Fever and sickness behaviours in rats following central administration of interleukin -1β

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Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Master of Science in Medicine in the field of Physiology.

Johannesburg, South Africa 2015.

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

Declaration

I, Tamzyn Lea Baartman, declare that the work contained in this dissertation is my own, except where otherwise specified. The work herein has not been submitted for a degree at any other university. It is being submitted for the degree of Masters of Science in Medicine in the field of Physiology within the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg.

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Signed on the 30 day of April , 2015.

Research Output

International Conferences

Baartman TL, Swanepoel T, Barrientos R, Laburn HP, Mitchell D, Dukhan T and Harden LM. Fever and sickness behaviours, including memory impairment, after central administration of interleukin-1ß. Presented at 5th International Symposium on the Physiology and Pharmacology of Temperature Regulation (PPTR), Skukuza, Kruger National Park, South Africa, 7-12 September 2014. Poster presentation.

Local Conferences

Baartman TL, Swanepoel T, Barrientos R, Laburn HP, Dukhan T, Mitchell D and Harden LM. Sickness responses in rats following central administration of Interleukin-1β. Presented at 41st Annual Congress of the Physiological Society of Southern Africa (PSSA), Pretoria, South Africa. 15-18 September 2013. Poster presentation, Johnny van der Walt poster competition, second place.

Baartman TL, Swanepoel T, Barrientos R, Laburn HP, Mitchell D, Dukhan T and Harden LM. The effect of centrally administered interleukin-1ß on fever and sickness behaviours, including memory impairment. Presented at the 6th Wits cross-faculty graduate symposium. 28 and 29 October 2014. Oral presentation, third place.

Ethics Clearance

My experiments were carried out in accordance with the regulations of the Animal Ethics and Control Committee of the University of the Witwatersrand and were approved by its Animal Ethics Screening Committee. See Appendix for clearance certificates. Clearance numbers: AESC 2012/08/04, AESC 2012/38/04, AESC 2013/28/04.

Abstract

Introduction

Upon entry into the central nervous system different pathogens are recognized by different pathogen receptors (e.g. *Escherichia coli* (*E. coli*) is recognized by Toll like receptor 4) present on the surface of innate immune cells. Once recognized, pathogens activate the innate immune system of the host which ultimately results in the synthesis and release of pro-inflammatory cytokines (e.g. Interleukin-1β (IL-1β)). Pro-inflammatory cytokines are the primary mediators of the acute phase response which consists of a suite of physiological and behavioural changes which include fever, lethargy, anorexia and in some cases memory impairment. Clinical cases of central bacterial infections (e.g. meningitis and brain abscesses) have shown that in addition to developing fever, lethargy and anorexia, one third of patients also present with cognitive deficits. Laboratory based studies simulating central bacterial infections using Lipopolysaccharide (LPS), the active fragment of *E. coli*, and IL-1β have well documented their effects on fever, anorexia, and lethargy. In addition centrally administered IL-1β has also been demonstrated to effect on memory, however no studies have examined the effect of centrally administered LPS on memory. Furthermore, to my knowledge, no studies have concurrently examined the impact of a simulated central bacterial infection using LPS or IL-1β on a suite of sickness responses including body temperature, activity, food intake, body mass and memory. Therefore I set out to concurrently examine body temperature, voluntary activity, food intake, body mass and memory after the central administration of either LPS or IL-1β and their appropriate vehicles.

Materials and methods

Male Sprague-Dawley rats (~200g) were anaesthetised and had radiotelemeters implanted intra-abdominally, which measured abdominal temperature and cage activity. Body mass and food intake were measured daily. The contextual and auditory fear conditioning paradigm was used to assess memory in rats. Fear conditioning entails conditioning/training of rats and performing two memory (hippocampal and amygdala dependent) tests. Rats were randomly assigned to receive a single intra-cisterna magna (ICM) injection of either LPS (10 µg or 100 µg), IL-1β (100 ng) or vehicle (phosphate buffered saline or 0.1% bovine serum albumin) in a total volume of 5 µl. Rats set to receive LPS and PBS were assigned to a protocol in which they were conditioned/trained, followed by an immediate ICM injection of either LPS or PBS. Rats set to receive IL-1β or BSA were assigned to one of two protocols: a) a protocol in which rats were conditioned/trained, followed by an immediate ICM injection of either IL-1β or BSA; b) a protocol in which rats first received an ICM injection of either IL-1β or BSA and then were conditioned/trained 2 h later. Sickness responses were measured for 7 days following injection and memory was assessed on day 7. In addition, hypothalamic and hippocampal concentrations of IL-1β for rats that received IL-1β and BSA were measured at several time points following injection.

Results

Following the administration of LPS, rats showed a dose-dependent hypothermia which preceded a fever, as well as a dose-dependent decrease in nocturnal activity, food intake and body mass ($P < 0.05$). However, regardless of dose, LPS administered immediately *after* conditioning had no effect on hippocampal-dependent memory assessed 7 days after LPS administration ($P > 0.05$). Moreover, following IL-1β administration, rats showed an increase in body temperature, a

decrease in activity and food intake and had significantly increased concentrations of IL-1β in the hippocampus and hypothalamus ($P < 0.05$). Furthermore, rats that received IL-1β immediately *after* conditioning had no effect on hippocampal-dependent memory ($P > 0.05$) however, rats that received IL-1β 2 h *before* being conditioned showed both hippocampal and amygdala-dependent memory impairment ($P < 0.05$).

Discussion and conclusion

The results of the present study suggest that a central infection severe enough to induce a suite of sickness responses including fever, anorexia and lethargy may occur without incurring memory impairment in rats. However, rats that received IL-1β 2 h *before* being conditioned were conditioned at a time when brain IL-1β concentrations were raised and whilst they were febrile, which suggests that memory impairment may occur, concurrently with other sickness behaviours, only when learning takes place during an immune challenge within the brain.

Acknowledgements

I would like to express my gratitude to the following people and organisations for their support throughout my Masters.

Firstly, to my supervisors Lois Harden and Tanya Swanepoel for your unwavering support, encouragement and seemingly boundless knowledge. You have both been a constant source of inspiration and have brought out the best in me. I could not have asked for two better mentors.

To my co-investigators and three great minds: Ruth Barrientos, Helen Laburn and Duncan Mitchell. My research has benefited greatly from the many discussions and years of wisdom you all contribute. Ruth thank you for coming all the way from Colorado to help set up our behavioural model, teaching us how to perform ICM injections as well as the many emails, your expertise, knowledge and advice. Helen you are greatly missed, your support and encouragement always came at the best of times when things felt as if they were falling apart. You were truly an inspiration and I feel so privileged to have had the opportunity to meet and work alongside you.

Tanusha Dukhan, Zipho Zwane, Adelaide Masemola and Anneri Kriel: for all of the early mornings, long days, weekends and hours of behavioural testing. Thank you so much for all of your help, friendship and support.

Monica Gomez and Margaret Badenhorst, thank you for your invaluable expertise and technical support during my ELISA's. Dr's Kennedy Erlwanger, Anna Haw and Sister Amelia Ramekwa for the hours of surgery and ICM injections, thank you for always finding a way to squeeze me into your busy schedules.

To the staff of CAS for your help with all of my procedures as well as for the provision and care of my animals. To the Brain Function Research Group for their research support and infrastructure.

To my family and friends: thank you for your undying support, love and understanding, for always believing in me and being my biggest cheerleaders even when I didn't believe in myself.

I gratefully acknowledge the South African Medical Research Council and the University of the Witwatersrand Friedel Sellschop award for the funding that made my research possible. Lastly I would like to thank the South African National Research foundation for funding me in my personal capacity.

Tamzyn

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Abbreviations

Anti-IL-1β – Interleukin-1β antibodies

BSA – Bovine Serum Albumin

CD-14 – Cluster of differentiation-14

CNS – Central nervous system

COX-2 - Cyclo-oxygenase-2

CRF – Corticotrophin releasing factor

CSF – Cerebral spinal fluid

CSN- Cold-sensitive neurons

E. Coli - Escherichia coli

GS - Glucose-sensitive

GR - Glucose-receptor

ICM – Intra-cisterna magna

ICV - Intracerebroventricular

IL-1α – Interleukin-1α

IL-1β – Interleukin-1β

IL-1r1 - Interleukin -1 receptor 1

IL-1rII - Interleukin -1 receptor 2

IL-1rIII - Interleukin -1 receptor 3

IL-1ra - Interleukin -1 receptor antagonist

IL-6 – Interleukin-6

IP – Intraperitoneal

ISN- Temperature insensitive neurons

IH - Intrahippocampal

LBP – Lipopolysaccharide-binding protein

LPS - Lipopolysaccharide

LTP - Long term potentiation

Myd88 – Myeloid differentiation factor

NO – Nitric oxide

NY-P – Neuropeptide-Y

PAMP – Pathogen associated molecular pattern

PG - Prostaglandin

PGE_2 – Prostaglandin-E_2

SC – Subcutaneous

TLR – Toll-like receptor

TLR-4 – Toll-like receptor-4

TNF-α - Tumor necrosis factor–α

TNZ – Thermoneutral zone

WSN - Warm-sensitive neurons

Chapter 1

Introduction

1.1. Central nervous system infections

The brain and central nervous system are considered to be sterile areas of the body not normally inhabited by pathogens (Agyeman *et al.*, 2014). The brain's innate protective barriers which include the blood brain barrier and the skull, protect the brain to a large extent from infection and inflammation (Agyeman *et al.*, 2014). However, central infections such as brain abscesses and bacterial meningitis can occur with potentially fatal consequences.

Upon entry into the central nervous system pathogens are detected by means of pathogen-associated molecular patterns (PAMPs) (Kapetanovic and Cavaillon, 2007, Kelley *et al.*, 2003). A PAMP is a specific, structurally conserved molecule, unique to the pathogen that enables its recognition by the innate immune system of the infected host (Heumann and Roger, 2002, Kapetanovic and Cavaillon, 2007, Kelley *et al.*, 2003). The detection of PAMPs in the brain is mediated by pattern-recognition receptors, including transmembrane proteins known as Toll–like receptors (TLR) (Dantzer, 2009, Heumann and Roger, 2002, Kapetanovic and Cavaillon, 2007). Pattern-recognition receptors are expressed on the surface of innate immune cells such as microglia, astrocytes, neurons and endothelial cells (Kapetanovic and Cavaillon, 2007).

Escherichia coli (*E. coli*) is a Gram-negative bacterium that frequently causes central infections (Agyeman *et al.*, 2014). Lipopolysaccharide (LPS) is the active fragment of *E. coli* and is frequently administered experimentally in order to mimic Gram-negative bacterial infections and associated inflammation (Conti *et al.*, 2004). Lipid A is the active structural moiety of LPS and it is the PAMP that is recognized by host immune cells (Heumann and Roger, 2002). The mechanism of LPS recognition in the host begins by the dissociation of LPS into a LPS-binding protein (LBP) complex which is then transferred to the functional membrane bound LPS receptor

cluster of differentiation 14 (CD14), which leads to early cellular activation (see Figure 1.1) (Conti *et al.*, 2004, Heumann and Roger, 2002). There is substantial evidence that LBP is specifically detected by Toll-like receptor – 4 (TLR-4) (Heumann and Roger, 2002). Once activated, TLR-4 transmits the LPS signal from CD14 to the cytoplasm (Heumann and Roger, 2002). Signaling from CD14 to the cytoplasm occurs via systematic recruitment of the myeloid differentiation factor (MyD88) which results in the activation of transcription factors. The transcription factors promote the expression of immune response genes which stimulate the release of a suite of pro-inflammatory cytokines, including interleukin (IL)-1α, IL-1β, IL-6, and tumor necrosis factor (TNF)-α (Conti *et al.*, 2004, Leon, 2004).

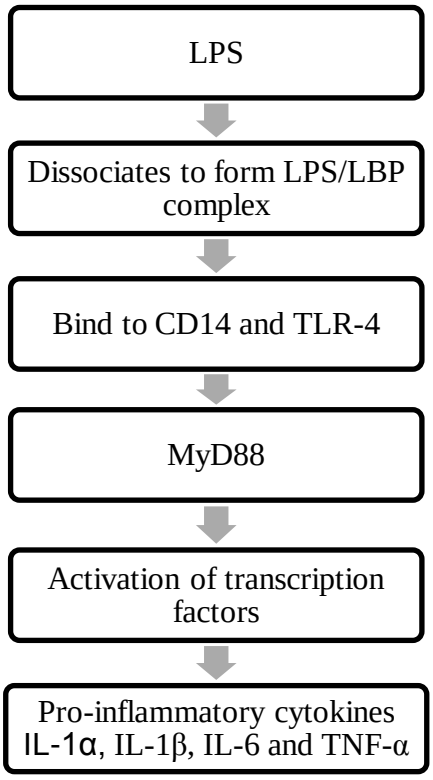


Figure 1.1 - The activation of the pro-inflammatory cascade after the central administration of LPS, adapted from Conti *et al.* (2004). Abbreviations: Lipopolysaccharide (LPS), Lipopolysaccharide-binding protein (LBP), Cluster of differentiation 14 (CD14), Toll-like receptor – 4 (TLR-4), Myeloid differentiation factor (MyD88), Interleukin-1α (IL-1α), Interleukin-1β (IL-1β), Interleukin-6 (IL-6) and Tumor-necrosis factor-α (TNF-α).

Pro-inflammatory cytokines are the primary mediators in the implementation of the host acute phase response (Dantzer *et al.*, 2008). The acute phase response consists of a suite of physiological and behavioural changes (Dantzer, 2009, Pugh *et al.*, 2001), some of which include an increase in core body temperature, as well as non-specific, non-thermal behavioural changes such as lethargy, anorexia, malaise, depression and in some cases even cognitive deficits (Begg *et al.*, 1999, Dantzer, 2009, van de Beek *et al.*, 2002). These changes are collectively referred to as sickness responses and are present in both mild and severe infections (Dantzer *et al.*, 2008). Generally, sickness responses are not considered to be maladaptive or undesirable, but rather as a change in the motivational state of an organism (Dantzer, 2009) that may be critical to fighting off an infection (Hart, 1988, Pugh *et al.*, 2001). However, chronic infections and inflammation can exacerbate the intensity and duration of sickness responses (Dantzer *et al.*, 2008) which may result in devitalisation of the host, a decreased quality of living, organ failure and death (Kelley *et al.*, 2003).

The implementation of sickness responses is also mediated by the action of pro-inflammatory cytokines (Dantzer, 2009). Pro-inflammatory cytokines do not work in isolation to implement sickness responses, once they have been released they induce the synthesis and secretion of other cytokines (Alheim *et al.*, 1997, Cartmell and Mitchell, 2005, Fantuzzi *et al.*, 1996), which have been shown to act synergistically and additively (Harden *et al.*, 2008, Pugh *et al.*, 2001, Sonti *et al.*, 1996). Although pro-inflammatory cytokines may work synergistically to induce sickness responses, the relative potency of individual cytokines has also been assessed in order to examine the predominant cytokines involved in mediating centrally-induced sickness responses. Cytokine potency has previously been determined by comparing the magnitude of sickness responses induced by individual cytokines administered at the same dose (Anforth *et al.*, 1998,

Busbridge *et al.*, 1989, Harden *et al.*, 2008). For example, several studies have reported that centrally administered IL-1β induces sickness responses (including fever, anorexia, lethargy and memory impairment) of a greater magnitude compared to the same dose of centrally administered IL-6 and TNF-α, which may indicate that IL-1β is a more potent mediator of the sickness responses (Goshen and Yirmiya, 2006, Harden *et al.*, 2008, Sonti *et al.*, 1996).

IL-1β is a member of the IL-1 family of pro-inflammatory cytokines. IL-1β is synthesized by innate immune cells (e.g. microglia, astrocytes and monocytes/macrophages) as a precursor protein and becomes biologically active after proteolytic cleaving via IL -1β converting enzyme (caspase-1) (Fantuzzi *et al.*, 1996, Zheng *et al.*, 1995). Mature IL-1β is then secreted from the innate immune cells in which it was synthesized (Dinarello, 2005; Fantuzzi *et al*., 1996; Zheng *et al*., 1995) and elicits its biological action through binding to specific IL-1 receptors (Dinarello, 2005). There are three IL-1 receptors, interleukin-1 receptor type 1 (IL-1rI), interleukin-1 receptor type 2 (IL-1rII) and interleukin-1 receptor type 3 (IL-1rIII) (Dinarello, 2000, Qian *et al.*, 2012), which are widely distributed throughout the brain indicating a broad spectrum of action for IL-1β (Farrar *et al.*, 1987, Qian *et al.*, 2012). IL-1r1 is a functional receptor responsible for signal transduction and initiation of biological activity for IL-1β (Dinarello, 2000). Upon interaction with IL-1r1 within the brain, IL-1β triggers a cascade of inflammatory events, which includes the release of secondary pro-inflammatory cytokines such as IL-6 and TNF-α as well as other pro-inflammatory markers and mediators, including Cyclooxygenase-2 (COX-2), Nitric oxide (NO) and Prostaglandin (PG) (Dinarello, 2000). IL-1rII is a functionally negative decoy receptor which binds IL-1β with a high affinity, inhibiting binding to IL-1rI and thus inhibiting agonistic biological activity (Dinarello, 2000). IL-1rIII is a truncated form of IL-1rI and acts as an accessory receptor to IL-1rI (Boraschi and Tagliabue, 2013, Qian *et al.*, 2012).

IL-1rIII does not bind directly to IL-1β, rather it binds to IL-1β-IL-1rI or IL-1β-IL-1rII complexes and increases the receptors affinity for IL-1β (Boraschi and Tagliabue, 2013). IL-1β activity is also endogenously mediated by IL-1ra, which is an endogenous antagonist (Zheng *et al.*, 1995). IL-1ra binds to IL-1r1 without initiating a biological response and subsequently inhibits the binding of IL-1β (Zheng *et al.*, 1995).

The acute phase response has a long evolutionary history that has been highly conserved in both humans and animals, therefore the acute phase response in humans and animals is very similar (Bauhofer *et al.*, 2001, Kluger, 1986), which enables the use of animal models of infection to explore aspects of the human sickness behaviour suite. During peripheral infections, pathogens colonize in the blood stream and activate peripheral immune cells (e.g. monocytes and macrophages) to release pro-inflammatory cytokines (Dantzer *et al.*, 2000). Peripherally-released cytokines then signal the brain via both blood-borne and neural (the vagus nerve) routes which leads to secondary production of pro-inflammatory cytokines in the brain (Agyeman *et al.*, 2014, Dantzer *et al.*, 2000). Laboratory based studies frequently mimic peripheral bacterial infections by peripherally administering live bacteria (e.g. *E. coli*) (Barrientos *et al.*, 2006), bacterial moieties (e.g. LPS) (Pugh *et al.*, 1998) or pro-inflammatory cytokines (e.g. IL-1β, IL-6 or TNF-α) (Begg *et al.*, 1999). In fact, the majority of our understanding regarding host immune activation and the acute phase response is derived from rodent models of peripheral infection. Central infections can also occur independently of peripheral infections and are often either the result of focal infections which occur close to the brain (e.g. sinusitis or ottitis media) or in cases where the integrity of the skull or meninges is compromised (Agyeman *et al.*, 2014). Central bacterial infections are induced in laboratory based studies by the direct administration of live bacteria (Barichello *et al.*, 2013, Barichello *et al.*, 2014b), bacterial moieties (McCarthy *et al.*,

1986) or pro-inflammatory cytokines (Harden *et al.*, 2008) into the cerebral spinal fluid (CSF) of animals (Begg *et al.*, 1999).

