SICKNESS-INDUCED COGNITIVE DYSFUNCTION: MOLECULAR, PHYSIOLOGICAL, AND BEHAVIOURAL CORRELATES

LISA THOMSON
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Canadian Centre for Behavioural Neuroscience, Department of Psychology and Neuroscience
University of Lethbridge
LETHBRIDGE, ALBERTA, CANADA

Abstract

Activated immune cells secrete proinflammatory cytokines that induce a generalized sickness response. The following experiments were designed to determine whether the proinflammatory cytokine IL-1β is the peripheral mediator of sickness-induced cognitive dysfunction, as well as to characterize the cognitive processes that are disrupted during illness. The effects of peripherally-administered IL-1β and LPS, an endotoxin that induces a peripheral immune response, are compared across learning and memory tasks, and on hippocampal LTP. LPS impaired memory consolidation and inhibited EPSP expression, whereas IL-1β had no effects across tasks, nor on LTP expression. However, IL-1β plus IL-6, inhibited hippocampal cell proliferation. Therefore, sickness-induced cognitive dysfunction is characterized by a disruption of memory consolidation and an inhibition in hippocampal LTP. In addition, whereas peripheral IL-1β, alone, is insufficient to disrupt learning and memory or hippocampal physiology, it may work with other proinflammatory cytokines, such as IL-6, to produce effects in the CNS.

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The first clue that peripheral cytokines could trigger cognitive dysfunction came from clinical observations of patients who had received cytokine therapy for cancer. Adams and colleagues (1984) observed these patients to exhibit mild to moderate cognitive dysfunction after having received daily treatment with the proinflammatory cytokine, interferon-alpha. Clear conclusions from these clinical observations concerning direct effects on cognition, however, could not be drawn because personality and mood changes were also observed. Clinicians could not be certain as to whether the repertoire of behavioural changes they observed in their patients were results of the cytokine therapy they had received or the cancer itself. Additional support for cytokine-induced cognitive dysfunction would come from behavioural observations of sick non-human animals.

Sickness behaviour in non-human animals

Hart and colleagues (1988) were the first to characterize a set of behavioural changes common to both sick animals and people. According to Hart *et al*. (1988), the most commonly recognized behavioral patterns of animals and people at the onset of febrile infectious diseases were lethargy, depression, anorexia, and reduction in grooming. Additional testing of laboratory animals suggested that sickness also induced anhedonia, or the lack of an ability to experience pleasure, hyperalgesia (i.e., enhanced responsiveness to pain), and cognitive dysfunction. These animals resembled in many ways the patients who had undergone cytokine therapy in treatment for their cancer, as initially described by Adams *et al*. (1984). However, rather than characterizing their behavioural observations as representing nothing more than debilitating side-effects of sickness, Hart and colleagues described their observations as a host of non-specific

behavioural changes accompanying illness that were fundamentally adaptive. That is, they theorized that, by re-routing bodily resources away from activities not necessary for immediate survival, the animal, or host, was able to provide more resources to mount the fever necessary to clear the body of infection (1988). Indeed, a few years later, Kluger described the febrile response as an *adaptive* homeostatic state that is characterized by an elevated set point in body temperature regulation (1992). Hart *et al*. (1988) significantly advanced the idea that sickness behaviour was a coordinated and adaptive central response to this elevated set point.

Sickness behaviour and the proinflammatory cytokines

Despite Hart *et al*.'s (1988) characterization of sickness behaviour and the earlier clinical suspicions that cytokines were responsible for causing it, no data were available to support a direct link between cytokines and the sickness response. The support came in 1992 when Kent and colleagues reported that laboratory rats who were injected with bacterial endotoxins or proinflammatory cytokines displayed several behavioural signs of sickness, such as depressed locomotor activity, decreased exploration of their physical and social environments, and reduced food and water intake (Kent, Bluthé, Kelly, & Dantzer, 1992). Their observations were sufficient to conclude that it was this peripheral release of the pro-inflammatory cytokines following immune activation that caused sickness behaviour.

The conclusion that a peripheral immune response could cause behavioural changes begot the question, then, of how the proinflammatory cytokines- molecules too large to directly cross the blood-brain barrier- gained access to the central nervous system (CNS). Though some proinflammatory cytokines can directly cross the blood-brain barrier

(Konsman, 2000), several studies indicate that the central response to peripheral immune activation is largely due to brain-produced cytokines, whose production and activation are induced by peripheral pro-inflammatory signaling.

The peripheral proinflammatory cytokines can indirectly produce brain cytokines through both humoral and nervous system pathways. The humoral route involves the activation of prostaglandins by peripheral proinflammatory cytokines. This signaling of prostaglandin production occurs at areas where the blood-brain barrier is either weak or absent, such as at the circumventricular organs. Prostaglandins diffuse into the brain parenchyma where they initiate a signaling cascade that culminates in the brain's own production of proinflammatory cytokines (Saper & Breder, 1994; Herkenhaum, Lee, & Baker, 1998; Laflamme & Rivest, 1999; Konsman, 2000).

The primary nerve responsible for transmitting peripheral proinflammatory- induced signals is the vagus nerve (Bluthé *et al*., 1994; Brady; Lynn, Herkenham, & Gottesfeld, 1994; Ericsson, Kovacs, & Sawchenko, 1994; Watkins *et al*., 1994; Ninjima, 1996; Gaykema *et al*., 1998; Goehler, Gaykema, Hammack, Maier, & Watkins, 1998; Konsman, Luheshi, Bluthé, & Dantzer, 2000). Peripherally activated immune cells induce the release of proinflammatory cytokines, which then activate vagal nerve fibers. This peripheral activation of the vagus nerve is transmitted to the point at which the vagus nerve terminates in the brain stem, the *nucleus tractus solitarius* (NTS). Glutamatergic projections from the NTS then sensitize different brain regions to the actions of brain-produced cytokines. Current opinion holds that it is the synergistic activation of both these humoral and nervous system pathways that is responsible for the

coordinated central response to peripheral immune activation (Konsman, Luheshi, Bluthé, & Dantzer, 2000; Dantzer, 2001).

Interleukin-1 beta (IL-1β)

IL-1β and Sickness Behaviour

As an understanding began to develop of how peripheral immune activation could cause behavioural changes, researchers began to focus on which, if any, of the proinflammatory cytokines was primarily responsible for inducing such changes. Because interleukin-1 beta (IL-1β) was observed to be one of the more potent proinflammatory cytokines and IL-1β receptors were located widely throughout the hypothalamus and hippocampus- brain structures largely responsible for orchestrating changes in mood and cognition- IL-1β became the focus of the majority of empirical investigation.

Indeed, growing investigation into cytokine-induced sickness behaviour supported the idea of IL-1β as the primary signaling molecule for sickness behaviour. For example, it was observed that peripheral IL-1β administration inhibited both social interaction (Bluthe, Dantzer, & Kelley, 1992; Luheshi *et al*., 2000) and induced anorexia (Kent, Bret-Dibat, Kelley, & Dantzer, 1996) in rats. From these promising studies, researchers began to look at IL-1β as the possible mediator of other sickness behaviours.

IL-1β and Cognitive Dysfunction

Cognitive dysfunction, though less explored than the other sickness behaviours, is also currently held to be induced by peripheral and central IL-1β. Cognitive processes in the rodent are investigated both behaviourally and physiologically. At the behavioural level, cognitive processes are often investigated using learning and memory tasks thought to

depend upon the cerebral cortex, most often the hippocampus. Examples of such tasks include the Morris water task (Morris, 1981) or the contextual fear conditioning (CFC) task. In the Morris water task, animals must learn to find a submerged platform in an opaque circular pool through the use of extra-pool cues. In the contextual fear conditioning task, rather than maintaining a contextual representation of environmental cues to navigate through space, animals are tested on their ability to mount an appropriate fear response to a context in which they had previously received a foot shock. Animals who display learned fear, evidenced by the amount of freezing they display in the original context in which they had received the shock, have acquired and maintained an association between contextual cues and shock. Animals who receive central injections of

IL-1β are impaired in both tasks (Oitzl *et al*., 1993; Barrientos *et al*., 2002), suggesting that IL-1β acts centrally to disrupt hippocampal learning and memory systems.

Investigation into *how* IL-1β acts to disrupt hippocampal functioning has led to studies of IL-1β effects on hippocampal long-term potentiation (LTP). LTP, a phenomenon initially described by Bliss and Lomo (1973), is an experimental manipulation by which high-frequency stimulation of a pre-synaptic neuron(s) causes an enhanced and long-lasting (i.e., hours to days) post-synaptic response. Because the enhanced post-synaptic response is only observed at synapses that have been artificially stimulated, LTP is held to tap the same or some of the same physiological processes that underlie normal learning. It is hypothesized by many that learning is the behavioural result of the physiological enhancement of synaptic efficacy based upon an experience-dependent LTP-like process.

Some evidence exists to suggest that IL-1β inhibits hippocampal LTP. *In vitro*, the administration of IL-1β either inhibits or completely blocks the induction of LTP in the CA1 (Bellinger, Madamba, & Siggins, 1993), CA3 (Katsuki *et al*., 1990), and dentate gyrus (Cunningham, Murray, O'Neill, Lynch, & O'Connor, 1996) regions of the hippocampus. *In vivo* studies on the anesthetized animal, show that the central administration of IL-1β inhibits LTP in the dentate gyrus (Murray & Lynch, 1998; Vereker, O'Donnell, & Lynch, 2000). Such observations have led researchers to hypothesize that the proinflammatory cytokine IL-1β most likely disrupts hippocampal learning and memory through a disruption of hippocampal LTP.

Hippocampal neurogenesis, like hippocampal LTP, is a cellular process that could contribute to sickness-induced cognitive dysfunction . Not only has hippocampal neurogenesis been observed to be up-or-down- regulated following different experiences (Kempermann, 2002, (review)), but hippocampal neurogenesis has also been functionally linked to a specific cognitive process, namely memory consolidation (Feng *et al*., 2001). It is plausible, therefore, that the experience of being sick with a bacterial infection disrupts hippocampal neurogenesis, and that this disruption contributes to hippocampal learning and memory impairments. In fact, the peripheral immune activation has been demonstrated to inhibit hippocampal neurogenesis (Monje, Toda, & Palmer, 2003); however, no experiments investigating the effects of the peripheral administration of IL-1β on hippocampal neurogenesis have been conducted. In summary, very recent investigation into the effects of the peripheral immune response on hippocampal neurogenesis support an inhibitory effect of the immune response on hippocampal

neurogenesis; however, no data are available to support whether peripheral IL-1β similarly inhibits this process.

Summary

Current Theory

To summarize, current theory holds that cognitive dysfunction represents an integral component of sickness behaviour. Similar to other sickness behaviours, such as anorexia and social withdrawal, cognitive dysfunction is held to be induced by the proinflammatory cytokines that are released by immune cells during a peripheral immune response to an infectious agent. Moreover, the pro-inflammatory cytokine, IL-1β, is held to be the primary peripheral and central signaling molecule of sickness-induced cognitive dysfunction (Danzer, 2001; Maier, 2003; Maier & Watkins, 2003). The current theory holds that, through humoral and nervous system pathways, the peripheral release of IL-1β induces brain IL-1β production. It is this brain production of IL-1β, in response to peripheral signaling by IL-1β, that then acts, at least in part, on hippocampal circuitry to disrupt learning and memory and its proposed physiological counterpart, LTP.

It is often the hope that a new theory of a biomedical process will lead to a new and improved medical treatment. Following the initial discovery linking proinflammatory cytokines to sickness behaviour, including sickness-induced cognitive dysfunction, the proinflammatory cytokines have been linked to the neurological impairments common to, not only to acute infection, but also to HIV infection (Wesselingh & Thompson, 2001 (review)); chronic pathological conditions, such as systemic lupus erythematosus ((SLE) Lauwerys & Houssiau, 2003 (review)); and the cognitive impairments that accompany aging (Wilson, Finch, & Cohen, 2002 (review)). Furthermore, several researchers have

supported anti-proinflammatory cytokine therapy for the treatment of inflammatory conditions. For example, interleukin-6 has been identified as a therapeutic target in the treatment of both systemic-onset juvenile arthritis (Yokota, 2003 (review)) and Crohn's disease (Ito, 2003 (review)). Anti-cytokine therapies have also been proposed for inflammatory bowel disease (Ogata & Hibi, 2003 (review)) and heart disease (Diwan, Tran, Misra, & Mann, 2003 (review)). It is conceivable, therefore, that anti-cytokine therapies can be developed to treat, not only sickness-induced cognitive dysfunction, but also the cognitive impairments that accompany other conditions that have an inflammation component (HIV infection, SLE, aging). The growing focus on the proinflammatory cytokine, IL-1β, as the primary signaling molecule of sickness-induced cognitive dysfunction has made it a most attractive target for the cognitive dysfunction that accompanies these other conditions. However, to successfully treat medical conditions, the correct targets for intervention need to be identified. Despite the prominent role played by peripheral IL-1β in theory, available data currently provide insufficient evidence to narrowing the identification of peripheral IL-1β as a sole target for the pharmacological treatment of cognitive dysfunction.

Weaknesses of Current Theory

The primary weakness of the current theory is whether or not the proinflammatory cytokine, IL-1β is, indeed, the primary signaling molecule of cognitive dysfunction. Certainly, this appears to hold true for brain IL-1β, as the central administration of IL-1β impairs performance in a variety of learning and memory tasks, including the Morris Water task (Oitzl *et al*., 1993) and the contextual fear conditioning task ((Barrientos *et al*., 2002); data reviewed in the *Introduction* to Experiments 3-7), but whether or not IL-

1β is the major signaling molecule in the *periphery* is less clear. The majority of empirical investigation into IL-1β effects on learning and memory has described learning and memory deficits following central IL-1β administration. Only one has published study has observed an inhibitory effect of peripheral IL-1β on performance in a hippocampal-dependent learning and memory task. In 1995, Gibertini and colleagues observed that peripheral administration of IL-1β produced performance deficits in the Morris water task. However, these deficits were later observed to disappear when the animals were subjected to different doses or training protocols (Gibertini, 1998), suggesting that the effects of peripheral IL-1β on learning and memory vary across dose and task conditions. More experiments, using a variety of tasks and doses, need to be done to determine the role of peripheral IL-1β in sickness-induced cognitive dysfunction.

Also, unlike global sickness behaviours, such as anorexia and reduced general motor activity, the measurement of cognition demands attention to its components. That is, several different aspects of learning and memory can be measured. For example, the learning or acquisition of a new task or a new set of cues can be dissociated from other cognitive processes, such as the short -or long-term retention of the task or cues. Few researchers have dissociated these processes in their investigations, and, as a result, it is unclear if sickness-induced cognitive dysfunction is the manifestation of a global impairment in cognition, or if it is a process-specific disruption. Moreover, cognitive impairments need to be dissociated,- through the use of appropriate motivational, perceptual, and sensori-motor controls,- from other non-cognitive effects that can impair learning and memory.

A final weakness of the current theory involves whether or not an IL-1β-induced disruption of hippocampal LTP underlies the learning and memory impairments observed in IL-1β-treated animals. The entirety of the investigation into IL-1β effects on hippocampal LTP has been *in vitro* or using *in vivo* anesthetized preparations. Both methods involve acute tissue damage, which is well-recognized to induce proinflammatory cytokine expression (Jankowsky, Derrick, & Patterson, 2000). Because both methods invoke a large-scale inflammatory response that complicates the interpretation of IL-1β effects on LTP, it is necessary to investigate the effects of proinflammatory cytokines on hippocampal LTP in an animal whose body has had sufficient time to recover from surgical-induced inflammation.

To summarize, then, available data only weakly support the current theory that the peripheral release of IL-1β by activated peripheral immune cells triggers the cognitive dysfunction component of sickness behaviour. Data from behavioural studies only weakly support the theory, namely, because not enough data has been collected on the learning and memory response to peripheral IL-1β administration. Because of the confounding effects of the preparations used to investigate the phenomenon, the available data also do not fully support hippocampal LTP disruption as a mechanim that underlies sickness-induced cognitive dysfunction. Furthermore, no experiments have been done to determine whether the peripheral administration of IL-1β inhibits hippocampal LTP in a freely-moving animal. Similarly, no experiments have been conducted to determine if the peripheral administration of IL-1β inhibits hippocampal neurogenesis, another cellular process empirically linked to learning and memory. Finally, the majority of investigators have failed to separately assess different cognitive processes in their

learning and memory protocols, and therefore, sickness-induced cognitive dysfunction has not been well-characterized.

Research Questions

The primary question that inspired the following set of experiments is: Is there a primary peripheral signaling role for the proinflammatory cytokine IL-1β in sickness-induced cognitive dysfunction? That is, will the peripheral administration of IL-1β, alone, be sufficient to trigger the sequence of events leading to disruption of learning and memory? Further, will hippocampal LTP prove sensitive to the peripheral inflammatory response and, if so, will it also be sensitive to the peripheral administration of IL-1β? Finally, what specific cognitive process is disrupted in sickness behaviour; is it a global impairment or a process-specific (i.e., acquisition, consolidation, retention) disruption?

In order to separately characterize the physiological and behavioural effects of a full peripheral inflammatory response from those that are induced by only peripheral IL-1β, effects of IL-1β are compared to effects rendered by the bacterial endotoxin, lipopolysaccharide (LPS). LPS is the cell wall (i.e. active component) of all gram-negative bacteria and induces a full peripheral immune response common to infection, including the release of several bioactive mediators, such as oxygen free-radicals, prostaglandins, platelet-activating factor, and several proinflammatory cytokines (Rietschei & Brede, 1992). Of the proinflammatory cytokines that are released in response to peripheral LPS administration, significant rises in plasma tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), in addition to IL-1β, have been observed (Rietschei & Brede, 1992; Kakizaki, Watanobe, Kohsaka, & Suda, 1999).

Because it is well-known that both peripheral LPS and peripheral IL-1β are capable of triggering changes within the CNS through previously described humoral (Saper & Breder, 1994; Herkenhaum *et al*., 1998; Laflamme & Rivest, 1999; Konsman, 2000).and neural mechanisms (Bluthé *et al*., 1994; Brady *et al*., 1994; Ericsson *et al*., 1994; Watkins *et al*., 1994; Ninjima, 1996; Gaykema *et al*., 1998; Goehler, *et al*., 1998; Konsman *et al*., 2000), indication of a centrally-mediated (i.e., feeding, fever, etc.) behavioural change following peripheral LPS and / or IL-1β adminstration is evidence that the peripherally-administered compound effectively signaled the central nervous system. If LPS and IL-1β are administered at doses that have been previously demonstrated to cause central effects, and LPS, but not IL-1β, induces changes in learning, memory, or in hippocampal physiology, then it can reasonably be inferred that peripheral IL-1β is *not* the primary signaling molecule of sickness-induced cognitive dysfunction. If this is the case, then other components of the peripheral immune response, such as TNF-α and IL-6, either working alone or synergistically with IL-1β, are responsible for sickness-induced cognitive dysfunction.

In the first set of experiments (Experiments 1-2), the effects of LPS are measured on: temperature, food consumption, body weight, and general motor activity. If the peripheral administration of LPS induces a full peripheral immune response, then it should induce changes across all of these measures. If LPS does induce a full peripheral inflammatory response, indicated by measurable signs of sickness, then effects of peripheral LPS and peripheral IL-1β on learning, memory, and hippocampal physiology can be compared. This comparison is necessary to the investigation of whether cognitive

dysfunction and disruption of hippocampal physiology constitute central components of sickness behaviour.

The second set of experiments (Experiments 3-7) is designed to test learning and memory across a variety of paradigms and doses to determine whether specific cognitive process(es) are sensitive to LPS and / or IL-1β. Again, if LPS administration causes changes in learning and memory that are not evident following systemic IL-1β administration, then other components of the peripheral immune response that act alone or in concert with IL-1β are primarily responsible for sickness-induced cognitive dysfunction.

The third set of experiments (Experiments 8-9) are designed to test whether hippocampal LTP in the freely-moving animal is sensitive to the peripheral immune response and, if so, whether the systemic administration of IL-1β is sufficient to disrupt its expression. A LPS or IL-1β-induced disruption of hippocampal LTP would support the theory of a mechanistic role for hippocampal LTP disruption in sickness-induced cognitive dysfunction, while the comparison of LPS- and IL-1β-induced effects will determine whether IL-1β is the primary peripheral signaling molecule of hippocampal LTP disruption.

The final experiment (Experiment 10) is designed to test the effects of the peripheral administration of IL-1β and IL-6 on hippocampal cell proliferation. Though not a direct assessment of hippocampal neurogenesis, the quantification of hippocampal cell proliferation following peripheral proinflammatory cytokine administration should indicate whether the acute administration of proinflammatory cytokines, such as IL-1β

and IL-6, exert inhibitory effects on a different (i.e., other than hippocampal LTP) hippocampal cellular process that correlates with learning and memory.

These experiments will answer the question of whether peripheral IL-1β is sufficient to trigger cognitive dysfunction and / or the disruption of hippocampal cellular processes. Furthermore, the experiments will attempt to characterize the learning and memory processes (i.e., acquisition, consolidation, retention) and underlying cellular processes (hippocampal LTP, hippocampal cell proliferation) that are disrupted in sickness-induced cognitive dysfunction.

General Animals and Housing

Male adult Long-Evans rats, supplied by Charles Rivers Laboratories (Montreal, Quebec) were used in all experiments. Rats were housed in pairs (individually housed in Experiments 8,9) and maintained on a constant light and dark schedule (light on 07:30 – 19:30) in the animal housing facilities at the Canadian Centre for Behavioural Neuroscience at the University of Lethbridge. Animals were allowed free access to both food and water throughout the experiments. Blood samples taken from sentinel animals at the termination of each experiment verified that the vivarium room was free from bacteria/viruses/parasites which could induce peripheral immune activation in the rats and obscure effects of the experimental treatments. All serology reports verified the vivarium room to be free from such organisms.

Sickness Behaviour (Experiments 1-2)

Introduction

Lipopolysaccharide (LPS), a potent endotoxin derived from gram-negative bacteria, should induce a full peripheral immune response in the rat (Rietschei & Brede, 1992;

Kakizaki *et al*., 1999). If sickness behaviour, described as a constellation of symptoms that accompanies illness (Hart, 1988), is a consistent behavioural phenomenon that accompanies infection, then several behavioural changes should occur in LPS-treated animals. Indeed, LPS-treated animals have been observed to show reduced social exploration and weight loss (Bluthé, Dantzer, & Kelly, 1992; Swiergiel, Smagin, Johnson, & Dunn, 1997), as well as anhedonia (Borowski, Kokkinidis, Merali, & Anisman, 1998). In addition, the systemic administration of an IL-1β antagonist blocks some behavioural signs of sickness, including loss of body weight and social withdrawal (Bluthé *et al*., 1992), implying that signaling by IL-1β is necessary to the induction of some components of the sickness response.

Prior to comparing the effects of peripheral LPS and IL-1β on cognition, it is necessary to confirm findings from previous studies that the peripheral administration of LPS does induce several sickness behaviours, such as decreased food intake and anhedonia (Bluthé *et al*., 1992; Swiergiel *et al*., 1997; Borowski, *et al*., 1998). Furthermore, it is necessary to confirm that LPS, at the same dose, produces a reliable inhibition of general motor activity. If it does, then it can be concluded that LPS induces a sufficient peripheral immune response,- effects of which can then be compared to those induced by IL-1β. In Experiment 1, the effects of LPS on food consumption and weight are measured. In Experiment 2, the effects of LPS on general motor activity are assessed. If LPS does reliably induce sickness behaviour, then reductions should be observed across all three measures.

