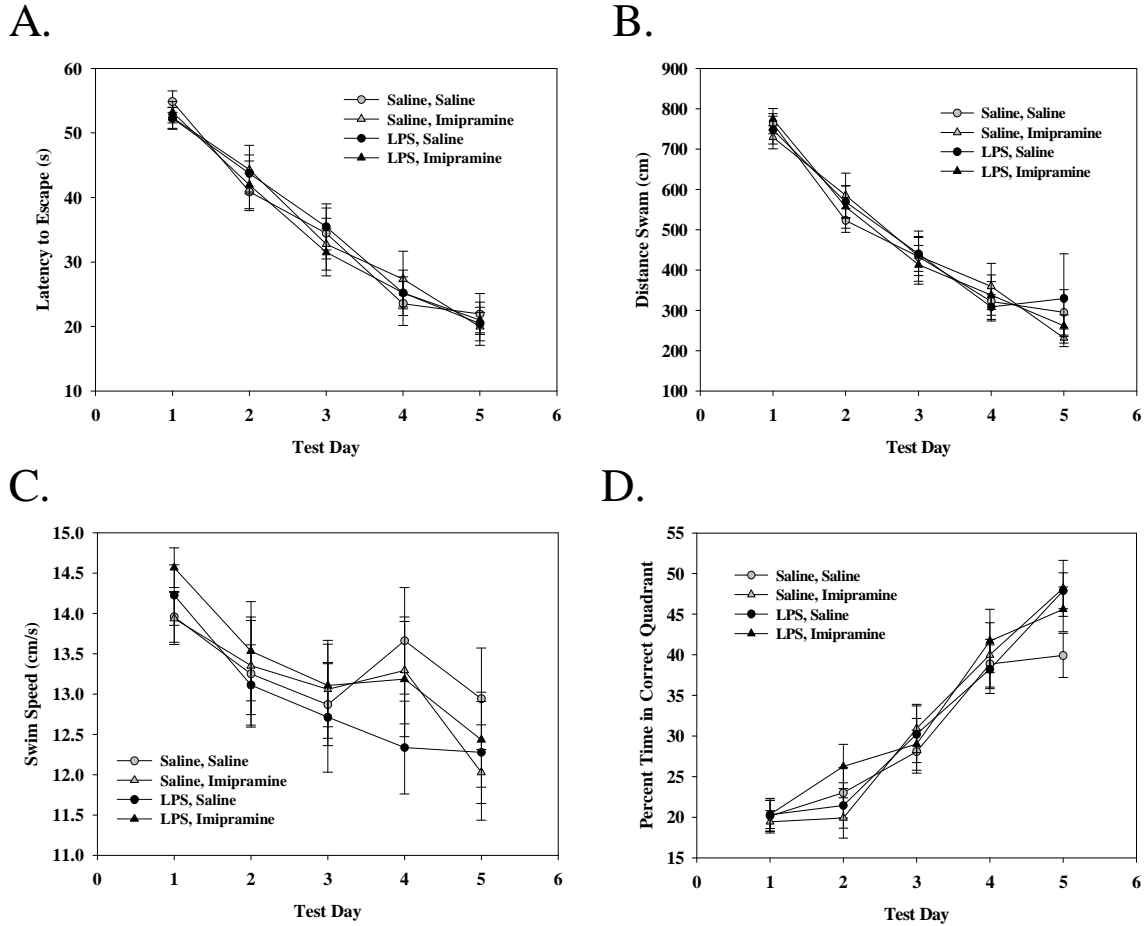
Figure 2. Administration of 4 bolus injections LPS did not have the hypothesized effects of increasing the latency to reach the platform (A), increasing distance swam (B), decreasing swim speed (C), and increasing percent time spent in the correct quadrant (D). Moreover, chronic imipramine had no effect on these variables, and no interactions were significant. Symbols represent mean ± SEM.

3.1.3. Experiment 1: Immunohistochemistry

Analyses for immunohistochemistry data indicated no main effect for Condition or Imipramine (($F(1,28)=0.05$; $F(1,28)= 1.10$, respectively; see Figure 3)), and no significant Condition X Imipramine interaction (($F(1,28)=1.86$, respectively; see Figure 3)), for the mean number of BrdU-labeled cells within the sampled regions of the dentate gyrus.

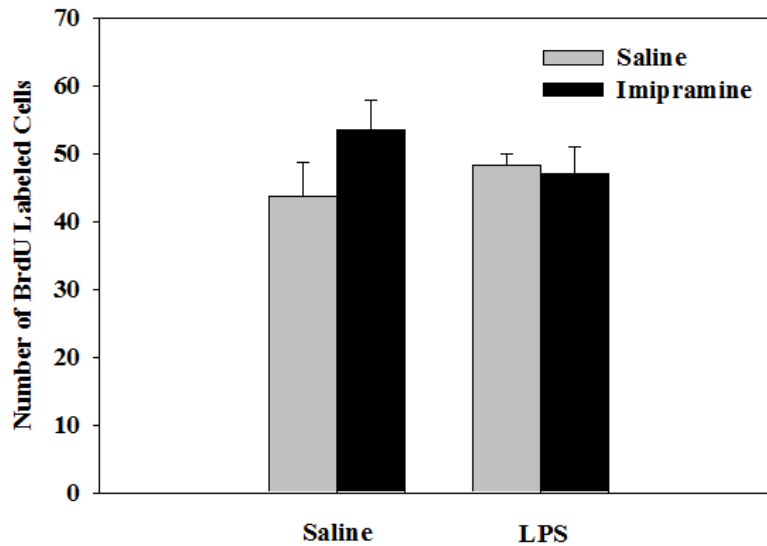Figure 3. Administration 4 bolus injections of LPS did not significantly decrease the mean number of BrdU-labeled cells in the sampled dentate as hypothesized. Moreover, contrary to the original hypothesis, chronic imipramine administration did not diminish the number of labeled cells. Bars represent mean ± SEM.