Due to immunological immaturity in children and age related immunological declines in the elderly, these two demographic groups are at the highest risk for central infections and inflammation (Fietta *et al.*, 1994, PrabhuDas *et al.*, 2011), however central infections are still prevalent in healthy and immunocompromised adults (Begg *et al.*, 1999). Laboratory based studies mimicking central bacterial infections have been well documented and have been studied at multiple developmental stages including neonatal (Bilbo and Schwarz, 2009), adolescence, adult hood (Harden *et al*., 2008) and old age (Barrientos *et al*., 2006). Interestingly, sickness responses are unique to each developmental stage and vary in terms of their intensity (Barrientos *et al.*, 2009). As stated above, the acute phase response is very similar in humans and animals which makes rodents an ideal model for studying central infections because of ethical considerations. Therefore, in order to avoid confounding species-specific and age-specific sickness responses as well as any inaccurate immunological responses brought about due to using an immunocompromised animal I will concentrate on data from acute, central bacterial infections simulated in otherwise healthy, adult rats and mice for the remainder of my dissertation.

Although laboratory based studies of fever and anorexia have been well documented following central bacterial infection and inflammation, little is known about lethargy and memory impairment in the context of central bacterial infections. In addition, to my knowledge, no studies have concurrently examined the impact of a central bacterial infection on a suite of physiological processes including body temperature, activity, food intake, body mass and memory. In this introductory chapter I will give a brief overview of the definitions and current

views in the literature regarding fever, anorexia, lethargy and memory impairment induced by simulated bacterial infection. Secondly, I will discuss the proposed mechanisms by which centrally administered LPS induces fever, anorexia, lethargy and memory impairment. Lastly I will discuss the proposed involvement of pro-inflammatory cytokines in mediating fever and sickness behaviours, with specific focus on the role of IL-1β.

1.2. Fever

During either pathogenic or simulated infection/inflammation, the host may develop a fever. Fever is a common accompaniment of the acute phase response and is characterized by an elevation in core body temperature (Conti *et al.*, 2004, Romanovsky *et al.*, 2005). Fever is the direct result of active thermogenic effectors and is achieved by an increase in heat production and/or by heat conservation mechanisms (Roth and Blatteis, 2014). Thermo-effector mechanisms are mediated by changes in neuronal activity localized in the preoptic nucleus of the anterior hypothalamus, which is the thermoregulatory centre of the brain (Conti *et al.*, 2004).

There are two thermosensitive neurons present in the pre-optic nucleus, namely warm-sensitive neurons (WSN) and cold-sensitive neurons (CSN). There are also neurons present in the hypothalamus that are not sensitive to changes in hypothalamic temperature, they are classified as temperature-insensitive neurons (ISN) (Boulant, 1998, Boulant, 2000). Whilst WSN appear to be intrinsically thermosensitive, evidence has suggested that CSN are not, instead they are dependent on synaptic input from neighbouring neurons (Boulant, 1998, Boulant, 2000, Hori *et al.*, 1988, Kobayashi and Takahashi, 1993). During hypothalamic warming, WSN increase their firing rates. Which leads to synaptic inhibition of CSN and consequently decreases their firing rates (Boulant, 2000, Hori *et al.*, 1988, Kobayashi and Takahashi, 1993). Inversely, during

hypothalamic cooling WSN decrease their activity, therefore synaptic inhibition to CSN will be reduced and resultantly CSN increase their firing rates (Boulant, 2000, Hori *et al.*, 1988, Kobayashi and Takahashi, 1993). There is also evidence which suggests that CSN are open to excitatory synaptic input from the ISN (Boulant, 1998). The ISN maintain a constant excitatory synaptic input to the CSN so during hypothalamic cooling when WSN reduce synaptic inhibition to CSN the ISN (which are insensitive to hypothalamic temperature changes) maintain excitatory synaptic input to the CSN, which thereby increases CSN firing rates (Boulant, 1998). Once activated temperature-sensitive neurons innervate premotor neurons that drive autonomic thermo-effector responses in the body such as sweating and shivering (Boulant, 2000; Dinarello, 2004). The everyday functioning of temperature-sensitive neurons under normal, non-pathological circumstances leads to the establishment and homeostasis of core body temperature (Boulant, 1998).

1.2.1. Fever in response to the central administration of LPS

Under pathological conditions, multiple substances may initiate a fever (Roth and Blatteis, 2014). Experimentally LPS is the most commonly used exogenous pyrogen used to study fever in the context of central infections. As shown in Table 1.1 the intracerebroventricular administration of LPS has been consistently demonstrated to induce a fever of rapid onset (~ 1 h) over a range of doses (10-60 ng) (Ledeboer *et al.*, 2002, McCarthy *et al.*, 1986). The direct administration of LPS into the cerebral ventricle of rats demonstrates the pyrogenic action of LPS, however it does not give any indication into the mechanism by which LPS induces fever. In fact, through radio-labelling studies it was discovered that pyrogens do not act directly via the pre-optic nucleus of the anterior hypothalamus to induce fever (Boulant, 1998, Boulant, 2000,

Hori *et al.*, 1988). Rather, upon entry into the brain LPS activates immune cells (e.g. microglia, astrocytes, neurons and endothelial cells), which release endogenous pyrogens, specifically pro-inflammatory cytokines (e.g. IL-1β, IL-6, TNF-α), which are the proximate mediators of fever (Roth and Blatteis, 2014).

Table 1.1: Summary of the studies that investigated the effect of LPS on core body temperature in rodents.

	Species	LPS dose and route	Effect on core body temp	Reference
Lipopolysaccharide				
Central administration of:				
LPS	Rats	10 ng, ICV	Increase	McCarthy *et al*., 1986
LPS	Rats	60 ng, ICV	Increase	Ledeboer *et al*., 2002
Lipopolysaccharide and blocking IL-1 action				
Peripheral LPS and central administration of:				
IL-1ra	Rats	100 μg/kg, IP	Attenuation	Cartmell *et al*,. 1999
IL-1ra	Rats	100 μg/kg, IP	Attenuation	Luheshi *et al*.,1996
Caspase-1 inhibitor	Rats	250 μg/kg, SC	Attenuation	Harden *et al*., 2011
IL-1β knockout mice	Mice	2.5 mg/kg, IP	Attenuation	Kozak *et al*., 1998
Anti-IL-1β	Rats	50 mg/kg, IP	Attenuation	Klir *et al*., 1994

IL-1β – Interleukin-1β, LPS – Lipopolysaccharide, IL-1ra – Interleukin -1 receptor antagonist, Anti-IL-1β – Interleukin-1β antibody, ICV – Intracerebroventricular, IP- Intraperitoneal, SC -Subcutaneous

Attenuation suggests that body temperature was significantly decreased in comparison to rodents that received LPS, but was still significantly elevated compared to rodents that received the control substance.

Once pro-inflammatory cytokines have been released into the brain they bind to immune cells and cytokine specific receptors present in the pre-optic anterior hypothalamic area where they induce the expression of COX–2 and hence, prostaglandin-E_2 (PGE_2) (Conti *et al.*, 2004, Roth and Blatteis, 2014). PGE_2 then diffuses into the pre-optic nucleus and acts on PGE_2 specific receptors present on thermosensitive neurons (Dinarello, 2004; Romanovsky *et al.*, 2005). PGE_2 inhibits WSN activity which reduces synaptic inhibition to CSN (Boulant, 1998, Boulant, 2000) and this interaction results in thermogenesis and heat retention mechanisms such as cutaneous vasoconstriction, shivering and increased metabolic activity of brown adipose tissue; these integrated mechanisms cause febrigenesis (the onset of fever) (Boulant, 2000). When endogenous pyrogen levels decrease, WSN activity returns to normal which in turn decreases CSN activity and results in heat loss mechanisms (Boulant, 1998, Boulant, 2000). The heat loss mechanisms include: vasodilation, panting and sweating and enable defervescence (the resolution of fever) (Boulant, 2000).

Studies in which LPS alone was administered centrally to rodents have demonstrated that LPS induces the synthesis of IL-1α, IL-1β, IL-6, and TNF-α, all of which are known to be intrinsically pyrogenic (Cunningham *et al.*, 2005; Gabellec *et al.*, 1995; Laye *et al.*, 1994; Quan *et al.*, 1998). These cytokines have been described as intrinsically pyrogenic based on their innate ability to act individually and directly on the pre-optic nucleus of the hypothalamus, in both humans and rodents, to induce fever (Anforth *et al.*, 1998, Busbridge *et al.*, 1989, Conti *et al.*, 2004, Dinarello, 1999, Harden *et al.*, 2008). To date the exact role of IL-1β in mediating fever during a central infection is still unclear. Table 1.1 shows the role of IL-1 in mediating fever induced by various doses (100 µg/kg – 50 mg/kg) of systemically administered LPS. There are no studies which have administered LPS centrally in conjunction with an IL-1 blocking

agent, therefore I included studies which administered LPS peripherally/systemically in conjunction with an IL-1 blocking agent. The proposed role of IL-1 in mediating fever was investigated using techniques which allowed for selective inhibition or blockade of IL-1. Studies in which brain IL-1 was blocked and which showed an attenuation of peripherally induced fever (Cartmell *et al.*, 1999, Harden *et al.*, 2011, Klir *et al.*, 1994, Kozak *et al.*, 1998, Luheshi *et al.*, 1996), indicate that IL-1 can act within the brain to mediate fever. In these studies, fever was measured by means of radiotelemetry (positioned in the abdomen of rodents) or by use of copper thermocouples (inserted into the rectum of rodents).

Table 1.2 summarizes the pyrogenic effect of intracerebroventricularly administered IL-1β on core body temperature. Many studies adminstered IL-1β directly into the brain (Anforth *et al.*, 1998, Busbridge *et al.*, 1989, Cao *et al.*, 2001, Harden *et al.*, 2008, Kent *et al.*, 1992, Li *et al.*, 2001, Montkowski *et al.*, 1997, Oitzl *et al.*, 1993) and have demonstrated that IL-1β consistently initiates fever over a range of doses (0.1 ng – 20 µg). Fever was measured using radiotelemetry (positioned in the abdomen of rodents) or by use of copper thermocouples (inserted into the rectum of rodents). Collectively these studies suggest that IL-1β is a likely mediator of fever during a central infection.

Table 1.2: Summary of the studies that investigated the effect of intracerebroventricularly administered IL-1β on core body temperature in rodents.

Species	IL-1β dose and route	Reference
Rats	0.1 ng, ICV	Montkowski *et al*., 1997
Rats	1 ng, ICV	Anforth *et al*.,1998
Rats	1 ng, ICV	Harden *et al*., 2008
Rats	5 ng, ICV	Anforth *et al*.,1998
Rats	5 ng, ICV	Busbridge *et al*., 1989
Rats	5 ng, ICV	Kent *et al*., 1992
Rats	10 ng, ICV	Anforth *et al*.,1998
Rats	25 ng, ICV	Cao *et al*., 2001
Rats	100 ng, ICV	Anforth *et al*.,1998
Rats	100 ng, ICV	Harden *et al*., 2008
Mice	100 ng, ICV	Li *et al*., 2001
Rats	100 ng, ICV	Montkowski *et al*., 1997
Rats	100 ng, ICV	Oitzl *et al*., 1993
Rats	20 µg, ICV	McCarthy *et al*., 1986

ICV – Intracerebroventricular

All studies reported an increase in body temperature following the intracerebroventricular administration of IL-1β

1.3. Anorexia

Central infections in both humans and animals are frequently accompanied by anorexia (Hart, 1988, Plata-Salamán, 1996). Anorexia manifests as a decrease in food intake as well as an associated decrease in body mass (Sonti *et al.*, 1996). The implementation of anorexia during illness is assumed to have both beneficial and deleterious consequences, dependent on its onset, duration and magnitude (Plata-Salamán, 1996). The potential for anorexia to have beneficial consequences seems paradoxical because implementation of the acute phase response constitutes a high energy demand. However, short-term anorexia is hypothesized to decrease the availability of macro and micro-nutrients to invading pathogens, which causes a decrease in proliferation of invading bacteria or viruses (Hart, 1988; Plata-Salamán, 1996). Long-term anorexia is associated with deleterious consequences. As energy expenditure exceeds energy input it can result in a net loss of body mass (Plata-Salamán, 1996). Long-term loss of body mass can result in cachexia which is characterized by muscle and adipose tissue wasting, malnutrition, organism devitalisation and ultimately may lead to death (Plata-Salamán, 1996).

The hypothalamus is hypothesized to be the primary site for regulation of feeding (Oomura, 1988, Plata-Salamán, 1996, Plata-Salamán *et al.*, 1988). However, the mechanisms of anorexia are multi-factorial; they involve hypothalamic modulation of feeding sites, modulation of endocrine release (insulin and glucagon) as well as gastro-intestinal modulation (gastric motility, emptying, acid secretion and intestinal motility) (Oomura, 1988, Plata-Salamán, 1996, Plata-Salamán *et al.*, 1988).

The hypothalamus has a dual center for the regulation of food intake (Plata-Salamán, 1998). These centers contain glucose-sensitive (GS) neurons and glucose-receptor (GR) neurons, which are present in the lateral hypothalamus and ventromedial hypothalamus respectively (Kuriyama *et al.*, 1990,

Oomura, 1988, Plata-Salamán *et al.*, 1988). The GS and GR neurons are reciprocally linked and alter their activity in response to glucose and other metabolites (Kuriyama *et al.*, 1990, Oomura, 1988, Plata-Salamán *et al.*, 1988). In the presence of glucose, GS neurons decrease their activity and GR neurons increase their activity. The change in neuronal activity in response to the presence of glucose integrates chemical signals from other brain areas involved with satiation and causes a decrease in gut motility, an increase in peripheral secretion of insulin and glucagon and ultimately results in appetite suppression (Kuriyama *et al.*, 1990, Oomura, 1988, Plata-Salamán, 1998, Plata-Salamán *et al.*, 1988). The inverse is true when glucose levels are low (Kuriyama *et al.*, 1990, Oomura, 1988, Plata-Salamán, 1998, Plata-Salamán *et al.*, 1988).

1.3.1. Anorexia in response to the central administration of LPS

A wide variety of pathogens have been shown to induce anorexia, but experimentally, LPS is one of the most commonly used pathogenic mimics used to study anorexia in the context of central bacterial infections. Intracerebroventricular administration of LPS, over a range of doses (3 ng – 2.5 µg), has consistently been shown to reduce both food intake and body mass (summarized in Table 1.3) (Bluthe *et al.*, 1999, Bluthé *et al.*, 2000, Burgess *et al.*, 1998, Castanon *et al.*, 2001, Faggioni *et al.*, 1995), which confirms the anorexigenic ability of LPS. However, LPS does not act directly on the hypothalamus to induce anorexia, rather upon entry into the brain LPS induces the release of pro-inflammatory cytokines which modulate anorexia (Plata-Salamán, 1996, Plata-Salamán, 1998).

Table 1.3: Summary of the studies that investigated the effect of LPS on food intake and/or body mass of rodents.

	Species	LPS dose and route	Effect on food intake/ body mass	Reference
Lipopolysaccharide				
Central administration:				
LPS	Mice	3 ng, ICV	Decrease	Bluthé *et al*., 2000
LPS	Rats	60 ng, ICV	Decrease	Bluthé *et al*., 1999
LPS	Mice	100 ng , ICV	Decrease	Burgess *et al*., 1998
LPS	Rats	100 ng, ICV	Decrease	Castanon *et al*., 2001
LPS	Mice	2.5 µg, ICV	Decrease	Faggioni *et al*., 1995
Lipopolysaccharide and blocking IL-1 action				
LPS	IL-1 type 1 knockout mice	3 ng, ICV	Attenuation	Bluthé *et al*., 2000
LPS	IL-1 converting enzyme deficient mice	100 ng, ICV	Ablation	Burgess *et al*., 1998

IL-1 – Interleukin-1, LPS – Lipopolysaccharide, IL-1ra – Interleukin -1 receptor antagonist, ICV – Intracerebroventricular

Attenuation suggests that food intake and/or body mass were significantly increased in knockout mice compared to wild type mice that received LPS

Ablation suggests that mice that were deficient in IL-1 converting enzyme were resistant to LPS and did not develop anorexia.

Pro-inflammatory cytokines may modulate anorexia either by direct (via neuronal mechanisms) or indirect (via modulation of brain chemistry) action on the hypothalamus (Plata-Salamán, 1996, Plata-Salamán, 1998). Pro-inflammatory cytokines that act directly on the hypothalamus increase the firing rate of GS neurons and decrease the firing rate of GR neurons, which will result in appetite suppression (Kuriyama *et al*., 1990; Oomura, 1988; Plata-Salamán *et al*., 1988). The hypothalamus also contains both neuropeptide and neurotransmitter systems which are related to feeding and appetite suppression. There is substantial data which shows that pro-inflammatory cytokines modulate hypothalamic chemistry by stimulating the synthesis and release of PGE_2 and by interacting with neuropeptides and neurotransmitters within the brain. Research has demonstrated that PGE_2 modulates anorexia by suppressing appetite (Dinarello, 2000, Levine and Morley, 1981). Pro-inflammatory cytokines have also been shown to up regulate the expression of corticotrophin releasing factor (CRF), which is an inhibitory neuropeptide that causes appetite suppression (Langhans and Hrupka, 1999, Plata-Salaman, 1998), and downregulate the expression of neuropeptide-Y (NP-Y), which is a potent promoter of feeding (Langhans and Hrupka, 1999, Plata-Salaman, 1998). Moreover, research has also demonstrated that the central administration of pro-inflammatory cytokines upregulates the secretion of catecholamines, serotonin and histamine, all of which contribute to the suppression of appetite (Langhans and Hrupka, 1999, Plata-Salaman, 1998). The collective modulation of brain chemistry via PGE_2, CRF, NP-Y, catecholamines, serotonin and histamine causes appetite suppression and a resultant decrease in food intake.

The pro-inflammatory cytokines that have been implicated in LPS-induced anorexia are IL-1β, IL-6 and TNF-α (Plata-Salamán, 1996; Sonti *et al.*, 1996*;* Harden *et al.*, 2008). Studies in which researchers centrally administered individual or multiple combinations of IL-1β, IL-6 and TNF-α have determined that these pro-inflammatory cytokines can either act individually or synergistically in order to induce a

decrease in food intake which results in anorexia (Sonti *et al.,* 1996; Harden *et al.,* 2008). The synergistic action of pro-inflammatory cytokines is additive and induces anorexia of greater magnitude than the individual action of each of the cytokines, which supports the hypothesis that LPS-induced anorexia is mediated by a multi-cytokine interaction. Table 1.3 details the role of IL-1, specifically in mediating anorexia when induced by central LPS administration, which was investigated using techniques which allowed for selective inhibition or blockade of IL-1. Studies in which IL-1β was completely neutralised during central infections (summarized in Table 1.3) indicate that IL-1β can act within the brain to mediate anorexia because they resulted in the attenuation or complete ablation of LPS-induced anorexia (Bluthé *et al.,* 2000, Burgess *et al.,* 1998). Anorexia was determined by measuring change in food intake and change in body mass.

Furthermore, studies in which IL-1β has been adminstered directly into the brain are summarized in Table 1.4 and have demonstrated that IL-1β is anorexigenic and consistently induces a decrease in both food intake and body mass over a range of doses (0.1-100 ng) (Bluthé *et al.,* 1995, Bluthé *et al.,* 2000, Harden *et al.,* 2008, Kent *et al.,* 1992, Kent *et al.,* 1994, Montkowski *et al.,* 1997, Sonti *et al.,* 1996). In these studies anorexia in rats and mice was assessed by measuring food consumed, meal size, meal latency, food motivated behaviour and loss of body mass. Collectively these studies suggest that IL-1β is a likely mediator of anorexia during a central infection.

Table 1 4: Summary of the studies that investigated the effect of intracerebroventricularily administered IL-1β on food intake and/or body mass of rodents.