Both experiments compare the effects of peripheral LPS to effects induced by peripheral IL-1β in order to establish a basis on which further comparisons of LPS and

IL-1β effects on cognition can be compared. An absence of an effect of peripheral IL-1β on cognition would signify an absence of an effect specifically on cognition, and not an inability of the compound to induce a central response.

Methods

Drugs For Experiments 1 and 2, LPS was supplied by Sigma Aldrich (LPS Ecoli 026:B6; Sigma, Lot no. 101K4080; 3 million endotoxin units per MG) and IL-1β was supplied by Research Diagnostics Inc.((RDI) catalog no. RDI-201b). Each compound was prepared in a pyrogen-free saline solution (Abbot Laboratories, Lot no. 79-613-DM-01) the morning of the experiment. A pyrogen is any substance capable of producing a fever. Therefore, in order not to obscure immune activation induced by the experimental treatment with saline-induced immune activation, pyrogen-free saline was used in all experiments.

Procedure: Experiment 1 Nine animals, divided equally into three groups (LPS, IL-1β, saline) were used in the first experiment to investigate the effects of LPS and IL-1β on food consumption and weight loss. Baseline measurements for both food consumption and weight were taken for two days prior to injections. Food consumption was measured by weighing the amount of food remaining in each animal's cage at the same time each morning.

Injections were administered after two days of baseline measurements. Animals received i.p. injections of: 100 μg/kg of LPS, 12μg/kg of IL-1β, or an equivalent volume of pyrogen-free saline. The dose of LPS was chosen based on a previous study demonstrating an anhedonic-inducing effect of LPS at a similar dose (Borowski, Kokkinidis, Merali, & Anisman, 1998). The dose of IL-1β was chosen based upon a

preliminary experiment showing an inhibitory trend in hippocampal LTP at this dose (see Appendix C). Presumably, these doses are sufficient to induce at least some central changes. If sickness behaviour is a robust response to systemic infection, the same dose should also cause a robust sickness response evidenced across several behavioural measures.

Post-injections measurements of both food and weight were taken 24 hours following injections.

Procedure: Experiment 2 10 animals were used in Experiment 2 (LPS- 4, IL-1β-6) to test the effect of peripheral LPS and IL-1β administration on activity. General motor activity was measured using activity-monitoring boxes (36 cm X 36 cm wire-mesh cages; manufactured in-house) individually equipped with infra-red sensors. Activity was measured in one-hour intervals. A 24-hour baseline measurement was taken in which the animals were allowed sufficient time to become familiar with their surroundings prior to injection.

On injection day, animals received injections of: 100 μg/kg of LPS, 12 μg/kg of IL-1β, or an equivalent volume of saline. Animals were returned to their cages immediately following injections and left for the remainder of the experiment (24 hours).

General activity was measured as the total number of cage crosses detected for each animal. Because the general activity monitoring system will often detect small movements by the animal and label them as crosses to the left or the right of the cage, these movements were not included in the analyses. Rather, the outcome measurement was the average difference in the total number of cage crosses for each animal between the 24 hour post-injection period and the 24 hour baseline period.

In addition to food consumption, weight, and general activity measurement, three three-hour measurements of brain temperature were recorded in an animal injected with: 250 μg/kg of LPS, 25 μg/kg of IL-1β, or an equivalent volume of pyrogen-free saline (see Appendix A). Though lower doses (2 μg/kg) have been observed to induce a febrile response (Yirmiya, Tio, & Taylor, 1996), these does have been demonstrated to induce a robust febrile response that dissipates after 24 hours (Konsman, Luheshi, Bluthé, & Dantzer, 2000).

Results

Experiment 1 There was a significant difference between saline and LPS- treated animals in both post-injection food consumption and body weight. LPS-treated animals ate significantly less ((t (4) = -3.9, $p \leq .05$); see Figure 1) and weighed significantly less ((t (4) = 3.7, $p \leq .05$; see Figure 2) than the saline-treated animals. Baseline food consumption did not differ between groups (t (4) = -.42, $p > .05$), with both groups leaving approximately the same amount of food in their trays (LPS M = 234 g, saline M = 234 g).

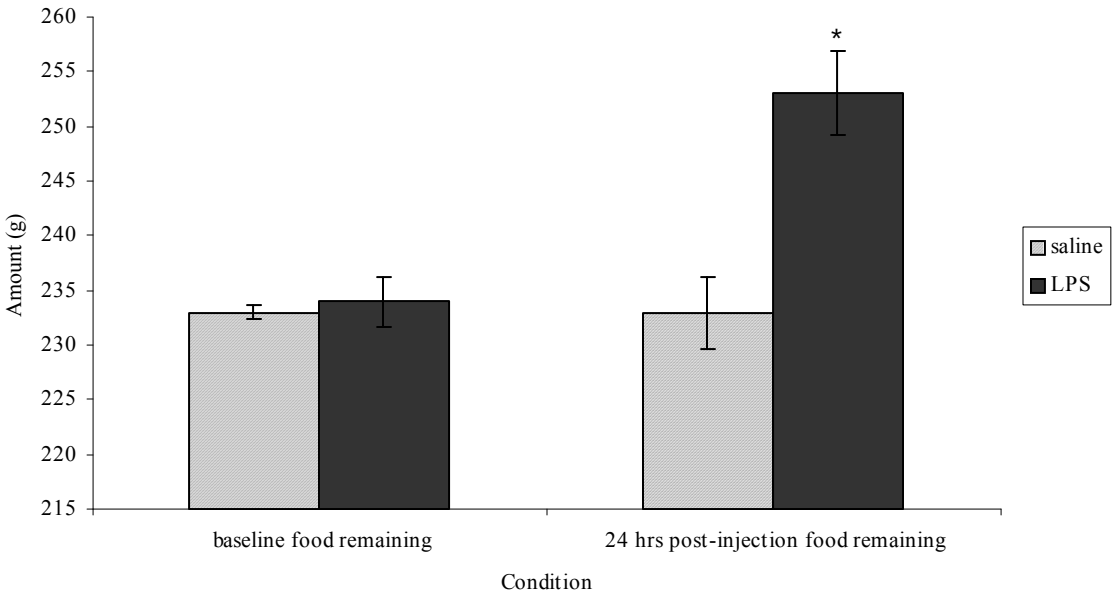


Figure 1. Effects of LPS on food consumption. Mean and SEM of the amount of food remaining (g) at baseline and following injections of 100 µg/kg of LPS and an equi-volume amount of pyrogen-free saline. *Significant difference in amount of food remaining 24 hours following injections ($p < .05$).

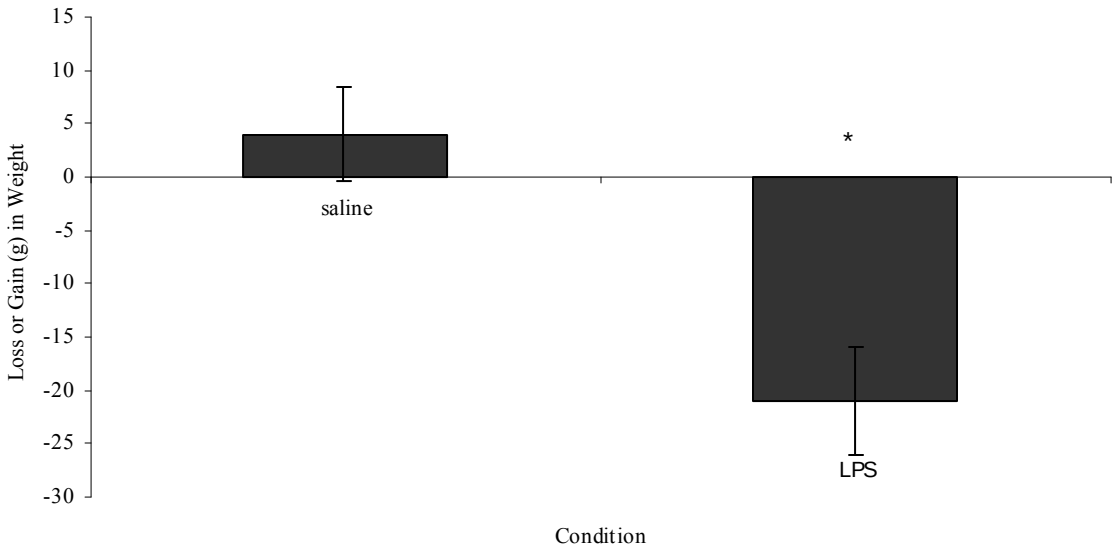


Figure 2. Change in weight 24 hours following LPS injection. Mean and SEM of change in weight (g) are depicted for animals injected with either 100 µg/kg of LPS or an equi-volume amount of pyrogen-free saline. *Significant difference between groups in amount of weight gained or lost over the 24 hour period following injections ($p < .05$).

As depicted in Figures 3 and 4, the peripheral administration of IL-1β also caused significant reductions in both food consumption ($t(4) = -2.9$, $p \leq .05$) and weight ($t(4) = 5.3$, $p \leq .05$). Though randomly assigned, a significant difference was also observed in baseline food consumption between treatment groups, with animals assigned to the IL-1β condition having eaten significantly *more* prior to injection than the animals who had been assigned to the saline condition ($t(4) = 4.8$, $p \leq .01$).

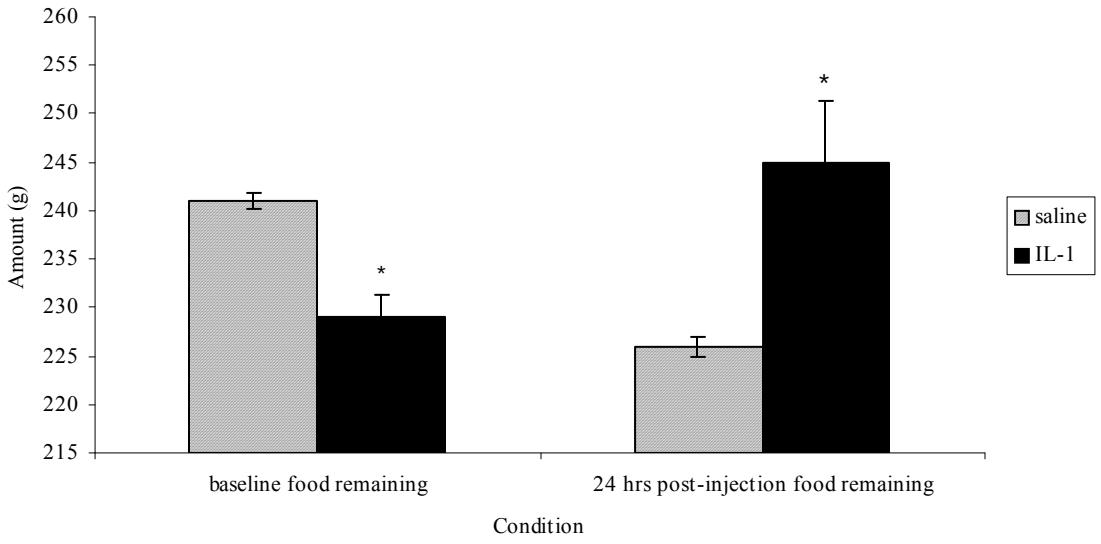


Figure 3. Effects of IL-1β on food consumption. Mean and SEM of the amount of food remaining (g) at baseline and following injections of 12 μg/kg of IL-1β and an equi-volume amount of pyrogen-free saline. *Significant difference in amounts of food remaining at baseline ($p \leq .01$) and 24 hours following injections ($p < .05$).

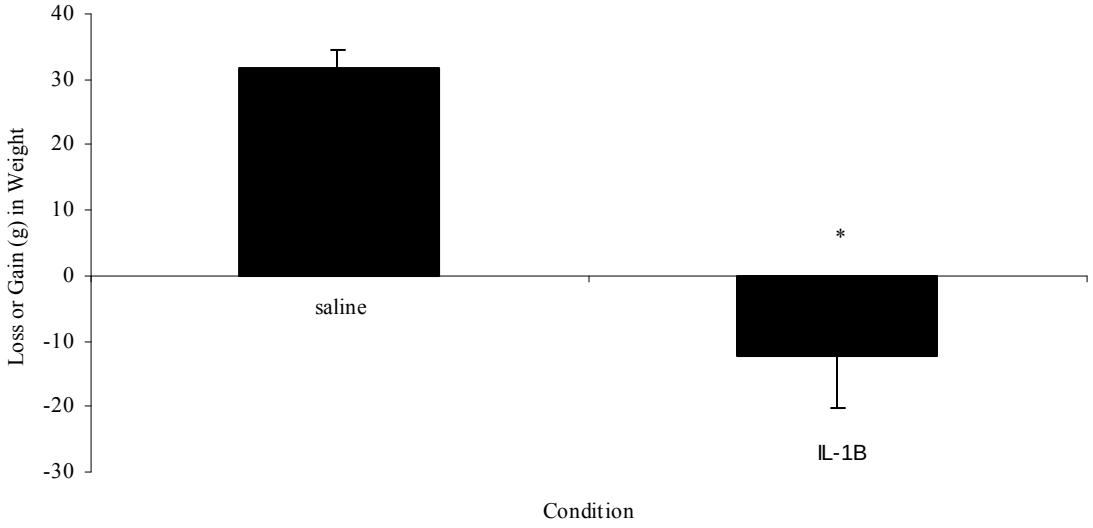


Figure 4. Change in weight 24 hours following IL-1β injection. Mean and SEM of the change in weight (g) are depicted for animals injected with either 12 μg/kg of IL-1β or an equi-volume amount of pyrogen-free saline. *Significant difference between groups in amount of weight gained or lost over the 24 hour period following injections ($p \leq .01$).

Experiment 2 As depicted in Figure 5, LPS-treated animals were less active than saline-treated animals (t (3) = -3.8, $p \leq .05$). Whereas the LPS-treated animals averaged approximately 30 *fewer* crosses following injections, saline-treated animals averaged 50 *more* crosses following injections.

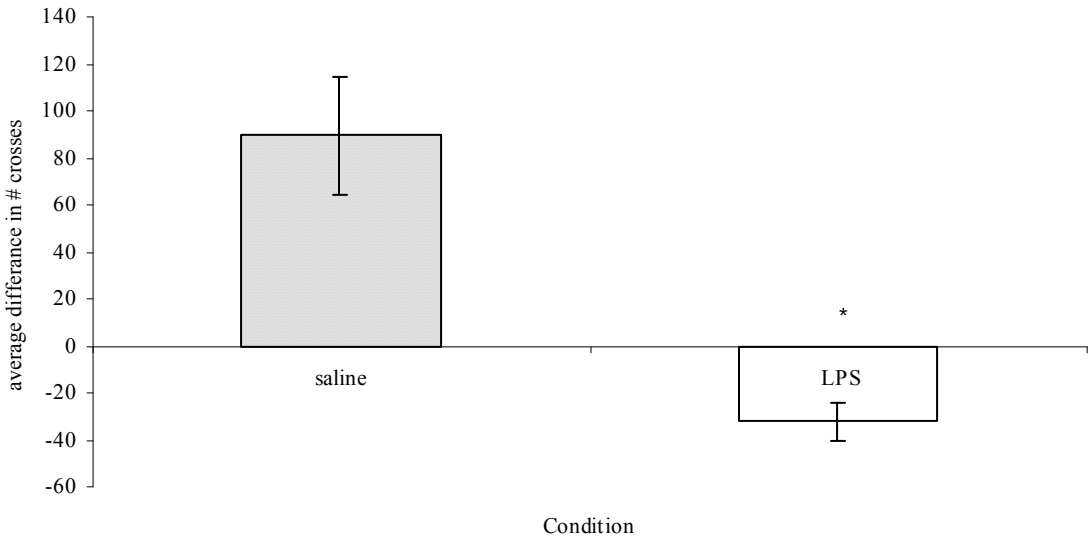


Figure 5. LPS effects on general motor activity. Mean and SEM are depicted of the difference in the total number of cage crosses before and after injections of either 100 μg / kg LPS or an equi-volume amount of pyrogen-free saline. *Significant difference observed in difference in total number of crosses between LPS and saline-treated animals ($p \leq .05$).

No significant differences in 24-hour general motor activity were observed in animals treated with IL-1β compared to saline-treated animals (t (5) = .47, $p \geq .05$). However, a closer look revealed a significant difference in general motor activity during the first eight hours following injection. Though treatment groups did not differ during the first eight hours of baseline activity (t (5) = -.35, $p \geq .05$), the groups did differ in general activity during the first eight hours following injection, with IL-1β-treated animals

averaging a significantly lower total number of crosses ((t (5) = 3.14, p ≤ .05; see Figure 6).

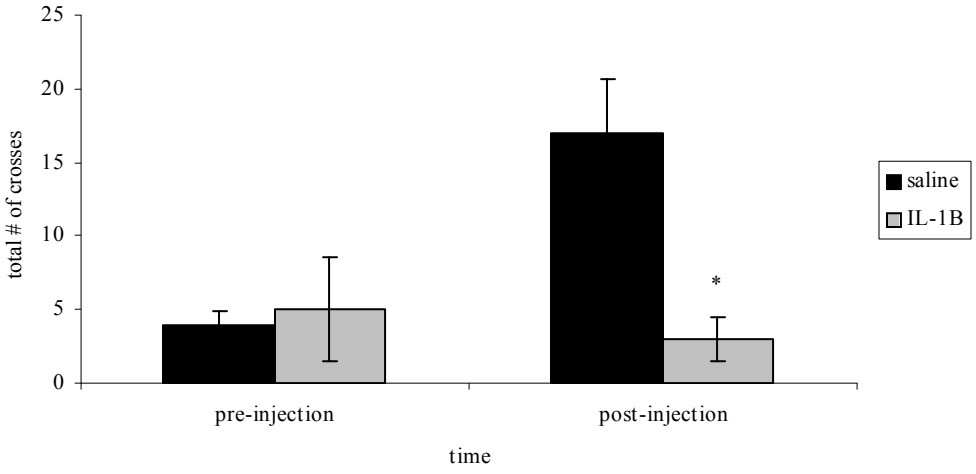


Figure 6. IL-1β effects on general motor activity during the first 8 hours following injection. Mean and SEM are depicted of the total number of crosses before and after injection with either 12μg/kg of IL-1β or an equi-volume amount of pyrogen-free saline. *Significant difference observed between groups in general activity level in first 8 hours following injection (p ≤ .05).

In addition to reductions observed in food consumption, weight, and general activity in both LPS and IL-1β-treated animals, brain temperature was elevated by both compounds (see Appendix A).

Conclusions: Experiments 1 & 2

Anorexia and fatigue are hallmark symptoms of infection. These symptoms are components of a centrally-organized adaptive response, coined *sickness behaviour*, that is employed by the host to conserve the additional energy it requires to mount the febrile response necessary to effective pathogen clearance (Hart, 1988; Kluger, 1992).

As observed in Experiments 1 and 2, the administration of LPS caused significant reductions in food consumption, weight, and in general motor activity. It can reasonably be inferred that these behavioural changes were fundamental to the animal's ability to mount the febrile response that was observed following the administration of endotoxin (Appendix A).

Not surprisingly, the effects of peripherally-administered IL-1β on food consumption and weight closely mirrored those rendered by LPS, as previous researchers have suggested a primary peripheral signaling role for IL-1β in these behaviours (Bluthé *et al*., 1992; Kent *et al*., 1996). In addition, though other proinflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor- alpha (TNF-α) act centrally on the hypothalamus to induce the febrile response to infection, peripheral IL-1β appears to be the primary signaling molecule for this central febrile response to infection (Luheshi & Rothwell, 1996).

Interestingly, peripherally-administered IL-1β produces similar, but not identical effects to LPS on behavior. This observation was most evident in Experiment 2, where LPS induced a general reduction in activity over a 24-hour interval, whereas IL-1β-induced reductions were only observed during the first eight hours following injection, suggesting that the magnitude of the suppressive effect of IL-1β and LPS on general motor activity is different. Indeed, other sickness behaviours, such as reduced food intake, which have been demonstrated to be induced by the peripheral administration of IL-1β (Bluthé *et al*., 1992; Kent *et al*., 1996), are attenuated, but not completely blocked by an IL-1β receptor antagonist (IL-1ra) following LPS adminstration. This lack of an ability of an IL-1ra to completely block LPS-induced sickness behaviours, suggests that

additional immune molecules and / or inflammatory events are involved in some sickness behaviours. Certainly, the observation of differential effects of LPS and IL-1β on general motor activity suggests this as well.

In summary, the intent with this first experimental set was to ensure that peripherally-administered LPS induced a full peripheral immune response to which the effects of peripherally-administered IL-1β could be compared. The behavioural and physiological changes observed following the peripheral administration of LPS confirms that LPS does initiate a sickness response indicative of peripheral immune activation. In addition, the peripheral administration of IL-1β also induces central effects, thereby allowing the inference that any absence of effects on cognition observed following IL-1β administration is not due to an inability of the compound to effect a central response, but rather is an inability of the compound to effect changes specific to cognition. Also, because both compounds reliably induce several sickness behaviours, effects of LPS and / or IL-1β on learning and memory can be dissociated from other sickness behaviours. If LPS induces a learning and memory deficit that is not present following the peripheral administration of IL-1β, then the learning deficit is likely *not* secondary to other illness processes.

Learning and Memory (Experiments 3-7)

Introduction

The purpose of this second set of experiments is to determine the role of peripheral IL-1β in cognitive disruption, as well as to determine the cognitive processes that are disrupted by the peripheral inflammatory response.

To date, most of the investigation into sickness-induced cognitive dysfunction has focused on the effects of LPS and IL-1β on hippocampal learning and memory systems. The hippocampus is a target worthy of investigation into sickness-induced cognitive dysfunction, not only because the hippocampal system is widely held to be integrally involved in numerous aspects of cognition, including configural learning (Rudy & Sutherland, 1989; Sutherland, McDonald, Hill, & Rudy, 1989); spatial navigation (Holscher, 2003 (review)); and visual discrimination, or object recognition (Nakamura & Kubota, 1996 (review)); but also because IL-1β receptors are up-regulated in the hippocampus following pathogen exposure, such as following exposure to LPS (Nagano, Takao, Nanamiya, & Hashimoto, 2000) or the envelop glycoprotein for the Human Immunodeficiency Virus ((HIV-1, gp120) Pugh *et al*., 2000). For these reasons, the majority of experiments investigating the effects of IL-1β and LPS on learning and memory involve the measurement of performance on tasks that require (i.e., sensitive to insult) the hippocampus.

Two learning and memory tasks demonstrated to require an intact hippocampus are the Morris water task ((Morris, 1981) Morris, Garrud, Rawlins, & O'Keefe, 1982; Sutherland, Kolb, & Wishaw, 1982; Sutherland, Wishaw, & Kolb, 1983) and contextual fear conditioning (Sutherland & McDonald, 1990; Opp, Obal, & Krueger,

1991; Seldon, Everitt, Jarrard, & Robins, 1991; Kim & Fanselow, 1992; Philips & LeDoux, 1994; 1995). The Morris water task is a spatial learning and memory task in which animals are trained to use distal cues to find their way to a submerged platform hidden in a circular pool of opaque water. The animal is released from different starting locations within the pool (NSEW), and so must learn to use the configural relationships among the different cues in the room to navigate to the platform successfully. This ability to navigate through an environment using topographical relationships among different objects within the environment has been demonstrated to be dependent upon the hippocampal formation, as animals with hippocampal damage show severe impairments in their ability to navigate to the submerged platform (Morris *et al*., 1982; Sutherland *et al*., 1992; 1993).