3.2. Experiment 2

3.2.1. Experiment 2: Weight Difference Scores

As in Experiment 1, there was a significant main effect of Condition ($(F(1,59)=$ 78.70, $p<0.0001$; see Figure 4)), in which LPS-treated animals overall had a higher difference score in weight compared to saline-treated animals. However, there was no main effect of Imipramine or Condition X Imipramine interaction for difference scores in weight $((F(1,59)= 3.13; F(1,59)= 0.17$, respectively; see Figure 4)). Moreover, there was a significant LPS X Timepoint interaction $((F(2,118)= 3.42, p<0.05$; see Figure 4)), but no Imipramine X Timepoint, or Condition X Imipramine X Timepoint interaction $((F(2,118)= 0.91, F(2,118)= 0.72, p<0.05$, respectively; see Figure 4)). Fisher's PLSD showed a LPS X Timepoint interaction, at Timepoint 1 ($p<0.0001$), Timepoint 2 ($p<0.001$), and Timepoint 3 ($p<0.005$). At all three timepoints LPS-treated animals had a higher difference in weight

(i.e., more weight loss) when comparing weight measured before LPS injection vs. 24 hours later, than did their saline-treated counterparts.

Examination of raw weights, data indicated there were no main effects of Condition, Imipramine, or a Condition X Imipramine interaction before LPS administration for Timepoint 1 (($F(1,59)= 0.27$; $F(1,59)= 0.05$; $F(1,59)= 2.46$, respectively; data not shown)). However, 24 hrs after LPS administration for Timepoint 1, there was a Condition effect (($F(1,59)= 0.21.45$, $p<0.0001$; data not shown)), revealing that LPS-treated animals had a significantly reduced body weight compared to their saline-treated counterparts. No significant Imipramine or Condition X Imipramine interactions were found (($F(1,59)= 0.72$; $F(1,59)= 0.52$, respectively; data not shown)). As for Timepoint 2 before LPS administration weights, and same as in Timepoint 1's before LPS administration weights, findings indicated there were no main effects of Condition, Imipramine, or Condition X Imipramine interaction before LPS was given (($F(1,59)= 1.20$; $F(1,59)= 0.02$; $F(1,59)= 0.88$, respectively; data not shown)). Once again, data revealed for 24 hrs after LPS administration on Timepoint 2, there was a Condition effect (($F(1,59)= 15.20$, $p<0.0005$; data not shown)), showing that LPS-treated animals had a significantly reduced body weight compared to their saline-treated counterparts. Looking at weight data for 24 hrs after LPS administration on Timepoint 2, there were no significant Imipramine or Condition X Imipramine interactions (($F(1,59)= 0.78$; $F(1,59)= 1.08$, respectively; data not shown)), as it was the case with Timepoint 1 24 hr post-LPS administration data. Finally, when examining weights before LPS was given at Timepoint 3, findings indicated at Timepoint 1 and Timepoint 2, before LPS administration weights, there were no main effects of Condition, Imipramine, or Condition X Imipramine interaction found (($F(1,59)= 2.02$; $F(1,59)= 0.39$; $F(1,59)= 1.79$, respectively; data not

shown)). Once again, as was time for Timepoint 1 and Timepoint 2 weights taken 24 hrs

after LPS administration, findings showed that on Timepoint 3 there was a Condition effect

$((F(1,59)= 12.39, p<0.001$; data not shown)), demonstrating that LPS-treated animals had a

significant reduced body weight compared to their saline-treated counterparts, and

furthermore no significant Imipramine or Condition X Imipramine interactions were found

$((F(1,59)= 0.78; F(1,59)= 1.08$, respectively; data not shown)). Note that data from