Species	IL-1β dose and route	Reference
Rats	0.1 ng, ICV	Montkowski *et al*., 1997
Rats	0.25 ng, ICV	Sonti *et al*., 1996
Rats	0.5 ng, ICV	Plata-Salamán, 1994
Rats	1 ng, ICV	Plata-Salamán, 1994
Rats	1 ng, ICV	Sonti *et al*., 1996
Mice	2 ng, ICV	Bluthé *et al*., 2000
Rats	2 ng, ICV	Plata-Salamán, 1994
Rats	4 ng, ICV	Plata-Salamán, 1994
Rats	5 ng, ICV	Kent *et al*., 1994
Rats	30 ng, ICV	Bluthé *et al*., 1995
Rats	30 ng, ICV	Kent *et al*., 1994
Rats	40 ng, ICV	Kent *et al*., 1992
Rats	90 ng, ICV	Castanon *et al*., 2001
Rats	100 ng, ICV	Harden *et al*., 2008
Rats	100 ng, ICV	Montkowski *et al*., 1997

ICV – Intracerebroventricular

All studies reported a decrease in body mass and/or food intake following the intracerebroventricular administration of IL-1β

1.4. Lethargy

Lethargy has been characterized as a change in the motivational state of an organism whereby it decreases its voluntary physical activity (Hart, 1988). Lethargy is a normal accompaniment of the acute phase response in both humans and animals and often accompanies fever (Harrington, 2012, Hart, 1988). It is hypothesized that lethargy plays a facilitative role in thermogenesis and a suppressive role in heat loss and energy expenditure (Hart, 1988). Lethargy results in a decrease in muscular activity accompanied by a decrease in energy demand which enables energy conservation for thermogenesis (Hart, 1988). Behavioural changes that accompany lethargy such as huddling and decreasing surface area also dramatically decrease heat loss (Hart, 1988). These changes in motility and position often negate the need for shivering and thus enable energy conservation (Hart, 1988).

It has recently been established that lethargy comprises a small component of a much larger disorder known as fatigue (Harrington, 2012). Fatigue in rodents comprises of several symptoms namely a decrease in spontaneous, voluntary activity, altered sleep patterns and social interactions, increased anxiety like behaviour as well as impaired cognition, memory and attention (Harrington, 2012).

The mechanisms of lethargy are largely unknown, however there are multiple neural networks that have been implicated in decreasing activity during infection or inflammation (Harrington, 2012). Associations have been made with the ventral tegmental area, the nucleus accumbens, the dorsal striatum and areas of the hippocampus (Harrington, 2012). Another school of thought has implicated arousal and motivational circuits in decreasing motivation for voluntary activity during pathological conditions by inhibiting input to the thalamus and cortex (Cheney, 1985, Harrington, 2012).

1.4.1. Lethargy in response to the central administration of LPS

Lethargy has been demonstrated after the administration of several experimental substances, including LPS, in several laboratories using rodent models of infection (Castanon *et al.*, 2001, Harden *et al.*, 2011, Kozak *et al.*, 1997, Leon *et al.*, 1996, Skinner *et al.*, 2009). The effects of peripherally administered LPS on lethargy have been well described; however very little research has explored the effects of centrally administered LPS on lethargy. Nevertheless, similarly to peripherally administered LPS (Harden *et al.*, 2011, Skinner *et al.*, 2009) intracerebroventricularly administered LPS (100 ng) has been demonstrated to induce a decrease in observed motility in rats (Castanon *et al.*, 2001) (see Table 1.5). Although the aforementioned studies show that LPS may induce lethargy, it is likely that LPS-induced pro-inflammatory cytokines are the proximate mediators of lethargy.

Table 1.5 summarizes the research findings discussed in the section above that describe the effect of intracerebroventricularly administered LPS on cage activity, wheel running or observed mobility, all of which are measures of lethargy. The table also details the role of IL-1 in mediating lethargy induced by systemic LPS administration, which was investigated using techniques which allowed for selective inhibition or blockade of IL-1. There are no studies which have administered LPS centrally in conjunction with an IL-1 blocking agent, therefore I included studies which administered LPS peripherally/systemically in conjunction with an IL-1 blocking agent. Studies which block brain IL-1 and show attenuation of a peripheral LPS-induced decrease in voluntary activity (Harden *et al.*, 2011, Kozak *et al.*, 1995, Leon *et al.*, 1996) give an indication that IL-1 can act within the brain to mediate lethargy. Although the exact role of IL-1β in mediating lethargy induced by central infections is unclear, studies in which IL-1β was antagonised or completely

neutralised during simulated peripheral infections using LPS (summarized in Table 1.5), show that IL-1β may or may not play a role in mediating lethargy because they caused either attenuation or had no effect on LPS-induced lethargy (Harden *et al.*, 2011, Kozak *et al.*, 1995, Leon *et al.*, 1996). Therefore lethargy appears to be mediated by a multi-cytokine interaction. Lethargy was quantitatively measured as a decrease in activity which is characteristic of a state of lethargy in rats and mice. Changes in voluntary activity were either monitored via video camera or measured using radio telemetry. Voluntary wheel running was measured using running wheels.

Table 1.5: Summary of the studies that investigated the effect of LPS on voluntary activity in rodents

	Species	LPS dose and route	Cage activity, observed mobility or wheel running	Effect on activity	Reference
Lipopolysaccharide					
Central administration:					
LPS	Rats	100 ng, ICV	Observed mobility	Decrease	Castanon *et al*., 2001
Lipopolysaccharide and blocking IL-1 action:					
Peripheral administration of:					
LPS	IL-1 type1 knockout mice	50 μg/kg, IP	Cage activity	-	Leon *et al*., 1996
LPS	IL-1β knockout mice	2500 μg/kg, IP	Cage activity	-	Kozak *et al*., 1995
LPS	IL-1 type 1 knockout mice	2500 μg/kg, IP	Cage activity	-	Leon *et al*., 1996
Peripheral LPS and central administration of:					
Caspase-1 inhibitor	Rats	250 μg/kg, SC	Wheel running	Attenuation	Harden *et al*., 2011

IL-1β – Interleukin-1β, LPS – Lipopolysaccharide, ICV – Intracerebroventricular, IP- Intraperitoneal, SC- Subcutaneous , - indicates no effect

Attenuation suggests that voluntary activity was significantly increased compared to rats that received LPS

As confirmation of the role that IL-1β plays as a mediator of lethargy during a central infection, studies in which IL-1β has been adminstered directly into the brain are summarized in Table 1.6 and have demonstrated that intracerebroventricularly adminstered IL-1β consistently induces a decrease in cage activity, wheel running and observed mobility in rats and mice, at a range of doses 0.1 ng – 10 ng (Bluthé *et al.*, 2000, Harden *et al.*, 2008, Montkowski *et al.*, 1997, Plata-Salamán, 1994). Voluntary activity was measured using running wheels, radiotelemetry or via subjective monitoring of observed activity. In addition a study in which IL-1β or IL-6 or a combination of the two pro-inflammatory cytokines were administered intracerebroventricularly to rats showed that IL-1β and IL-6 can either act individually or synergistically to induce lethargy (Harden *et al.*, 2008). However, the combined effect of IL-1β and IL-6 on lethargy was additive and resulted in lethargy of a greater magnitude than when compared to the individual effects of IL-1β and IL-6 (Harden *et al.*, 2008), which lends credence to the hypothesis that lethargy is mediated by a multi-cytokine interaction.

Collectively the data suggests that rodents in which IL-1β production is intact may rely partly on the expression of IL-1β for the development of lethargy. However, mice which are deficient in IL-1β or IL-1r1 may rely on different cytokine interactions in order to develop lethargy after the administration of LPS.

Table 1.6: Summary of the studies that investigated the effect of intracerebroventricularly IL-1β on voluntary activity in rodents

Species	IL-1β dose and route	Cage activity, observed mobility or wheel running	Reference
Rats	0.1 ng, ICV	Cage activity	Montkowski *et al*., 1997
Rats	0.1 ng, ICV	Wheel running	Harden *et al*., 2008
Rats	0.25 ng, ICV	Cage activity	Plata-Salamán, 1994
Rats	0.5 ng, ICV	Cage activity	Plata-Salamán, 1994
Rats	1 ng, ICV	Wheel running	Harden *et al*., 2008
Mice	2 ng, ICV	Observed mobility	Bluthé *et al*., 2000
Rats	2 ng, ICV	Cage activity	Plata-Salamán, 1994
Rats	4 ng, ICV	Cage activity	Plata-Salamán, 1994
Rats	90 ng, ICV	Observed mobility	Castanon *et al*., 2001
Rats	100 ng, ICV	Wheel running	Harden *et al*., 2008
Rats	100 ng, ICV	Cage activity	Montkowski *et al*., 1997

ICV – Intracerebroventricular

All studies reported a decrease in voluntary activity following the intracerebroventricular administration of IL-1β

1.5. Memory impairment

In clinical cases of central bacterial infections (e.g. meningitis and brain abscesses), in addition to developing fever, lethargy and anorexia, one third of patients also present with cognitive deficits (Nau and Bruck, 2002). The cognitive deficits in humans following central bacterial infections may include cognitive slowness, impairment of psychomotor and visuoconstructive performance and learning and memory deficits (Nau and Bruck, 2002, Nau *et al.*, 1999, van de Beek *et al.*, 2006, van de Beek *et al.*, 2002). Learning and memory deficits are not only seen in clinical cases of central bacterial infections; experimental models have shown that rodents may also develop learning and memory impairment as a consequence of central bacterial infections (Barichello *et al.*, 2014a, Barichello *et al.*, 2013, Barichello *et al.*, 2010, Barichello *et al.*, 2014b).

Memory does not exist as a single entity; there are several types of memory (see Figure 1.3) including short-term and long-term memory (Morris, 2001). Long-term memory is further sub-divided into declarative and non-declarative memory (Phelps, 2004). Declarative memory refers to memories that are factual in nature and can be recalled (Phelps, 2004). There are several structures within the brain that are responsible for declarative memory namely the hippocampal formation, the basal ganglia and the prefrontal cortex; as such declarative memory is often referred to as hippocampal-dependent memory (Phelps, 2004). Non-declarative memory is described as implicit in nature and refers to memories that are emotional and procedural (Phelps, 2004). The brain structure predominantly involved in non-declarative memory is the amygdala, hence non-declarative memory is often referred to as amygdala-dependent memory (Phelps, 2004). For the purposes of my study I will be focusing on hippocampal-dependent and amygdala-dependent memory, with specific focus on contextual and auditory-cued memory, for the remainder of my dissertation.

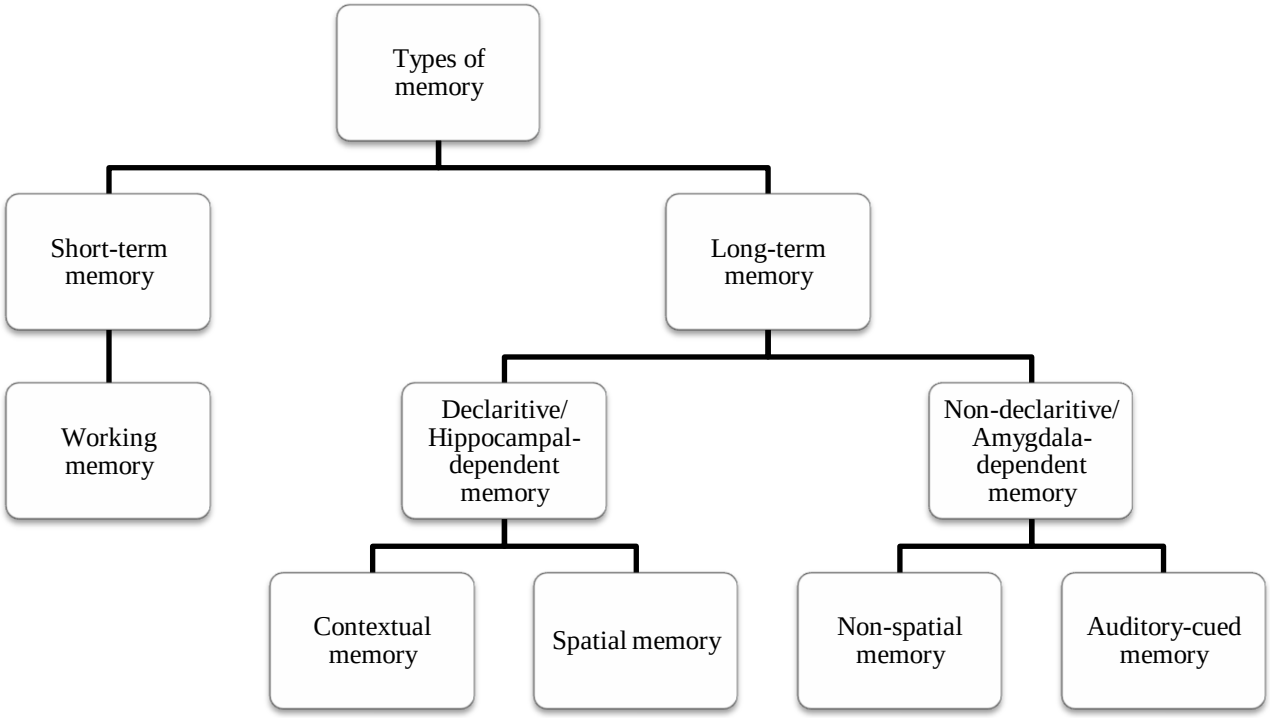


Figure 1.2: The types of memory adapted from (Morris, 2001).

Learning, and ultimately the formation of a stable long-term memory involve the culmination and integration of a cascade of processes (see Figure 1.3). Memory acquisition is the initial process involved in the formation of a memory, it is the process of learning or acquiring new information (Abel and Lattal, 2001). After acquisition the early memory is in a labile state (Abel and Lattal, 2001) it then undergoes a period of consolidation which can take place anywhere between a few minutes to 24 hours (Abel and Lattal, 2001, Barrientos *et al.*, 2002, Rudy *et al.*, 2004). Consolidation allows the memory to be cemented into a fixed state and it is during this stage that

memories may either be interrupted or facilitated, thereafter the memory will either be stored as a complete or incomplete memory (Abel and Lattal, 2001, Barrientos *et al.*, 2002). Memory retrieval is the last process in the cascade of memory processes and it is the point at which memories are recalled and the strength of their associations is tested (Abel and Lattal, 2001).

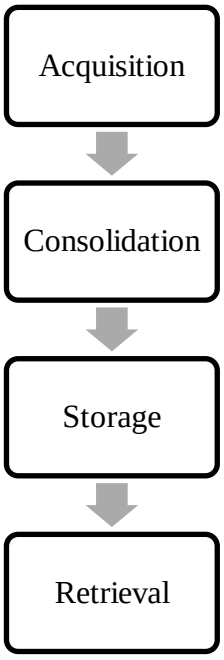


Figure 1.3: Flow diagram showing the basic processes involved in memory formation.

Memory impairment refers to an interruption in one or more of the processes involved in the formation of a memory. Multiple studies have explored the early processes involved in memory formation (i.e. acquisition and consolidation) in order to determine the stage at which it is most likely to be disrupted during infection or inflammation (Abel and Lattal, 2001, Thomson and Sutherland, 2006). The timing of pathogen administration relative to learning a task may indicate which memory process could be affected. Studies in which a pathogen is administered *before* learning a task will cause the animal being studied to be under the influence of the pathogen during and after learning and so acquisition or consolidation may be impaired (Cunningham and

Sanderson, 2008, Thomson and Sutherland, 2006). Whereas studies in which a pathogen is administered *after* learning a task will result in the animal being under the influence of the substance during memory consolidation and therefore memory consolidation may be impaired (Thomson and Sutherland, 2006).

There are many experimental paradigms employed to specifically test long-term memory, one of the most well established and widely used in rodents is the contextual and auditory fear conditioning paradigm (Rudy *et al.*, 2004), which measures both contextual and auditory-cued fear (Rudy *et al.*, 2004). During fear conditioning, immediately once a rat is placed into a novel environment, the rat will sample all the individual features of the environment and bind them together to form a contextual representation (Rudy *et al.*, 2004). In the case of the fear conditioning task rats also experience a shock whilst they are in the novel environment and so the contextual representation for that environment will have a negative association (Rudy *et al.*, 2004). Fear conditioning is theoretically based on Pavlovian (classical) conditioning (Maren, 2001) which takes into account an rodents instinctive survival behaviour to form environmental associations with aversive stimuli (Bouton and Moody, 2004, Phillips and LeDoux, 1992, Sanders *et al.*, 2003). In the face of an aversive stimulus a rodent will exhibit its dominant fear response, namely freezing. Freezing is a common measure of conditioned fear in a rodent and is defined by the absence of all visible movement, excluding regular respiration (Barrientos *et al.*, 2002). Freezing often appears in conjunction with increased heart-rate, piloerection and defecation (Phillips and LeDoux, 1992, Rudy *et al.*, 2004).

1.5.1. Memory impairment in response to the peripheral administration of LPS

Using several different memory paradigms and routes of administration, several laboratories have

examined the effects of LPS on the formation of memory in order to examine its effect on either memory acquisition or memory consolidation. To my knowledge no animal studies have examined the effect of centrally administered LPS on memory acquisition or memory consolidation using fear conditioning therefore I will discuss studies which examined the effects of peripherally administered LPS on hippocampal-dependent memory in rodents. Interestingly, to my knowledge no studies have examined the effect of peripherally administered LPS specifically on memory acquisition using fear conditioning. However, to date three studies measured hippocampal-dependent memory acquisition following the peripheral administration of LPS using other paradigms (e.g. the cued lever-press task (Aubert *et al.*, 1995), the passive avoidance paradigm (Sparkman *et al.*, 2005) and the Morris water maze (Sparkman *et al.*, 2006)). Although in all studies LPS was administered peripherally and immediately before acquisition the effect of on memory acquisition was different; in two of the studies LPS caused inhibition of memory acquisition (Aubert *et al.*, 1995, Sparkman *et al.*, 2005), whereas another study reported that LPS had no impact on memory acquisition (Sparkman *et al.*, 2006). Together these studies conclude that the relationship between peripherally administered LPS and memory acquisition is complex. Unlike the relationship between centrally administered LPS and other sickness responses, LPS does not seem to consistently induce hippocampal-dependent memory impairment.

The complex relationship between LPS administration and memory impairment was echoed in studies that examined the effect of peripherally administered LPS specifically on memory consolidation: in these studies LPS was administered immediately or 30 min after fear conditioning (Pugh *et al.*, 1998, Terrando *et al.*, 2010) (see Table 1.7). As seen in Table 1.7, the peripheral administration of LPS to rats selectively induced hippocampal-dependent memory impairment (Pugh *et al.*, 1998, Terrando *et al.*, 2010). Interestingly at the low dose of 0.5mg/kg and the high

dose of 2 mg/kg, peripherally administered LPS had no effect on memory, whereas the midrange dose of 1 mg/kg caused hippocampal-dependent memory impairment (Pugh *et al.*, 1998, Terrando *et al.*, 2010). Collectively these studies suggest that LPS may act on hippocampal-dependent memory in a U-shaped dose response. One possible explanation for this phenomenon was suggested by Pugh *et al.* (1998); high doses of LPS may cause the prolonged release of corticosterone, which has previously been demonstrated to enhance performance in the fear conditioning paradigm (Pugh *et al.*, 1997). Furthermore, LPS is known to induce the expression of pro-inflammatory cytokines, including IL-1β (Conti *et al.*, 2004), a high dose of LPS may have induced a significant overexpression of IL-1β, which has in some instances been shown to enhance hippocampal-dependent memory performance (Goshen *et al.*, 2007).