In the contextual fear conditoning task, animals are conditioned to associate both a context and auditory tone with a foot shock. The animals' retention for the context and tone are later assessed, via the assessment of the animals' fear response (freezing) to either the tone (auditory-cue fear conditioning) and / or the context (contextual fear conditioning). Typically, a conditioning chamber consists of a clear, plexi-glass ceiling, four walls, and a floor consisting of stainless-steel rods. By removal of the floor and / or the insertion of plastic sides to cover the clear walls, the context can be altered. This alteration permits the dissociation of context and tone retention, which, in turn, allows for the clear identification of hippocampal-specific effects of a treatment. An intact hippocampus is necessary to successful task performance through its proposed ability to store a representation of a set of features bound into a unitary representation, referred to as a *conjunctive representation* (Rudy & O'Reilly, 2001 (review)). This proposal is

supported by observations that animals with hippocampal damage exhibit selective impairments in retention for the original context in which they had experienced the shock, but show normal retention for the tone presented in an alternate environment (Opp, Obal, & Krueger, 1991; Jarrard, & Robins, 1991; Kim & Fanselow, 1992; Philips & LeDoux, 1994; 1995). This dissociation suggests that animals with compromised hippocampal functioning are impaired specifically in their ability to create and store representations of contextual information, not in their ability to retain information regarding the aversive quality of the shock, or their ability to mount an appropriate fear (i.e., freezing) response.

The use of the contextual fear conditioning task also allows for the additional dissociation of the animals retention specifically *for* the context from the animals' retention for the aversive quality of the shock *within* the context. The ability to construct a representation of the features and the ability to associate this representiation with the aversive qualities of the shock are held to constitute two distinct processes, with only the former demonstrated to depend on the hippocampus (Young, Boheneck, & Fanselow, 1993; Rudy, 1996; Pugh, Tremblay, Fleshner, & Rudy, 1997; Pugh *et al*., 1998; Barrientos *et al*., 2002). The dissociation between the two processes can be made by briefly (i.e, couple of minutes) pre-exposing the animals to the context in which they will later receive the foot shock. Pre-exposure presumably allows the animal to acquire a stable representation of the environment, as several studies have demonstrated that impairments in context retention that had been previously observed following hippocampal damage and / or insult, were abolished if the animals were pre-exposed to the context prior to damage and / or insult (Young *et al*., 1993; Rudy, 1996; Pugh *et al*.,

1997, 1998; Barrientos *et al*., 2002). It has been proposed, and supported with data, that the hippocampus is necessary to the beneficial effects of brief pre-exposure through its ability to acquire a conjunctive representation of the context (Rudy & O'Reilly, 1999; Rudy, Barrientos, & O'Reilly, 2002). Therefore, the use of the contextual fear conditioning task can be used to reveal learning and memory effects that are specific to effects on hippocampal functioning, as well as to identify non-hippocampal treatment-induced effects.

Available data do not provide for a clear picture of which, if any, cognitive processes are impaired by the peripheral inflammatory response, as it is induced by the administration of endotoxin. In one of the few studies to investigate the effects of peripherally administered LPS on Morris water task performance in the rat, Shaw and colleagues (2001) observed a LPS-induced inhibitory effect on latency to reach the platform on days four and eight following a single LPS injection (100 μg/kg). However, this inhibitory effect was not observed on any other of the testing days, making difficult the extraction of any meaningful conclusions. Furthermore, Shaw *et al*. (2001) used a fixed release point in their training protocol. This is important because the use of a fixed release point does not require the animal to alter its swim path in accordance with extra-maze cues. In fact, Eichenbaum and colleagues (1990) demonstrated that animals with hippocampal damage can perform as well on the fixed-start version of the Morris water task as control animals. To conclude, then, the data provided by Shaw *et al*. (2001) suggest that peripherally-administered LPS may disrupt Morris water task performance via a LPS-induced inhibition of hippocampal learning and memory systems, but they cannot be considered strong support for the proposal.

The strongest evidence of a process-specific effect of LPS on learning and memory comes from a series of experiments on Pavlovian contextual fear conditioning conducted by Pugh and colleagues (1998). Pugh *et al*. observed that peripheral administration of LPS immediately after conditioning impaired retention of contextual fear, but not fear of an auditory-cue. This dissociation suggests that LPS disrupts a hippocampal-specific learning and memory process. Furthermore, Pugh and colleagues report that brief pre-exposure to the context eliminated the inhibitory effect of LPS on context retention. However, this impairment re-emerged if LPS was administered immediately after the context pre-exposure. Taken together, these results suggest that LPS disrupts a short-term, post-trial memory consolidation process. Because context pre-exposure eliminated the inhibiting effect LPS had on context retention, LPS appears to specifically disrupt a hippocampal-dependent ability to acquire a representation of the context, and *not* the animal's ability to retain a representation of the aversive quality of the shock (Young *et al*., 1993; Rudy, 1996; Pugh *et al*., 1997, 1998; Barrientos *et al*., 2002). This evidence, then, suggest that LPS interferes with retrograde (i.e., memories acquired immediately prior to endotoxin administration) and not with anterograde (i.e., memories acquired following endotoxin administration) learning and memory processes. It appears necessary, then, to separately assess the effects of LPS on these two processes in the Morris water task.

In addition to the clarification of the cognitive processes that are disrupted by the administration of endotoxin and the consequent peripheral inflammatory response, it is necessary to determine if the proinflammatory cytokine IL-1β is the primary peripheral signaling molecule of sickness-induced cognitive dysfunction. Evidence exists to suggest

that IL-1β is the primary *central* signaling molecule of sickness-induced cognitive dysfunction. Animals who received an intracerebroventricular (i.c.v) administration of IL-1β one hour prior to training in a fixed-platform version of the Morris water task showed longer latencies to reach the hidden platform the following day (Oitzl, van Oers, Schobitz, & de Kloet, 1993). However, because the cytokine was administered prior to the training episode, it cannot be concluded whether central IL-1β disrupts acquisition or retention. A later study conducted by Barrientos and colleagues (2002) suggests that central IL-1β impairs learning and memory by disrupting memory consolidation. Barrientos *et al* (2002) observed that animals who received an injection of IL-1β into the dorsal hippocampus immediately following a brief pre-exposure to the environment in which they would receive a foot shock, later showed impaired retention for the context. These observations suggest that, not only does central IL-1β inhibit a hippocampal-dependent learning and memory process, but that IL-1β may also be the central signaling molecule of the post-trial consolidation-specific effect of LPS that had been earlier observed by Pugh and colleagues (1998).

Peripherally, the actions of IL-1β on hippocampal learning and memory systems are not clear. Gibertini and colleagues (1995) observed an inhibitory effect of peripherally-administered IL-1β on Morris water task performance in mice. Using a fixed-platform version of the task, Gibertini peripherally administered IL-1β prior to training on two consecutive days. After being left unperturbed for a week, the IL-1β-injected animals were slower to reach the hidden platform. However, because Gibertini *et al*., (1995) administered IL-1β prior to training, effects of peripheral IL-1β on learning cannot be dissociated from IL-1β effects on retention. Moreover, a later experiment by the same

group (Gibertini, 1998) revealed that IL-1β-injected animals were no longer impaired in their ability to locate the hidden platform using a different training protocol, water temperature, or dose. A spaced training protocol (i.e., greater delay between training blocks), colder pool water, and a higher dose of IL-1β did not induce longer escape latencies in the animals during retention testing (Gibertini, 1998). Therefore, if peripheral IL-1β is sufficient to induce learning and memory impairments, the impairments may not be global nor robust.

Importantly, the effects of peripherally administered IL-1β on Morris water task performance in rats have not yet been investigated. Because differences in performance in the Morris water task have been observed within strains of a species (Klapdor & van der Staay, 1996; Gleason, Dreiling, & Crawley 1999) and across species, such as the mouse and the rat (Frick, Stillner, & Berger-Sweeney, 2000), the effects of peripherally administered IL-1β on Morris water task performance in the rat should be investigated prior to forming any conclusion regarding the role of peripheral IL-1β in sickness-induced cognitive dysfunction.

Taking the foregoing observations into consideration, it appears necessary to address the question of the key components, and key cognitive consequences, of the peripheral inflammatory response more systematically than has been done previously. First, it is necessary to distinguish anterograde from retrograde learning and memory effects induced by LPS and / or IL-1β. Second, it is necessary to compare the effects of the two compounds across learning and memory paradigms. It is possible that one or both compounds might have an effect on performance in a task, such as the contextual fear conditioning task that assesses configural learning and memory, but not on performance

in a task, such as the Morris water task that assesses the ability to use configural associations to accurately navigate to a hidden target. In order for results to successfully converge on a single hippocampal-depended process, similar impairments in both tasks would be necessary. The pattern of results should reveal which cognitive processes are sensitive to the peripheral inflammatory response, as it is induced by LPS, and whether the peripheral actions of IL-1β, alone, are sufficient to trigger similar changes.

These questions are addressed in the following experimental set (Experiments 3 – 7). In Experiments 3 and 4, the effects of peripheral LPS and IL-1β are measured on post-trial memory consolidation processes in the Contextual fear conditioning task. If LPS disrupts post-trial memory consolidation processes, then LPS, administered immediately after conditioning, should inhibit context retention (replicating Pugh *et al*., 1998). If IL-1β is the primary peripheral signaling molecule of this effect, then a similar impairment in context retention should be observed following peripheral IL-1β administration.

In Experiments 5-7, the effects of peripherally-administered LPS and IL-1β on learning and memory performance in the Morris water task are assessed in both the anterograde and retrograde directions. If sickness-induced cognitive dysfunction is a robust memory consolidation-specific effect, an impairment should also be evident in animals who are administered LPS following the acquisition of new spatial location information. In addition, if IL-1β is the primary peripheral signaling molecule of this effect, then IL-1β -treated animals should exhibit a similar performance deficit.

Experiments 3 & 4: LPS and IL-1β Effects on Contextual Fear Conditioning

Methods

Apparatus. Testing chambers were supplied by Med Associates Inc (MED-AFC-R1). Each chamber (25.4 cm L X 31.8 cm W X 22.9 cm H) consisted of: removable floors of stainless steel rods (0.5 cm in diameter, spaced 1.0 cm apart), a metal floor pan, and a speaker through which a 1000 Hz tone was presented (conditioning and tone retention). A 2 s. 1.0 mA shock was delivered through the rods. The chambers were altered during tone retention testing by removing the floor rods and inserting three white plastic panels to cover the clear plexi-glass top and two sides of the chamber. Additionally, the ambient light in the room was dimmed. A video camera that delivered images to data analysis software was placed approximately 1.5 meters from the chambers. Chambers were disinfected between trials.

Data analysis. Freezeframe (Actimetrics, Inc.) software was used to acquire video images of the animals and to quantify each animal's freezing response. As recommended by the software developers, the freezing threshold was chosen by examining motion index histograms generated for each animal and determining the value at which the trough in the histogram met the broad peak (represents activity). After each trial was individually reviewed to ensure that one value was valid for all animals, a freezing threshold of *20* was chosen. This value was within the range (20-30) recommended by the software developers.

Drugs. In Experiment 3, lipopolysaccharide (LPS Ecoli 026:B6); Sigma, Lot no. 101K4080; 3 million endotoxin units per MG) was injected i.p. at doses of 2μg/kg and 4μg/kg. These doses were matched, based on endotoxin levels, to the doses used by Pugh

et al. (1998). In Experiment 4, doses of IL-1β were chosen to incorporate a wide (albeit not all-inclusive) dose range, including a low, moderately-low, moderate, and high dose. Human recombinant interleukin-1beta ((hrIL-1β) Research Diagnostics Inc.(RDI); catalog no. RDI-201b) was injected i.p. at doses of: .1 μg/kg (low), 2 μg/kg (mod-low), 4 μg/kg (mod), and 100 μg/kg (high). All vehicle controls received i.p. injections of an equivalent volume of sterile, pyrogen-free saline (Abbot Laboratories, Lot no. 79-613-DM-01).

Procedure. Eighteen (6 per group) animals were used in Experiment 3 and thirty-five animals (seven per group) were used in Experiment 4. The experiments were conducted during the light cycle, between 09:00 and 14:00. The same procedure was used for both experiments. During the conditioning period, animals were placed in the conditioning chamber for five minutes. Two tone-shock pairings were presented during this time, one at 120 s. and one at 400 s. For each pairing, a two-second shock was presented during the last two seconds of a twenty-second tone. Following the second tone-shock presentation, animals were left in the chamber for 30 s. before being removed. Immediately upon removal, animals were injected with: Experiment 3: 2 μg/kg LPS, 4 μg/kg LPS, or saline; Experiment 4: .1 μg/kg IL-1β, 2 μg/kg IL-1β, 4 μg/kg IL-1β, 100 μg/kg IL-1β, or saline. Following injections, animals were returned to their home cage.

Retention for the context and tone was tested 48 hours following the conditioning period, allowing time for any direct effects of the injections to dissipate. Context retention testing was conducted prior to tone retention testing. During context retention testing, animals were placed into the original conditioning chamber for five minutes. After all animals had been tested for context retention, they were placed into the altered

chamber for six minutes to test for tone retention, during which the original tone was absent for the first three minutes of testing and present during the last three minutes of testing. Following the cessation of tone retention testing, all animals were returned to their home cage.

Results

Experiment 3. In an attempt to directly replicate the dose-specific effects of LPS on context retention as observed by Pugh *et al.* (1998), planned *t*-test were conducted on the percentage of time spent freezing during context retention for both the 2 μg/kg LPS and saline-treated groups, and the 4 μg/kg LPS saline-treated groups. As Figure 7 depicts, a significant impairment in context retention was observed in animals treated with 2 μg/kg of LPS ($t(10) = 2.67$; $p \leq .05$) compared to saline-treated controls. The 2 μg/kg LPS dose resulted in roughly a 40 percent decrease in freezing behaviour (M(saline) = 68%, M(2 μg/kg LPS) = 43%). A context retention impairment was not observed in animals treated with 4 μg/kg of LPS ($t(10) = 0.95$; $p \geq .05$) compared to saline-treated animals, though these animals showed a decreased mean freezing response (non-significant) compared to saline-treated controls (MU(4 μg/kg) LPS = 54%). When both LPS-treated groups were combined (n = 12) and compared to the saline-treated animals in Experiments 3 and 4 (n = 13), a significant impairment in context retention was similarly observed in the LPS-treated combined group ($t(23) = 2.42$, $p \leq .05$).

As depicted in Figure 8, no differences were observed among groups in their freezing response prior to and during the presentation of the tone as revealed by an absence of a group-by- time effect ($F(1,15) \leq 1$, $p > .05$). However, all animals froze significantly more during the presence of the tone, as revealed by a significant effect of time ((pre-

and-post tone presentation) ($F(1,15) = 61$, $p \leq .001$). Furthermore, a repeated-measures test on the combined data (2 μg/kg + 4 μg/kg LPS vs. Exp. 3 & 4 saline-treated animals) revealed similar effects, with an absence of a group-by- time effect ($F(1, 30) = 000.22$, $p > .05$) and the presence of a significant effect of time ($F(1, 30) = 159.93$, $p < .001$).

Figure 7. Retention for context 48 hours post-LPS. Mean and SEM of percentage of time spent freezing in original context are depicted for animals injected with: 2 μg/kg LPS, 4 μg/kg LPS, or saline.. *Significant difference between 2 μg/kg LPS and controls ($p \leq .05$).

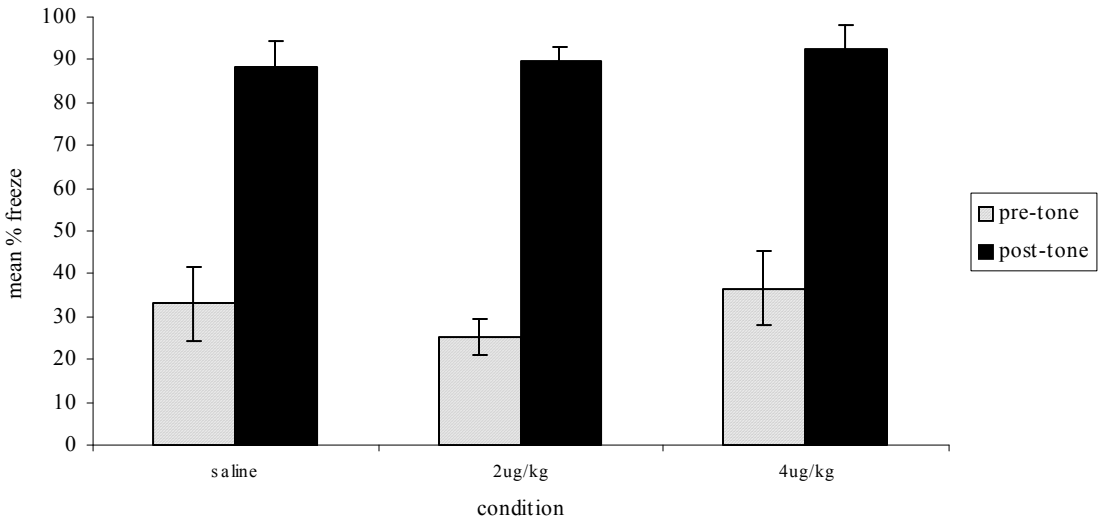


Figure 8. Retention for tone 48 hours post-LPS. Mean and SEM of percentage of time spent freezing before and after tone presentation for animals injected with: 2 µg/kg LPS, 4 µg/kg LPS, or saline. No significant differences among groups ($p > .05$).

Experiment 4. As depicted in Figure 9, no differences were observed among groups in their freezing response to the context ($F(4, 37) = 1.27$, $p > .05$). In addition, as depicted in Figure 10, no differences were observed among groups in their freezing response prior to and during the presentation of the tone ($F(4, 37) < 1$, $p > .05$) , with all animals freezing significantly more during the presentation of the tone ($F(4, 37) = 174$, $p \leq .001$).

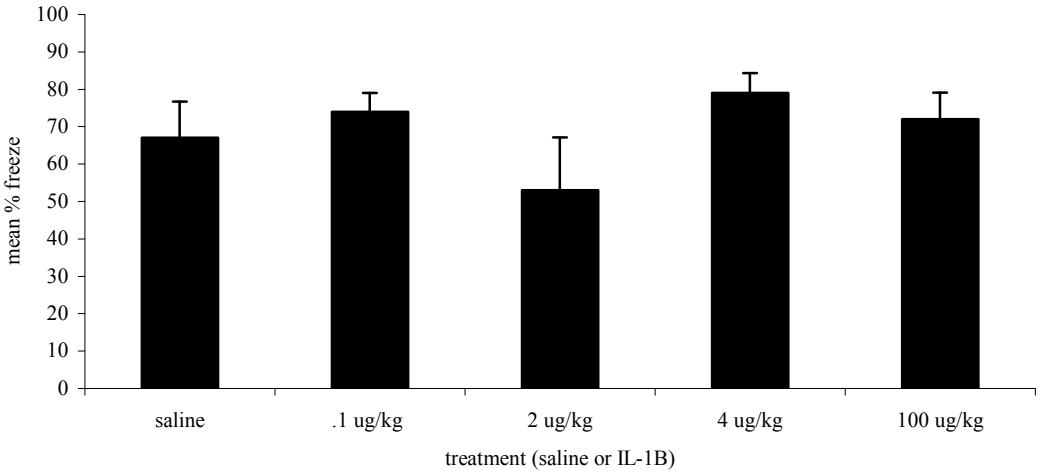


Figure 9. Retention for context 48 hours post-IL-1β. Mean and SEM of percentage of time spent freezing in original context are depicted for animals injected with: .1 µg/kg IL-1β, 2 µg/kg IL-1β, 4 µg/kg IL-1β, 100 µg/kg IL-1β, or saline. No significant differences observed among groups ($p > .05$).

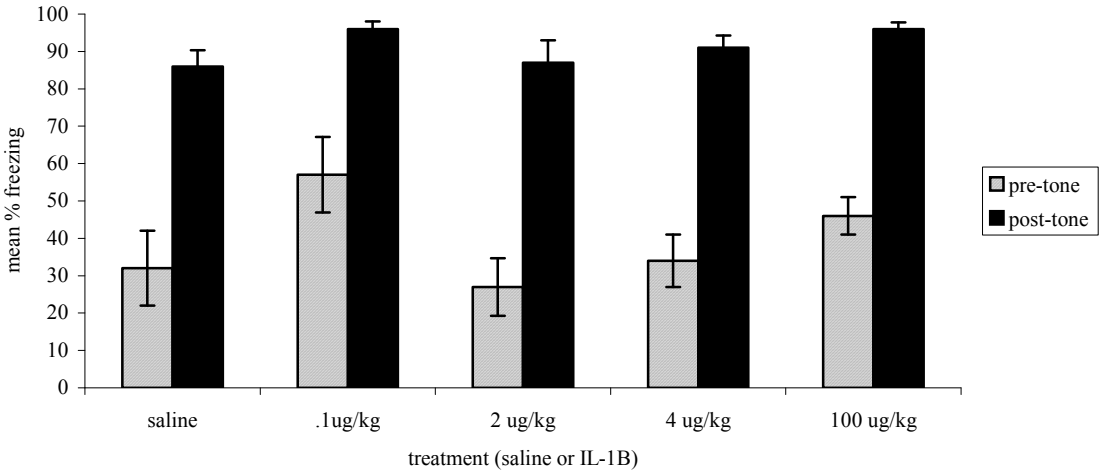


Fig. 10. Retention for tone 48 hours post-IL-1β. Mean and SEM of percentage of time spent freezing before and after tone presentation for animals injected with: .1 ug/kg IL-1β, 2 µg/kg IL-1β, 4 µg/kg IL-1β, or saline. No significant differences among groups ($p > .05$).

Conclusions: Experiment 3 & 4

Animals injected with a 2μg/kg LPS dose exhibited a significant impairment in context retention compared to the saline-treated animals, replicating effects observed by Pugh and colleagues (1998). Unlike Pugh and colleagues (1998), however, a significant inhibition of freezing behaviour in response to the original context was not observed in the animals treated with the higher dose of LPS (4μg/kg LPS), though these animals did exhibit a non-significant inhibitory trend. The difference in results could derive from differences in biological activity of the compound across batches, despite having been matched for equivalent endotoxin levels (Sigma Aldrich, technical support, scientific communication).

Because the hippocampus appears to be necessary to context retention and not to the retention and / or expression of fear, (Opp *et al*., 1991; Seldon *et al*., 1991; Kim & Fanselow, 1992; Philips & LeDoux, 1994; 1995), these results suggest that LPS, at certain doses, specifically disrupts hippocampal-dependent post-trial memory consolidation processes. The observation of a non-significant trend toward inhibition in the animals treated with the higher dose of LPS suggests that there may be a U-shaped dose-response relationship between LPS and memory consolidation, with moderate doses inhibiting memory more than lower or higher doses. Indeed, this type of relationship has been suggested by other researchers. Pugh and colleagues (1998) demonstrated that, whereas two of their two tested doses (.125 mg/kg, .25 mg/kg) inhibited context retention, animals injected with saline or a higher LPS dose (.5 mg/kg) showed normal context retention.

Unlike the LPS-treated animals, animals injected with four different doses-

.1 μg/kg, 2 μg/kg, 4 μg/kg, and 100 μg/kg- of IL-1β did not exhibit any significant differences in freezing behaviour during context retention testing compared to the saline-treated animals. A non-significant trend in inhibition of freezing behaviour during context retention was observed in the animals treated with 2 μg/kg of IL-1β, suggesting that systemic IL-1β may have an inverted-U dose-response relationship to learning and memory similar to LPS. This observation is consistent with previous observations that low levels of the pro-inflammatory cytokines are necessary for learning and memory (Gibertini, 1998; Yirmiya, Wincour, & Goshen, 2002; Brennen, Beck, & Servatius, 2003). It is plausible that moderate doses of systemic IL-1β - though not sufficient to impair learning and memory- are more likely than other dosage levels to impair learning and memory.