Experiment 2 weights for Timepoint 3, 24 hrs post-LPS administration differ from

Experiment 1 data, in which there were no significant differences on Timepoint 3 post-LPS

administration weights for the main effect of Condition.
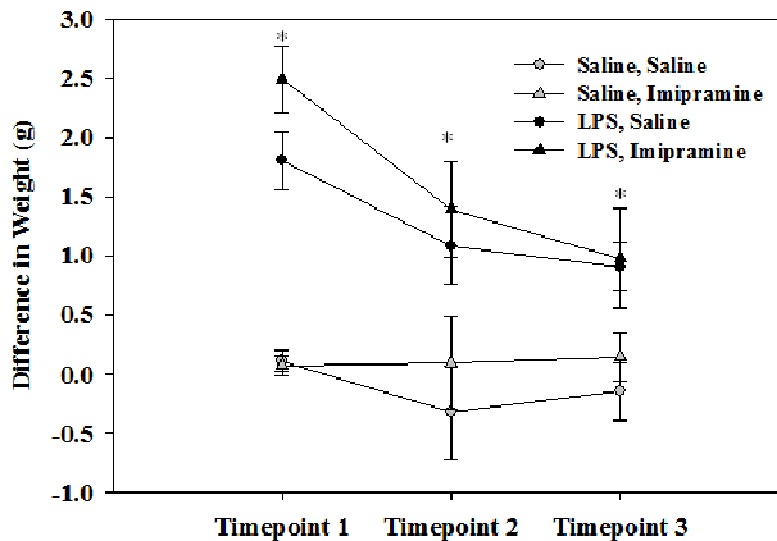
Figure 4. Administration of chronic imipramine and 4 bolus injections of LPS significantly increased difference (before compared to 24 hrs after LPS administration) in body weight relative to saline-treated subjects, in 3-month-old animals across all three timepoints ($p<0.005$). * indicates a significant difference from saline-treated subjects. Symbols represent mean ± SEM.

3.2.2. Experiment 2: Immunohistochemistry

Analyses for immunohistochemistry data indicated no significant main effects of

Condition, Imipramine, or Timepoint for the mean number of BrdU-labeled cells within the

sampled regions of the dentate gyrus (($F_{(1,53)}=3.21$; $F_{(1,53)}=1.28$; $F_{(1,53)}=433.76$, respectively; see Figure 5). Moreover, there were no significant Condition X Imipramine, Condition X Timepoint, or Imipramine X Timepoint interactions (($F_{(1,53)}=0.25$; $F_{(1,53)}=1.71$; $F_{(1,53)}=3.20$, respectively; see Figure 5. Lastly, there was not a significant Condition X Imipramine X Timepoint interaction (($F_{(1,53)}=0.03$; see Figure 5)).
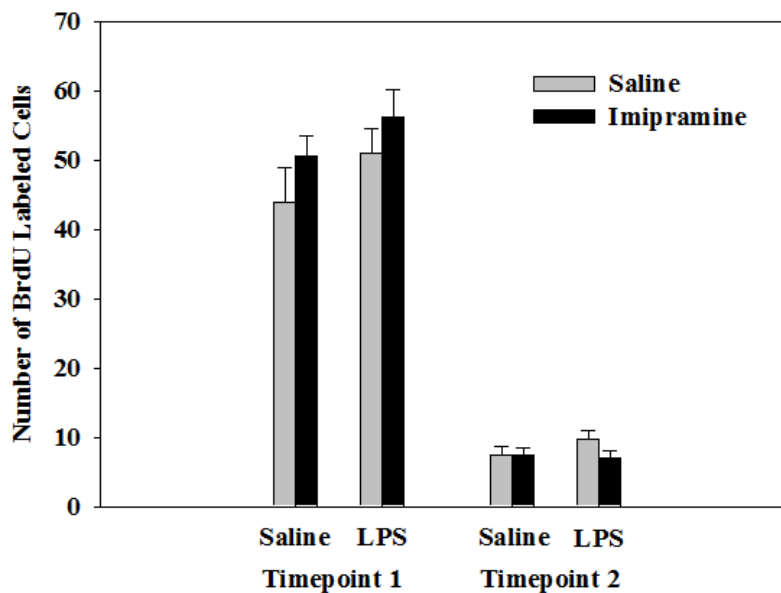
Figure 5. Administration of chronic imipramine did not significantly increase the mean number of BrdU labeled cells in the sampled dentates at Timepoint 1, and did not have an effect on the survival of cells (i.e., Timepoint 2) as hypothesized. Moreover, contrary to the original hypothesis, 4 bolus injections of LPS did not diminish the number of cells labeled at either timepoint. Bars represent mean ± SEM.

3.3. Experiment 3

3.3.1. Experiment 3: Morris Water Maze

As in Experiment 1, there were no main effects of Condition for the latency to find the platform, distance traveled, speed, or percent time in the correct quadrant (($F_{(1,49)}=0.16$; $F_{(1,49)}=0.02$; $F_{(1,49)}=0.25$; $F_{(1,49)}=0.01$), respectively; see Figures 6A–D)). Moreover, there were no main effects of Imipramine for latency to find the platform, distance traveled,