Table 1.7: Summary of the studies that investigated the effect of peripherally administered LPS on learning and memory in rodents

	Species	LPS dose and route	Time of injection	Memory paradigm	Effect on memory	Reference
Lipopolysaccharide						
Peripheral administration of:						
LPS	Rats	0.5 mg/kg, IP	Immediately after conditioning	Fear conditioning	No effect	Pugh *et al*., 1998
LPS	Rats	1 mg/kg, IP	Immediately after conditioning	Fear conditioning	HD memory impairment	Pugh *et al*., 1998
LPS	Mice	1 mg/kg, IP	30 min after conditioning	Fear conditioning	HD memory impairment	Terrando *et al*,. 2010
LPS	Rats	2 mg/kg, IP	Immediately after conditioning	Fear conditioning	No effect	Pugh *et al*., 1998
Peripherally administered Lipopolysaccharide and blocking IL-1 action						
Peripheral administration of:						
IL-1ra	Rats	0.5 mg/kg, IP	Immediately after conditioning	Fear conditioning	Attenuation - HD memory	Pugh *et al*., 1998
IL-1ra	Mice	1 mg/kg, IP	30 min after conditioning	Fear conditioning	Attenuation - HD memory	Terrando *et al*,. 2010

HD memory – Hippocampal-dependent memory, IL-1β – Interleukin-1β, LPS – Lipopolysaccharide, IL-1ra – Interleukin -1 receptor antagonist, IP- Intraperitoneal

The central action of several pro-inflammatory cytokines induced by LPS, specifically IL-1β, IL-6 and TNF-α, have been shown to play a neuromodulatory role in memory (Goshen and Yirmiya, 2006). Although no studies have administered an IL-1 blocking agent in conjunction with central LPS injection, Table 1.7 summarizes the effect on memory following peripherally administered LPS in conjunction with a peripherally administered selective IL-1 antagonist. These studies, which attempted to block IL-1, showed attenuation of peripheral LPS-induced memory impairment (see Table 1.7) and may indicate that IL-1 is a likely mediator of memory impairment during infection. The contextual and auditory fear conditioning paradigm was used in order to assess memory in these studies (Pugh *et al.*, 1998, Terrando *et al.*, 2010).

The role of IL-1β in mediating hippocampal-dependent memory in rodents has been extensively studied in many laboratories, using several different memory paradigms. There are no studies to my knowledge which have examined the effect of centrally administered IL-1β on memory acquisition using fear conditioning. However, several studies have examined the effect of centrally administered IL-1β on memory acquisition using other memory paradigms (e.g. the lever-press avoidance task (Brennan *et al.*, 2004), eye-blink response test (Servatius and Beck, 2003), the Morris water maze (Gibertini, 1998) and the inhibitory avoidance paradigm (Depino *et al.*, 2004)) which also test hippocampal-dependent memory. Although in all studies IL-1β was administered centrally, immediately before acquisition the effect of IL-1β on memory acquisition was different; in three of the studies IL-1β caused facilitation of acquisition (Brennan *et al.*, 2004, Gibertini, 1998, Servatius and Beck, 2003) however, one study showed that centrally administered IL-1β caused inhibition of memory acquisition (Depino *et al.*, 2004). Together these studies suggest that, similar to LPS, IL-1β also has a complex relationship with memory and does not consistently induce memory impairment.

The complex relationship between IL-1β and memory was also seen in studies that examined the effect of centrally administered IL-1β specifically on memory consolidation (see Table 1.8). In all these studies IL-1β was administered immediately after and up to 24 h after fear conditioning. The majority of studies listed in Table 1.8 have demonstrated that the acute intracerebroventricular or intrahippocampal administration of IL-1β over a range of doses (2 ng - 20 ng), caused hippocampal-dependent memory impairment (Barrientos *et al.*, 2002, Barrientos *et al.*, 2004, Goshen *et al.*, 2007, Hein *et al.*, 2007, Pugh *et al.*, 1999). However, despite the findings that elevated levels of IL-1β in the brain may be detrimental to memory consolidation, the intracerebroventricular administration of a low dose (1 ng) of IL-1β caused an improvement in hippocampal-dependent memory (Goshen *et al.*, 2007), suggesting IL-1β may also be beneficial to memory. Moreover, studies which blocked basal levels of IL-1β (see Table 1.8), using pharmacological and genetic means, caused hippocampal-dependent memory impairment (Avital *et al.*, 2003, Goshen *et al.*, 2007), suggesting that low and/or basal levels of IL-1β may be necessary for memory consolidation. Collectively, all the data discussed above suggests that IL-1β exerts its influence on memory consolidation in an inverted U-shaped pattern (Goshen and Yirmiya, 2006). The inverted U-shaped hypothesis suggests that low or basal levels of IL-1β in the brain are necessary for memory consolidation and may even play a beneficial role in improving memory (Goshen and Yirmiya, 2006). However, deviation from the basal level of IL-1β either by excessively elevated levels or by antagonizing IL-1β may have a detrimental effect on memory and subsequently cause memory impairment (Goshen and Yirmiya, 2006).

Table 1.8: Summary of the studies that investigated the effect of centrally administered IL-1β on learning and memory in rodents.

	Species	IL-1β dose and route	Time of injection	Memory paradigm	Effect on memory	Reference
Interleukin - 1β						
Central administration:						
	Mice	1 ng, ICV	Immediately after conditioning	Fear conditioning	HD memory improvement	Goshen *et al*., 2007
	Rats	2 ng, IH	Immediately, 3 h, and 24 h after conditioning	Fear conditioning	HD memory impairment	Barrientos *et al*., 2002
	Rats	10 ng, IH	Immediately after conditioning	Fear conditioning	HD memory impairment	Barrientos *et al*., 2004
	Mice	10 ng, ICV	Immediately after conditioning	Fear conditioning	HD memory impairment	Goshen *et al*., 2007
	Rats	10 ng, IH	Immediately after conditioning	Fear conditioning	HD memory impairment	Hein *et al*., 2007
	Rats	10 ng, ICV	Immediately after conditioning	Fear conditioning	HD memory impairment	Pugh *et al*., 1999
	Rats	20 ng, ICV	Immediately after conditioning	Fear conditioning	HD memory impairment	Pugh *et al*., 1999
Blocking basal levels of IL-1β						
IL-1ra	Mice	-	Immediately after conditioning	Fear conditioning	HD memory impairment	Goshen *et al*., 2007
IL-1 type 1 knockout mice	Mice	-	-	Fear conditioning	HD memory impairment	Avital *et al*., 2003
IL-1ra over-expression	Mice	-	-	Fear conditioning	HD memory impairment	Goshen *et al*., 2007

HD memory – Hippocampal-dependent memory, IL-1β – Interleukin-1β, IL-1ra – Interleukin -1 receptor antagonist, ICV – Intracerebroventricular, IH - Intrahippocampal

1.6. Dissertation hypotheses and aims

I hypothesize that immune activation in the brain will concurrently induce fever, anorexia, lethargy and memory impairment.

The findings presented in the sections above have highlighted that the acute, central administration of LPS seems to consistently induce fever, anorexia and lethargy in rodents. Although peripheral administration of LPS has been shown to induce memory impairment in rodents, no study to date has examined the effect of centrally administered LPS on memory in rodents. Additionally no study has examined the effects of central LPS-induced fever, lethargy and food intake, whilst concurrently examining memory.

Therefore I undertook an experiment which aimed at concurrently examining the dose-dependent effect of LPS administered via the cisterna-magna on body temperature, activity, food intake, body mass and memory of rats. Memory was assessed using the fear conditioning task, which measures both hippocampal-dependent and amygdala-dependent memory. Importantly, conditioning/learning took place immediately *before* rats received an intra-cisterna magna injection of either LPS or vehicle. Following the intra-cisterna magna injection of LPS rats became febrile, lethargic and anorexic however, surprisingly rats did not show memory impairment.

Having failed to reject the null hypothesis and identifying a dissociation between fever, lethargy, anorexia and memory processes when rats were conditioned *before* receiving LPS, I undertook a second experiment aimed at investigating the dynamics of this dissociation and developed a new hypothesis.

I hypothesize that immune activation in the brain will differentially affect abdominal temperature, voluntary activity, food intake and body mass as compared to cognitive function.

During the second experiment I injected rats centrally with IL-1β, as central IL-1β has been demonstrated to be a predominant mediator in the initiation of fever, anorexia, lethargy and also memory impairment. Body temperature, activity, food intake, body mass and memory were measured following a single ICM injection of IL-1β. Importantly, conditioning/learning took place either immediately *before* or 2 h *after* the rats received an intra-cisterna magna injection of IL-1β. The purpose of conditioning the rats at different time points, i.e. either before or after receiving IL-1β, was to examine the time course by which memory interference could occur, relative to increased IL-1β concentrations in the brain, in conjunction with the presentation of the other sickness responses. In addition, I also measured the time course of IL-1β synthesis in the hippocampus and hypothalamus after the ICM injection of IL-1β.

Chapter 2

Materials and Methods

2.1. Experimental animals and housing

Adolescent male Sprague-Dawley rats (initial body mass 80-100g) were obtained from the National Health Laboratory Service (NHLS, Johannesburg, South Africa) and housed individually in cages in the Central Animal Service at the University of the Witwatersrand. The rats were housed at an ambient temperature of 22 ± 1 °C on a 12:12 light-dark cycle. Rats were initially housed on a light-dark cycle from 07:00-19:00. However, for behavioural experiments the light-dark cycle was changed to 03:00-15:00 to accommodate injections occurring during working hours. For brain sample collection a different group of rats were housed on a light-dark cycle from 12:00-24:00, for ease of termination at the 8 h time point which took place at 17:00. The changes in light-dark cycles of the rats occurred 3 weeks before surgery (described below) in order for the rats to acclimatize to the new cycle. Food (pelleted rat chow, Epol, Johannesburg, South Africa) and water were provided to the rats *ad libitum*. All experimental procedures were performed in accordance with the regulations described in the University of the Witwatersrand guide for the care and use of laboratory animals and approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (AESC2012/08/04; AESC2012/38/04; AESC2013/28/04).

2.2. Surgery

All rats weighed approximately between 150-200g, and were considered adults at the time of surgery, for intraperitoneal implantation of precalibrated temperature and activity sensitive radiotransmitters (TA101A-F40, Data Sciences, St. Paul, MN, USA). Rats were anaesthetized with an intramuscular injection of a combination of ketamine hydrochloride (100 mg/kg) (Anaket-V, Bayer, SA) and xylazine (5mg/kg) (Chanazine, Bayer, SA). Once anaesthetized, the surgical area was shaved and cleaned with veterinary disinfectant (Health and Hygiene, Sunninghill, South Africa). A 50 mm incision was made through the skin and muscle, into the peritoneal cavity. The

radiotransmitter (30x15x10 mm; ~8g) was inserted into the abdomen and left as "free floating" in the peritoneal cavity, thereafter the wound was sutured. After surgery the animals received a single injection of atipamezole hydrochloride (100 mg/kg) (Pfizer, Sandton, South Africa) to promote recovery from anaesthesia. After surgery the rats were housed in a warm recovery room (~27°C) for 5 hours and then they were returned to their cages for an additional seven day period of recovery.

2.3. Body temperature and activity

Abdominal temperature and cage activity were measured continuously using implanted radiotransmitters (TA101A-F40, Data Sciences, St. Paul, MN, USA). The output from the implanted radiotransmitters (frequency in Hz) was monitored using a receiver plate (33x23x3 cm) (RPC-1, Data Sciences, St. Paul, MN, USA), placed under each rat's cage. The data from the radiotransmitters were logged every 5 min into a processor (DP-24, Data Port, Vital View, MiniMitter, Sunriver, OR, USA) and the signal relayed to a computer. The radiotransmitters were calibrated to an accuracy of 0.01 °C in a water bath at 35 °C and 39 °C by the manufacturer. The temperature was encoded in the radiotransmitter pulse repetition rate, which was recorded at each temperature using the 2-calibration-point method. The calibration values were calculated for each transmitter by the manufacturer.

2.4. Food intake and body mass

Food intake and body mass of rats were measured daily at the same time every day approximately 4 hours before the beginning of the dark cycle using an electronic scale (Avery weigh-tronix, UK) accurate to 1g. Rats were weighed and their food containers filled during their day time a few hours before the beginning of their dark cycle as they are largely inactive and do not consume much food

during this time. Food containers were filled daily with 200 g of standardized, pelleted rat chow and food intake of rats was determined by subtracting the food left in the food container and on the cage floor from the initial amount of food given (200 g) at the preceding time each day.

2.5. Memory

2.5.1. Contextual and auditory fear conditioning

Fear conditioning was used as an experimental tool for measuring memory in rats. The contextual and auditory fear conditioning paradigm is a well established model used to test for learning and memory and has been used in many laboratories (Barrientos *et al.*, 2002, Goshen *et al.*, 2007, Hein *et al.*, 2007, Pugh *et al.*, 1999).

2.5.1.1. Apparatus and behavioural procedure

The contextual and auditory fear conditioning apparatus used in my study consisted of two large, isolation chambers (106 x 76 x 80 cm), each housing another smaller chamber (30 x 25 x 30cm) (see Figure 2.1). One chamber (A) is referred to as the "context chamber" and was equipped with a stainless steel grid floor as well as a speaker, which was located at the back of the chamber. Another chamber, referred to as the "auditory chamber" (B), also was equipped with a speaker but differed significantly from the context chamber: it consisted of a smooth, perspex floor and a cage divider (5 cm in height) that divided the cage diagonally. The walls of the auditory chamber were covered by a zebra print pattern and the cage floor was lined with tissue paper. A red block and two egg cartons were placed onto the chamber floor. The purpose of having two different chambers was to test for the two different types of memory.

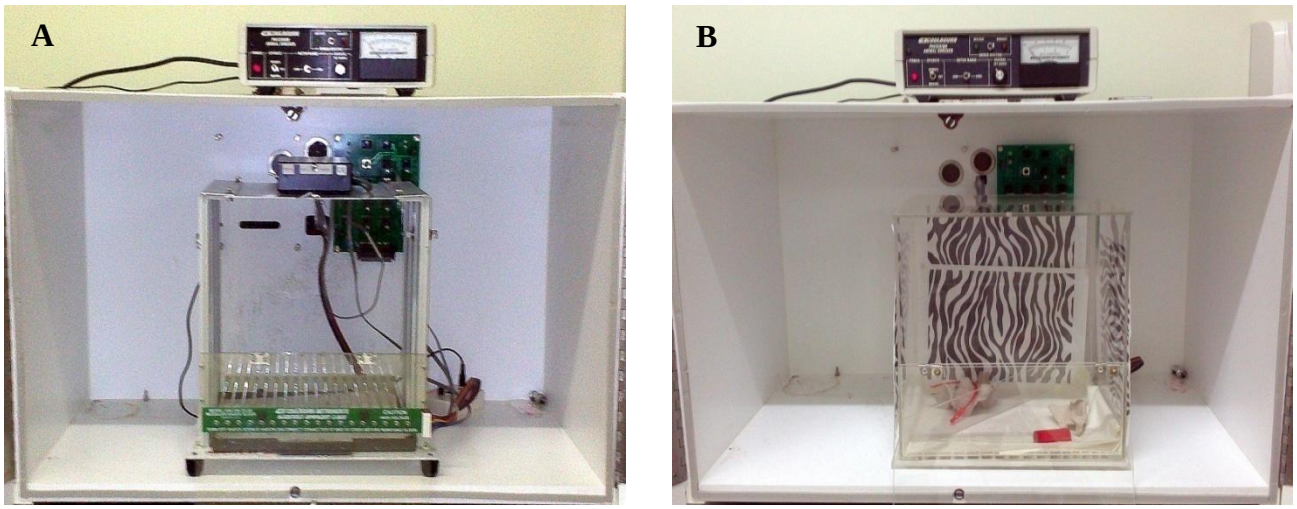


Figure 2.1: Illustration of the experimental setup for contextual and auditory fear conditioning and testing in our laboratory. The context chamber (A) is used for testing contextual fear conditioning, while the auditory chamber (B) is used to test for auditory fear conditioning.

I followed a typical fear conditioning protocol, which consisted of three phases: a conditioning phase and two testing phases. During conditioning a rat was placed into the context chamber (A) for 2 min after which a 15 s tone was presented over the speaker, followed by a single, 2 s foot shock. The tone was produced by a frequency generator set at 1 kHz and amplified to 70 db. The amplitude of the tone was measured at the entrance to the chamber using a sound level meter (TES-1357, TES Electrical Electronic corp, Tapei, Taiwan). The foot shock (1.5 mA) was produced by a current generator (Coulbourn Instruments, Allentown, PA) and delivered via the grid floor.

During the conditioning phase rats were individually transported in black buckets with lids into our fear conditioning laboratory and placed into the context chamber (A) where they were presented with a tone and received a foot shock. Rats were then immediately removed and transported back to their home cages (again in black buckets with lids). We used black buckets with lids to transport the rats to and from the fear conditioning laboratory to ensure that the rats could not see out of the

bucket; the bucket with the lid became a contextual cue which indicated to the rats that they were being transported to and from the context chamber. The context chamber (A) was cleaned with disinfectant (F10 Veterinary disinfectant, Health and Hygiene, Sunninghill, South Africa) before the next rat was conditioned.

Seven days after conditioning two memory tests were performed on each rat. The first test, namely the contextual fear test, assessed hippocampal-dependent memory of rats after their initial exposure to the context chamber (A). Similarly to the conditioning phase, the rats were individually transported (in black buckets with lids) to the fear conditioning laboratory and placed into the context chamber (A). Once rats were placed into the context chamber they were allowed to explore freely for 6 min, during the entire duration of this test rats did not experience either a tone or a shock. During the 6 min period rats were scored, every 10 s, as either active or freezing by two observers blind to the experimental intervention. After the 6 min testing-period rats were immediately removed from the context chamber and transported back to their home cages (again in black buckets with lids). After testing each rat, the context chamber was cleaned with disinfectant (F10 Veterinary disinfectant, Health and Hygiene, Sunninghill, South Africa).

The second test, namely the auditory fear test, assessed amygdala-dependent memory of rats and took place 3 hours after the contextual fear test. During this test rats were transported two at a time, using a white bucket with no lid, from their home cages to the fear conditioning laboratory and placed into one of two, separate auditory chambers (B). Once rats were placed into their respective auditory chambers they were allowed to explore freely for 3 min; this part of the test is referred to throughout my dissertation as the pre-tone period. Exploration was followed by presentation of a tone for 3 min; this part of the test is referred to throughout my dissertation as the post-tone period. For the entire 6 min period, rats were scored every 10 s for freezing behaviour. At cessation of the

test, rats were removed immediately and transported back to their home cages (two at a time in a white bucket with no lid). Rats were transported in twos in a white bucket with no lid in order to provide the rats with a different experience whilst being carried to the auditory chamber, this was important to ensure that the rats did not associate the new method of transportation with the negative experience of the foot shock they had in the context chamber. After testing each rat the auditory chamber was cleaned with water, as opposed to disinfectant, to ensure that the cage did not smell the same as the context chamber, in this case smell could also act as a reminder of the negative experiences in the context chamber.

I created the protocol for fear conditioning and testing using Graphic state Notation 4 software (Coulbourn Instruments, Allentown, PA). Presentation of both the tone and foot shock were controlled by a Habitest self-powered linc (Coulbourn Instruments, Allentown, PA).

Table 2.1: Summary of the procedures followed in each of the three phases of fear conditioning

	Conditioning	Contextual fear test	Auditory fear test
Transport of rats:	Individual Black bucket with lid	Individual Black bucket with lid	Two rats White bucket, no lid
Timing:	Exploration – 2 min Tone – 15 s Shock 2 s	Exploration - 6 min	Exploration (pre-tone) – 3 min Exploration with tone – 3 min
Scoring:	Every 10 s for 2 min	Every 10 s for 6 min	Every 10 s for 6 min
Cleaning:	Context chamber and bucket cleaned with disinfectant	Context chamber and bucket cleaned with disinfectant	Auditory chamber and bucket cleaned with water

2.5.1.2. Validation of fear conditioning

Fear conditioning is not a novel behavioural method for assessing memory experimentally, however it was novel in our hands. In order to validate fear conditioning and testing in our laboratory, I made use of a pharmacological challenge using scopolamine hydrobromide (Sigma-Aldrich, St Louis, MO, USA), a known amnesic drug. Scopolamine is an anti-cholinergic drug (Gravius *et al.*, 2006, Sanders *et al.*, 2003) that is frequently used in studies as a positive control, to induce short-term memory impairment in laboratory animals (Gravius *et al.*, 2006, Rudy, 1996, Sanders *et al.*, 2003). Both hippocampal-dependent and amygdala-dependent memory seem to be mediated by cholinergic input from the medial septal nucleus and nucleus basalis magnocellularis, respectively (Rudy, 1996). Scopolamine is thought to affect both of these central pathways by interrupting post trial processing that leads to memory formation (Rudy, 1996).