In summary, LPS, at low-moderate doses, disrupts memory consolidation. IL-1β showed a non-significant U-shaped relationship between dose and inhibition of memory consolidation. However, IL-1β, alone, is insufficient to cause reliable inhibition. As previously described, LPS is a potent endotoxin that induces the release of several proinflammatory cytokines in addition to IL-1β, such as IL-6 and tnf-α (Rietschei & Brede, 1992; Kakizaki, Watanobe, Kohsaka, & Suda, 1999). It is plausible, then, that the synergistic actions of these cytokines are required in the periphery to cause the inhibition of memory consolidation that we, and Pugh *et al*. (1998), observed.

Experiments 5-7: LPS and IL-1ß Effects on Morris water task Performance

Methods

Apparatus. The Morris water task apparatus consisted of a pool (1.5m diameter) filled to within 20 cm of the top of the wall with water (20 $\pm 1^0$ C) that was rendered opaque by skim milk powder. The wall of the pool was uniformly white. In addition, the pool was located in a room rich with distal cues, which remained intact and unobstructed throughout the duration of the experiment. During the hidden platform trials, a hidden platform, constructed of clear plexi-glass (13 X 13 cm), was submerged 1.5 cm under the surface of the water.

In all experiments, each trial began with the rat being placed in the pool at one of the 4 cardinal compass positions around the perimeter of the pool according to a pseudo-random sequence, such that each starting location was used once per block of four trials. The maximum duration of each swim trial was 60 seconds (s.). If the rat found the platform within this 60 s. period, it was allowed to remain on the platform for 8 additional seconds. If it did not find the platform during the allotted time, then it was manually placed onto the platform for 8 s, before being placed back into its holding cage. Following each swim trial, each rat was placed back into a holding cage where it was allowed to rest for at least five minutes before the start of the next swim trial.

Data analysis. Data were collected using a video camera + Windows-based microcomputer automated system, which included a HVS Image Analysis system with video monitoring and storage capabilities. In addition to overall latencies to find the platform, the system calculated: total swimming path distance, initial heading direction

(after swimming 13 cm), and proportion of swimming in each quadrant of the pool. The main outcome measure used for all experiments was the average latency of each group to navigate to the hidden platform. In addition, probe and visible platform trials were conducted for each experiment. For the probe trial, the platform was removed from the pool and each animal was allowed to swim for 30 s. before being removed from the pool. The percentage of time the animal spent in the quadrant in which the platform had been previously located was calculated for each animal. For the visible platform trial, the pool was drained approximately 3.0 cm so that the platform could be seen by the animals. It was placed directly across from one fixed release point and one trial was conducted for each animal. The visible platform trial was used as a non-hippocampal-dependent control task, as well as to control for any sensori-motor effects that could be induced by either or both of the compounds. The time to mount the visible platform was used as the dependent variable.

Drugs. In the following experiments, lipopolysaccharide (LPS Ecoli 026:B6); Sigma, Lot no. 101K4080; 3 million endotoxin units per MG) was injected intraperitoneally (i.p.) at doses of 2μg/kg (Experiment 5) and 100 μg/kg (Experiment 6). Human recombinant interleukin-1beta (hrIL-1β) was injected i.p at a dose of 2 μg/kg for all experiments (explanations for the dosage levels are provided in the *procedure* sections for each experiment). For Experiment 5, the cytokine was purchased from Research Diagnostics Inc.((RDI) catalog no. RDI-201b), and for Experiments 6 and 7, the cytokine was generously donated by the National Institutes of Health National Cancer Institute Biological Resources Branch (NIH NCI BRB) Preclinical Repository. The compounds were determined to have equivalent *in vivo* biological activity. All vehicle controls

received i.p. injections of an equivalent volume of pyrogen-free saline (Abbot Laboratories, Lot no. 79-613-DM-01).

Procedure: Experiment 5. A moving platform protocol was used in Experiment 5 to test the effects of peripherally-administered LPS and IL-1β on post-trial memory consolidation of spatial location information. This experiment was conducted in order to test whether the LPS-induced inhibitory effect in memory consolidation of context conditioning, observed in the previous experiment, generalized to an inhibition in memory consolidation in a spatial navigation task. In this experiment, animals (N = 21, 7 animals per group) were initially trained for five days, with one block of trials a day (4 trials per rat), to locate a hidden platform that was moved every other day to a new location within the pool. So, the sequence was: Day 1(new), Day 2(same), Day 3(new), Day 4(same), Day 5(new). Immediately following the completion of the fourth trial on the fifth day, animals were injected with: 2μg/kg of LPS, 2μg/kg of IL-1β, or pyrogen-free saline. These doses were based on observations from the previous experiment (Experiment 3) that these doses were most likely to inhibit memory consolidation. In addition, these doses have been previously demonstrated to induce symptoms of sickness, such as fever (Yirmiya, Tio, & Taylor, 1996). Following injections, animals were returned to their home cage and allowed to rest for 24 hours.

On testing day, all animals were first subjected to a probe trial in which the percentage of time spent in the quadrant in which the platform was located on Day 5 was calculated and used as the dependent variable. Following the completion of the probe trial, all animals received an additional block of trials (4 trials/rat) during which they had to locate

the platform, hidden in the same location as Day 5. Finally, all animals performed a visible platform trial.

Procedure: Experiment 6. Whereas experiment 5 tested the effects of LPS and IL-1β on retrograde learning and memory processes (i.e, memory consolidation), experiment 6 tested the effects of both compounds on anterograde learning and memory processes. In this experiment, animals were injected with either LPS or IL-1β *prior* to having to learn the location of a new platform location. Two separate groups of animals were run in this experiment (group 1 receiving LPS (N = 10, 5 animals per group); group 2 receiving IL-1β (N = 12, 6 animals per group). However, because the experimental procedure for both experiments was identical, both data sets are included in this section.

In Experiment 6, animals were trained to navigate to a hidden platform that was moved daily to a different location in the pool. Each animal received 1 block (4 trials) of training per day. Animals were trained until their average escape latencies reached asymptote. On testing day, animals were injected with: 100 μg/kg of LPS, 2 μg/kg of IL-1β, or an equi-volume amount of pyrogen-free saline. A higher dose of LPS was used in this experiment in an attempt to replicate Shaw *et al*. (2001) who had observed the previously discussed inhibitory effect of LPS at this dose on ability to learn the location of the platform in the Morris water task. At one hour, animals were required to navigate to a recently-moved hidden platform. A block of 4 trials was conducted in which the difference between the average escape latencies for trials 1 and 2 was calculated for each animal and averaged across treatment groups. In addition, probe and visible platform trials were conducted upon the completion of the fourth trial.

Additional testing was conducted for four days following injections in an effort to replicate Shaw *et al*. (2001) who observed a LPS-induced inhibitory effect on Day 4 performance.

Procedure: Experiment 7. A fixed platform version of the Morris water task was used in this experiment to test the effects of peripherally-administered IL-1β on the retention of a well-learned spatial location. Because evidence suggests (Gibertini *et al*, 1995) that IL-1β might interfere with the retention of spatial location information, effects were measured of peripherally-administered IL-1β on retention of a platform's location after it had been well-learned over a course of several days. Animals (saline = 4 animals, IL-1β = 5 animals) were trained to find the location of a fixed hidden platform until their average escape latencies reached asymptote. On testing day, animals were injected with 2 μg/kg of IL-1β or an equivalent volume of pyrogen-free saline. Again, this dose of IL-1β was chosen because there was a non-significant trend observed in memory consolidation in Experiment 3. Approximately 75 minutes following injections, animals were tested over the course of four trials (1 Block) to locate the hidden platform. After the completion of the block, both probe and visible platform trials were conducted.

Results

Experiment 5. As Figure 11 depicts, no significant differences were observed among treatment groups in their ability to successfully navigate to the platform during trials 1 through 4. A one-way analysis of variance (ANOVA) on the percentage of time spent in the quadrant in which the platform had been located the previous day revealed the three treatment groups averaged approximately the same amount of time of the 30 second

probe trial in the correct quadrant (F (2,18) = .38, p > .05; M(saline) = 36 %; M (IL-1β) = 40 %; M(LPS) = 33 %). In addition, a repeated-measures ANOVA revealed that, though all treatment groups evidenced learning over the course of the four trials, as indicated by a significant effect of trial (F(2, 18) = 7.92, p ≤ .01), no differences were observed among groups in their ability to locate the platform, as evidenced by the absence of a treatment effect (F(2, 18) = 2.46, p > .05) or of a trial by treatment interaction (F (2,18) = .17, p > .05).

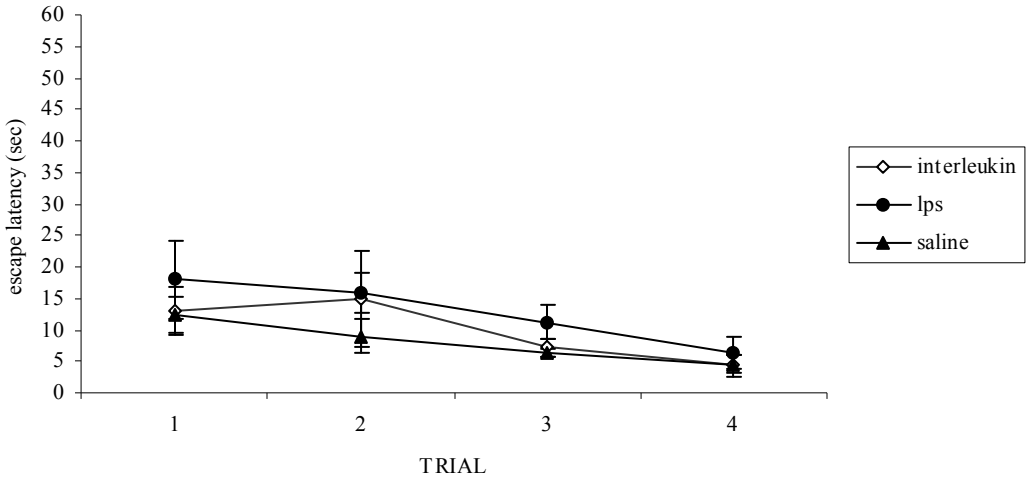


Figure 11. Effects of LPS and IL-1β on post-trial memory consolidation. Mean and SEM of average escape latency (sec to reach platform) are depicted for animals injected with: 2 μg/kg IL-1β, 2 μg/kg LPS, or saline. .No significant differences among groups (p > .05).

Experiment 6. As Figures 12 and 13 depict, both saline and LPS – treated animals were able to learn the location of the moving platform equally well. A paired-samples *t*-

test revealed no significant differences in the difference in escape latencies for trials one and two between groups, neither an hour following (t (9) = -1.59, p > .05), nor twenty-four hours following (t (9) = -1.18, p > .05) injections.

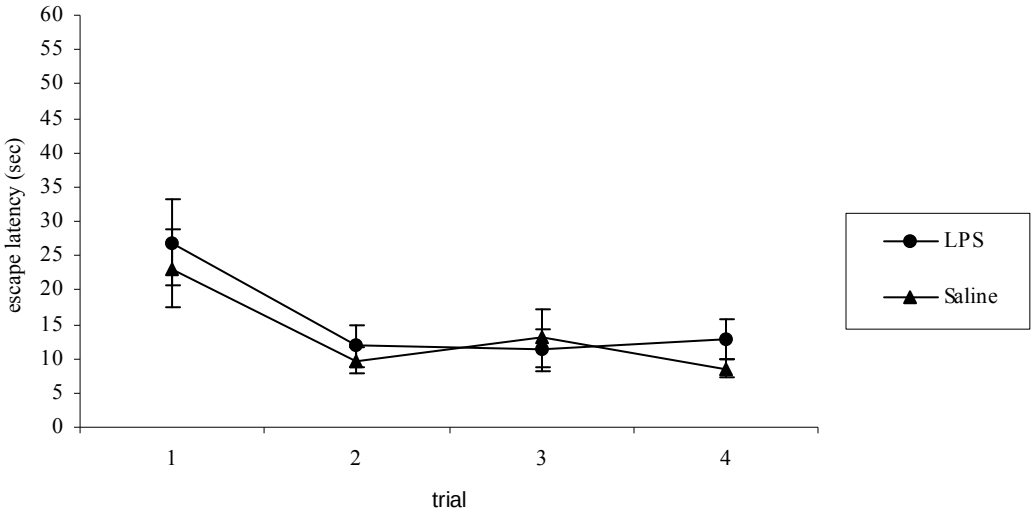


Figure 12. Effects of LPS on acquisition of new platform location 1 hour following injection. Mean and SEM of average escape latency (sec.) are depicted for animals injected with 100 µg/kg of LPS or an equi-volume amount of pyrogen-free saline. No significant differences among groups(p > .05).

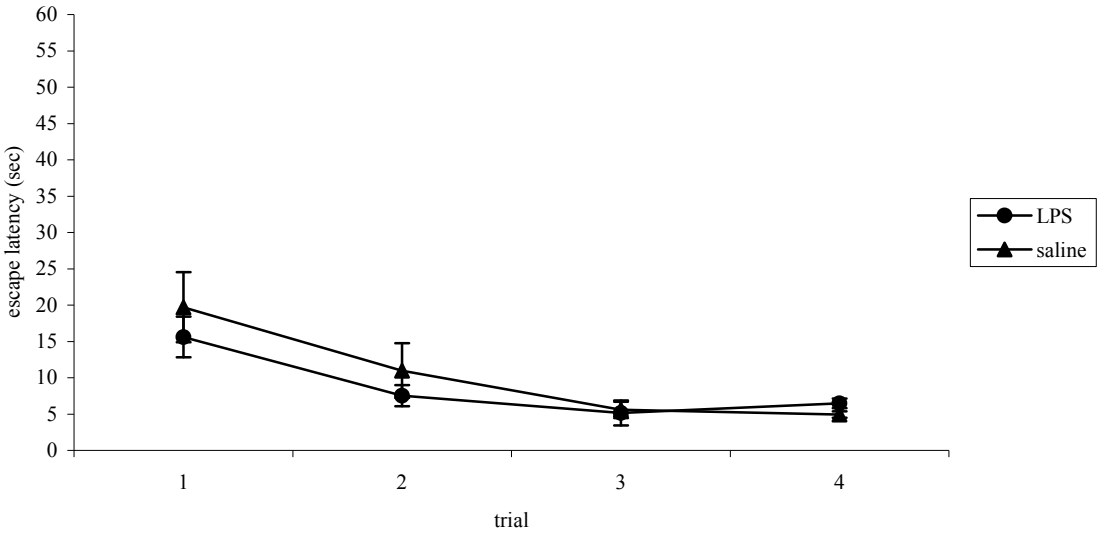


Figure 13. Effects of LPS on acquisition of new platform location 24 hours following injection. Mean and SEM of average escape latency (sec.) are depicted for animals injected with 100 μg/kg of LPS or an equi-volume amount of pyrogen-free saline. No significant differences among groups ($p > .05$).

Similar performance in LPS and saline-treated animals was observed for four days following injection; no differences were observed between groups (data not shown).

As depicted in figures 14 and 15, IL-1β and saline-treated animals also performed equally well. A paired-samples *t*-test revealed no significant differences in the difference in escape latencies for trials 1 and 2 between groups, neither an hour following ($t (11) = .20$, $p > .05$), nor twenty-four hours following ($t (11) = -.32$, $p > .05$) injections.

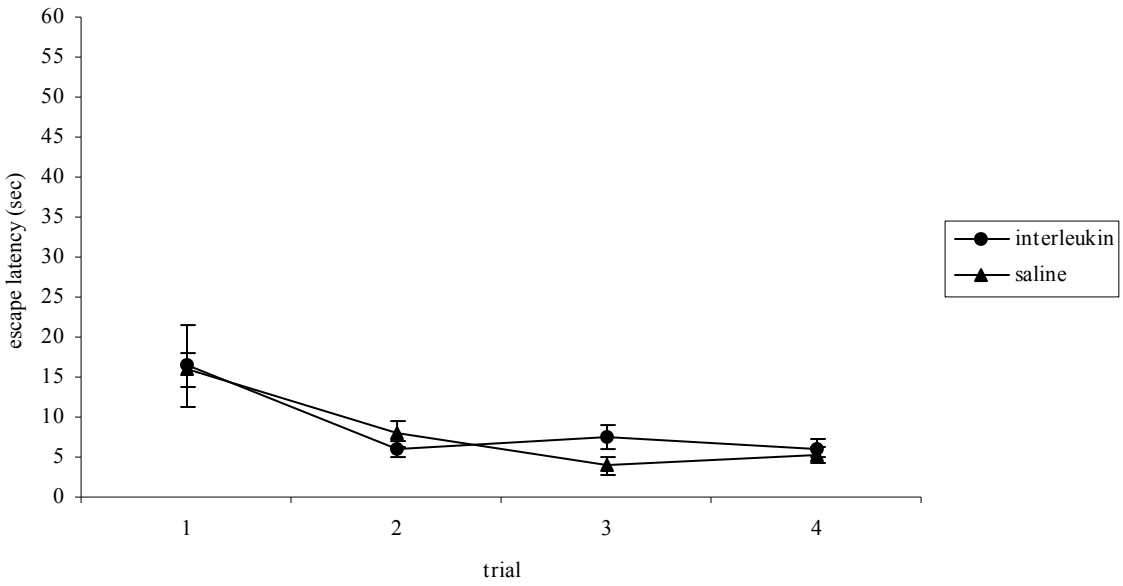


Figure 14. Effects of IL-1β on acquisition of new platform location 1 hour following injection. Means and SEM of average escape latency (sec) are depicted for animals injected with 2μg/kg of IL-1β or an equi-volume amount of pyrogen-free saline. No significant differences observed between groups ($p > .05$).

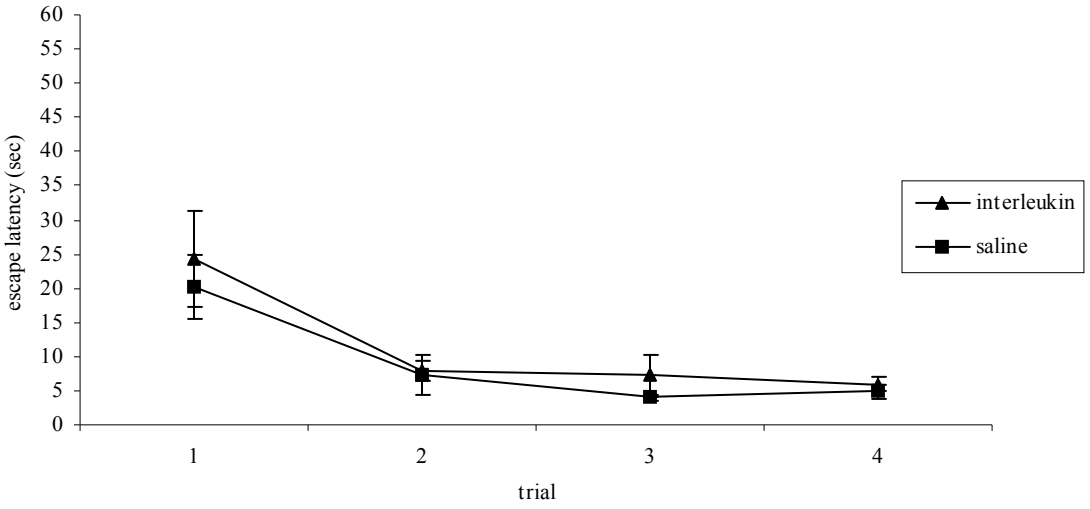


Figure 15. Effects of IL-1β on acquisition of a new platform location 24 hours following injection. Mean and SEM of average escape latency (sec) are depicted for animals treated with 2μg/kg of IL-1β or an equi-volume amount of pyrogen-free saline. No significant differences observed between groups ($p > .05$).

Experiment 7. As depicted in figures 16 and 17, IL-1β- and saline treated animals were both equally able to retain the well-learned location of the hidden platform. No differences were observed between groups in their ability to locate the platform one hour following injections, as evidenced by the absence of a treatment effect ($F(1, 8) = .68$, $p > .05$), or of a trial by treatment interaction ($F(3,6) = .63$, $p > .05$). The two groups also produced similar escape latencies 24 hours following injections, as evidenced by the absence of a treatment effect, ($F(1, 8) = 1.10$, $p > .05$), or of a trial by treatment interaction ($F(3,6) = 2.29$ $p > .05$).

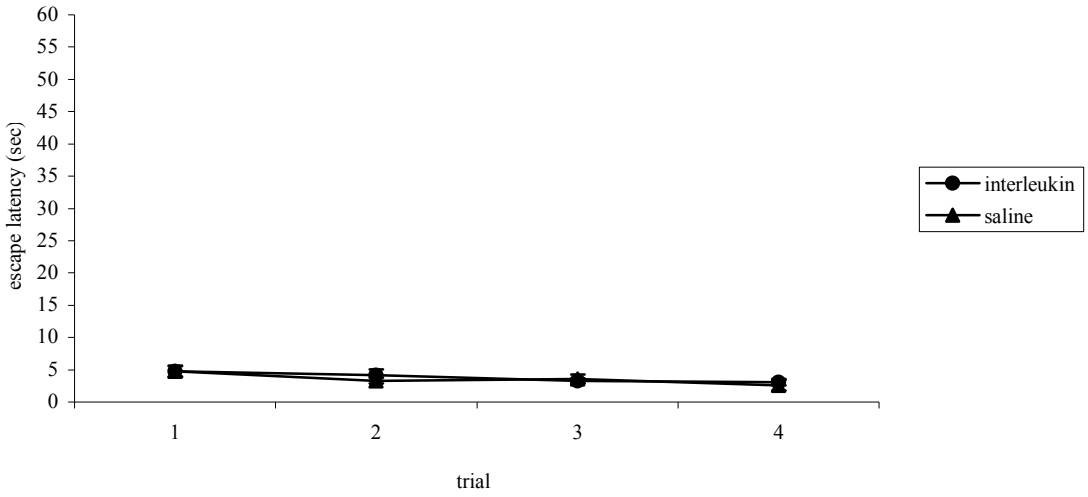


Figure 16. Effects of IL-1β on retention for a fixed platform 1 hour following injection. Mean and SEM of average escape latency are depicted for animals injected with 2 ug/kg of IL-1β or an equi-volume amount of pyrogen-free saline ($p > .05$).

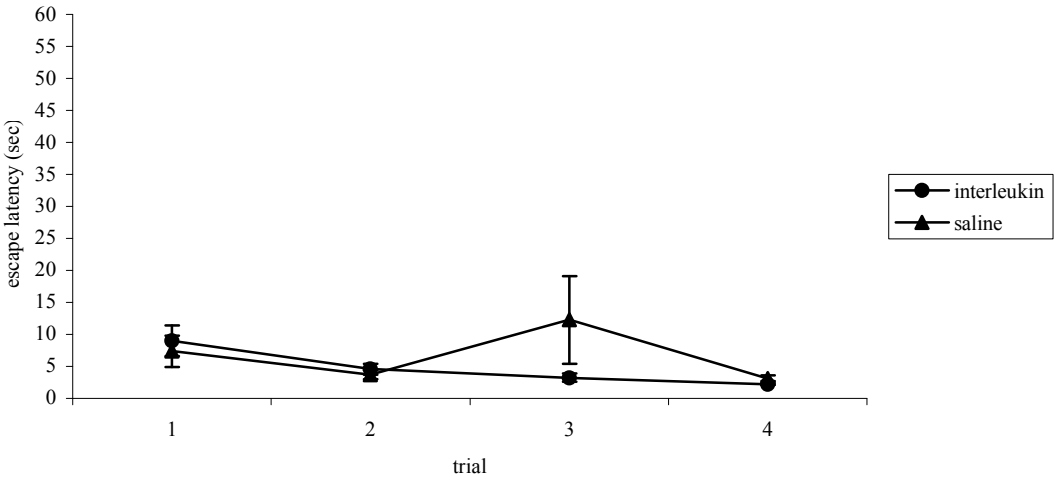


Figure 17. Effects of IL-1β on retention for fixed platform 24 hours following injection. Mean and SEM of the average escape latency are depicted for animals treated with 2μg/kg of Il-1β or pyrogen-free saline ($p > .05$).