speed, or percent time in the correct quadrant (($F_{(2,49)}=0.35$; $F_{(2,49)}=0.26$; $F_{(2,49)}= 0.12$; $F_{(2,49)}= 0.02$, respectively; see Figures 6A–D)), or any Condition X Imipramine interactions (($F_{(2,49)}=1.44$; $F_{(2,49)}=1.78$; $F_{(2,49)}= 2.90$; $F_{(2,49)}= 2.16$, respectively; see Figures 6A–D)). Furthermore, there were no Condition X Day interactions for latency to find the platform, distance traveled, speed, or percent time in the correct quadrant (($F_{(4,196)}=0.43$; $F_{(4,196)}=0.76$; $F_{(4,196)}= 3.41$; $F_{(4,196)}= 1.39$, respectively; see Figures 6A–D)), Imipramine X Day interactions (($F_{(8,196)}=0.71$; $F_{(8,196)}=0.57$; $F_{(8,196)}= 0.20$; $F_{(8,196)}= 0.18$, respectively; see Figures 6A–D)), or Condition X Imipramine X Day interactions (($F_{(8,196)}=0.86$; $F_{(8,196)}=0.79$; $F_{(8,196)}= 0.49$; $F_{(8,196)}= 0.97$, respectively; see Figures 6A–D)).
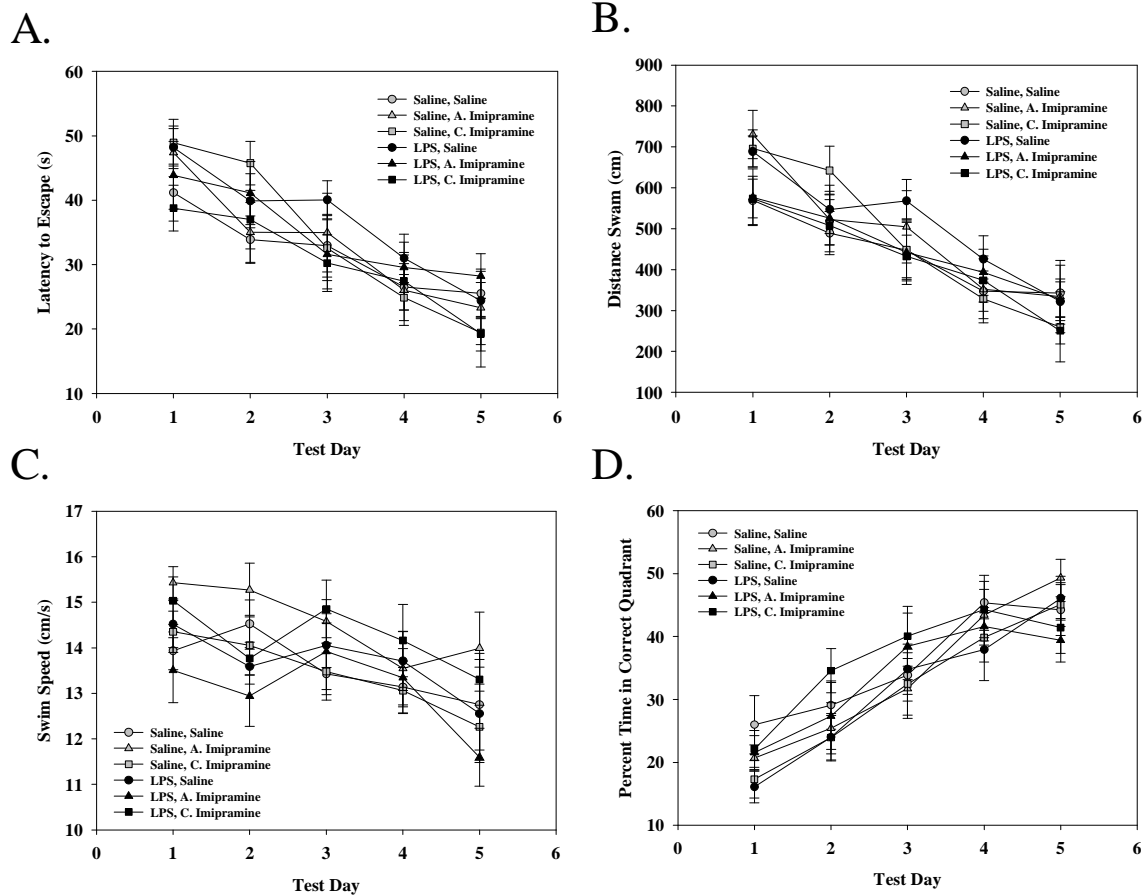
Figure 6. Administration of 1 bolus injection of LPS did not have the hypothesized effects by increasing the latency to reach the platform (A), increasing distance swam (B), decreasing swim speed (C), and increasing percent time spent in the correct quadrant (D). Moreover, acute or chronic imipramine had no effect on these variables, and no interactions were significant. Symbols represent mean ± SEM.

3.4. Experiment 4

3.4.1. Experiment 4: Contextual Fear Conditioning

As hypothesized, there were no significant main effects or interactions of Condition or Imipramine for day 1 (i.e., the training day) (($F(1,25)=0.10$; $F(2,25)=0.12$; $F(2,25)=1.05$, respectively; see Figure 7A)), for mean amount freezing. However, data for day 3 (i.e., test day) revealed that there was a main effect of Condition (($F(1,25)=5.99$; see Figure 7B)), in which LPS treated animals froze more than animals treated with saline. No main effect of

Imipramine ((F(2,25)=0.23; see Figure 7B)) was observed, nor were there any significant

Condition X Imipramine interaction ((F(2,25)=0.51; see Figure 7B)).