The dose 0.8 mg/kg of scopolamine hydrobromide (Sigma-Aldrich, St Louis, MO, USA) was chosen for my validation study based on a study whereby rats were injected peripherally with 0.8 mg/kg of scopolamine hydrobromide and showed significant memory impairment (Rudy, 1996). The timing of scopolamine administration (before or after conditioning) was also chosen based on a study wherein the researcher stated that peripherally administered scopolamine induced sufficient amnesia, whether it is injected (10 or 30 min) before or after conditioning (Rudy, 1996). I chose to inject scopolamine 30 min before conditioning as well as 30 min before the contextual and auditory fear tests based on a study which demonstrated that serum levels of peripherally administered scopolamine hydrobromide peak approximately 30 min after injection (Ebert *et al.*, 1998). Moreover, whether scopolamine is administered peripherally or centrally does not seem to influence its effect on memory (Wallenstein and Vago, 2001), therefore I chose to administer scopolamine peripherally.

Figure 2.2 shows that the peripheral administration of scopolamine hydrobromide induced significant memory impairment in rats. Figure 2.2 A shows that during the contextual fear test, rats which received scopolamine froze significantly less than rats that received saline ($t_{(11)}=12.18$, $P < 0.001$), which gives an indication that rats that received scopolamine had hippocampal-dependent memory impairment. Figure 2.2 B shows the freezing behaviour of rats during the auditory fear test. During the pre-tone period rats that received scopolamine and saline froze to a similar extent ($t_{(6)}=1.81$, $P > 0.05$). However, during the post-tone period rats that received scopolamine froze significantly less than rats that received saline ($t_{(7)}=12.31$, $P < 0.001$), which indicates that rats which received scopolamine had amygdala-dependent memory impairment.

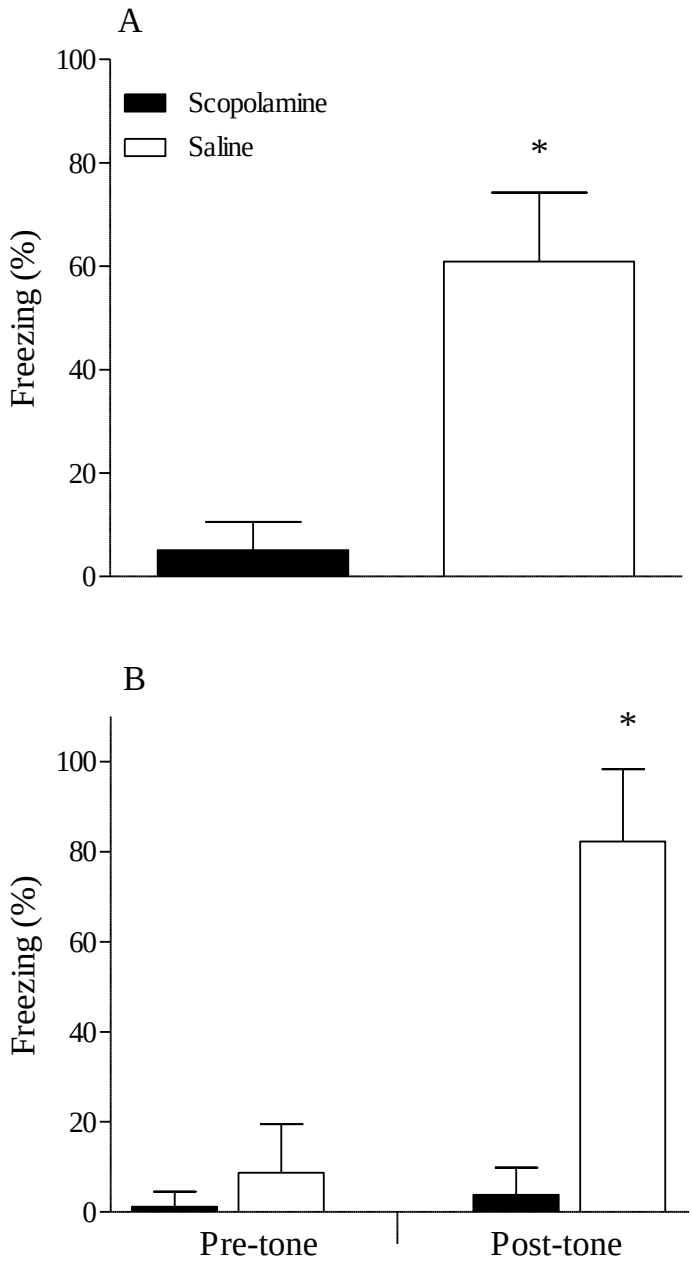


Figure 2.2: Freezing behaviour during the contextual fear test (A) and the auditory fear test (B) for rats that received a single IP injection of 0.8 mg/kg of scopolamine hydrobromide (n = 10) or 1 ml/kg saline (n = 10). During each test each rat was either judged as freezing or mobile every 10 s at the instant the measurement was taken. The number of positive freezing scores were summed for each observer and an average freezing score between the observers was obtained. The average freezing score was divided by the number of sampling bocks for each test and multiplied by 100 to express the freezing behaviour as a percentage. Significant differences: * Saline vs Scopolamine. The results are represented as means ± SD.

2.6. Intra-cisterna magna injections

Administration of substances to rodents via the cisterna magna have been commonly used in other laboratories (Barrientos *et al.*, 2012, Frank *et al.*, 2010, Proescholdt *et al.*, 2000). However, it was the first time that this procedure was setup and performed in our laboratory. For my study I injected lipopolysaccharide (LPS), interleukin - 1β (IL-1β) and their vehicles, phosphate buffered saline (PBS) and 0.1% bovine serum albumin (BSA) respectively, into the cisterna magna of rats.

Before all intra-cisterna magna (ICM) injections rats were placed into a gas chamber and anaesthetized using isoflurane, an inhalable anesthetic (see Figure 2.3A). Once anesthetized the dorsal aspect of the rats' skull was shaved and cleaned with 70 % ethanol (see Figure 2.3B). A 26-gauge needle (Kendon Medical Supplies, Johannesburg, South Africa) which was connected to a 50 µl Hamilton gastight microlitre syringe (Hamilton, Switzerland) by polyethylene tubing (0.38 mm inner diameter, 1.09 mm outer diameter) (see Figure 2.3C), was inserted into the cistern magna (see Figure 2.3 D and E). To confirm the correct positioning of the needle in the cistern magna, 3 µl of CSF was drawn up: if the CSF was clear of blood it verified the correct entry into the cistern magna. Rats that had blood in the CSF were excluded from all data analysis, as entry of the drug into the cistern magna could not be verified (n = 12). After verification of entry into the cistern magna 5 µl of the appropriate experimental substance (LPS or IL-1β) or vehicle (PBS or BSA) was administered and the needle was left in the cisterna magna, for 30 s, to ensure total dispersal of the substance. After recovery from anesthesia each rat was returned to its home cage.

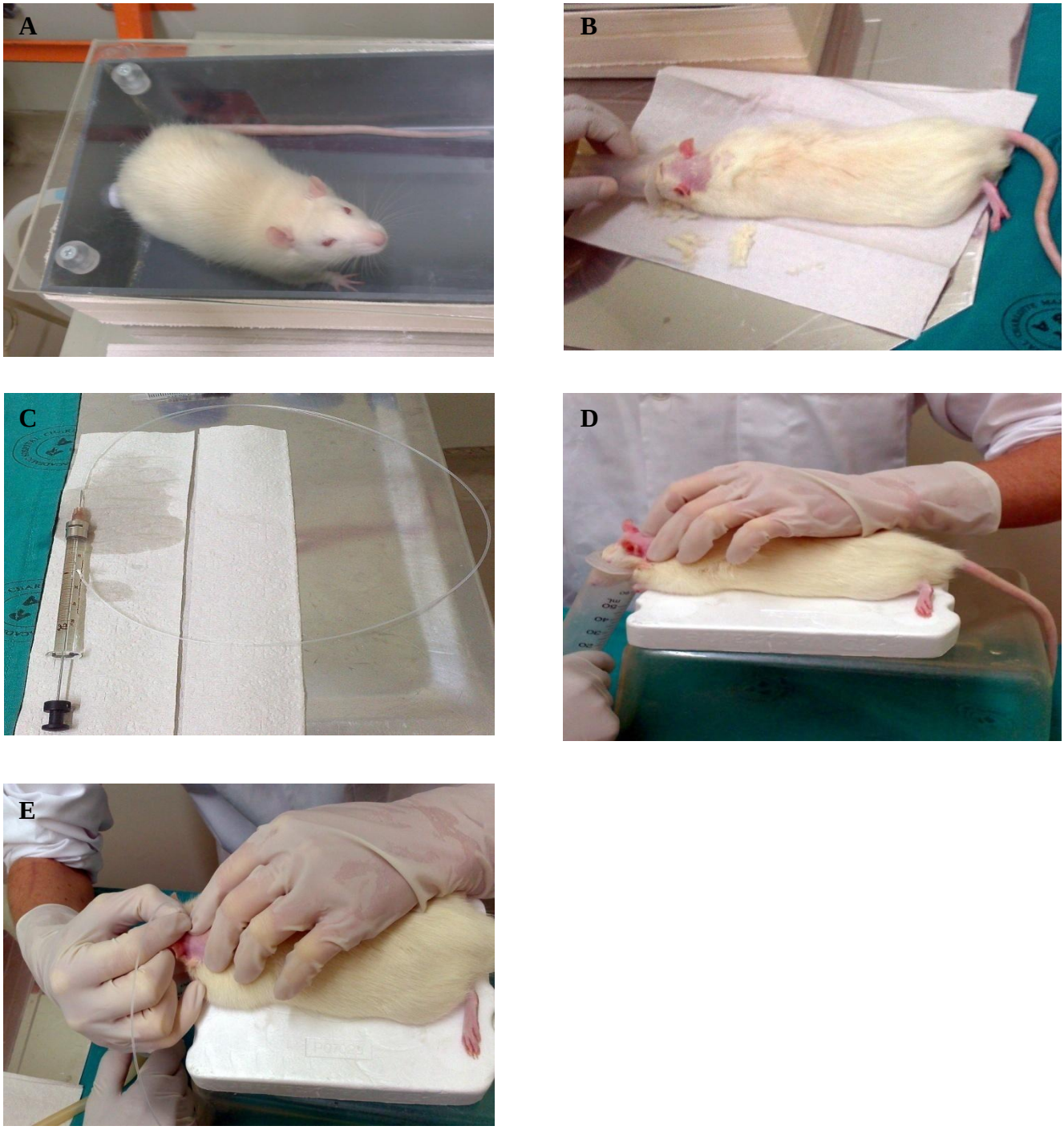


Figure 2.3: Preparation for intra-cisterna magna injections. (A) Anaesthesia given in a gas-chamber using inhalable isoflurane. (B) Preparation of the rat's skull for ICM injection. (C) Example of a 50 µl Hamilton syringe used during ICM injection. (D) Locating the cisterna magna at the posterior part of the skull at the midline of the ears. (E) Insertion of the needle into the cisterna magna at a 90°angle.

2.6.1 Pyrogens and control substances

LPS derived from *E. coli* endotoxin (serotype 011:B4, Sigma, St Louis, MO, US) was reconstituted in sterile PBS and injected via the cisterna magna at either 10 or 100 µg. The doses of LPS were chosen based on studies which peripherally administered LPS to adult rats at 10 and 100 µg (Cartmell *et al.*, 1999, Luheshi *et al.*, 1996) and in neonatal studies in which rat pups were injected intracerebroventricularly with 10 µg of LPS (Pang *et al.*, 2003). Both peripheral and intracerebroventricular injections of LPS have been shown to induce the synthesis and release of pro-inflammatory cytokines in the brain including IL-1β (Conti *et al.*, 2004, Pang *et al.*, 2003). Rat recombinant IL-1β (501-RL, R&D Systems, Minneapolis, MN, USA) was reconstituted in sterile 0.1% BSA and injected via the cisterna magna at a dose of 100 ng. The 100 ng dose of IL-1β was chosen based on previous studies in which IL-1β (100 ng) was administered intracerebroventricularly to rats and induced fever, lethargy and anorexia (Anforth *et al.*, 1998, Harden *et al.*, 2008).

2.7. Brain tissue collection

In order to measure IL-1β in the brains of rats injected via the cisterna magna with IL-1β, I perfused the rats and collected hypothalamus and hippocampus samples. Rats were perfused at 1 h, 2 h, 4 h, 8 h and 24 h after intra-cisterna magna injection. These time points were chosen based on temperature profiles obtained in my study following the intra-cisterna magna administration of IL-1β to rats. The basic fever profile following the intra-cisterna magna administration of IL-1β (100 ng) to rats included: the rising phase typically starting ~ 1 h after intra-cisterna magna injection, the plateau phase occurring between 4 - 6 h and defervescence at ~ 8 h.

For perfusions rats were anaesthetized with a single intaperitoneal injection of 0.8 ml sodium pentobarbital (Euthapent, 200 mg/ml; Kyron Laboratories (Pty) Ltd., South Africa). The chest cavity of the rat was exposed, a catheter was inserted through the left ventricle into the aorta and the rat was transcardially perfused using cold, sterile saline (0.9 %) for approximately 3 min. The rat was then decapitated and the brain was removed from the skull. The hypothalamus and both hippocampi were quickly dissected on an ice-cold plate and placed into individual, sterile, plastic tubes, which were snap frozen in liquid nitrogen. All tissue samples were stored at – 80 °C until used in the enzyme-linked immunosorbent assays (ELISA) described below.

2.8 Interleukin-1β analysis

Hippocampi and hypothalamus samples were removed from the – 80 °C freezer to thaw and kept on ice at approximately 4 °C. The use of the left or right hippocampus was randomized between rats for IL-1β analysis. Thereafter 300 µl of sonication buffer (50 mM Tris base, 100 mM Amino-n-caproic acid, 1 mM Ethylenediaminetetraacetic acid, 5 mM Benzamidine and 0.2 mM Phenylmethylsulfonylflouride) was pipetted into each tube containing the tissue. Each tube was placed individually into a beaker full of ice and the tissue was sonicated for approximately 10 s using an ultrasonic cell disruptor (Microson XL 2000, Newton, CT, USA) to dissociate the tissue. Sonicated tissue samples were then centrifuged at 4 °C at 14000 rpm for 10 min. After centrifugation approximately 250 µl of the supernatant was placed into a 1.5 ml sterile, plastic tube, which was kept on ice (~ 4 °C) until used in the ELISA. Bradford protein assays (Bradford, 1976) were performed to determine total protein concentrations in each sonicated sample. The Bradford standards were prepared using a serial dilution of 0.1% BSA and distilled water. Concentrations used were 1000 µg/ml, 800 µg/ml, 600 µg/ml, 400 µg/ml, 200 µg/ml, 100 µg/ml and 50 µg/ml.

Protein samples were prepared using a 1:20 dilution. Once the samples had been diluted 5 µl of each diluted sample was pipetted into individual wells on a 96 well-plate. Thereafter 250 µl of Bradford reagent (Sigma-Aldrich, Saint-Louis, Missouri, USA) was pipetted into each well and the plate was incubated for 15 min at room temperature. After incubation protein absorbance was measured at 595 nm.

Concentrations of IL-1β were determined using a commercially-available Quantikine ELISA kit (R&D Systems, Minneapolis, MN) for rat IL-1β. The microplate used in the ELISA was pre-coated with an affinity purified polyclonal antibody specific for IL-1β. Fifty microlitres of the provided assay diluent was added to each well followed by 50 µl of sample, standard or control (samples were tested in duplicate). The plate was then covered and incubated at room temperature for 2 hours. After washing away any unbound substances, rat conjugate IL-1β (100 µl) was added to each well and incubated at room temperature. After another wash to remove any unbound conjugate, substrate solution (100 µl) was added to each well and the plate was protected from light and incubated for 30 min. Thereafter a stop solution (100 µl) was added to each well in order to terminate the enzyme reaction. Sample optical density was determined using a microplate reader set at 450 nm and 540 nm and IL-1β concentrations were calculated by interpolation from a standard curve obtained from rat recombinant IL-1β (31.2-100 µg/ml). Data were corrected for protein concentration and the results expressed as pictograms of IL-1β per milligram of protein.

2.9 Experimental procedures

2.9.1 Experiment 1 – Lipopolysaccharide

All rats were implanted intraperitoneally with radiotransmitters and allowed to recover for 7 days (see Figure 2.4). Thereafter, rats were randomly assigned to either an experimental group which received LPS (10 µg (n = 10) or 100 µg (n = 3)) or a control group which received PBS (n = 9). Following recovery from surgery rats were conditioned in the fear conditioning task (~12:00) (as described in section 2.5). Immediately (~ 5 min) after conditioning, rats received a single intra-cisterna magna injection (5 µl) of either LPS (10 µg or 100 µg) or PBS. Abdominal temperature, activity, food intake and body mass were measured for 7 days after injection. By day 7 the sickness behaviours experienced by the rats that received LPS had resolved and then memory of rats was tested in both the contextual and auditory fear tests. Contextual fear of rats was tested at 7:00, 8 h before the onset of the daily dark phase (15:00). Auditory fear of rats was tested 3 h after testing for contextual fear. Rats were excluded from data analysis if blood was present in the CSF during intra-cisterna magna injection (n = 4) or if rats presented with a significant hypothermia below 32 °C (n = 2).

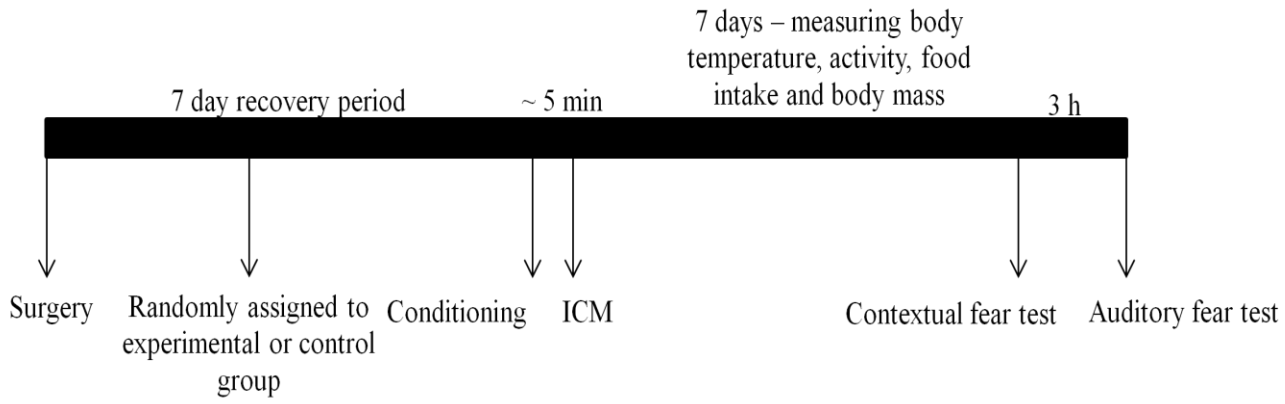


Figure 2.4: Basic flow diagram showing the order and timing of surgery and behavioural experiments for rats which were conditioned *before* receiving an ICM injection.

2.9.2. Experiment 2 – Interleukin-1β

2.9.2.1. Part A - Behavioural experiments

All rats were implanted intraperitoneally with radiotransmitters and allowed to recover for 7 days. Thereafter, rats were randomly assigned to either an experimental group in which they received IL-1β (100 ng) or a control group in which they received 0.1% BSA. Following recovery from surgery, rats were either conditioned immediately *before* (IL-1β: n = 13, BSA: n = 5) (see Figure 2.4) or 2 h *after* (IL-1β: n = 10, BSA: n = 9) (see Figure 2.5) receiving a single intra-cisterna magna injection (5 µl) of IL-1β (100 ng) or 0.1% BSA. The two hour delay in conditioning was chosen based on temperature profiles which showed that by 2 h after intra-cisterna magna injection rats were in the rising phase of fever. Abdominal body temperature, activity, food intake and body mass were measured for 7 days. By day 7 the sickness behaviours experienced by the rats that received IL-1β had resolved and then memory of rats was tested in both the contextual and auditory fear tests. Contextual fear of rats was tested at 7:00, 8 h before the onset of the daily dark phase (15:00).