Conclusions: Experiments 5-7

Data from Experiments 5-7 demonstrate that animals are able to acquire and maintain spatial information quite well. The observation that both IL-1β and LPS-treated animals

performed as well as saline-treated control animals suggests that neither compound acts peripherally to interfere with these abilities.

The impairments observed in the LPS-treated animals in Experiment 3 did not generalize to the Morris water task. As observed in Experiment 5, animals treated with the same dose of LPS as used in Experiment 3 using the contextual fear conditioning task did not show impairments in their ability to locate the platform the following day. Because the animals were injected with LPS immediately after a training block in which they had to learn the location of a recently moved hidden platform, the effects of LPS on immediate memory consolidation presumably are restricted to the contextual fear conditioning task. Explanations for this dissociation are discussed at the end of this paper.

Experiments 6 and 7 were attempts to replicate, using a a proper random release schedule, a previous observation that LPS interferes with acquisition of new spatial location information (Shaw *et al*., 2001), and another observation that IL-1β interferes with the retention of well-learned spatial location information (Gibertini *et al*., 1995). The data from both experiments did not support either scenario. LPS-treated animals performed at a level on par with saline-treated animals in their ability to successfully learn the new location of a hidden platform; and furthermore, these animals were just as able as the saline-treated animals to retain information regarding the new location across the remaining three trials in the block. One difference in the present protocol compared to that used by Shaw and colleagues (2001) was the use of random release points. It is possible that the impairment observed by Shaw *et al* (2001) was caused by an extra-spatial processing impairment in LPS- treated animals that became occluded when the

animals were subjected to the presumably more difficult, hippocampal-dependent spatial task used in these experiments.

In comparison to Gibertini and colleagues (1995), a deficit was not observed in IL-1β-treated animals in long-term retention version of the Morris water task. Unlike Gibertini *et al*. (1995), Long-Evans rats were used rather than mice. Also unlike Gibertini *et al*. (1995), animals were trained for more days prior to injection. This longer training schedule is necessary to be able to separately assess IL-1β effects on memory retention from consolidation. Though the time course of hippocampal-dependent consolidation and long-term retention processes is a hotly debated ongoing theme in the learning and memory field, asymptotic escape latencies are a well-recognized behavioural marker that animals have successfully learned the task. It can be reasonably concluded, therefore, that IL-1β, when it is administered after animals have acquired a stable memory of a hidden platform location, does not interfere with the ability to successfully navigate toward this location.

Novel testing of IL-1β on memory consolidation in the Morris water task (Experiment 5) and on acquisition of a new location in the Morris water task (Experiment 6) revealed no significant IL-1β-induced effects. The absence of an impairment in memory consolidation in the Morris water task is consistent with the absence of an impairment in the contextual fear conditioning task, as observed in Experiment 4. The absence of an IL-1β-induced impairment on acquisition in the Morris water task suggests IL-1β similarly does not interfere with new learning.

In contrast to the limited number of previous experiments that have investigated the effects of peripherally-administered IL-1β or LPS on Morris water task performance,

Experiments 5 through 7 were designed to separately assess: the ability to learn the new location of a moving hidden platform (acquisition); the ability to retain well-learned information regarding the location of a fixed platform (retention), and the ability to consolidate new spatial information after peripheral LPS or IL-1β administration. These functionally distinct memory processes have been assessed using appropriate training (i.e, duration) and testing (i.e, random release points) methods, with no effects on learning and memory evident following neither LPS nor IL-1β administration. Importantly, doses that were previously demonstrated in Experiments 1 and 2 to make an animal ill, did not cause deficits in learning and memory in the present set of experiments. In addition, doses that were lower than those tested in Experiments 1 and 2, but which have also been demonstrated to induce a central response, such as fever (Yirmiya *et al*., 1996), did not produce performance deficits across learning and memory tasks. Taken together, these observation suggests that cognitive dysfunction may not represent as reliable a response to infection as it has previously been considered to represent. Of course, the range of doses used in these experiments is not exhaustive, nor have all possible training schedules been explored. Nonetheless, it appears that sickness-induced learning and memory deficits, as induced by LPS,- though present- do not generalize to all learning and memory tasks that tap hippocampal function. Nor, it appears, are they peripherally mediated by IL-1β.

Cytokines and Long-Term Potentiation in the Freely-Moving Rat (Experiments 8-9)

Introduction

In Experiment 3, animals treated with LPS immediately following conditioning later displayed inhibited retention of the original context in which they had been conditioned. The following experimental set was done in order to test one central hypothesis on the physiological mechanism by which the peripheral immune response, as induced by LPS, could impair hippocampal- dependent learning and memory processes. To date, the most viable physiological mechanism underlying hippocampal-dependent learning and memory is hippocampal long-term potentiation (LTP).

Hippocampal LTP was first discovered in 1973 by Bliss and Lomo who demonstrated that high-frequency stimulation of perforant path fibres to the dentate area of the hippocampus resulted in a long-lasting increase in the post-synaptic response, as evidenced in an increased population postsynaptic excitatory potential (EPSP) and population action potential (pop spike). Because the increase in postsynaptic response was limited to the synapses which had been artificially stimulated, Bliss and Lomo theorized that this long-lasting potentiation represented a form of synaptic plasticity- possibly the form that is required for information storage within the hippocampus (1973). In other words, the ability of both human and non-human animals to retain information regarding current experience could be partly or wholly dependent upon the ability of the synapses within the hippocampus to modify their responses to activation invoked by environmental stimuli.

Indeed, several experiments conducted since the early 1970s have successfully linked hippocampal LTP to learning and memory. Morris and colleagues (1986) discovered that

the pharmacological blockade of hippocampal LTP induction, via the infusion of a N-methy-D-aspartate (NMDA) receptor antagonist, produced a selective impairment in place learning in animals, an ability previously demonstrated to depend upon the hippocampus (O'Keefe, Nadel, Keightley, & Kill, 1975). In addition, two separate research groups demonstrated that spatial learning, another ability dependent upon the hippocampus (Morris *et al*., 1982; Sutherland *et al*., 1982, 1983), was impaired following the artificial *in vivo* saturation (i.e., blockade) of hippocampal LTP (McNaughton, Barnes, Rao, Baldwin, & Rasmussen, 1986; Moser, Krobert, Moser, & Morris, 1998). It was further demonstrated that this spatial impairment disappeared at approximately the same rate as it took the saturation of LTP in the dentate gyrus to recover (Castro, Silbert, McNaughton, & Barnes, 1989). Recently, the use of knock-out mice, with a targeted gene mutation in the hippocampus causing the delayed clearance of elevated calcium ($Ca^{2+)}$ following depolarization, have provided yet another link between hippocampal LTP and learning and memory. These mice show both a lower threshold for LTP induction and a corresponding enhancement in performance in several hippocampal learning and memory tasks (Jeon *et al*., 2003)

Though substantial evidence indicates that hippocampal LTP processes may be necessary to some forms of memory storage (McNaughton *et al*., 1986; Moser *et al*., 1998; Jeon *et al*., 2003) other studies have demonstrated a dissociation between the physiological phenomenon and the cognitive processes it is acclaimed to underlie. Jo and colleagues observed a dissociation between hippocampal LTP and spatial learning: Knock-out mice with a targeted gene mutation showed enhanced LTP in the CA1 region of the hippocampus as compared to wild-type mice, but both mutant and wild-type mice

performed equally well in the Morris water task (1998). Other researchers claim that spatial impairments, such as those previously observed by Morris *et al*. (1986), were sensori-motor artifacts induced by the NMDA receptor antagonist, and were not selective spatial impairments induced by the intended target- the blockade of LTP (Keith & Rudy, 1990). In a detailed set of experiments investigating the effects of hippocampal LTP on hippocampal learning and memory tasks, Cain and colleagues (1993) demonstrated that hippocampal LTP saturation did not adversely affect performance in the Morris water task. In contrast, performance was adversely affected in animals in which hippocampal seizures were experimentally induced. Because the saturation methods used to block LTP can also induce hippocampal seizures, this suggests that previously observed hippocampal-dependent performance deficits following LTP saturation (McNaughton, *et al*., 1986; Moser, & Morris, 1998; Castro *et al*., 1989) could have been caused by the temporary, seizure-induced perturbation of normal brain function.

Though still unresolved and quite controversial, the hippocampal LTP phenomenon remains the best candidate for a physiological substrate of learning and memory. Indeed, empirical investigation into cytokine effects on learning and memory have also involved cytokine effects on hippocampal LTP.

The majority of empirical investigation into cytokine effects on hippocampal LTP have been done *in vitro*. The consensus of these experiments is that of a robust inhibitory effect of both LPS and IL-1β on the induction and /or maintenance of hippocampal LTP. The *in vitro* administration of LPS has been observed to inhibit LTP induction in the CA1 region of the hippocampus (Jo, Park, Lee, Jung, & Lee, 2001). Moreover, *in vitro* administration of IL-1β has been observed to inhibit and/or block LTP induction in the

CA1 (Bellinger *et al*., 1993) CA3 (Katsuki *et al*., 1990), and dentate gyrus (Cunningham, Murray, O'Neill, Lynch, & O'Connor, 1996) regions of the hippocampus, suggesting that IL-1β is the mediator of hippocampal LTP inhibition.

The only *in vivo* preparations used to investigate the effects of cytokines on hippocampal LTP have been that of the anesthetized animal; however, the inhibitory effect of both LPS and IL-1β administration on hippocampal LTP observed in this preparation are consistent with the previous *in vitro* observations. Commins and colleagues observed an inhibition in LTP induction in the CA1 region of the hippocampus following peripheral LPS administration (2001). Moreover, Kelly and colleagues (2003) observed an inhibition in LTP induction in the dentate gyrus region of the hippocampus following i.p. LPS administration. Similar to the observations made using *in vitro* preparations, the administration of IL-1β appears sufficient to induce this impairment. The intracerebroventricular (i.c.v) administration of IL-1β inhibits hippocampal LTP induction (Murray & Lynch, 1998; Vereker, O'Donnell, & Lynch, 2000). Results from both *in vitro* and *in vivo* anesthetized preparations strongly suggest that LPS disrupts hippocampal LTP, and that the pro-inflammatory cytokine, IL-1β, is the probable central mediator of this disruption.

A later study conducted by Jankowsky and colleagues (2000), however, cast considerable doubt on the methods previously used to examine cytokine effects on hippocampal LTP. Because both *in vitro* recordings and those taken from anesthetized animals involve acute tissue damage, which is well-recognized to induce cytokine expression, Jankowsky *et al*. (2000) compared the expression of various cytokines following: hippocampal slice preparation (*in vitro*), acute electrode implantation in intact

brains (*in vivo, anesthetized*), and chronic electrode implantation in intact brains (*in vivo, freely-moving*). They observed that, whereas both the hippocampal slice preparation and the acute implantation preparation caused a dramatic increase in a number of different cytokines, including IL-1β, the increase returned to basal levels when the animals were allowed sufficient time to recover from the surgical procedure, as in the chronic implantation preparation. That is, the chronic *in vivo* preparation appears to eliminate the confounding effects of injury from the study of synaptic activity. Given this, it appears, not only worthwhile, but necessary, to study the effects of LPS and IL-1β on hippocampal LTP in the freely-moving animal before drawing any conclusions of a cytokine-initiated inhibition of hippocampal LTP.

Experiments 8 and 9 were designed to separately assess the effects of peripheral LPS and IL-1β on hippocampal LTP in the freely-moving rat. First, pilot data were collected on the effect of successively higher amounts of peripherally-administered LPS, IL-1β, or saline on the amplitudes of both the pop spike and EPSP following high-frequency stimulation ((HFS) see Appendix C). From this dose-response curve, the dose of LPS and IL-1β that caused the smallest increase in both measures of postsynaptic activity was chosen for the following experiments.

Methods

Drugs Lipopolysaccharide (LPS Ecoli 026:B6); Sigma, Lot no. 101K4080; 3 million endotoxin units per MG) was injected intraperitoneally (i.p.) at a dose of 12 μg/kg. Human recombinant interleukin-1beta (hrIL-1β) was purchased from Research Diagnostics Inc.((RDI) catalog no. RDI-201b), and injected also at a dose 12 μg/kg . All

saline-treated animals received i.p. injections of an equivalent volume of sterile, pyrogen-free saline (Abbot Laboratories, Lot no. 79-613-DM-01).

Procedure

Electrode implantation. Rats were anesthetized with isoflurane and implanted, using aseptic surgical techniques, with a single stimulation electrode and a single recording electrode. The recording electrode was implanted in the hilar region of the dentate gyrus (Paxinos & Watson coordinates: 3.5 mm posterior to the bregma, 1.8 mm lateral to the midline, and 3.6 mm below the top of the skull; see Appendix B for depiction of recording electrode trace) and the stimulating electrode was implanted in the ipsilateral perforant path (Paxinos & Watson coordinates: 8.1 mm posterior to the bregma, 4.3 mm lateral to the midline, and 3.0 mm below the top of the skull). The positions of both depth electrodes were optimized under electrophysiological guidance using single pulse stimulation (pulse duration = 100 μs, amplitude = 500 μA, frequency = 1 per 30 s). Two stainless-steel, jewelers' screws were tapped into the skull to serve as reference and ground components of the differential recording circuit. Additional jewelers' screws were tapped into the skull to provide structural support for the electrode assembly, which was held in place by dental acrylic cement. The skin incision was closed with veterinary glue and the rats received injections of penicillin G (60,000 I.U. subcutaneous(sc)) and buprenorphine (0.05 mg/kg i.p.). Animals were then individually housed and remained unperturbed until electrophysiological recording. Importantly, though single-housing may induce several physiological alterations in the rat (see Plaut & Friedman, 1982 for review), it was necessary to individually house the animals post-operatively in order to ensure full recovery from the electrode implantation procedure.

Electrophysiology apparatus. All electrodes were constructed of stainless-steel, insulated with teflon, with an outside diameter of 114 μm. Gold-plated amphenol pins on the rat's head served as connectors to the recording leads. The recording leads passed through a commutator and into a differential preamplifier (Grass model P15D) and thence to a Neurolog filter and amplifier. The signal was filtered (1/2 amplitude low frequency - 1 HZ; 1/2 amplitude high frequency = 10 kHz) and the total amplification was 200X. Stimulation was provided by an AMPI Master 8 pulse former and an Isoflex optically isolated constant current stimulator. Signals were displayed on a Nicolet digital storage scope, continuously monitored on a Grass audio monitor, and sent to a microcomputer running the BrainWave (version 3.3) data acquisition and storage system. The Workbench analysis package was used to measure the amplitude of the dentate evoked potential.

Electrophysiology procedures. Approximately one week after implantation, daily electrophysiological recording sessions began. Six animals in a within-subjects design (six animals per group) were used in Experiments 8 and 9. An input-output curve was produced for each animal, in which the size of the evoked potential was measured at each of 6 stimulus pulse intensities (50, 100, 200, 300, 400, 500 μA) - each intensity was repeated 10 times at the rate of 1 pulse per 30 s. To find the optimal baseline intensity for each animal, an intensity was chosen which produced a pop spike amplitude that was between 50 and 75 percent of the size of the pop spike amplitude generated by the 500 μA stimulus pulse. This intensity was then used to collect 20 additional evoked potentials. Three continuous days of individual IO curves and baseline recordings were taken for each animal.

Following the baseline recording on the third day, animals received one of three injections: 12 μg/kg of LPS (Experiment 8), 12 μg/kg of IL-1β (Experiment 9), or an equivalent volume of pyrogen-free saline. For each experiment, the order of injections (LPS/ IL-1β or saline) was counterbalanced across animals, and one week passed between injections. One hour following injections, another baseline recording was taken in order to determine if the injection procedure or the injected compound itself produced any changes in the evoked field potential. Immediately following the post-injection recording, animals received tetanic stimulation at the stimulation site, which consisted of 10 trains of 10 pulses (frequency = 400 Hz, intensity = 500 μA), with 30 s between each train. Stimulation parameters were then returned to the baseline values and three additional recordings were taken: immediately after high-frequency stimulation, or HFS; 75 minutes following HFS, and 24 hours following HFS.

Two measures were taken of each evoked potential: the slope of the field EPSP (EPSP), measured at approximately the middle of the rising slope at a fixed time after the stimulus artifact and the amplitude of the trough (population spike (pop spike)) using the tangent method (voltage difference between a tangent to the two positive peaks and the trough of the negative-going wave component).

An example of a hippocampal evoked potential is provided in Figure 18.

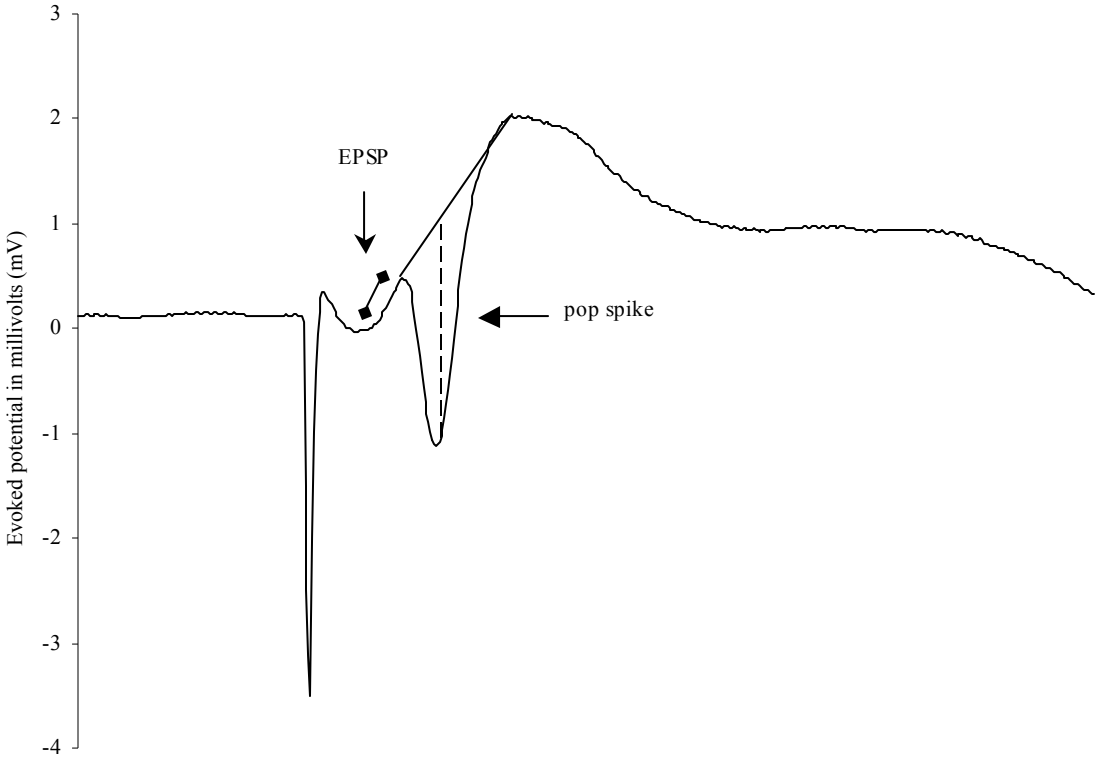


Figure 18. Sample hippocampal evoked potential. The amplitude of an evoked potential is assessed by the size of the slope of its EPSP and amplitude of its pop spike

Changes in the evoked responses were expressed as fractional changes from the original values measured at the pre-injection baseline. Animals who did not show a greater than or equal to 50 percent increase (.5 AFC) in the pop spike amplitude and a greater than or equal to 10 percent increase (.10 AFC) in the EPSP at any of the three time points following HFS (immediately following HFS, 75 minutes, 24 hours) were considered not capable of showing LTP and were eliminated from the final analyses (not all animals show an enhanced synaptic response to high-frequency stimulation (Bliss & Lomo, 1973)). Animals that did not exhibit a measurable population spike and/or EPSP

were also excluded from the final analysis. To address the possibility that excluded animals might have different evoked potentials compared to animals who did not meet the above criteria, additional analyses were done on all animals, regardless of the response of their evoked potential to high-frequency stimulation.

Results

Experiment 8 Hippocampal LTP in the freely-moving rat is sensitive to the peripheral immune response, as it is invoked by the peripheral administration of LPS. A paired-samples *t*-test performed on the difference between the average fractional change (AFC) in the EPSP between LPS and saline-treated animals at each of the three time points following HFS revealed a significant difference between groups at 24 hours ($t(3) = 5.82$, $p \leq .01$). However, this effect was not evident at 75 minutes ($t(3) = 1.5$, $p > .05$) or immediately after ($t(3) = 1.4$, $p > .05$) HFS (see Figure 19). In addition, no differences were observed in the EPSP of LPS and saline-treated animals following injection ($t(3) = 1.39$, $p \geq .05$). A similar trend was observed when all animals were included in the analysis, regardless of the size of the EPSP response to high-frequency stimulation (post-injection: $t(5) = 1.79$, $p > .05$; HFS: $t(5) = 1.50$, $p > .05$; 75 min.: $t(5) = 2.16$, $p > .05$; 24 hrs.: $t(5) = 4.14$, $p \leq .01$). Figures 20 and 21 depict sample evoked potentials from a saline-and-LPS-injected animal. The saline-injected animal (Figure 20) shows a larger-than-baseline EPSP 24 hours after HFS, whereas the LPS-injected animal (Figure 21) shows a smaller-than-baseline EPSP 24 hours after HFS.

No differences were observed in the AFC of the pop spike amplitude between groups at any of three time points (HFS: $t(4) = -.64$, $p > .05$; 75 min.: $t(4) = -.70$, $p > .05$; 24 hrs.: $t(4) = -.41$, $p > .05$ (see Figure 22)). In addition, no differences were observed in

the population spike in LPS and saline-treated animals after the injection ($t(4) = -1.0$, $p > .05$). Similarly, no between groups differences were observed in the population spoke when all animals were included in the analysis, regardless of the response of the population spike to high-frequency stimulation (post-injection: $t(5) = -1.22$, $p > .05$; HFS: $t(5) = .10$, $p > .05$; 75 min.: $t(5) = .67$, $p > .05$; 24 hrs.: $t(5) = .47$, $p > .05$).