A.   B.
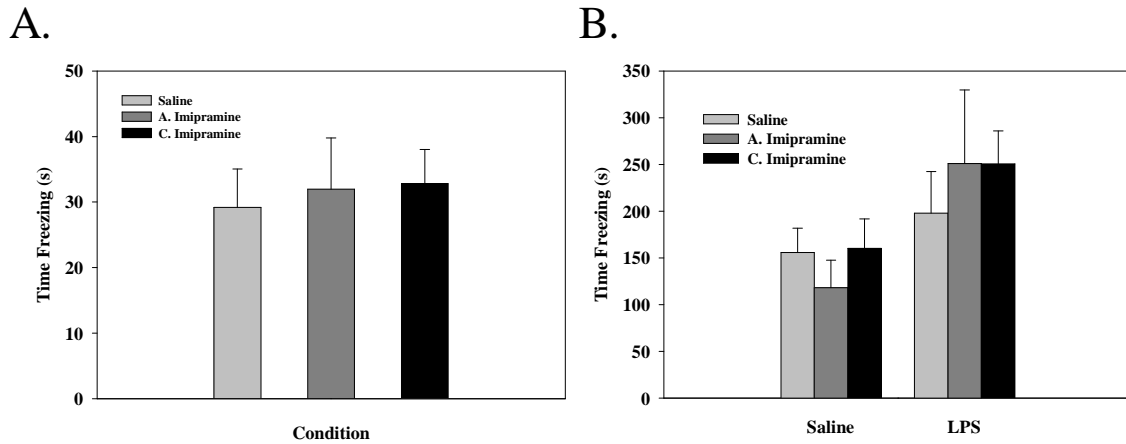
Figure 7. Prior to the administration of LPS on the training day, administration of acute or chronic imipramine had no significance among groups (A). Contrary to our original hypotheses, 1 bolus injection of LPS immediately after testing on training day, LPS administration had no effect on freezing behavior on test day (B). Moreover, chronic or acute imipramine did not have the abrogating effect as originally hypothesized. Bars represent mean ± SEM.

4. DISCUSSION

The first two experiments were designed to investigate the effects of 4-repeated bolus

injections of LPS, followed by a three day break, on learning and memory in a spatial

learning task (i.e., MWM) and hippocampal neurogenesis. Moreover, these experiments

investigated whether the hypothesized deficits in learning and neurogenesis could be rescued

with chronic antidepressant treatment (i.e., imipramine). The second two experiments were

designed to determine whether a single bolus injection of LPS would produce deficits in

spatial memory consolidation performed in MWM and contextual fear conditioning, and test

the hypothesis that deficits after LPS administration can be ameliorated by either acute or

chronic administration of an antidepressant.

Generally, our results failed to support our original hypotheses. The findings indicated that repeated (experiments 1 and 2) or acute (experiments 3 and 4) bolus injections of LPS did not show the hypothesized deficits in MWM learning/memory, or the hypothesized decrease in mean number of hippocampal progenitor cells (either at initial or survival timepoints). Further, the data showed that both chronic (in all 4 experiments) and acute (experiments 3 and 4) administration of imipramine did not show any statistically significant effects, either alone or with the co-administration of LPS. However, as hypothesized, LPS-treated animals did show a significant drop in weight at most timepoints measured, though this effect was diminished with repeated injections.

Although numerous studies have tested the acute effects that cytokines have on behavior (e.g., Aubert et al., 1995; Pugh et al., 1998; Sparkman et al, 2005; Barrientos et al., 2002) when cytokines are elevated, very few have examined the lasting effects of repeated LPS administration (after a break and during which cytokines have returned to basal levels). The first study to examine learning and neurogenesis in this way was conducted by Wu et al. (2007). Our lab sought to replicate and extend the findings of Wu et al. by using imipramine instead of treadmill exercise to rescue memory and neurogenesis from the long-term effects of LPS.

Specifically, Experiment 1 examined the effects that chronic imipramine administration had on hypothesized LPS-induced learning decrements after four bolus injections evenly spaced apart. Following 21 days of imipramine administration, and four (weekly) bolus LPS injections, MWM testing began after a three day break (i.e., examining potential long-term effects of LPS, after cytokine levels had gone back down). Six days later perfusions were performed for BrdU immunohistochemical analyses. It was hypothesized

that chronic imipramine administration for 21 days straight would ameliorate the LPS-induced reduction in the latency to find the platform, increase in the overall distance swam, reduction in swim speed, and reduced percent time spent in the correct quadrant where the platform was located. Contrary to our original hypotheses based upon the findings of Wu et al. (2007), the data obtained from experiment 1 failed to support our original hypotheses. As for mean number of BrdU-labeled neurons in the dentate gyrus, once again despite previous findings reporting an LPS mediated drop in neurogenesis (Wu et al., 2007), LPS did not produce the hypothesized decrease in mean number of labeled cells. Moreover, imipramine did not affect neurogenesis either by itself or in combination with LPS. Clearly an obvious question to be answered was whether or not our LPS injections elevated cytokine levels in the brain or periphery. Although we did not measure cytokine production directly in these studies, the presence of central cytokines can be inferred through measures of sickness behavior (i.e., diminished feeding and weight loss). As expected, LPS affected weight by increasing the weight differences before LPS injection and 24 hrs later. This effect was seen at the first two timepoints measured, but there was no statistical difference between LPS-treated animals and weight differences of saline-treated animals at Timepoint 3. Even though pre-injections weight were obtained for the last LPS injection (Timepoint 4), weights 24 hrs later unfortunately were not, so Timepoint 4 was unable to be analyzed for this experiment or for Experiment 2.