Auditory fear of rats was tested 3 h after testing for contextual fear. . Rats were excluded from data analysis based on three criteria, blood present in the CSF (n = 6), abnormal temperature data, such as the lack of fever (n = 2) or being an outlier as calculated using the Grubbs test (n = 1) for any of the memory tests.

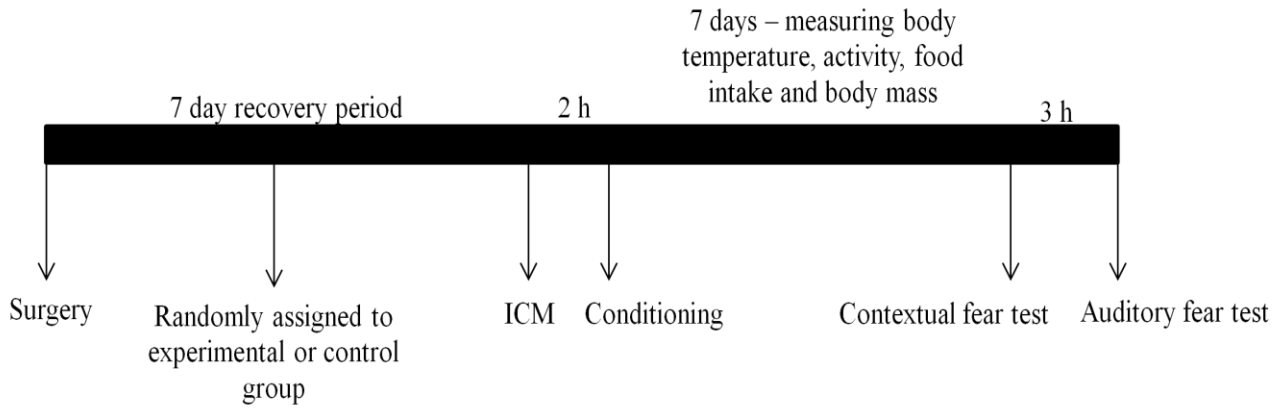
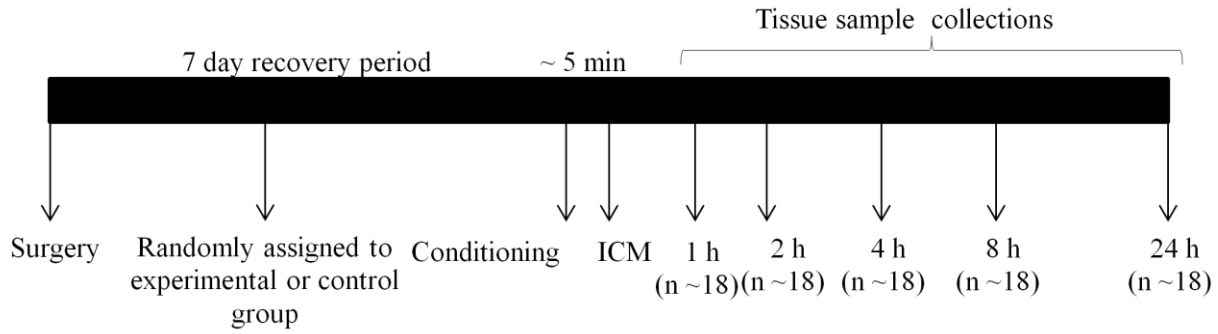


Figure 2.5: Basic flow diagram showing the order and timing of surgery and behavioural experiments for rats which were conditioned *after* receiving an ICM injection

2.9.2.2. Part B - Sample collection

All rats were implanted intraperitoneally with radiotransmitters and were allowed to recover for 7 days. Thereafter, rats were randomly assigned to either an experimental group which received 100 ng of IL-1β or a control group which received 0.1% BSA. Following recovery from surgery rats were either conditioned immediately *before* (IL-1β: n = 28, BSA: n = 16) or 2 h *after* (IL-1β: n = 34, BSA: n = 27) receiving a single intra-cisterna magna injection (5 µl) of IL-1β (100 ng) or 0.1% BSA. Rats were then terminated and brain tissue samples (hypothalamus and hippocampi) were collected at 1 h, 2 h, 4 h, 8 h and 24 h after intra-cisterna magna injection (see Figure 2.6).

A



B

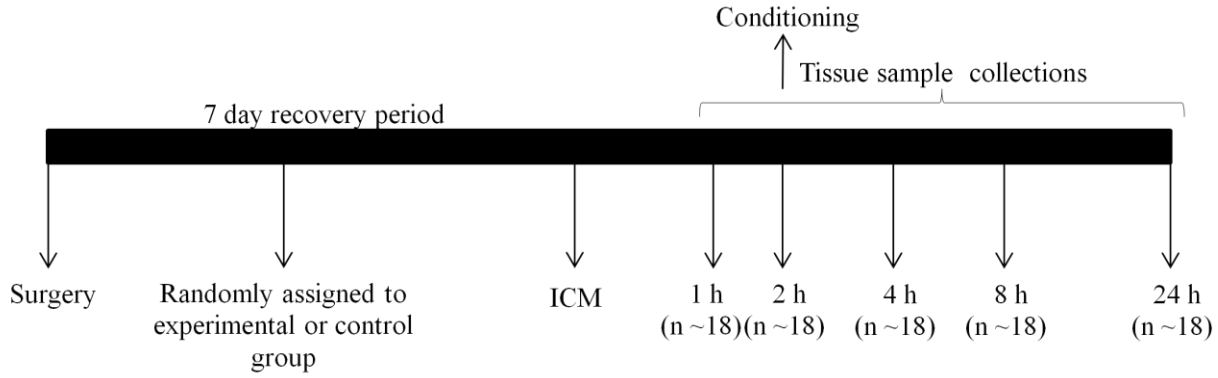


Figure 2.6: Basic flow diagrams showing the order and timing of sample collections for rats which were conditioned *before* (A) or *after* (B) receiving an ICM injection.

2.10. Data analyses

Temperature responses for rats were plotted as abdominal temperature-time curves in 1 or 2 hour intervals. For statistical analysis the original 5-min temperature recordings for each rat were averaged over 2 hours. The 2-hourly means were then analyzed by means of a two-way repeated measures ANOVA (with intervention and time as main effects), with a Bonferroni *post hoc* test when the ANOVA showed significance.

Change in nocturnal cage activity (15:00-3:00) of rats was calculated as the difference between the sum of night-time activity following injection and the average sum of night-time activity in the three day period before injection and expressed as a percentage of the pre-injection activity. Food intake was measured daily and calculated as grams of food consumed in 24 hours per 100 g of body mass and expressed as a percentage change from the mean daily food intake measured over three days before injection. Twenty-four hour change in body mass was determined by subtracting the body mass measured on each day after the injection from the body mass measured immediately before the injection and expressed as the change in body mass (g) per 100 g of rat body mass. Changes in nocturnal activity, food intake and body mass were compared by means of a two-way repeated measures ANOVA (with intervention and time as main effects), with a Bonferroni *post hoc* test when the ANOVA showed significance.

To assess memory, freezing behaviour during conditioning, the contextual fear test and the auditory fear test was assessed individually for each rat by 3 observers, blind to the experimental intervention. Inter-rater reliability, i.e. the consensus between scorers, exceeded 95% for all experiments. During conditioning and both memory tests, each rat was judged every 10 s as either mobile or freezing at the instant the sample was taken. Scoring began 10 s after the rat was placed into the relative context and continued every 10 s for 2 min during conditioning and 6 min during

the contextual fear and auditory fear (3 min pre-tone and 3 min post-tone) tests. The number of positive freezing scores were summed for each observer and an average freezing score between the observers was obtained. The average freezing score was divided by the number of samples taken for each test and multiplied by 100 to express the freezing behaviour as a percentage. Scores were then compared using a one-way ANOVA with a Bonferroni *post-hoc* test when the ANOVA showed significance.

Brain IL-1β concentrations were compared over 5 time points (1 h, 2 h, 4 h, 8 h and 24 h) using a two-way ANOVA (with intervention and time as main effects) with a Bonferroni *post hoc* test to determine inter and intra-group interactions when the ANOVA showed significance. Concentrations of IL-1β were compared separately for the hypothalamus and the hippocampus.

Data were expressed in terms of means and standard deviations and statistical significance was set at $P < 0.05$.

Chapter 3

Results

3.1. Experiment 1 - Lipopolysaccharide

3.1.1. Abdominal temperature

Figure 3.1 shows the abdominal temperature responses over 72 h for rats that received intra-cisterna magna injections of PBS or LPS (either 10 µg or 100 µg) (main effect of time ($F_{(36,684)}$ = 41.7, P < 0.001), intervention ($F_{(2,19)}$ =3, P > 0.05) and interaction ($F_{(72,684)}$ = 30.5 , P < 0.001)). After injection, the abdominal temperatures of rats that received both doses of LPS started to decrease significantly within 1-2 h after injection (P < 0.05, Bonferroni). Approximately 2 h after injection, in comparison to rats that received PBS, the abdominal temperatures of the rats that received 10 µg and 100 µg of LPS differed by ~ 1 °C and ~ 1.5 °C respectively. By the end of night 1 the LPS-induced hypothermia had resolved for both doses of LPS (10 and 100 µg). By day 1 after injection rats that received 10 µg of LPS had a significantly greater abdominal temperature than rats that received PBS. The fever noted in the rats that received 10 µg of LPS peaked at 38.1 ± 0.4 °C, ~ 10 h after injection (P < 0.05, Bonferroni). By day 2 the fever induced by 10 µg of LPS had resolved and rats that received 100 µg of LPS began to show a significantly greater abdominal temperature than rats that received PBS and 10µg of LPS (P < 0.05, Bonferroni). The fever induced by 100 µg of LPS peaked at 38.2 ± 0.2 °C, ~ 36 h after injection and resolved by the end of day 3 (P < 0.05, Bonferroni). The dose-dependent effect of LPS (10 µg and 100 µg) was evident throughout the three days after injection. The hypothermia induced by 100 µg of LPS reached lower temperatures and lasted longer (~ 14 h) than the hypothermia induced by 10 µg of LPS (~ 6 h) (P < 0.05, Bonferroni). Both doses of LPS induced a fever of similar magnitude and duration, however rats that received 10 µg of LPS reached the peak of the fever sooner (~10 h) than rats that received 100 µg of LPS (~36

h). The abdominal temperatures of rats, 48 h prior to injection, were not significantly different from each other.

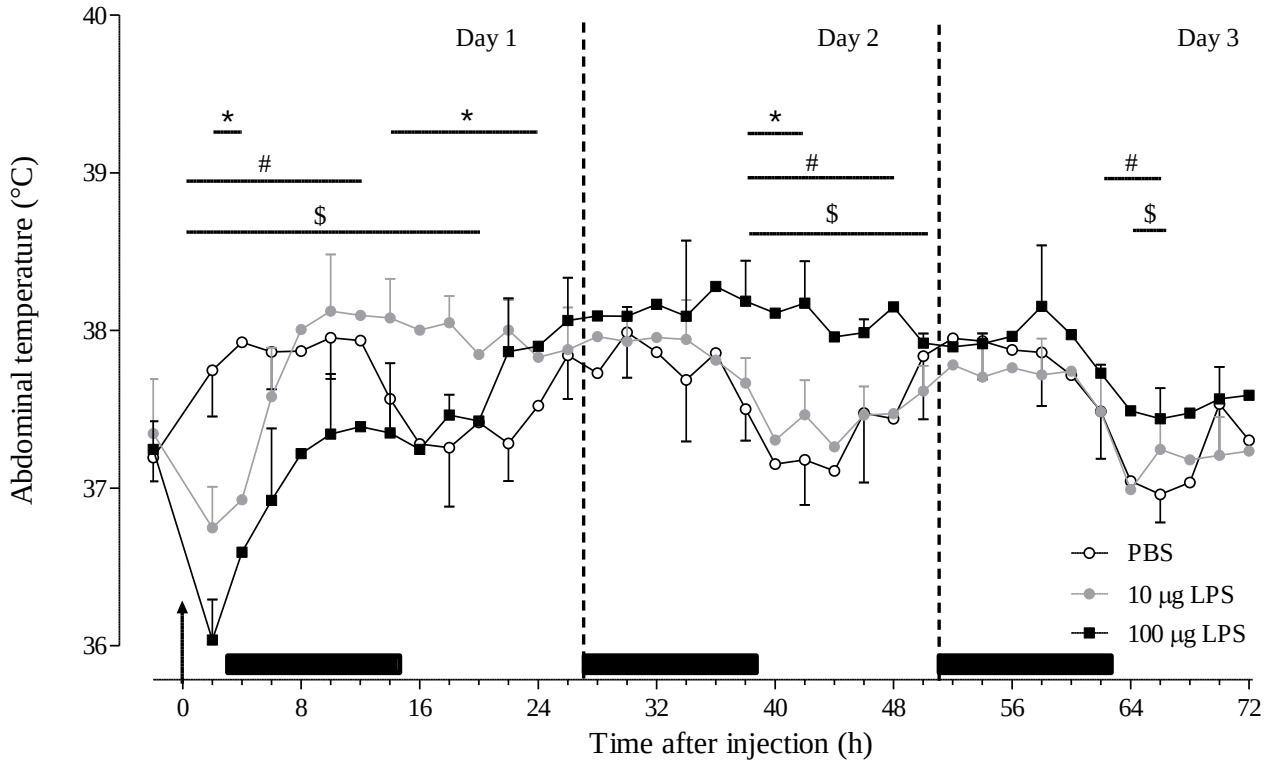


Figure 3.1 - The abdominal temperatures of rats 72 h after receiving a single ICM injection of 5 µl of PBS (n = 9), 10 µg of LPS (n = 10) or 100 µg of LPS (n = 3). The abdominal temperatures of rats were plotted in 2 h intervals. Statistical analysis was conducted on the original 5-min temperature recordings for each rat which were averaged over 2 hours. The arrow indicates the time of injection and the black bars indicate lights off. Significant differences: * PBS vs 10 µg LPS; # PBS vs 100 µg LPS; $ 10 µg LPS vs 100 µg LPS. Results are represented as mean ± SD.

3.1.2. Nocturnal cage activity

Figure 3.2 shows that the central administration of LPS caused a decrease in nocturnal cage activity for 4 nights after injection compared to rats that received PBS (main effect of time ($F_{(5,95)}$ = 54.9, P < 0.001), intervention ($F_{(2,19)}$ = 29, P < 0.001) and interaction ($F_{(10,95)}$ = 6.4, P < 0.001)). On the first night after injection, irrespective of dosage, LPS induced a maximum decrease in nocturnal cage activity of ~ 80% (P < 0.001, Bonferroni). The decrease in nocturnal cage activity was dose-dependent only on night two after injection and rats that received 100 µg of LPS showed a larger decrease in nocturnal activity than rats that received 10 µg of LPS (P < 0.01, Bonferroni). Thereafter rats gradually became more active and by night 5 nocturnal activity was not significantly different between groups.

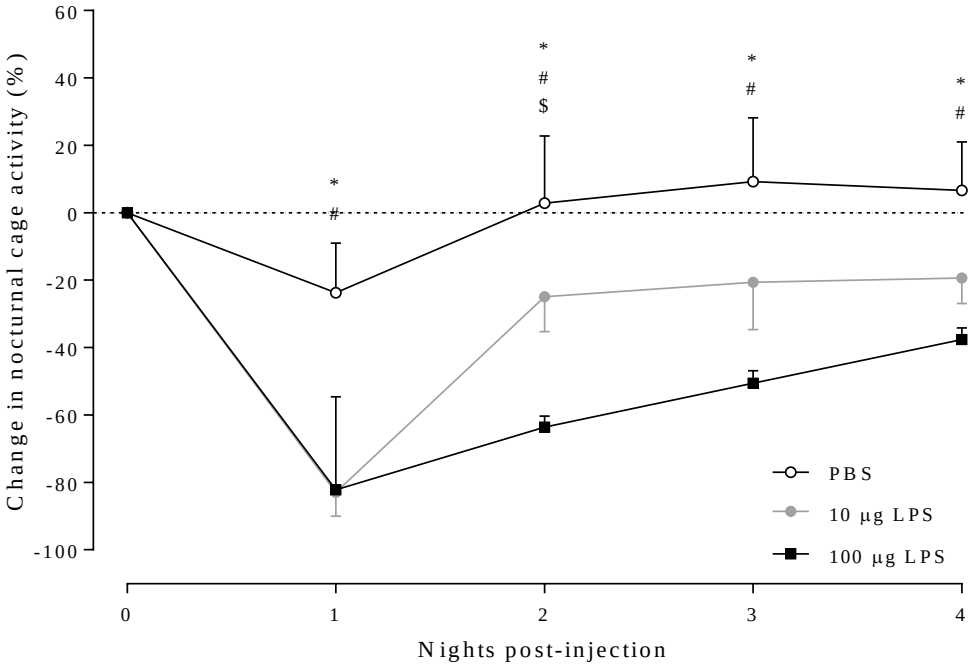


Figure 3.2 - Nocturnal cage activity of rats after receiving a single ICM injection of 5 µl of PBS (n = 9), 10 µg of LPS (n = 10) or 100 µg of LPS (n = 3). Change in nocturnal cage activity was calculated as the difference between the sum of night-time activity following injection and the average sum of night-time activity in the three day period before injection and expressed as a percentage of the pre-injection activity, with – 100% indicating a complete cessation of nocturnal cage activity. Significant differences: * PBS vs 10 µg LPS; # PBS vs 100 µg LPS; $ 10 µg LPS vs 100 µg LPS. Results are represented as mean ± SD.

3.1.3. Food intake and body mass

On average, rats consumed between 9 and 11 g of food, per 100g of body mass, per day during the 3 days before experimental intervention. Figure 3.3 shows that LPS induced a decrease in food intake (A) and body mass (B). Rats that received LPS (10 and 100 µg) consumed less food for 2 days after injection when compared to rats that received PBS (main effect for time ($F_{(5,95)}$= 191.3, $P < 0.001$), intervention ($F_{(2,19)}$= 18.2, $P < 0.001$) and interaction ($F_{(10,95)}$= 49.5, $P < 0.001$). The magnitude of the decrease in food intake was dose-dependent and rats that received 100 µg of LPS consumed significantly less food on day 1 and 2 as compared to rats that received 10 µg of LPS ($P < 0.05$, Bonferroni). Following the initial decrease in food intake rats that received LPS gradually began to eat more food and on days 3 and 4 after injection food intake was similar between groups ($P > 0.05$, Bonferroni). As shown in Figure 3.3B, rats that received LPS also showed a significant, dose-dependent, loss of body mass for 4 days after injection when compared to rats that received PBS (main effect for time ($F_{(3,57)} = 36.8$, $P < 0.001$), intervention ($F_{(2,19)} = 156$, $P < 0.001$) and interaction ($F_{(6,57)} = 3$, $P < 0.05$)). Rats that received 100 µg of LPS lost more body mass over the 4 days after injection as compared to rats that received 10 µg of LPS ($P< 0.05$, Bonferroni). Thereafter, rats gradually started to regain body mass and body mass was not significantly different between groups.