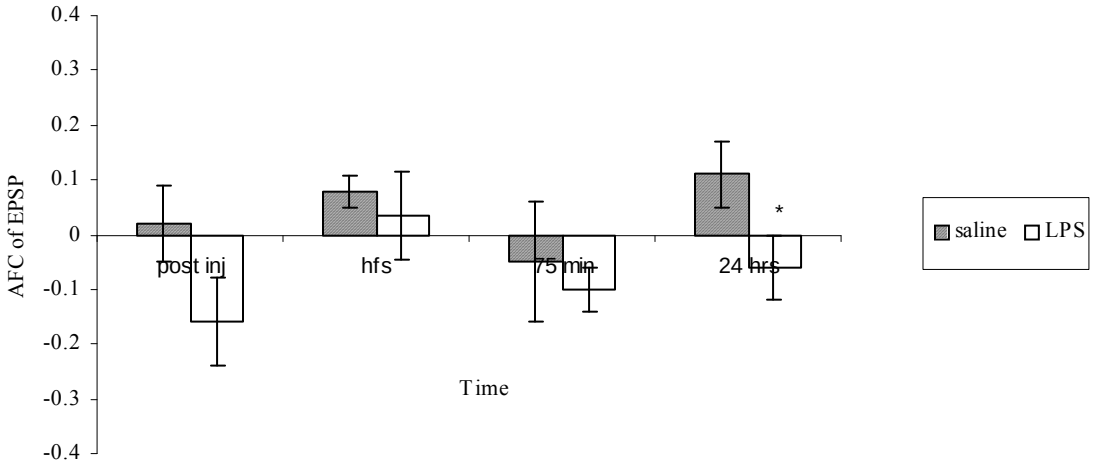
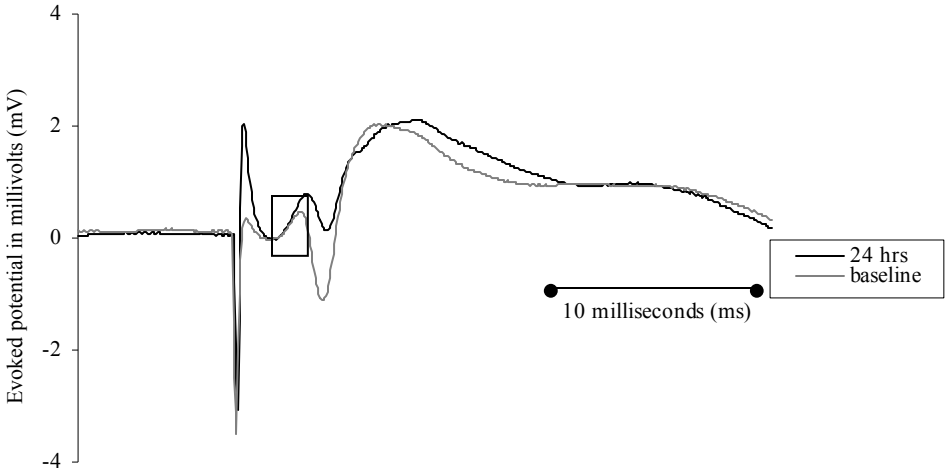
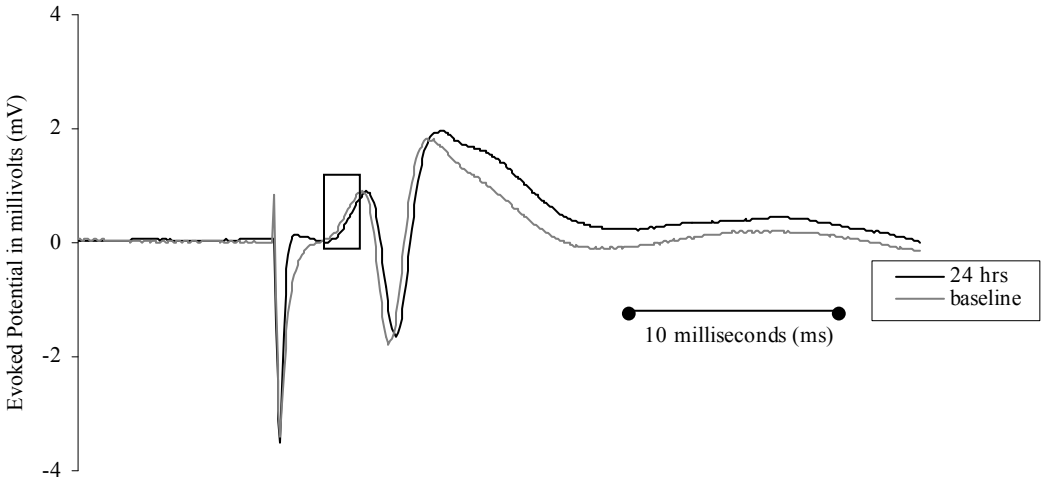


Figure 19. LPS effects on the EPSP of the evoked potential following HFS. The mean and SEM of the average fractional change (AFC) of the EPSP are depicted for animals injected with 12 µg/kg of LPS or an equi-volume amount of sterile pyrogen-free saline. * = significant difference between groups ($p \leq .05$)



Figures 20. Sample evoked potential from a saline-treated rat. Twenty-four hours after HFS, this animal shows a potentiated EPSP (i.e., greater slope) in response to baseline stimulus parameters (("Box" = EPSP slope); the decline in pop spike amplitude at 24 hours was not consistently observed across rats)).



Figures 21. Sample evoked potential from a LPS-treated rat. Twenty-four hours after HFS, this animal shows a smaller EPSP in response to baseline stimulus parameters and no change in pop spike ("Box" = slope of EPSP).

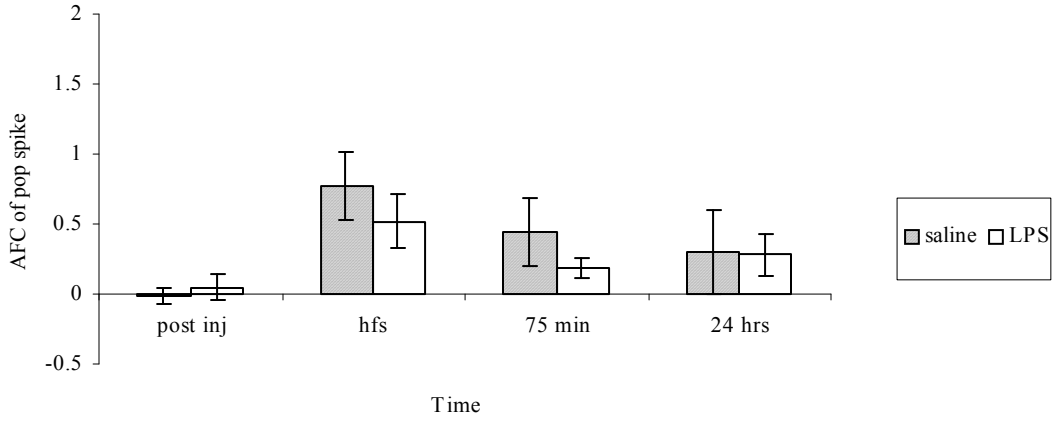


Figure 22. LPS effects on the pop spike amplitude of the evoked potential following HFS. The means and SEMs of the average fractional change (AFC) of the pop spike amplitude are depicted for animals injected with 12 µg/kg of LPS or an equi-volume amount of sterile pyrogen-free saline. No significant differences observed between groups ($p > .05$).

Experiment 9 No significant differences were observed between IL-1β and saline-treated animals in the evoked field potential following HFS. IL-1β-treated animals showed no significant difference in the AFC of their EPSP compared to the saline-treated controls, neither immediately following ($t(3) = -.15$, $p > .05$), 75 minutes ($t(3) = -1.46$, $p > .05$), nor 24 hours following ($t(3) = 1.83$, $p > .05$) HFS (see Figure 23). Furthermore, no significant differences were observed between groups in the EPSP following injection ($t(3) = .38$, $p > .05$). Similarly, no significant differences were observed between groups when all animals were included in the analysis, regardless of the EPSP response to high-frequency stimulation (post injection: $t(5) = .27$, $p > .05$; HFS: $t(5) = .08$, $p > .05$; 75 mins.: $t(5) = -.77$; $p > .05$; 24 hrs.: $t(5) = 1.45$; $p > .05$).

No significant differences were observed between IL-1β and saline-treated animals in the AFC of their pop spike amplitude compared to the saline-treated controls, neither immediately following ($t(5) = 1.38$, $p > .05$), 75 minutes ($t(5) = .05$, $p > .05$), nor 24 hours following (($t(5) = 1.21$, $p > .05$) HFS (see Figure 24)). Also, no differences were observed between groups in the population spike following injection ($t(5) = -.87$, $p > .05$).

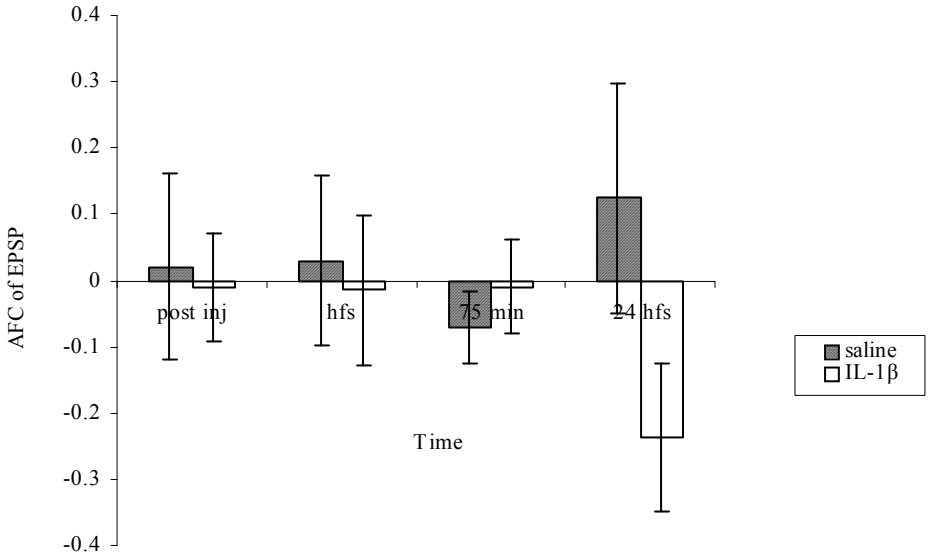


Figure 23. IL-1β effects on the EPSP of the evoked potential following HFS. The mean and SEM of the average fractional change (AFC) of the EPSP are depicted for animals injected with 12 μg/kg of IL-1β or an equi-volume amount of sterile pyrogen-free saline. No significant differences observed between groups ($p > .05$).

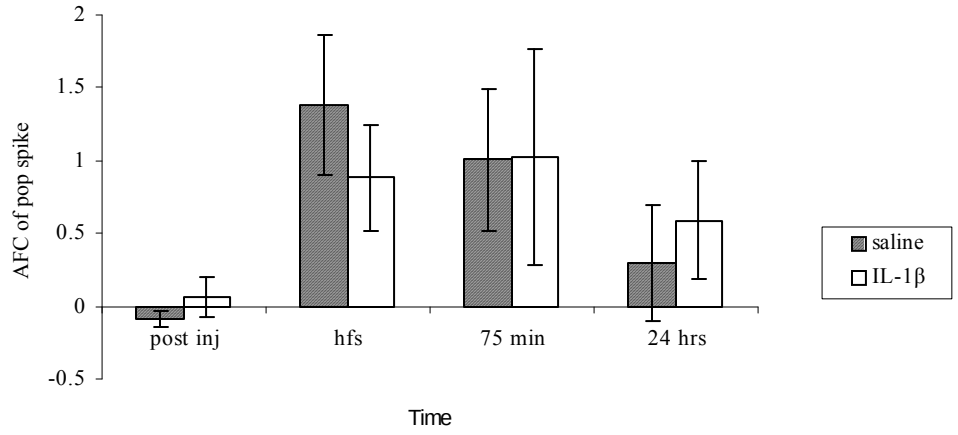


Figure 24. IL-1β effects on the pop spike amplitude of the evoked potential following HFS. The mean and SEM of the average fractional change (AFC) of the pop spike amplitude are depicted for animals injected with 12 μg/kg of IL-1β or an equi-volume amount of sterile pyrogen-free saline. No significant differences observed between groups ($p > .05$).

Conclusions: Experiments 8 & 9

The peripheral immune response, as elicited by the peripheral administration of LPS, significantly inhibits the EPSP 24 hours following HFS. This is the first demonstration that hippocampal LTP is sensitive to the peripheral administration of endotoxin in the freely-moving rat. This is significant because the chronic implantation preparation allows for testing electrophysiological effects after cytokine activation produced by tissue damage has subsided. Earlier work using *in vitro* hippocampal slice preparations and *in vivo* acute recording preparations are confounded by the release of endogenous cytokines, as demonstrated by Jankowsky and colleagues (2000). It appears that hippocampal

synaptic plasticity is disrupted by the induction of the peripheral immune response. The mechanism by which LPS affects EPSP slope may be through an NMDA-inhibitory effect. Jo *et al*. (2001) work in patch-clamp recording revealed that LPS inhibits the EPSP specifically by blocking calcium (Ca^{2+}) entry through post-synaptic NMDA receptors.

The present demonstration that the EPSP is inhibited by the peripheral administration of LPS is consistent with Vereker *et al*.'s observation that, in the anesthetized animal, the peripheral administration of LPS significantly inhibits the EPSP (2000). However, unlike Verker *et al*. who observed an inhibition in the persistent rise in the EPSP over a forty-minute period following HFS (2000), LPS was not observed to significantly inhibit the EPSP immediately following, nor 75 minutes following HFS. This difference may be ascribed to inherent differences involved in acute and chronic *in vivo* preparations. Rick and Milgram (1999) observed that, in acute, anesthetized preparations, the continuous application of moderate-intensity stimulation pulses (not HFS) induced increases in both the population spike amplitude and EPSP slope, while chronically-implanted freely-moving animals showed significant increases in the pop spike amplitude, but significant decreases in the EPSP slope. If the EPSP slope is already inhibited to a certain degree in the freely- moving animal, then it is conceivable that it would not be as sensitive (i.e, "not as much size to lose") to the administration of LPS as it would be in an anesthetized animal.

No significant differences were observed in the evoked potential in IL-1β-treated animals. This observation contrasts previous observations that IL-1β inhibits LTP in the hippocampus *in vitro* (Bellinger *et al*., 1993; Katsuki *et al*., 1990; Cunningham *et al*.,

1996), or that the central administration of IL-1β inhibits hippocampal LTP in the anesthetized animal (Vereker *et al*., 2000; Murray & Lynch, 1998). Experiment 9 was the first experiment to test the effects of IL-1β on hippocampal LTP in the freely-moving animal. Moreover, Experiment 9 was the first experiment to test the effects of *peripherally*-administered IL-1β on hippocampal LTP. The observation, then, that the peripheral administration of IL-1β has no effects on the evoked potential following HFS could be attributed to either the absence of an effect of peripheral IL-1β on hippocampal synaptic plasticity, or an absence of an effect of IL-1β,- be it peripheral or central,- on hippocampal syntaptic plasticity in the freely-moving animal. Certainly, additional studies are needed to separate the effects of central administration of IL-1β from peripheral administration of IL-1β on hippocampal LTP in the freely-moving rat. Nonetheless, the observation that peripheral administration of IL-1β has no effect on hippocampal LTP in the freely-moving rat strongly supports the argument that the peripheral actions of the pro-inflammatory cytokine, IL-1β, do not have a primary peripheral signaling role in the disruption of hippocampal LTP. The peripheral administration of IL-1β does cause a similar, albeit non-significant, trend in the EPSP as compared to the LPS-treated animals (see Figure 21). It may be the case that, in addition to IL-1β, the actions of other proinflammatory cytokines are required in the periphery to disrupt hippocampal LTP.

Of additional interest is the observation that saline-treated animals in experiments 8 and 9 had an EPSP below–baseline-value at 75 minutes (and at 24 hours in Experiment 9). To explore the possibility that experimental handling, necessary to the connection of the animal to the recording apparatus, confounded the change in the EPSP following

HFS, evoked potentials following HFS were continuously recorded from an unperturbed animal for two hours following HFS, and then at 24 hours after HFS (see Appendix D). The change in the EPSP over the two- hour period mirrored that of the majority of animals in the experiments. That is, the initial rise in the AFC of the EPSP following HFS slowly diminished over the course of one hour until it fell below baseline values. It then returned to higher-than-baseline values at 24 hours. Reasonably, the depressed EPSP observed at 75 minutes in the control animals was not an artifact of experimental handling, but rather could be a result of differences in the animals' body core temperatures as a function of activity state.

While measuring temperature change following different injections (see Appendix A), it was observed that the saline-treated animal's temperature steadily decreased as it fell asleep (activity state was observed by experimenter and not systematically recorded). Cain and colleagues (1994) observed that the evoked field potential in the dentate gyrus, by stimulation of the perforant path, varied with both behaviour and brain temperature. Specifically, they observed that slow-wave sleep correlated with a decreased field EPSP slope and an increased population spike amplitude. It could reasonably be inferred, therefore, that the animals who showed inhibited EPSPs at 75 minutes were in slow-wave sleep. If animals' temperatures were experimentally controlled, either by increasing the ambient temperature in the testing room to over-ride sleep-induced hypothermia or by artificially maintaining the animals in a state of constant activity, then it is suspected that the decrease in the EPSP slope, as observed in the present experiments at the 75 minute time point, would be abolished. Additional studies are required to test this hypothesis.

If the argument is followed, then, that slow-wave sleep inhibits the EPSP slope, it is possible that the inhibited EPSP slope observed in the LPS-treated animals could be an indirect effect of an LPS-induced increase in slow-wave sleep, rather than a direct LPS-induced inhibitory effect on hippocampal synaptic plasticity. Though the febrile effects of LPS have been observed to dissipate after 8 hours (Yirmiya, Tio, & Taylor, 1996), results from Experiment 2 suggests that LPS-induced reductions in general motor activity persist for as along as 24 hours. Additional studies of LPS and hippocampal LTP require the separate assessment of LPS-induced soporific effects from LPS-induced inhibitory effects on hippocampal physiology.

In summary, data from Experiment 8 demonstrate for the first time that hippocampal LTP in the freely-moving animal is disrupted by peripheral LPS administration. The idea that this disruption might be an indirect result of the sedative consequences of infection is an intriguing one and warrants further investigation. No differences were observed in the pop spike amplitude in the LPS - treated animals compared to controls. However, this was not the first observation of a dissociation between the EPSP and the population spike, as previous researchers have observed similar dissociations following different experimental manipulations, including pharmacological (Munro, Walling, Evans, & Harley, 2001) and dietary (Bronzino, Austin-Lafrance, Mokler, & Morgane, 1997) manipulations. Notably, some researchers (Verker *et al*., 2000) measure just the EPSP as an assessment of hippocampal LTP. Though the functional consequence of an inhibited EPSP, yet normal pop spike, remains to be elucidated, the LPS-induced EPSP inhibition observed in Experiment 8 is consistent with the LPS-induced disruption of memory consolidation observed in Experiment 3, suggesting that sickness-induced disruption of

memory consolidation may have a physiological basis in disrupted hippocampal synaptic plasticity.

Data from Experiment 9 demonstrate that IL-1β it not the primary peripheral signaling molecule linking peripheral inflammation to disruption of hippocampal synaptic plasticity. The observation of a trend (non-significant) for a lower 24-hour EPSP in the IL-1β-treated animals that is similar to the inhibition (significant) in the 24-hour EPSP observed in the LPS-treated animals, suggests that additional proinflammatory molecules are required to induce a statistically-reliable EPSP inhibition. This observation is consistent with the observation from Experiment 4 that a similar dose of IL-1β caused a non-significant, inhibitory trend in context retention similar to that of LPS. Notably, the dose of IL-1β that was used in Experiments 1 and 2 to produce reliable changes in food consumption, body weight, and general motor activity was insufficient in the present experiment to reliably disrupt hippocampal LTP. This observation suggests that cognitive dysfunction, though it has demonstrated behavioural (Experiment 3) and physiological correlates (Experiment 8), does not appear to share similar signaling molecules (i.e., IL-1β) as other sickness behaviours, nor does it appear to be *as* central of a component of the sickness response as other sickness behaviours.

Cytokines and Hippocampal Cell Proliferation

Introduction

Results from Experiment 8 support an LPS-induced inhibition of long-term potentiation in the freely-moving rat. The absence of a significant effect, but presence of an IL-1β-induced inhibitory influence on hippocampal LTP, suggests that IL-1β, alone, is not sufficient to reliably inhibit hippocampal LTP in the freely- moving rat. Rather, it appears that additional proinflammatory cytokines are required in the periphery to induce a statistically reliable inhibitory effect on hippocampal physiology.

Interleukin-6 Interleukin-6 (IL-6) is a proinflammatory cytokine that likely plays a role in peripheral inflammation-induced hippocampal inhibition. There is anatomical and functional evidence, coupled with theoretical reason, to believe that IL-6 is necessary to inducing an inhibitory effect on hippocampal physiology. First, IL-6 and IL-6 receptor (IL-6R) mRNA has been localized in the pyramidal and granular neurons of the hippocampus (Schobitz, Voorhuis, & de Kloet, 1992; Schobitz, de Kloet, Sutanto, & Holsboer, 1993). Second, peripheral exposure to endotoxin stimulates the biosynthesis of IL-6R within the hippocampus (Vaillieres & Rivest, 1997), indicative that the peripheral inflammatory response induces the central hippocampal expression of IL-6.

The theoretical reason for suspecting IL-6 has a role in the inhibition of hippocampal function is not based on empirical results, but is none-the-less compelling. As described previously, the term *sickness behaviour* emerged as a term that comprised those behaviours- eating, drinking, socializing, *learning*- that are compromised in times of illness, in order to save the additional metabolic energy required to mount the febrile

response necessary to pathogen clearance (Kluger, 1992). In other words, sickness-induced cognitive dysfunction is organized around,- or occurs in order to accommodate,- the febrile response. Experimental manipulations, then, that result in the greatest febrile response, should also cause greater inhibitions of hippocampal physiology and function.

A series of experiments conducted by Cartmell and colleagues (2000) demonstrated that, though the administration of peripheral IL-1β alone was sufficient to induce a fever (IL-1β is an endogenous pyrogen as well as an inducer of IL-6 production), the peripheral co-administration of IL-1β with IL-6 produced a fever of greater magnitude and of greater duration than that which developed from the peripheral administration of either cytokine alone. Moreover, animals pre-treated intraperitoneally with IL-6 antiserum failed to show a febrile response to the peripheral administration of LPS. From these data, the authors conclude that IL-6 acts in concert with IL-1β at the local site of peripheral inflammation before entering the circulation. Once it enters the circulation, the authors conclude that IL-6 activates central nervous system mechanisms to produce a febrile response during illness. Because the peripheral administration of IL-6 augments one of the central responses to illness, it follows that the peripheral administration of IL-6 might indeed augment other central responses to illness, including the inhibition of learning and memory, and possibly, hippocampal functioning.

Hippocampal Cell Proliferation Experiment 10 was done to determine if IL-1β, when co-administered with IL-6, would inhibit hippocampal cell proliferation. This study is necessary to the investigation of cytokine effects on cognition for two reasons. First, the quantification of hippocampal cell proliferation is an indirect assessment of hippocampal neurogenesis, a cellular process that has been observed to represent a correlate of learning

and memory, and, more specifically, to memory consolidation (Feng *et al*., 2001; Kempermann, 2002 (review)).

Similar to hippocampal LTP, hippocampal neurogenesis proves attractive as a physiological counterpart to learning and memory because it can be up-or down-regulated following different experiences (see Kempermann, 2002 for review). Perhaps the most compelling argument for a role of neurogenesis in learning and memory comes from Feng and colleagues (2001) who show that transgenic mice lacking a gene necessary to post-learning enrichment neurogenesis display a normal fear response to the auditory cue in the contextual fear conditioning paradigm, but show a heightened fear response to the context compared to control animals. Importantly, the transgenic animals who were exposed to an enriched environment *prior* to conditioning showed a level of retention similar to enriched non-transgenic animals. From these data, the authors argue that, rather than being necessary to the formation of new memories, neurogenesis is necessary to memory consolidation. That is, the authors argue that hippocampal neurogenesis aids memory consolidation by degrading temporarily stored memory traces in the hippocampus once extra-hippocampal memory consolidation has taken place. If these memory traces are not cleared from the hippocampus, the authors argue that the hippocampus becomes overloaded and unable from acquiring and processing new memories (Feng *et al*., 2001). Therefore, it is possible that dysregulation of hippocampal neurogenesis may be a correlate of memory consolidation dysregulation, such as that which was observed by Pugh and colleagues (1998) and replicated in Experiment 3. In the least, hippocampal neurogenesis is a correlate of hippocampal physiology, and its

investigation will provide insight as to whether cytokines act on a physiological level to disrupt learning and memory.