Experiment 2 examined the effects that four bolus injections of LPS on adulthood hippocampal neurogenesis after a three day break (Timepoint 1, initial timepoint) and 4 weeks later (Timepoint 2, survival timepoint), and what effect chronic administration of imipramine would have on neurogenesis at the initial timepoint and the survival timepoint.

The differences between this experiment and Experiment 1 are that in Experiment 2 there was no behavioral testing (i.e., MWM) between the 3 day break and assessment of neurogenesis. This was altered to control for the potential effects of the testing procedure itself. Additionally, Experiment 2 looked at the survival of BrdU-labeled cells 1 month after the initial assessment. Once again, based on the report of Wu et al. (2007), we hypothesized that LPS-treated animals would have a decreased number of BrdU-labeled cells at the initial timepoint of collection, and potentially for the survival timepoint (which Wu et al. did not report). Further, based on several other prior reports (Chiou et al., 2006; Huang et al., 2007; Peng et al., 2008), we hypothesized that imipramine may be able to rescue the decline in neurogenesis. The results once again failed to support these hypotheses. However, examination of weight data showed that there was a main effect of Condition, as in Experiment 1, revealing that LPS-treated animals had a greater drop in body weight, than saline-treated animals. This effect was evident between timepoint 1 and the other two timepoints, decreasing with subsequent injections of LPS. Examining weight data more closely, both before LPS administration and 24 hrs after, weights before all three timepoint injections of LPS were not statistical different among groups. As for weights 24 hrs after LPS injections, there was statistical significance between animals receiving LPS and those receiving saline injections at all three timepoints, for which LPS-treated animals had a reduced weight in comparison to saline-treated animals. These data are slightly different than the weight data in Experiment 1, when looking raw weights. In Experiment 1, at timepoint 3, there were no statistical differences among groups 24 hrs after LPS administration, despite a clear trend in that direction. Despite the effect on sickness behavior, the biological data obtained failed to support our original hypotheses. In our hands

LPS injections neither diminished learning nor diminished neurogenesis. Further, our chronic administration of imipramine had no effect upon neurogenesis.

In Experiment 3, the effect of a single bolus injection of LPS directly after day one of MWM testing (memory consolidation) was examined. Furthermore, the effects of both acute (given 1 day before bolus injection of LPS) and chronic imipramine administration (21 days straight) on the hypothesized decrements in memory consolidation were examined. In addition to having received only a single LPS challenge, Experiment 3 differed from Experiment 1 in that there was no three day post-injection break preceding behavioral testing. In other words, this experiment examined the effect of imipramine administration on short term effects of LPS administration. Our hypothesis for this experiment was that animals receiving chronic imipramine administration for 21 days straight, but not acute imipramine given 24 hrs before LPS injection, would have an abrogated LPS-induced memory consolidation deficit. Again, the data failed to support our hypotheses that LPS-induced animals would show a decrement in memory consolidation. In other words, there was no adverse LPS effect from which subjects could be rescued by imipramine. No direct effects of imipramine on MWM learning were hypothesized, nor were any found.

The design for Experiment 4 and hypothesized results were much like those of Experiment 3, but instead of MWM testing, animals were exposed to a contextual fear conditioning paradigm. Results obtained were once again negative, as they were in Experiment 3.

Our negative findings in the water maze are surprising and run counter to the effects that Wu et al. (2007) reported, including decreased performance in MWM, evidenced by a reduction in the latency to find the platform, compared to the saline control group. Wu et al.

did not report distance swam, or percent time spent in the correct quadrant during testing measures. They only reported this measure during the probe trial (when the platform was removed), for which there were significant differences found between LPS-treated animals and saline controls. What was similar with the current MWM studies and what Wu et al. found, is there were no differences in swim speed when comparing LPS-treated animals and their saline treated counterparts. Furthermore, running counter to the findings of Wu et al., LPS-treated animals did not show the hypothesized reduction in hippocampal neurogenesis compared to the saline-treated animals.

There are many possible explanations for the negative findings we obtained that were inconsistent with Wu et al. First, the serotype of LPS that Wu et al. (2007) used was different than the one we utilized, and possibly expressed different antigens on the cell surface leading to a different receptor affinity. Although this is an unlikely candidate for the many differences found between studies, it may be play a role in the inconsistencies in findings. Additionally, the experimental design in the Wu et al. paper was the first that has used repeated bolus doses of LPS followed by a break (when cytokines usually diminish to baseline levels) to assess learning/memory and neurogenesis; no other groups have replicated these findings. The authors administered repeated bolus injections of LPS over a 21 day period. Their experiment differed from our design in that they used treadmill exercise to increase hippocampal neurogenesis rather than an antidepressant. This may have served to be a crucial difference between our design and Wu et al., in that our experiment involved the use of frequently repeated injections, whereas theirs did not. Another possible reason for the inconsistencies between Wu et al.'s findings and ours was that our mice may have exhibited endotoxin tolerance to LPS by end of the final injection. Wu et al. do not report their weight

data or other measurements of potential sickness behavior, we cannot be certain whether or not similar behavior indicators of endotoxin tolerance were observed in their study as well.