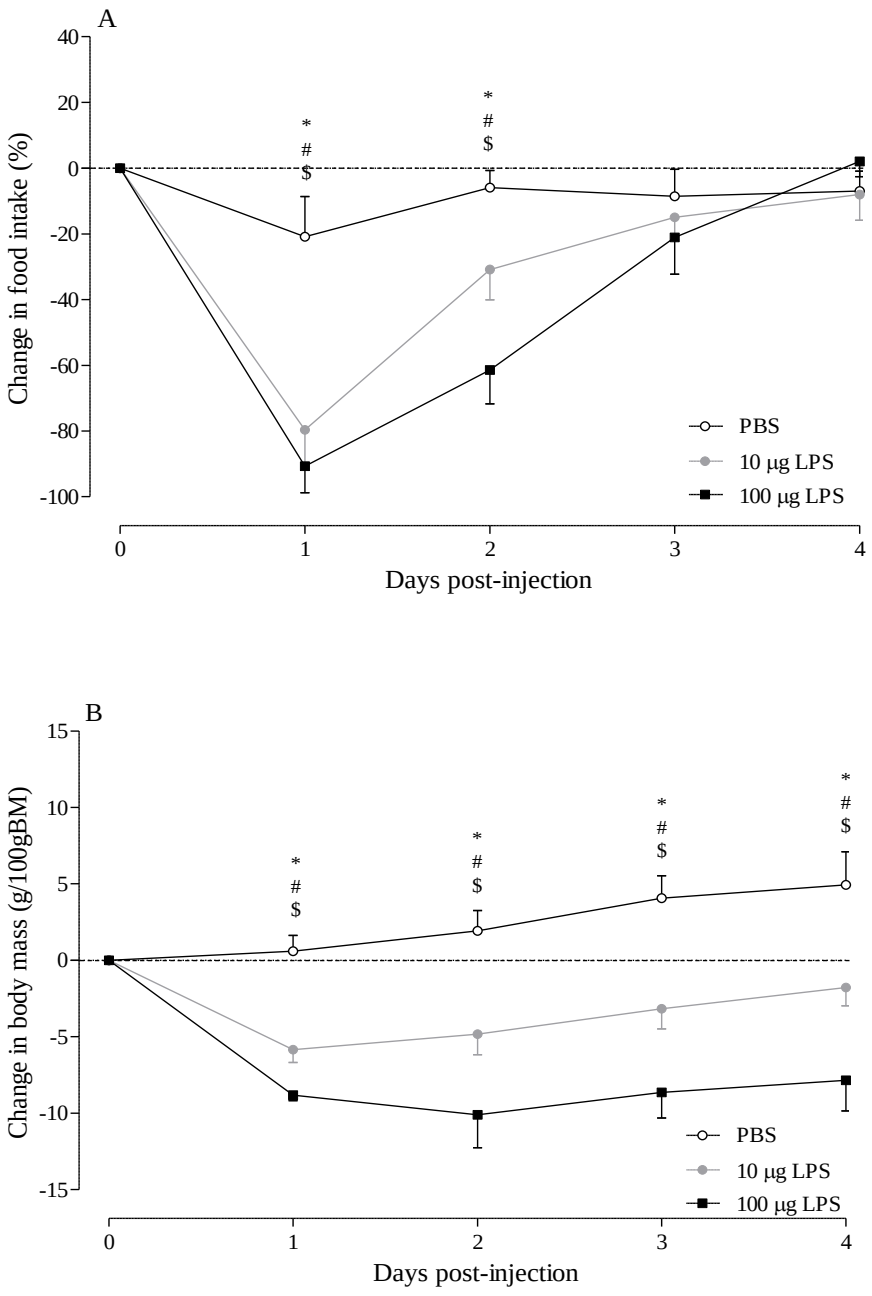


Figure 3.3 - Food intake (A) and body mass (B) of rats that received a single ICM injection of 5 µl of PBS (n = 9), 10 µg of LPS (n = 10) or 100 µg of LPS (n = 3). Food intake was calculated as the grams of food consumed in 24 hours per 100 g of body mass and expressed as a percentage change from the mean daily food intake measured over three days before injection. Twenty-four hour change in body mass was determined by subtracting the body mass measured on each day after the injection from the body mass measured immediately before the injection and expressed as the change in body mass (g) per 100 g of rat body mass. Significant differences: * PBS vs 10 µg LPS; # PBS vs 100 µg LPS; $ 10 µg LPS vs 100 µg LPS. Results are represented as mean ± SD.

3.1.4. Memory

Figure 3.4 shows the freezing behaviour of rats during conditioning (A), the contextual fear test (B) and the auditory fear test (C). Figure 3.4A shows that during conditioning, which took place immediately *before* any intervention, all rats were between 95 - 100% active in the context chamber, although rats which received 100 µg of LPS froze significantly more (~ 5 %) than rats that received PBS and 10 µg LPS ($F_{(2,18)}$= 12.7, $P < 0.05$). Figure 3.4B shows that during the contextual fear test rats that received LPS (10 and 100 µg) and PBS showed similar freezing behaviour (between ~ 55 - 85 %) ($F_{(2,18)}$ = 3.2, $P > 0.05$). Figure 3.4C shows that during the pre-tone period of the auditory fear test rats which received LPS (10 and 100 µg) froze to a similar extent (~ 40 – 60 %). Moreover, rats which received 10 µg of LPS froze to a similar extent to rats that received PBS (~ 25 - 40%). However, rats which received 100 µg of LPS froze significantly more (~35 %) than rats that received PBS ($F_{(2,19)}$= 5, $P < 0.05$). There were no differences in freezing behaviour between rats that received LPS (10 and 100 µg) and PBS during the presentation of the tone ($F_{(2,19)}$= 0.04, $P > 0.05$).

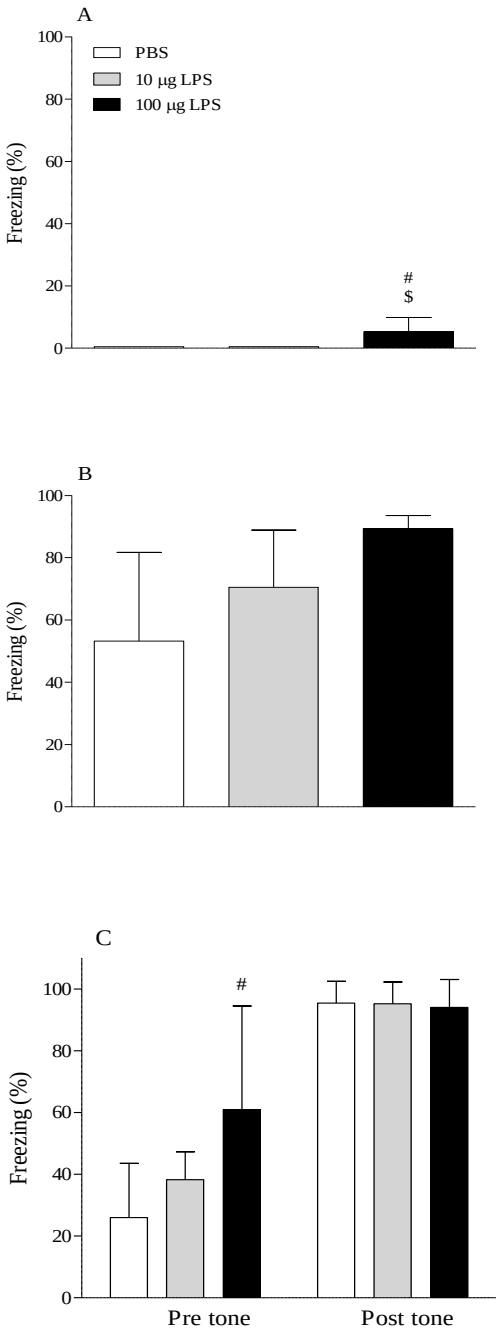


Figure 3.4 - Freezing behaviour during conditioning (A), the contextual fear test (B) and the auditory fear test (C) for rats that received a single ICM injection of PBS (n = 9), 10 µg of LPS (n = 10) or 100 µg of LPS (n = 3). During each test each rat was either judged as freezing or mobile every 10 s at the instant the measurement was taken. The number of positive freezing scores were summed for each observer and an average freezing score between the observers was obtained. The average freezing score was divided by the number of sampling bocks for each test and multiplied by 100 to express the freezing behaviour as a percentage.Significant differences: # PBS vs 100 µg LPS; $ 10 µg LPS vs 100 µg LPS. Results are represented as mean ± SD.

3.2. Experiment 2 - Interleukin-1β

3.2.1. Part A – Behavioural experiments

3.2.1.1. Abdominal temperature

Figure 3.5 shows that rats which received a single ICM injection of IL-1β had a significant rise in abdominal temperature after a latent period of ~ 2 h compared to rats which received BSA (main effects of time ($F_{(24,840)}$ = 127.4, $P < 0.001$), intervention ($F_{(1,35)}$ = 135.7, $P < 0.001$) and interaction ($F_{(24,840)}$ = 34.5, $P < 0.001$)). The abdominal temperature of rats injected with IL-1β peaked at 38.9 ± 0.3 °C, ~ 6 h after injection and remained significantly elevated compared to rats that received BSA during day 1 and day 2 after injection ($P < 0.05$). By the end of day 2 the IL-1β-induced fever appeared to resolve and the abdominal temperatures of rats that received IL-1β were not significantly different to rats that received BSA ($P > 0.05$). The abdominal temperatures of rats, 48 h prior to injection, were not significantly different from each other. The abdominal temperatures for rats that were conditioned immediately *before* or 2 h *after* they received BSA were not significantly different from each other and subsequently, the data for the groups were combined. Similarly, the abdominal temperatures for rats that were conditioned *before* or 2 h *after* they received IL-1β were not significantly different from each other and therefore the data for the groups were combined.

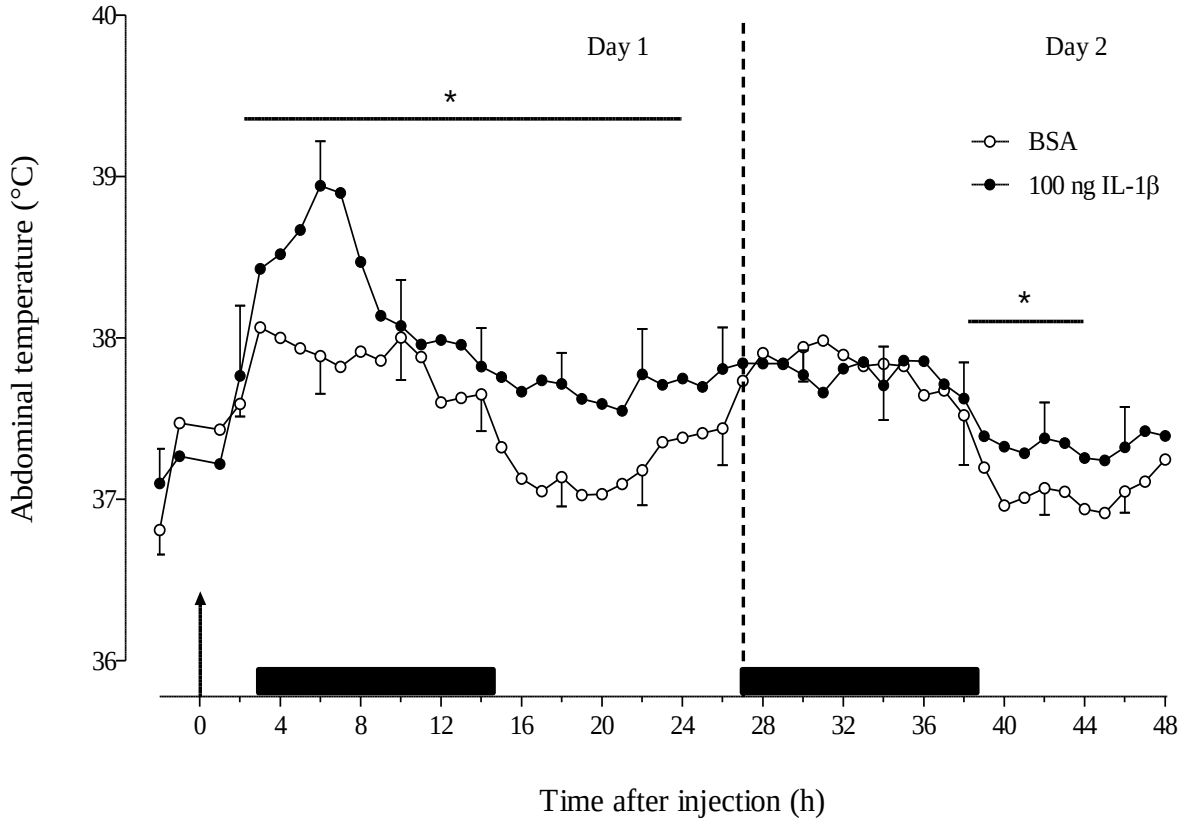


Figure 3.5 - The abdominal temperatures of rats 48 h after receiving a single ICM injection of 5 µl of 100 ng of IL-1β (n = 23) or 0.1% BSA (n = 14). Abdominal temperature was plotted in 1 hour intervals. Statistical analysis was conducted on the original 5-min temperature recordings for each rat which were averaged over 2 hours. The arrow indicates the time of injection and the black bars indicate lights off. Significant differences: * BSA vs 100 ng IL-1β. Results are represented as mean ± SD.

3.2.1.2. Nocturnal cage activity

Figure 3.6 shows that rats that received a single ICM injection of IL-1β had a decrease in nocturnal cage activity for two nights after injection compared to rats that received BSA (main effect of time ($F_{(3,105)} = 43.7$, $P < 0.001$), intervention ($F_{(1,35)} = 22.1$, $P < 0.001$) and interaction ($F_{(3,105)} = 6.6$, $P < 0.05$)). During night 1 after injection, IL-1β induced a maximum decrease in nocturnal activity of 62 ± 17 % ($P < 0.001$, Bonferroni). Thereafter rats gradually became more active and by night 3 after injection nocturnal cage activity was not significantly different between rats that received BSA and IL-1β. The nocturnal cage activities for rats that were conditioned immediately before or 2 h after they received BSA were not significantly different from each other and subsequently, the data for the groups were combined. Similarly, the nocturnal cage activities for rats that were conditioned before or 2 h after they received IL-1β were not significantly different from each other and therefore the data for the groups were combined.

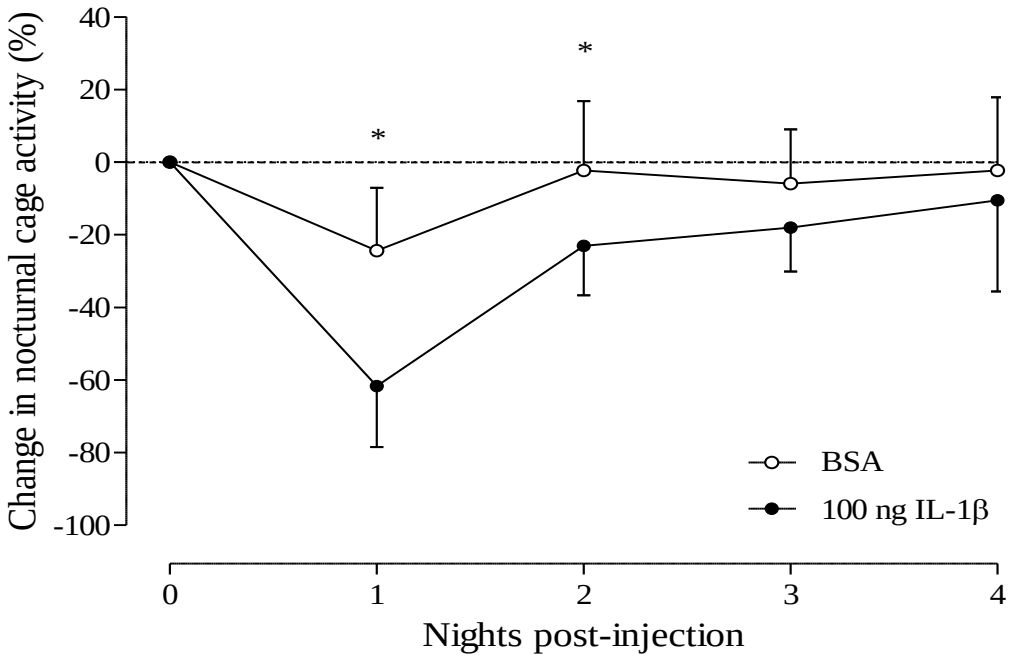


Figure 3.6 - Percentage change in nocturnal cage activity of rats after receiving a single ICM injection of 5 µl of 100 ng of IL-1β (n = 23) or 0.1% BSA (n = 14). Change in nocturnal cage activity was calculated as the difference between the sum of night-time activity following injection and the average sum of night-time activity in the three day period before injection and expressed as a percentage of the pre-injection activity, with – 100% indicating a complete cessation of nocturnal cage activity. Results are represented as mean ± SD.

3.2.1.3. Food intake and body mass

On average, rats consumed between 8 and 12 g of food, per 100 g of body mass, per day for 3 days before experimental intervention. Figure 3.7 shows that rats which received IL-1β had a decrease in both food intake (A) and body mass (B). Figure 3.7A shows that the central injection of IL-1β caused rats to consume less food than rats that received BSA (main effect of time ($F_{(3,105)}$= 131.1, $P < 0.001$), intervention ($F_{(1,35)}$= 16.3, $P < 0.05$) as well as interaction ($F_{(3,105)}$= 21.2, $P < 0.001$)) for 2 days after injection ($P < 0.001$, Bonferroni). Figure 3.7B shows that rats which received IL-1β also lost body mass compared to rats that received BSA (main effect of time ($F_{(3,105)} = 28.3$, $P < 0.001$), intervention ($F_{(1,35)} = 32.8$, $P < 0.001$) and interaction ($F_{(3,105)} = 13.2$, $P < 0.001$)). The body mass of rats which received IL-1β remained decreased for 3 days compared to rats that received BSA ($P < 0.001$, Bonferroni). Following the first day after injection rats that received IL-1β began to regain body mass and by day 4 their body mass was similar to the body mass of rats that received BSA. The food intake and body mass of rats that were conditioned immediately before or 2 h after they received BSA were not significantly different from each other and subsequently, the data for the groups were combined. Similarly, the food intake and body mass of rats that were conditioned before or 2 h after they received IL-1β were not significantly different from each other and therefore the data for the groups were combined.

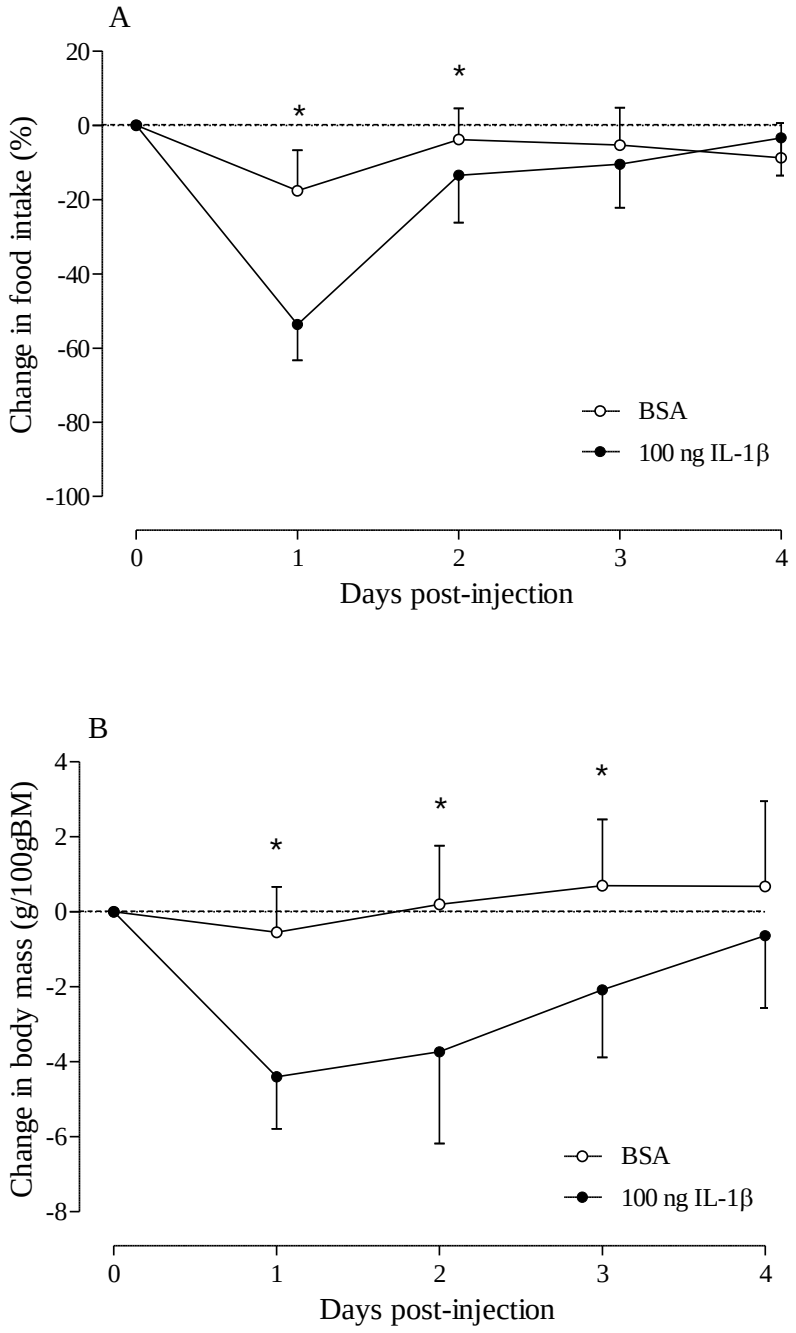


Figure 3.7 – The food intake (A) and body mass (B) of rats that received a single ICM injection of 5 µl of 100 ng of IL-1β (n = 23) or 0.1% BSA (n = 14). Food intake was calculated as the grams of food consumed in 24 hours per 100 g of body mass and expressed as a percentage change from the mean daily food intake measured over three days before injection. Twenty-four hour change in body mass was determined by subtracting the body mass measured on each day after the injection from the body mass measured immediately before the injection and expressed as the change in body mass (g) per 100 g of rat body mass. Significant differences: * BSA vs 100 ng IL-1β. Results are represented as mean ± SD.