Hypothesis This experiment is designed to assess whether the peripheral administration of two proinflammatory cytokines,- IL-1β and IL-6,- will have an inhibitory effect on hippocampal cell proliferation. Because this experiment was designed to assess the short-term (i.e., few hours) effects of an acute injection of IL-1β and IL-6 on hippocampal physiology, animals were perfused the same day as injections. Presumably, animals that show less hippocampal cell proliferation should also show less hippocampal neurogenesis. If illness-induced cognitive dysfunction arises from a direct, inhibitory effect of proinflammatory cytokines on hippocampal physiology, then reduced cell proliferation should be observed in animals pre-treated with IL-1β and IL-6. In contrast, if illness-induced cognitive dysfunction arises from an indirect effect of cytokines on an extra-hippocampal system, then no differences in cell proliferation should be observed between cytokine and saline-treated animals.

Quantification of cell proliferation. To quantify the genesis of new cells in the dentate gyrus, BrdU labeling will be used. 5-Bromo-2'-Deoxyuridine (BrdU) is a thymidine analog that is incorporated into the DNA of dividing cells (Miller & Nowakowski, 1988) and is considered a marker for cell genesis. BrdU can be visualized with immunhistochemical techniques and quantitatively assessed with stereological counting techniques. Thus, in the following experiment, hippocampal cell proliferation is assessed as the number of cells that are labeled with BrdU.

Methods

Procedure

Drugs. Cytokine-treated animals (n = 5) received a 20 μg/kg injection of recombinant human interleukin-6 ((rhIL-6), PeproTech Inc., Canada, catalog no. 200-06, lot no. 02016)), followed one hour later by an .2 μg/kg injection of recombinant human interleukin-1β ((rhIL-1β), PeproTech Inc., Canada, catalog no. 200-01B, lot no. 037951). The doses, timing, and order of the injections were chosen to maximize the febrile response that had previously been observed by Cartmell and colleagues (2000). Saline-treated animals (n = 5) received equivalent volumes of pyrogen-free saline (Abbot Laboratories, Lot no. 79-613-DM-01). One hour following the last IL-1β injection, all animals received an injection of 150 mg/kg of BrdU (Sigma Aldrich; lot no. 71K1172).

Tissue processing. Three hours following BrdU injections, all animals were perfused transcardially with phosphate buffered saline (PBS) followed by Lanas fix (paraformaldehyde (PFA), phosphate buffer (PB), picric acid (PA)). All brains were removed and post-fixed in individual containers with Lanas fix and stored at 4° C. Using a vibratome, serial sections of the brains were cut through the hippocampus (plates 26-40; Paxinos and Watson, 1998) in 50 μm sections into 0.1 M PBS. Tissue was collected in microcentrifuge tubes filled with PB and stored at 4° C until processed for immunohistochemistry.

Immunohistochemistry. Every fifth section of the hippocampus was processed for BrdU. For BrdU immunohistochemistry, free-floating tissue sections were incubated in 2N HCL for 30 minutes at 50° C. After being washed in PB, sections were incubated in

rat anti-BrdU (Accurate, 1:175), goat serum (3 drops/5 ml), and 3% Triton-X (1:20) overnight at 4° C. Sections were then washed in PB and incubated overnight at 4° C in goat anti-rat solution (Chemicon, 1:1000). After another wash with PB, sections were treated overnight with anti-mouse Alex-4888-conjugated secondary antibodies (Molecular Probes, 1:500 in PBS), washed in PB, and incubated overnight in Streptavidin 568-conjugated secondary antibodies. Sections were then washed a final time with PB, mounted onto 1% gel slides and cover-slipped with anti-fading mountant.

Unbiased stereology. The stereological technique used to quantify the number of BrdU-labeled cells was based on the optical fractionator method described by West (1993). A randomly selected serial series comprised of every fifth 50 μm section through the hippocampus was analyzed from each animal. A Zeiss LSM 410 Laser fluorescent microscope was used to z-section images at 1 μm intervals. Z-sectioning was controlled by Zeiss LSM 5 image browser software. All BrdU-labeled cells within the hilus, subgranular zone, and the granular cell layer of the dentate gyrus were counted. Four sides (eight sections) were counted from each animal. To estimate the total number of BrdU-positive cells within the hippocampus, an average of the total number of BrdU-positive cells was calculated for each section and multiplied by five.

Results

As depicted in Figure 25, more BrdU-positive cells were observed in the dentate gyrus of saline-treated animals compared to cytokine-treated animals ($t(7) = 8.46$; $p < .001$; refer to Figure 26 to view immunhistochemical stain of BrdU-labeling).

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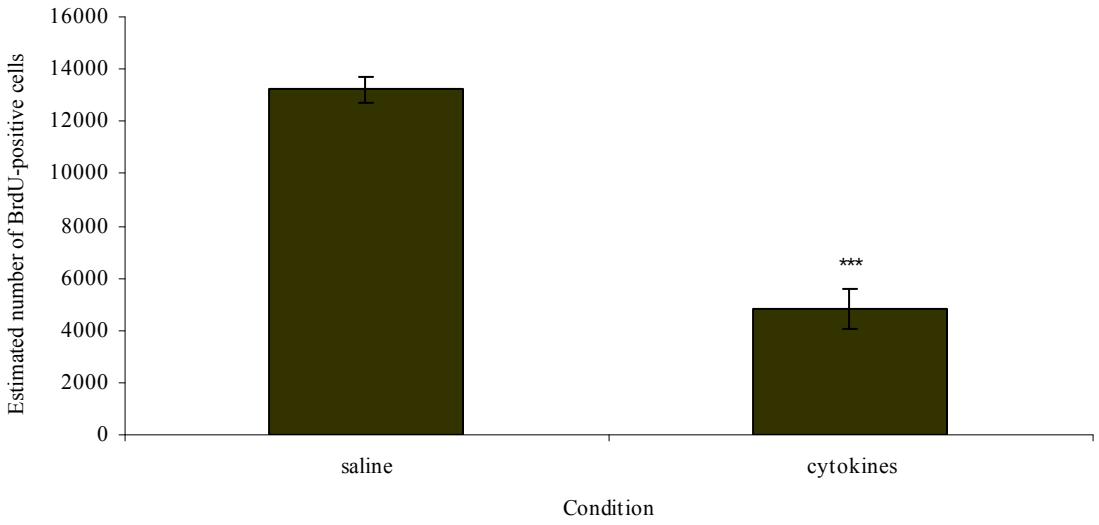

Figure 25. Effects of cytokines on dentate gyrus cell proliferation. Mean and the SEM of the estimated number of BrdU-positive cells are depicted for animals treated with 20μg/kg of IL-6 + .2μg/kg of IL-1β or an equi-volume amount of pyrogen-free saline. *** Significant difference observed between groups ($p < .001$); statistical analysis performed on raw data; one animal from cytokine-treated group excluded from analysis due to a poor perfusion).

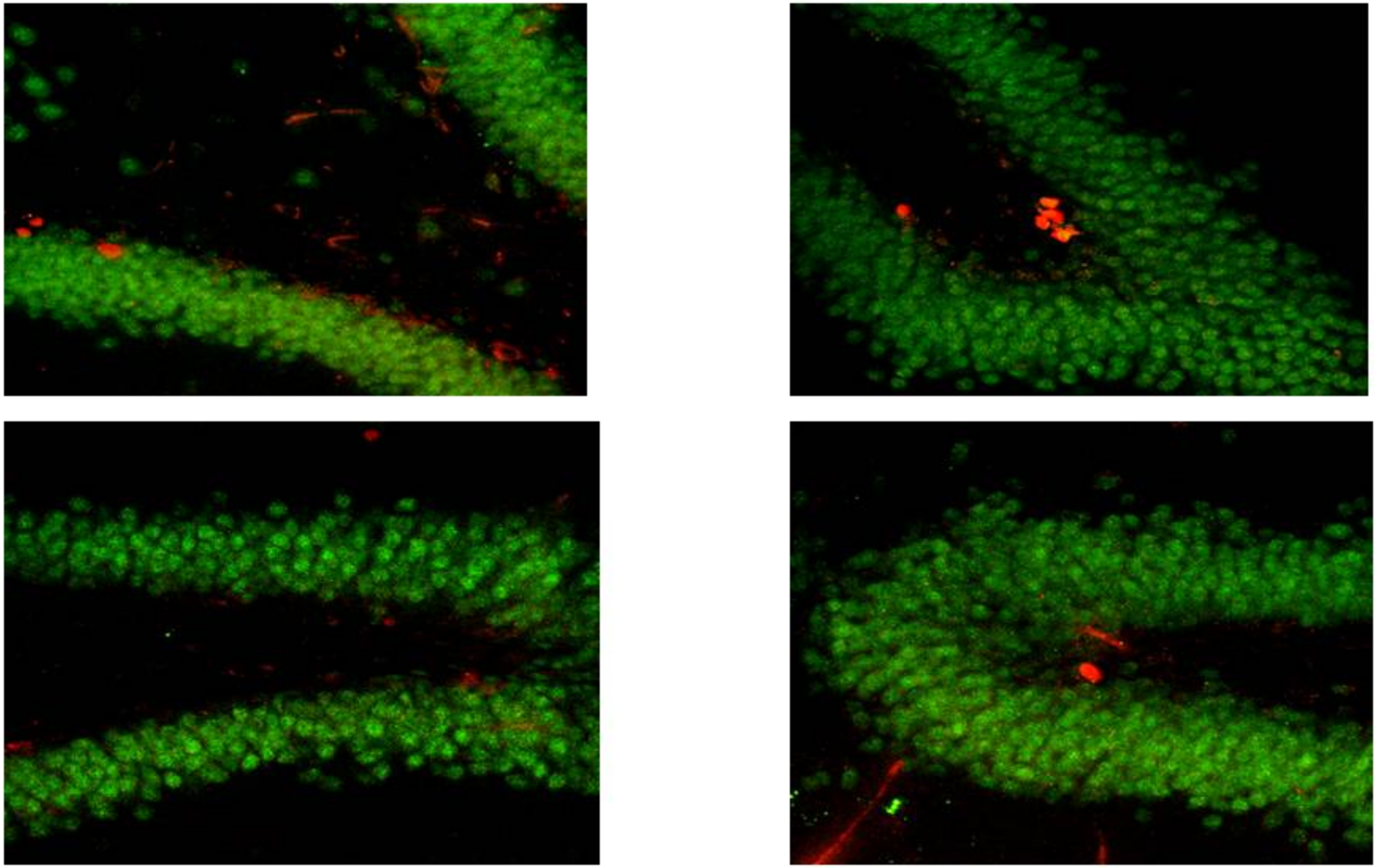

Figure 26. Pictures from the dentate gyrus of a rat treated with saline (top) and the cytokines IL-6 and IL-1B (Bottom). The colors represent: green=granule cells, red=proliferating cells (BrdU+). Picture from saline-treated rat shows mean BrdU-positive (proliferating) cells.

Conclusions: Experiment 10

The peripheral administration of the proinflammatory cytokines IL-1β and IL-6 cause significant reductions in hippocampal cell proliferation. This is the first experiment to show significant reductions in hippocampal cell proliferation resulting from the peripheral administration of proinflammatory cytokines. These results suggest that the acute administration of the proinflammatory cytokines IL-1β and IL-6 induce a relatively rapid (i.e., less than 4-5 hrs) suppression of hippocampal cell proliferation. These data are consistent with a previous observation that the peripheral inflammatory response, induced by the peripheral administration of LPS, causes reduced hippocampal neurogenesis (Monje, Toda, & Palmer, 2003). In addition, Vaillères and colleagues (2002) observed reduced hippocampal neurogenesis in adult transgenic mice with chronic astrocytic productic of IL-6, suggesting that chronic, in addition to the acute, administration of IL-6 reduces hippocampal neurogenesis.

One limitation of the use of BrdU-labeling method to quantify hippocampal cell proliferation is the difficulty in assessing whether the experimental treatment caused changes in cell proliferation *per se*, or whether the experimental treatment caused changes in blood-brain-barrier permeability which, in turn, caused altered labeling patterns across groups. Though possible, it is unlikely that altered blood-brain barrier permeability in the cytokine-treated group caused the direction of results that were observed in the present experiment. Though no study has investigated the effects of an acute peripheral injection of IL-1β and / or IL-6 on blood-brain barrier permeability, available data suggest that the peripheral inflammatory response increases blood-brain barrier permeability (Mark & Miller, 1999; Arsenijevic *et al*., 2000; Stanimirovic &

Satoh, 2000 (review)). It is reasonable to conclude, therefore, that if altered blood-brain barrier permeability caused differential labeling across groups, then this effect would have been observed in the opposite direction (i.e., cytokine-treated group would show greater hippocampal cell proliferation). Because an effect in the opposite direction was not observed, cytokines most likely act by directly inhibiting hippocampal cell proliferation.

In addition, though the intent of this present experiment was to assess the short-term effects of an acute proinflammatory injection on hippocampal physiology, it cannot be concluded with certainty that the cytokine-treated animals would also show reduced neurogenesis had they been injected with BrdU for several days. Though implausible, it is possible that a greater percentage of the newly-generated cells in the cytokine-treated group compared to the saline-treated group would differentiate into new neurons. Again, though no data are available to support this scenario, additional experiments should investigate the effects of an acute injection of proinflammatory cytokines on hippcampal neurogenesis.

The observation that the peripheral administration of IL-1β and IL-6 causes reduced hippocampal cell proliferation has several implications. First, because it can be inferred that reduced hippocampal cell proliferation correlates with reduced hippocampal neurogenesis (with the above caveats in mind), and because hippocampal neurogenesis has been empirically linked to memory consolidation (Feng *et al.*, 2001), it is possible that the LPS-induced impairment in post-trial memory consolidation observed by Pugh and colleagues (1998), and replicated in Experiment 3, is a behavioural correlate of inhibited hippocampal physiology.

Secondly, the observation that peripheral cytokine administration has a direct inhibitory effect on a hippocampal process supports the observation in Experiment 8 of a LPS-induced inhibitory effect on hippocampal LTP. Though the possibility of an indirect suppression of hippocampal LTP via a LPS-induced enhancement of slow-wave sleep remains, the data from this experiment make a stronger case for a direct, cytokine-induced inhibitory effect on hippocampal physiology.

Finally, the data from this experiment highlight the importance of IL-6 as a potential peripheral mediator of hippocampal inhibition and consequent cognitive dysfunction. Though additional experiments are required to separately assess the effects of IL-6 from IL-1β on hippocampal neurogenesis, peripheral IL-6 may play more of a role in sickness-induced cognitive dysfunction than has previously been considered, and possibly, *more* of a peripheral role than the much-maligned IL-1β.

General Conclusions

Re-statement of current theory

The experiments included in this dissertation were completed to determine whether the proinflammatory cytokine IL-1β is the major peripheral mediator of sickness-induced cognitive dysfunction, as well as to characterize the components and underlying mechanisms of sickness-induced cognitive dysfunction. To re-state the current generally accepted theory in this field: Cognitive dysfunction is one of several behavioural consequences of the peripheral inflammatory response, and represents a principle component of the organized behavioural response to peripheral inflammation. Moreover, the potent pro-inflammatory cytokine, IL-1β, is both the primary central and peripheral mediator of sickness-induced cognitive dysfunction (Danzer, 2001; Maier, 2003; Maier & Watkins, 2003).

Though generally accepted, this theory holds little empirical ground. Certainly, it appears to be true that the central release of IL-1β,- experimentally induced by the i.v. or i.c.v administration of IL-1β,- impairs learning and memory across a variety of paradigms (Oitzl *et al*., 1993; Barrientos *et al*., 2002). In addition, *in vitro* (Jo *et al*., 2001; Bellinger *et al*., 1993; Katsuki *et al*., 1990; Cunningham *et al*., 1996), and *in vivo* studies done in the anesthetized animal (Commins *et al*., 2001; Kelly *et al*., 2003; Vereker *et al*., 2000; Murray & Lynch, 1998), have suggested hippocampal LTP inhibition as a possible mechanism by which the inflammatory response impairs learning and memory.

Though tantalizing, experiments such as these do not provide adequate support for the argument that it is the peripheral inflammatory response, of which IL-1β has been proposed as the principle mediator, that impairs learning and memory. Of the few

experiments that have investigated the effects of LPS and/or IL-1β on learning and memory, the majority have involved central administration (Oitzl *et al*., 1993; Barrientos *et al*., 2002), while the few that have involved peripheral administration have produced weak (Shaw *et al*., 2001) or mercurial (Gibertini, 1998) results. In addition, the timing of compound administration relative to training used in these learning and memory experiments render it impossible to determine what component of cognition is impaired (i.e., acquisition (new learning), consolidation of recent learning, or long-term retention). The *peripheral* administration of LPS and IL-1β, the separate assessment of their effects on different cognitive components, and the convergence of evidence across learning and memory paradigms are required to establish the characteristics of sickness-induced cognitive dysfunction, as well as to determine the *peripheral* role of IL-1β in the induction of cognitive dysfunction.

Prior hippocampal LTP work offers only weak support for the current theory. The *in vitro (*e.g., Jo *et al*., 2001) and *in vivo* (e.g., Commins *et al*., 2001) preparations that were used to investigate the effects of LPS and / or IL-1β on hippocampal LTP are confounded because of the massive cytokine response to tissue injury (Janowsky *et al*., 2000). Cytokine response to tissue injury aside, no experiments have investigated the effects of LPS and/or IL-1β on hippocampal LTP in the awake, freely moving (and freely sleeping) rat. Such experiments are essential to the understanding of whether LPS and/or IL-1β have a physiological role in sickness-induced disruption of hippocampal synaptic plasticity.

Summary of Experiments 1-10

Following the confirmation that peripheral LPS induced a robust and reliable sickness response (Experiments 1 and 2), Experiments 3-9 were done in an effort to characterize the components of cognition that are impaired by sickness, as well as to determine whether or not peripheral IL-1β has a primary, physiological role in triggering sickness-induced cognitive dysfunction and / or the disruption of hippocampal synaptic plasticity.

Before the components and role of peripheral IL-1β in sickness-induced cognitive dysfunction could be determined, however, it had to be established whether or not the peripheral administration of LPS did, in fact, induce cardinal physiological and behavioural signs of sickness, which include: reduction in general motor activity, reduction in food consumption, loss of weight, and a febrile response. Indeed, in Experiments 1 and 2, the peripheral administration of LPS reliably induced all these signs of sickness. Peripheral administration of IL-1β also induced these sickness symptoms. Therefore, it can be concluded from Experiments 1 and 2 that the peripheral administration of LPS does render an animal ill, and that peripheral IL-1β is sufficient to induce several symptoms of illness.

So, is the peripheral administration of IL-1β sufficient to induce learning and memory deficits? A wide range of doses were used to test the effects of peripherally-administered IL-1β on learning and memory in the contextual fear conditioning task and the Morris water task. Importantly, the doses that were used had previously been demonstrated to induce a sickness response (Experiments 1,2; Yirmiya *et. al*., 1996). Yet, these doses did not produce deficits in performance in Experiments 3-7. So, these animals were ill, yet were able to learn and retain information at a level on par with healthy controls.

Together, these observations demonstrate that peripheral IL-1β is not sufficient to induce cognitive dysfunction.

So, what cognitive processes,- if any,- are affected by the peripheral inflammatory response? Again using a wide range of doses, performance was measured on a variety of learning and memory tasks following peripheral LPS administration. Evidence already existed to suggest that the peripheral administration of LPS would impair memory consolidation (Pugh *et al*., 1998), so an attempt was made to replicate this experiment in Experiment 3. Importantly, animals injected with a low-moderate dose (2 :g/kg) of LPS immediately following conditioning did show a significant impairment in context retention, as had been previously demonstrated by Pugh and colleagues (1998). However, this impairment in post-trial memory consolidation did not generalize to an impairment using a consolidation moving-platform version of the Morris water task, another hippocampal-sensitive memory task. The different effects observed across the tasks may be due to different task requirements. For example, the contextual fear conditioning task required the animal to rapidly acquire (during the 5 minute conditioning period) a configural representation of the environment. LPS only disrupted the consolidation of this representation if the animal had *not* been previously exposed to the environment, implying that LPS only disrupts recently acquired representations (Pugh *et al*., 1998). In contrast, the Morris water task required that the animal learn the location of a recently-moved hidden platform in a room with a stable cue-set. These animals may have had already acquired a stable representation of the environment in which they had to find the platform, making their representations less sensitive to the disruptive effects of LPS. These animals "only" had to update their representation to include a new platform

location, but they did not have to acquire a completely new configural representation. Task differences such as these point to the necessity of testing learning and memory across a variety of paradigms. There is empirical support for an LPS-induced inhibition of memory consolidation (Pugh *et al*., 1998; Experiment 3). However, it appears that this impairment is sensitive to the task (Experiment 4), suggesting that sick animals are able to overcome sickness-induced learning and memory impairments under certain conditions. Certainly, it appears then, that the cognitive dysfunction component of the constellation of symptoms referred to as sickness behavior is not, necessarily, a principle component. Animals rendered ill by LPS, not only are capable of acquiring and retaining information regarding a familiar environment, but they do so quite well.

Experiments 8 and 9 were designed to test whether the induction of hippocampal LTP would be sensitive to the peripheral immune response in the freely-moving rat, and, if so, whether the peripheral administration of IL-1β would be sufficient to disrupt hippocampal LTP. For the first time, it was demonstrated that hippocampal LTP in the freely-moving animal is, indeed, disrupted by the peripheral administration of LPS. However, no disruption in LTP was observed following the peripheral administration of IL-1β. Consistent with previous observations that peripherally-administered IL-1β has no effect on the acquisition, consolidation, or retention of information, it also appears that IL-1β has no primary peripheral signaling role in the disruption of hippocampal synaptic plasticity. It is interesting to note, however, that, though not statistically reliable, the peripheral administration of IL-1β causes a similar downward shift in the EPSP at 24 hours following injection, similar to the peripheral administration of LPS. It is possible, therefore, that peripheral IL-1β has a role in the disruption of hippocampal synaptic

plasticity, but that the actions of additional proinflammatory cytokines are required in the periphery to reliably disrupt synaptic plasticity.

As observed in Experiment 10, the combined administration of IL-6 and IL-1β significantly inhibited hippocampal cell proliferation, suggesting, in part, that IL-6 may have an important peripheral role in hippocampal modulation of learning and memory.

In conclusion, the results of the present series of experiments do not support the current theory regarding peripheral IL-1β and sickness-induced cognitive dysfunction. Data from the present series of experiments confirm: 1) Sick (i.e., febrile) animals share several behaviours, including reductions in food consumption, body weight, and general motor activity; 2) Sick animals show some cognitive dysfunction; 3) Sickness-induced cognitive dysfunction may have a cellular basis in the disruption of hippocampal LTP; and 4) Peripheral IL-1β is sufficient to induce some sickness behaviours (e.g., anorexia, lethargy).