Endotoxin tolerance has been shown numerous times using taste aversion paradigms. One such study showed that pre-exposure to LPS changes the response to a subsequent LPS challenge by blunting peripheral proinflammatory cytokines and ameliorating the reduction in LPS-saccharine associated decrease in fluid intake (Pacheco-Lopez et al., 2007). Our lab has found evidence of behavioral indicators of endotoxin tolerance in MWM (Sparkman et al., 2005b), showing animals that received daily injections of LPS for 5 consecutive days were similar in swim speed to saline-treated animals by day 4 of testing. Appetitive and consummatory feeding behavior are reduced (i.e., anorexia) by peripheral immune stimulation primarily through TNFα release (Asarian & Langhans, 2005; Porter et al., 1998). It should also be noted, elevated peripheral cytokines affect learning and behavioral responses after immune activation as stated previously. There is a strong associative learning process that occurs when food/fluid consumption is paired with a preceding immune activation, even after a single pairing (Exton et al., 1995). In a study conducted by Pacheco-Lopez et al. (2007), researchers gave repeated LPS challenges to rats over the course of 5 consecutive days and compared them to animals that received saline injections. The data revealed that, in the LPS-tolerant group, the taste-LPS association did not occur compared to LPS-naïve rats (animals that did not receive the LPS/saccharine pairing). The authors also reported a blunted peripheral cytokine response for IL-1β, IL-6, and TNFα. In the current set of studies, repeated LPS administration could have led to our negative results obtained for both acquisition learning (Experiment 1) and memory consolidation (Experiment 2) in MWM due to endotoxin tolerance, as evidenced by reduction in the weight difference scores

over the course of the LPS injection regimen. As noted previously, Wu et al. did not give weights throughout experimentation so we unable to compare their data with ours to show that our animals may have exhibited endotoxin tolerance and theirs did not. As with the behavioral measures, the lack of an LPS-induced decrease in BrdU-labeled cells in Experiment 1 and 2 could be attributed to the blunting of peripheral and central cytokines effects following endotoxin tolerance.

Endotoxin tolerance is considered an adaptive response to prevent endotoxic shock upon future encounters with a pathogen. One of the hallmarks of a decreased cytokine response to later LPS challenges is a diminished monocytic synthesis of TNFα (Randow et al., 1997). It has also been shown in human peripheral blood mononuclear cells that priming cells with LPS before a later LPS challenge causes LPS-induced desensitization or hyporesponsivness (i.e., endotoxin tolerance). The main effect of the priming of monocytes is a reduction in TNFα and IL-6 and to a lesser degree IL-1β (Erroi et al., 1993). Along the same lines, Nomura et al. (2000) found that monocytes and immune cells from monocyte lineage (e.g., macrophages) not only produce less cytokines in animals that exhibit endotoxin tolerance, but that TLR-4 receptors (the receptor for LPS) are down-regulated on the cell surface. Nomura concluded that this may explain one of the mechanisms for LPS tolerance. Not only do TNFα and TLR-4 play a role in endotoxin tolerance but the up-regulating of IRAK-M (interleukin 1 receptor-associated kinase M; a negative regulator of TLR and IL-1R signaling) and down regulation of IRAK-1 (interleukin 1 receptor-associated kinase 1; partially responsible for TLR up-regulation of the NF-kappa B pathway) are contributing factors (van 't Veer et al., 2007). TNFα is one of the major proinflammatory cytokines to be up-regulated after a peripheral immune challenge with LPS, and it is known to cause both

sickness behavior and learning/memory deficits (Cunningham et al., 2009), as well as decreased hippocampal neurogenesis (Seguin et al., 2009). Seguin et al. specifically found that systemic administration of TNFα reduced hippocampal BrdU labeling in the dentate gyrus. It is possible that our negative findings in the MWM may be due to decreased monocytic synthesis of TNFα after repeated immune challenges, thereby reducing the chances for cytokines entering the brain and exerting their deleterious effects.

These studies, among others, have shown that cytokines are reduced after repeated immune challenges. From these studies, it may be hypothesized that with the reduction in peripheral cytokines after repeated endotoxin or cytokine exposure, a reduction in sickness behavior (as well as learning and memory) may also be seen. Sickness behavior includes many adaptive symptoms which include decreased feeding, anhedoina, and anorexia (Hart, 1988; Kent, et al., 1995; Konsman et al., 2002; Maier & Watkins, 1998). Along with these symptoms, weight loss is often observed in LPS-treated animals.

As noted previously, weight differences were analyzed for Experiment 1 and 2 and data indicated that there were significant differences for a main effect of Condition and a Condition X Timepoint interaction. Animals that received 4 bolus injections of LPS regardless of imipramine showed a greater significant difference in weight at all three timepoints, and this difference was reduced at the later timepoints. Unfortunately, a timepoint 4 could have been taken but was not. However, given the trajectory of weight difference scores, we hypothesize that this trend would have shown even a smaller difference at Timepoint 4. Analysis of the raw weight data showed, as hypothesized, that there were no statistically significant differences between the animals that received LPS and the animals that received saline injections, at all three injection timepoints before LPS was administered.

Examining weights after the first two timepoints but not at the third (contrary to our hypothesis), data revealed, as hypothesized, that there were significant differences between animals that received LPS versus saline-treated controls. However, these data clearly show that animals are having reduced sickness behavior the more injections of LPS are given, showing that they may be exhibiting endotoxin tolerance.

Yet another possible reason for negative findings in analyses of BrdU-labeled cells is that the somewhat stressful MWM procedure in of itself has been shown to decrease proliferation in the hippocampus. Gould and Tanapat (1999) note that chronic stress can actually change the morphology of the dentate gyrus through the release of glucocorticoids from the adrenal cortex and cognitive impairments may arise as a result of this increase. Specifically, Namestkova et al. (2005) showed that after 15 days of acquisition testing in MWM there was a significant reduction in proliferating cells in the granular cell layer of the dentate gyrus. They concluded that stress, from acquisition training in the MWM, was the cause for the reduction in proliferating cells in the granular cell layer of the dentate gyrus. However, this finding is contrary to the results obtained by Lemaire et al., who showed an increase in the number of proliferating cells in the hippocampus after 5 days of spatial task learning in the MWM (Lemaire et al., 2000). A possible significant difference between these studies and the current experiments is the added stressor of multiple injections. Multiple injections might be considered a potential chronic mild stressor, together with the Morris procedure, which can cause a decrease in proliferating cells in the hippocampus. One of the obvious reasons that chronic intraperitoneal injections can be stressful is the mild pain component to them. Duric and McCarson (2006) showed that chronic inflammatory stimulus (complete Freund's adjuvant) injected three times spaced one week apart over 21 days in the

hind paw of rats produced a significant reduction in BrdU-labeled cells in the dentate gyrus. The negative effects found in MWM, CFC and neurogenesis of the current studies that ran contrary to our hypotheses may be due to chronic stress and/or chronic pain associated with multiple injections over 21 days before cognition and neurogenesis were assessed.

In conclusion, the large majority of the present results failed to confirm our original hypotheses. Reductions in the difference between weight taken before LPS administration showed that central cytokines were elevated at least during the initial phase of each experiment. However, repeated endotoxin challenges, and hypothesized endotoxin tolerance, may have engendered peritoneal monocyte priming, reductions in cell surface expression of TLR-4 and other modulators of endotoxin tolerance. Further, a stressful spatial learning task, and chronic pain as a result from multiple injections, may have also contributed to a general failure to confirm our hypotheses. Though not an exhaustive list, these factors may have contributed, to some degree, to the lack of significance differences between LPS-treated animals and their saline controls. Clearly more research is needed to determine whether or not repeated bolus injections of LPS can cause manifestations of endotoxin tolerance, similar to what we have observed.

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EFFECTS OF CHRONIC ANTIDEPRESSANT COADMINISTRATION ON ACQUISITION, MEMORY CONSOLIDATION, AND NEUROGENESIS AFTER REPEATED LIPOPOLYSACCHARIDE ADMINISTRATION

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Neurogenesis within the hippocampus along with learning/memory are altered by many factors including stress, immune activation, and depression. Chronic dosing with antidepressants has been shown to alleviate many of the symptoms following immune activation. To examine interactions between depression, immune activation, and cognitive dysfunction more closely, the following experiments were conducted. Generally, the current set of experiments examined the effects that repeated bolus injections of LPS have on spatial learning in MWM, memory consolidation in both CFC and MWM, and hippocampal neurogenesis. The effects of chronic (all experiments), or acute imipramine (Experiments 3 and 4) were also examined to see if they influenced the hypothesized LPS-induced decrements. Experiment 1 looked at the effects of chronic imipramine (10mg/kg for 21 days) and 4 bolus injections of LPS (1mg/kg) have on Morris water maze acquisition, and hippocampal neurogenesis after a 3-day break (past the point that central or peripheral cytokines are present). Experiment 2 addressed the effects that chronic imipramine and bolus injections of LPS have on neurogenesis after a 3-day break (i.e., prior to any potential effects of behavioral testing). Experiment 3 examined the effects acute verses chronic imipramine coadministration with a single

acute bolus injection of LPS (1mg/kg) on memory consolidation in Morris water maze. Experiment 4 was much like Experiment 3 but instead of MWM, a CFC paradigm was used. Our hypotheses were that LPS-treated animals would show acquisition deficits in MWM, memory consolidation deficits in both MWM and CFC, and show a decline in neurogenesis (at both the initial and survival timepoints). Moreover, we hypothesized that chronic but not acute administration of imipramine would ameliorate all decrements in MWM, CFC, and neurogenesis as the result from repeated or acute bolus LPS administration. The data revealed that LPS did not show the hypothesized effects nor did imipramine in all four studies. We attribute our negative findings may be due to endotoxin tolerance, monocytic priming, TLR-4 down-regulation and/or stress from both behavioral testing and repeated injections.