However, the same data *do not* confirm that peripheral IL-1β is sufficient to induce learning and memory impairments or to reliably disrupt hippocampal LTP. If a peripheral role exists for IL-1β in sickness-induced cognitive dysfunction, then it must work in concert with other proinflammatory cytokines . Additionally, the data do not confirm that cognitive dysfunction represents an integral component of the sickness response. Rather, the data support the idea of a sickness-induced impairment in the consolidation of recent learning that is sensitive to the requirements of the task-at-hand. Furthermore, the observation of a cytokine-induced disruption in hippocampal LTP and hippocampal cell proliferation supports a cellular basis for sickness-induced learning and memory impairments.

Future research

When the proinflammatory cytokines were first experimentally linked to sickness behaviour, interest grew in the proinflammatory cytokines as potential psychotherapeutic targets (e.g., Reichenberg *et al*., 2001; Kent *et al*., 1992). The idea of proinflammatory cytokine antagonists as therapeutic agents was, not only a novel concept, but an extremely attractive one, as the proinflammatory cytokines were believed to underlie the neurological impairments common to: acute infection (Reichenberg *et al*., 2001), HIV infection (Wesselingh & Thompson, 2001); systemic lupus erythematosus (Lauwerys & Houssiau, 2003); and aging (Wilson *et al*., 2002). Also, anti-cytokine therapy had already been demonstrated to successfully treat several pathologies with a large inflammation component, such as SLE, heart disease, and inflammatory bowel disease (Yokota, 2003 (review); Ito, 2003 (review); Ogata & Hibi, 2003 (review); & Diwan, *et al*., 2003 (review)). It was, - and is,- highly conceivable that anti-cytokine therapy could be used to treat the cognitive dysfunction that accompanies other pathologies marked by aberrant immune activation.

However, it appears that in the race of drug discovery, two key elements have been left by the road side. First, cognitive dysfunction may not constitute as primary a role in sickness behaviour as had first been suggested. Certainly, data from Experiments 3-7 suggest that sick animals show some cognitive impairments, but the same data also suggest that this impairment is acutely sensitive to LPS dose and task requirements. This is not the case with other behavioural and physiological symptoms of sickness. The animals in Experiments 1 and 2 displayed several symptoms of sickness, such as reduced food consumption and consequent weight loss, across a range of doses and task

conditions. It appears from these observations, then, that the physiological mechanisms underlying sickness-induced cognitive dysfunction are functionally discrete from those underlying other sickness behaviours, and, as a consequence, should be put under a different "microscopic lens" in empirical investigation.

The second key element that has not been suitably addressed in the drug discovery effort is that of fundamental differences between the central and peripheral inflammatory response. As previously discussed, evidence exists to strongly suggest that the proinflammatory cytokine, IL-1β, has a fundamental and primary role in cognitive dysfunction resulting from a *central* inflammatory response (Oitzl *et al*., Barrientos *et al*., 2002). However, there is a glaring lack of evidence that the same can be said for cognitive dysfunction resulting from a *peripheral* inflammatory response. Experiments 3-9 do not support a role for peripheral IL-1β in cognitive dysfunction across a range of doses, nor across different learning and memory tasks. If proinflammatory cytokine antagonists are going to be used to treat inflammation-induced cognitive dysfunction in a human population- be it in the form of a bolus injection or a continuous infusion- they are going to be administered peripherally. Moreover, the discovery of anti-cytokine therapy that targets a single proinflammatory cytokine is preferable to one that induces a general immune suppression, as the non-specific effects of immunosuppressive drugs have been demonstrated to be greatly reduced by using cytokine-specific inhibitors (Weckmann & Alcocer-Varela, 1996). It is for these reason that empirical investigation into cytokine-induced cognitive dysfunction needs to be re-directed to the peripheral inflammatory response, as well as to other proinflammatory cytokines.

Data from the present series of ten experiments suggest the direction in which empirical investigation into the cytokine-cognitive dysfunction relationship should proceed. First, because IL-1β does not appear to be the primary peripheral mediator of sickness-induced cognitive dysfunction, the effects of other proinflammatory cytokines on learning and memory should be investigated. The observation that the peripheral administration of IL-6 inhibits hippocampal cell proliferation suggests that peripheral IL-6 may have an important role in hippocampal learning and memory systems.

Another proinflammatory cytokine that warrants investigation is tumor necrosis factor- alpha (tnf-α). Following peripheral LPS administration, tnf-α has been observed to be up-regulated in the hippocampus to a higher degree than IL-1β (Sunter *et al*., Society for Neuroscience, 2003). Also, in addition to having higher plasma levels than IL-1β following i.p. LPS administration, *both* IL-6 and tnf-α are up-regulated in the paraventricular nucleus (PVN) of the hypothalamus to a higher degree than IL-1β (Kakizaki *et al*., 1999).

In the present set of experiments, it was observed that IL-1β caused similar inhibitory trends in learning and memory and on hippocampal LTP as LPS, but, alone, was not sufficient to induce reliable inhibition. It follows from this observation that other proinflammatory cytokines are required in the periphery to induce cognitive dysfunction. It is more than reasonable, then, to suspect tnf-α and / or IL-6 as having a stronger peripheral role in sickness-induced cognitive dysfunction than that of IL-1β. Certainly, additional experiments should investigate the effects of peripheral IL-6 and tnf-α on learning, memory, and on hippocampal physiology via a drug "cocktail"-like approach.

Second, it is evident from the data, that sickness-induced cognitive dysfunction is not reliably produced across tasks by the peripheral administration of LPS. The strongest evidence for a LPS-induced learning and memory impairment (Pugh *et al*., 1998; Experiment 3) support a role for LPS in the disruption of recently acquired representations. Additional experiments should investigate whether this impairment generalizes to other tasks. Though an attempt was made in Experiment 5 to investigate whether the LPS-induced impairment observed in Experiment 3 generalized to a consolidation version of the Morris Water task, the tasks demands differed. Using a moving-platform version of the MWT, it would be worthwhile to investigate whether LPS, when administered immediately after one block of training, would disrupt retention for the platform's location in a different environment than the one in which animals had received the training. A task such as this should reveal whether the LPS-induced impairment in memory consolidation (Experiment 3; Pugh *et al*., 1998) is specific to recently-acquired representations across different tasks.

In addition, though an inhibitory effect of LPS on the EPSP was observed in Experiment 8, this effect was noticeable solely at the 24-hour time point. It would be worthwhile to investigate if this LPS-induced inhibition in EPSP maintenance is the result of a direct inhibitory effect of LPS on hippocampal synaptic plasticity, or if it is the result of an indirect enhancing effect of LPS on slow-wave sleep, which in turn, inhibits hippocampal synaptic plasticity. Results from the final experiment suggest that peripheral cytokines do have direct, inhibitory effects on hippocampal physiology; however, additional experiments that investigate the effects of peripheral administration

of LPS on LTP in an animal whose activity state was artificially maintained would strengthen this assumption.

Certainly, more empirical investigation needs to be done in order to fully understand the effects of the peripheral immune response on cognition. Data from Experiments 1-10 have characterized the cognitive process that is disrupted during infection and have demonstrated a cytokine-specific disruption of two physiological processes within the hippocampus linked to learning and memory. Additionally, the data have shown peripheral IL-1β to be virtually ineffective at disrupting cognition across a variety of doses and paradigms. Finally, data from Experiment 10 have provided preliminary evidence of additional proinflammatory cytokines acting in the periphery to disrupt central learning and memory processes. Additional experiments that are re-directed toward answering the questions posed above should provide for a more sound basis on which anti-cytokine therapy for sickness and inflammation-induced cognitive dysfunction can be discovered.

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Appendix A: LPS and IL-1β Effects on Brain Temperature

In addition to food consumption, weight, and general activity measurement, three three-hour measurements of brain temperature were recorded in an animal injected with: 250 μg/kg of LPS, 25 μg/kg of IL-1β, or an equivalent volume of pyrogen-free saline. The rise and fall of brain temperature has been observed to closely mirror that of core body temperature (Sundgren-Andersson, Ostlund, & Bartfai, 1998), and so it can be expected that if LPS and / or IL-1β provoke a peripheral immune response, it should be evident in a rise in brain temperature.

Implantation

Rats were anesthetized with isoflourane (O2 flow rate 1.5 l/min at 1.5-2% isoflourane) for chronic implantation. Rats were implanted, using aseptic surgical techniques, with two temperature probes (physitemp, custom 29 ga needle probe, T-107D). Both probes were aimed at the hilar region of the bilateral dentate gyrus (Paxinos & Watson coordinates: 3.5 mm posterior to the bregma, 2.0 mm lateral to themidline, and 3.5 mm below the top of the skull). Three stainless-steel,jewelers' screws tapped into the skull provided structural support for the probes, which were held in place by dental acrylic cement. The skin incision was closed with veterinary glue and the rats received injections of penicillin G (60,000 I.U. subcutaneous(sc)) and buprenorphine (0.05 mg/kg i.p.). The animal was allowed to recover for one week prior to testing.

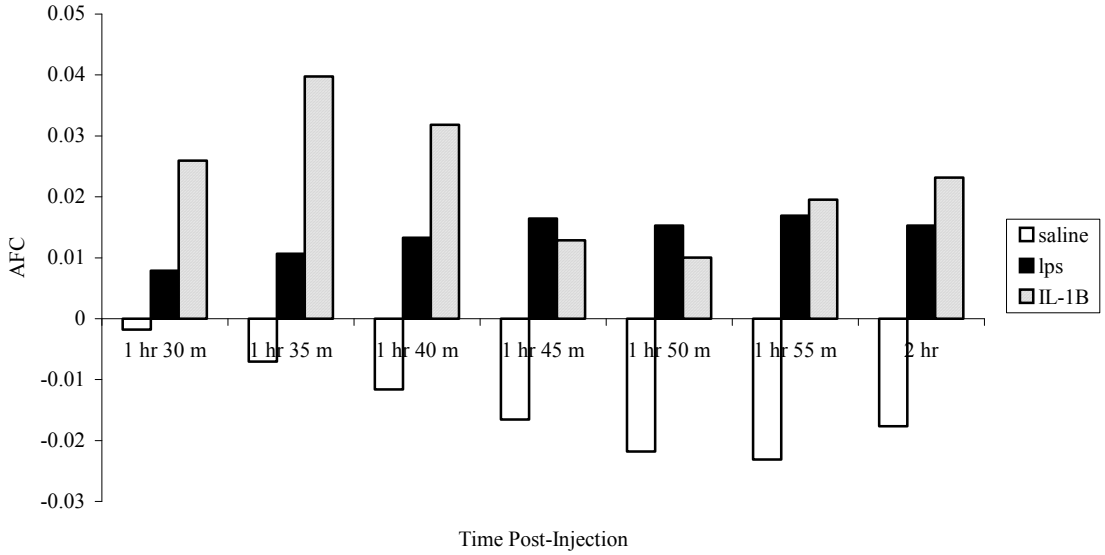
Temperature Monitoring

Brain temperature was recorded on a monitor (Physitemp, thermalert model TH-8 temperature monitor) while the animal was allowed to freely move around in its cage. Temperature was recorded in twenty-second intervals during a one-hour baseline recording, and during the two hours following the injection.

Injections

The animal was injected with either: 250 μg/kg of LPS ((Ecoli 026:B6); Sigma, Lot no. 101K4080; 3 million endotoxin units per MG), 25 μg/kg IL-1β ((RDI) catalog no. RDI-201b), or an equivalent volume of pyrogen-free saline (Abbot Laboratories, Lot no. 79-613-DM-01). The doses were chosen based on a previous experiment that had observed changes in temperature following these doses (Konsman *et al*., 2000); however, doses as low as 2 μg/kg of IL-1β have been observed to induce a febrile response (Yirmiya, *et al*. 1996). The order of injections was: saline, LPS, IL-1β. Each injection was spaced one day apart. Because temperature has previously been observed to return to baseline values within eight hours after injection (Yirmiya, *et al*. 1996), 24 hours between testing is more than sufficient to allow any increases in temperature to return to baseline values.

Results



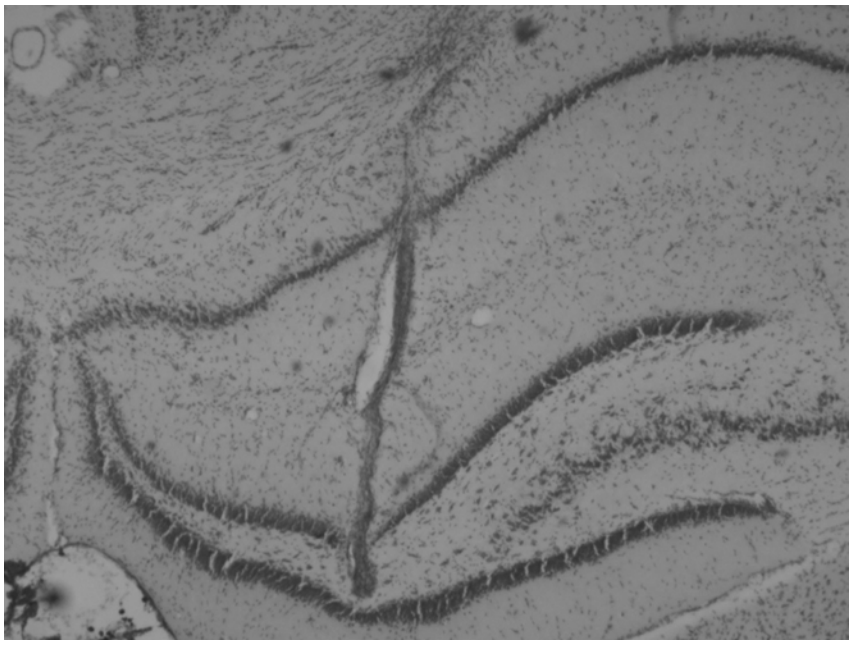
Appendix A. Effects of 250 µg/kg LPS, 25 µg/kg IL-1β, or an equi-volume a mount of pyrogen-free saline on brain temperature, expressed as the average fractional change (AFC) from baseline. N = 1.

Conclusion

Brain temperature began to rise approximately 1 hour and 30 minutes following an injection with LPS and IL-1β; whereas, in the saline-treated animal, brain temperature began to drop as the animal started to fall asleep (activity state only observed by the experimenter, and not video-monitored).

Because brain temperature has been observed to closely mirror the temperature of the peritoneum (Sundgren-Andersson *et al*., 1998), it can be concluded that both LPS and IL-1β induce a febrile response in the rat.

Appendix B: Verification of Recording Electrode Placement



Appendix B. Coronal section showing trace of the recording elelectrode, aimed at the hilar region of the dentate gyrus (Paxinos & Watson coordinates: 3.5 mm posterior to the bregma, 1.8 mm lateral to the midline, and 3.6 mm below the top of the skull)

Appendix C: Dose-Response Curve for LPS and IL-1β Effects on Hippocampal Long-Term Potentiation (LTP) in the Freely-Moving Rat

Because no previous experiments have measured the effects of LPS or IL-1β on LTP in the freely-moving rat, a dose-response curve was conducted to find the dose(s) at which LPS and / or IL-1β would most likely inhibit hippocampal LTP in a larger experiment.

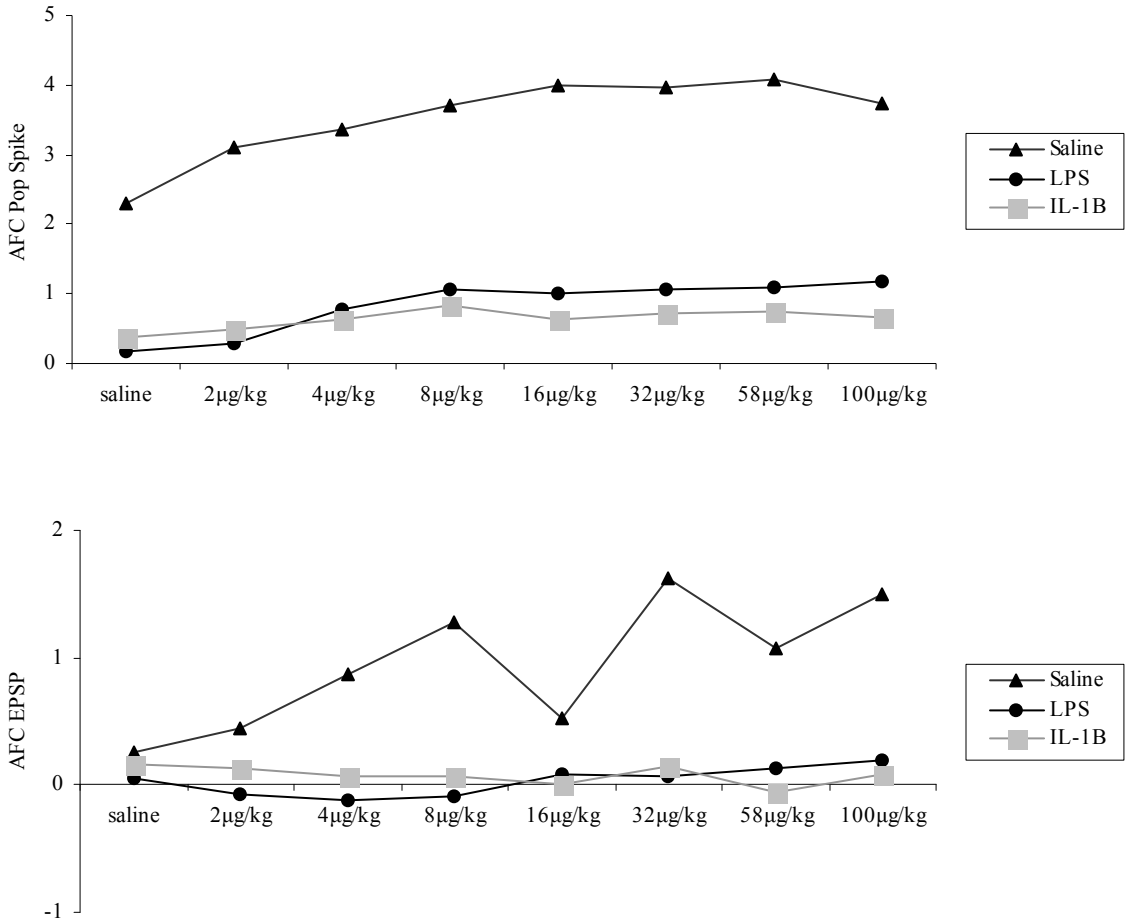
The implantation method, electrophysiology apparatus, and recording method were all identical to that which was described in the *Procedure* section of Experiments 8 and 9.

On testing day, an input-out (I-O) curve was produced for each animal to determine the baseline stimulus intensity. A pre-injection baseline recording was then taken. All animals were then injected with saline, after which high-frequency stimulation (HFS) was delivered.

Injections

Following the first delivery of HFS, animals were injected i.p. with cumulative doses of LPS ((Ecoli 026:B6); Sigma, Lot no. 101K4080; 3 million endotoxin units per MG) or IL-1β ((RDI) catalog no. RDI-201b). Cumulative doses ranged from 2 μg/kg to 100 μg/kg and were spaced approximately one hour apart. Each delivery of HFS was spaced approximately 45 minutes after each injection, and stimulation parameters were returned to baseline values after each HFS delivery. Changes in the evoked responses were expressed as fractional changes from the original values measured at the pre-injection baseline, as has been previously described.

Results



Appendix C. Effects of cumulative doses of LPS, IL-1β, and saline on hippocampal LTP in the freely-moving rat. LTP is expressed as the average fractional change (AFC) in pop-spike amplitude (top) and EPSP slope (bottom). N=3.

Conclusions

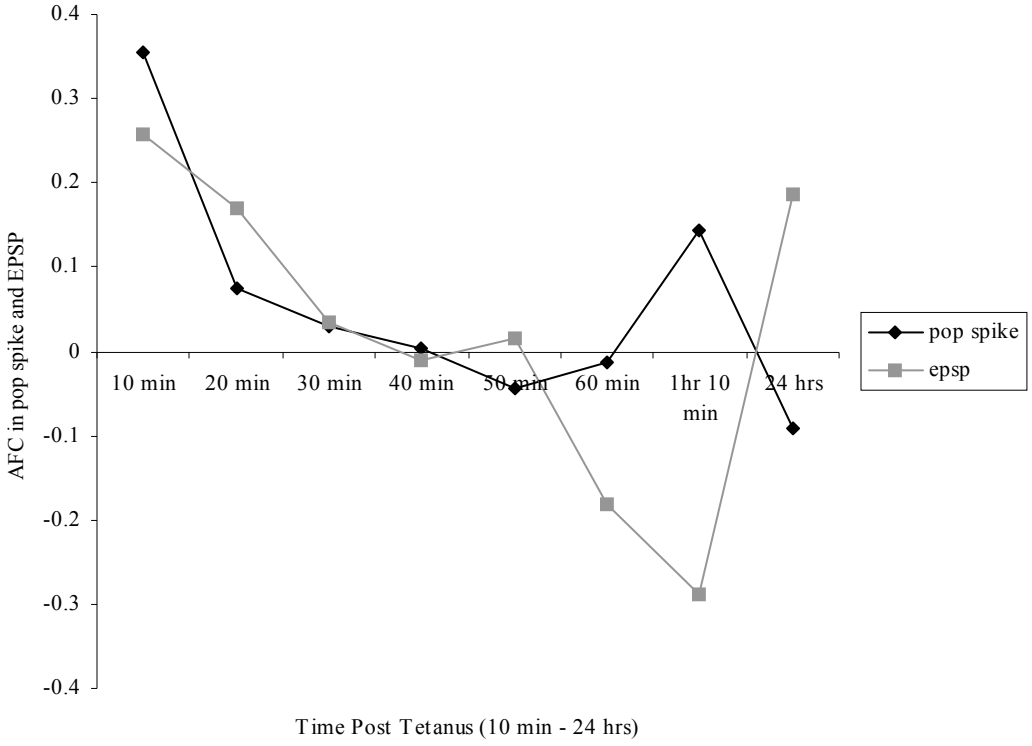
A dose of 12 µg/kg was chosen for both LPS and IL-1β as the dose most likely to inhibit hippocampal LTP. This dose was chosen because it fell mid-way between the doses which appeared to cause the most inhibition in the EPSP (8 µg/kg – 16 µg/kg) and the population spike, as evidenced by the downward deflection of the curve at these values.

Appendix D: Hippocampal Long-Term Potentiation (LTP) in the Freely-Moving Rat: Continuous Recording

To explore the possibility that experimental handling, necessary to the connection of the animal to the recording apparatus, confounded the change in the EPSP following HFS (Experiments 8,9), the evoked potential following HFS was measured continuously for approximately two hours, and then again at 24 hours. The animal was left unperturbed in its cage for the duration of the experiment.

The implantation method, electrophysiology apparatus, and recording method were all identical to that which was described in the *Procedure* section of Experiments 8 and 9.

On testing day, an input-out (I-O) curve was produced for the animal to determine the baseline stimulus intensity. A 30-pulse baseline recording was then collected. Forty-five minutes later, high-frequency stimulation (HFS) was delivered. Parameters were then returned to baseline values and remained there for two hours, and again at 24 hours. Changes in the evoked responses were expressed as fractional changes from the original values measured at the pre-injection baseline.



Appendix D. LTP expression in freely-moving, unperturbed rat. LTP is expressed as the average fractional change (AFC) in pop spike amplitude and EPSP slope following tetanus. N = 1.

Conclusions

The change in the EPSP over the 2 hour period mirrored that of the majority of animals in Experiments 8 and 9. That is, the initial rise in the AFC of the EPSP

following HFS slowly diminished over the course of one hour until it fell below baseline values. It then returned to higher-than-baseline values at 24 hours. Presumably, then, the depressed EPSP that was observed at 75 minutes in saline-treated animals in Experiments 8 and 9 was not an artifact of experimental handling, but rather was a result of differences in the animals' body core temperatures as a function of activity state. This is further explained in the *Conclusions* section of Experiments 8 and 9.