3.2.1.4. Memory

Figure 3.8 shows that the freezing behaviour seen during conditioning (A), the contextual fear test (B) and the auditory fear test (C) was dependent on the time point at which rats were conditioned relative to receiving an ICM injection of IL-1β. Figure 3.8A shows that rats which received BSA and rats that were conditioned *before* they received IL-1β explored the context with equal interest during conditioning and were active most of the time ($F_{(2,27)}$= 7.4, $P < 0.05$). However, rats that were conditioned 2 hours *after* receiving IL-1β explored the context with less freedom and froze for approximately 13 % of the time during conditioning, which was significantly more than rats that were conditioned *before* they received IL-1β and rats that received BSA ($P < 0.001$, Bonferroni). Figure 3.8B shows the freezing behaviour of rats during the contextual fear test. Rats that were conditioned 2 hours *after* receiving IL-1β froze approximately 45% less than rats that received BSA and rats that were conditioned *before* receiving IL-1β ($F_{(2,34)}$= 17.4, $P < 0.001$). However, rats that received BSA and rats that were conditioned *before* receiving IL-1β showed similar freezing behaviour (~ 65 %) to each other ($P < 0.001$, Bonferroni). Figure 3.8C shows that during the auditory fear test rats which were conditioned 2 h *after* receiving IL-1β froze significantly less during both the pre-tone ($P < 0.001$, Bonferroni) and post tone ($P < 0.001$, Bonferroni) periods than rats that received BSA and rats that were conditioned *before* receiving IL-1β. Again rats that received BSA and rats that were conditioned *before* receiving IL-1β froze similarly to each other during both the pre-tone (~ 30 %) ($F_{(2,34)}$= 15.1, $P < 0.001$) and post tone periods (~ 85 %) ($F_{(2,34)}$= 17.5, $P < 0.001$). Rats that were conditioned immediately before or 2 h after they received BSA froze similarly during conditioning, the contextual fear test and the auditory fear test, subsequently the data for the groups were combined for each test.

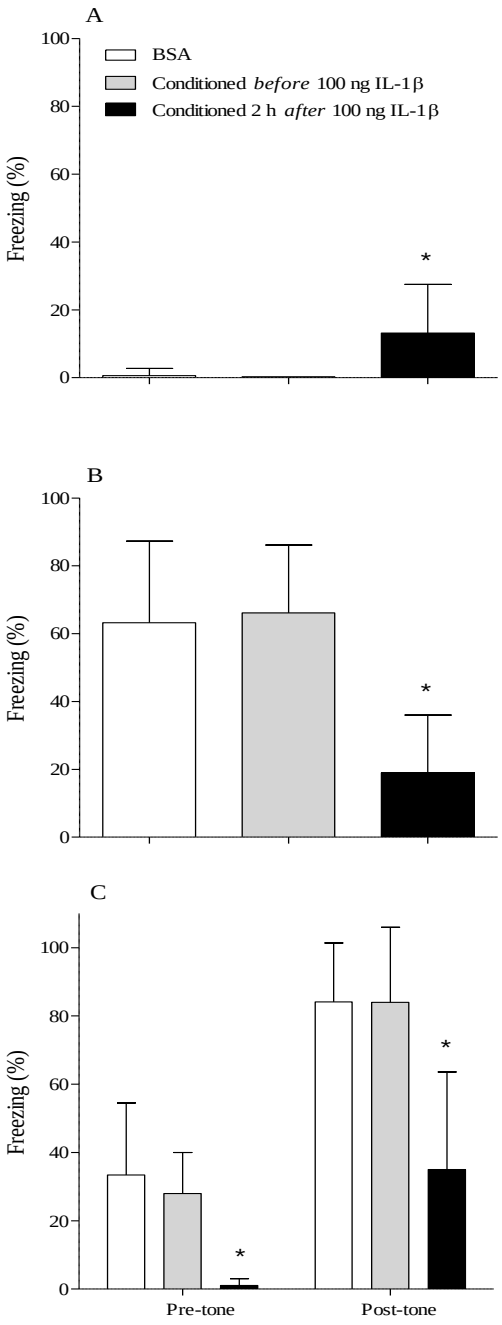


Figure 3.8 – Freezing behaviour during conditioning (A), the contextual fear test (B) and the auditory fear test (C) for rats that were: (1) conditioned immediately *before* receiving a single ICM injection of 100 ng of IL-1β (n = 13); (2) conditioned 2 h *after* receiving a single ICM injection of 100 ng of IL-1β (n = 10) or that received 0.1% BSA (n = 14). During each test each rat was either judged as freezing or mobile every 10 s at the instant the measurement was taken. The number of positive freezing scores were summed for each observer and an average freezing score between the observers was obtained. The average freezing score was divided by the number of sampling bocks for each test and multiplied by 100 to express the freezing behaviour as a percentage. Significant difference: *Rats conditioned *after* receiving 100 ng of IL-1β compared to rats receiving BSA and rats conditioned *before* receiving 100 ng IL-1β. Results are represented as mean ± SD.

3.2.2. Part B – Sample collections

3.2.2.2. Concentration of IL-1β in the brain

Figure 3.9 shows that a single ICM injection of 100 ng of IL-1β induced a significant increase in IL-1β concentrations in two brain areas at several different time points after injection. Figure 3.9A shows that rats which received IL-1β had significantly increased IL-1β concentrations in the hypothalamus compared to rats that received BSA (main effect of time ($F_{(4,83)} = 17.6$, $P < 0.001$), intervention ($F_{(1,83)} = 34.2$, $P < 0.001$) and interaction ($F_{(4,83)} = 2.7$, $P < 0.05$)) at 1 h, 4 h and 8 h after injection ($P < 0.05$, Bonferroni). However, there were no significant differences in hypothalamic IL-1β concentrations of rats that received IL-1β between 1 – 8 h after injection ($P < 0.05$, Bonferroni). There also were no significant differences in hypothalamic IL-1β concentrations of rats that received BSA from 1 – 24 h after injection ($P > 0.05$, Bonferroni). Figure 3.9B shows that rats which received IL-1β had significantly increased IL-1β concentrations in the hippocampus compared to rats that received BSA (main effect of time ($F_{(4,83)} = 6.8$, $P < 0.001$), intervention ($F_{(1,83)} = 43.9$, $P < 0.001$) and interaction ($F_{(4,83)} = 2.8$, $P < 0.05$)) at 1 h, 4 h and 8 h after injection ($P < 0.05$, Bonferroni). There were no significant differences in hippocampal IL-1β concentrations of rats that received IL-1β between 1 – 8 h after injection ($P < 0.05$, Bonferroni). There were no significant differences in hippocampal IL-1β concentrations of rats that received BSA from 1 – 24 h after injection ($P > 0.05$, Bonferroni). The hypothalamic and hippocampal IL-1β concentrations of rats that were conditioned immediately before or 2 h after they received BSA were not significantly different from each other and subsequently, the data for the groups were combined. Similarly, the hypothalamic and hippocampal IL-1β concentrations of rats that were conditioned before or 2 h

after they received IL-1β were not significantly different from each other and therefore the data for the groups were combined.

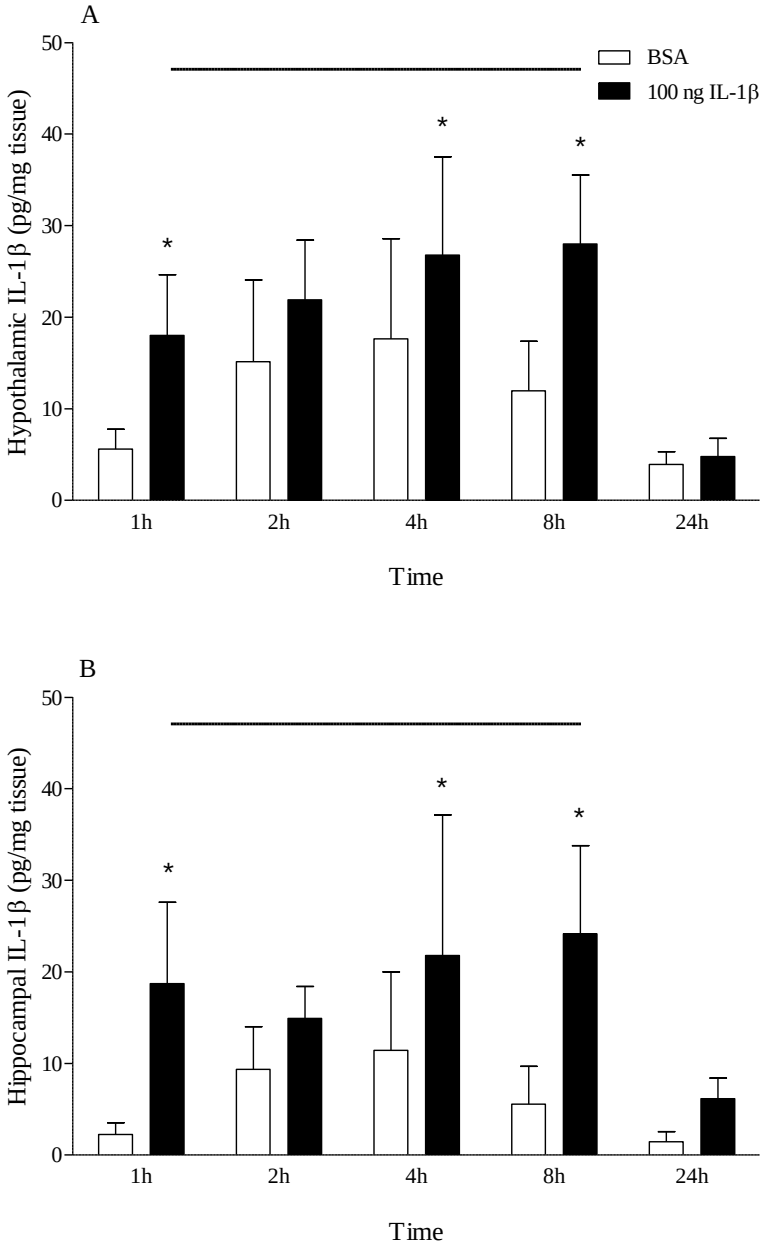


Figure 3.9 - Hypothalamic (A) and hippocampal (B) IL-1β concentrations in rats at several time points following a single ICM injection of 5 µl of 100 ng of IL-1β (n = ~ 11 per time point) or 0.1% BSA (n = ~ 7 per time point). The black line indicates the period during which there was no significant difference in IL-1β concentrations in the brain for rats receiving IL-1β after injection. Significant differences: *BSA vs 100 ng IL-1β. Results are represented as mean ± SD.

Chapter 4

Discussion

The results of the present study have shown that the intra-cisterna magna administration of LPS to rats dose-dependently induces profound sickness responses including: a biphasic temperature response, lethargy and anorexia. Interestingly, even though the rats injected with LPS developed profound sickness responses, their hippocampal-dependent memory remained intact. Although the findings that centrally administered LPS induces fever, anorexia and lethargy is not novel, to my knowledge this is the first study that concurrently examined the effect of LPS administered via the cisterna magna to rats on a suite of sickness responses. Furthermore, my study is the first to have examined the effect of centrally administered LPS on memory consolidation in rats using fear conditioning.

Moreover, I have also shown that the intra-cisterna magna administration of IL-1β to rats induces fever, lethargy and anorexia, as well as an increase in hypothalamic and hippocampal IL-1β concentrations for up to 8 h following injection. Additionally, I demonstrated that the time point at which IL-1β is administered to rats, in relation to when they were conditioned (i.e. when learning takes place), plays a significant role in whether a rat will develop memory impairment or not. I found that IL-1β administered 2 h *before* conditioning induced both hippocampal and amygdala-dependent memory impairment, whereas IL-1β administered immediately after conditioning had no effect on memory. While the characterization of sickness responses after the central administration of IL-1β is not novel, what is novel in my study was the concurrent examination of the effect of IL-1β administered via the cisterna magna on body temperature, activity, food intake, body mass and memory. Furthermore, my study was the first to characterize the expression of IL-1β in the brain following an intra-cisterna magna injection of IL-1β.

Despite all the studies undertaken to date which used the third cerebral ventricle as the route of central injection for pathogenic substances to rodents, I chose to inject both LPS and IL-1β via the

cisterna magna of rats. Research on cannulae implantation, which is required for intracerebroventricular injection, showed that the implantation of cannulae causes activation of inflammatory transcription factors within the brains of rodents (Damm *et al.*, 2013) and requires a 3-4 week recovery period (Pugh *et al.*, 1999). Intra-cisterna magna injections do not require cannulae implantation but rather the direct injection of substances into the cisterna magna of rodents, therefore rodents only require a short recovery from anesthesia. Moreover, to my knowledge no studies have determined that intra-cisterna magna injection causes either immune or microglial activation.

4.1. LPS-induced sickness responses

My findings support the view that centrally administered LPS is a potent exogenous pyrogen and can act within the brain to induce fever. Previous research has consistently shown that the intracerebroventicular administration of LPS at a range of doses (10 – 60 ng) induces a monophasic fever (Ledeboer *et al.*, 2002, McCarthy *et al.*, 1986), however the temperature profiles attained in my study after the intra-cisterna magna administration of LPS (10 and 100 µg) showed a dose-dependent hypothermia followed by the development of a fever (see Figure 3.1). Although hypothermia may be uncommon after the central administration of LPS, the temperature profile in my study is very similar to the mild hypothermias which precede fevers often seen after intermediate doses (0.5 – 5 mg/kg) of peripherally or intravenously administered LPS (Leon, 2004, Liu *et al.*, 2012, Romanovsky *et al.*, 1996, Romanovsky *et al.*, 1997, Romanovsky and Szekely, 1998). The most common reasons for the hypothermia which precedes fever are unfavourable experimental conditions during or after inflammatory insult such as an ill animal, a low ambient temperature (below the animals thermoneutral zone (TNZ)) or a very strong inflammatory insult (Leon, 2004, Romanovsky *et al.*, 2005). The TNZ for a rat lies between 27 – 30 °C (Leon, 2004,

Maloney *et al.*, 2014, Romanovsky *et al.*, 1996) and normal laboratory ambient temperatures generally range between 20 – 24 °C (Leon, 2004, Maloney *et al.*, 2014). Rodents are particularly vulnerable to an inflammatory insult when they are housed in an environment with an ambient temperature lower than their TNZ, this is due to their high surface area to body mass ratios (Leon, 2004). A study in which LPS (5 mg/kg) was administered peripherally to rats showed that if LPS was administered to rats at an ambient temperature within their TNZ (28 °C) rats developed fever, however when the same dose of LPS was administered to rats at an ambient temperature below their TNZ (22 °C) it resulted in hypothermia (Liu *et al.*, 2012). Both hypothermia and fever are the direct result of active thermogenic effectors within the brain and are achieved by an increase in heat gain or a decrease in heat loss mechanisms respectively (Leon, 2004). After an inflammatory insult, heat production mechanisms compete with heat loss mechanisms to induce fever (Rudaya *et al.*, 2005). However when infection occurs at a low ambient temperature, heat gain mechanisms become harder to activate and heat loss mechanisms are predominate, which results in the development of a moderate hypothermia, followed by a fever of low magnitude and a delayed onset (Rudaya *et al.*, 2005). For the duration of my study the ambient temperature in my laboratory ranged between 22 - 23 °C. Furthermore, although the doses of LPS used in my study (10 and 100 µg) are low compared to the doses of LPS administered in simulated peripheral infections (0.5 – 10 mg/kg) (Leon, 2004, Romanovsky *et al.*, 1996, Romanovsky and Szekely, 1998), the intra-cisterna magna doses I used are 1000 to 10000 fold higher than those used in other studies which simulated central infections using LPS (10 – 60 ng) (Ledeboer *et al.*, 2002, McCarthy *et al.*, 1986). Therefore, in light of the conditions under which my study was conducted (high doses of LPS administered at a low ambient temperature) this could explain the reason for the biphasic temperature response seen after the intra-cisterna magna administration of LPS to rats in my study.

Both the peripheral and central administration of LPS induce the release and synthesis of multiple pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α (Conti *et al.*, 2004, Leon, 2004) all of which are classified as endogenous pyrogens and are involved in febrigenesis (Cunningham *et al*., 2005; Gabellec *et al*., 1995; Laye *et al*., 1994; Quan *et al*., 1998). However, TNF-α can also act as an endogenous cryogen and has been demonstrated to be the predominant mediator of hypothermia in both peripheral and central infections (Leon, 2004, Matsui *et al.*, 2006, Romanovsky *et al.*, 2005). The cyrogenic ability of TNF-α is demonstrated by several factors: (1) centrally administered TNF-α has shown to induce hypothermia in rodents (Holt *et al.*, 1989), (2) LPS induced TNF-α release correlates with hypothermic events (Blanque *et al.*, 1996) and (3) TNF-antiserum has shown to attenuate LPS induced hypothermia (Derijk and Berkenbosch, 1994).

Furthermore, a study in which LPS was administered peripherally to rats demonstrated that increased serum concentrations of TNF-α, which were associated with hypothermia, also played a role in mediating LPS-induced anorexia (Tollner *et al.*, 2000). Clinically, hypothermia is associated with severe infections and patients that present with hypothermia are prone to organ failure and have a higher incidence of mortality than patients that present with fever (Clemmer *et al.*, 1992, Marik and Zaloga, 2000). Therefore, because hypothermia is often associated with severe infections it may also be associated with an increase in inflammatory responses and so may produce exaggerated sickness responses.

In contrast to the differences observed for body temperature between the intra-cisterna magna and the intracerebroventricular administration of LPS, my finding that the intra-cisterna magna injection of LPS induced lethargy and anorexia in rats has previously been shown in studies wherein LPS was administered intracerebroventricularly to rats and mice. Previous research has demonstrated that the intracerebroventricular administration of LPS (100 ng) potently induces lethargy in rats (Castanon *et*

al., 2001), my data are in line with this study and showed that the intra-cisterna magna administration of LPS (10 and 100 µg) dose-dependently induced lethargy which was maintained for up to 4 nights after injection (see Figure 3.2). In addition, I also confirmed the anorexigenic effects of intracerebroventricularly administered LPS (3 ng – 2.5 µg) described in the literature (Bluthe *et al.*, 1999, Bluthé *et al.*, 2000, Burgess *et al.*, 1998, Castanon *et al.*, 2001, Faggioni *et al.*, 1995) by showing that the intra-cisterna magna administration of LPS (10 and 100 µg) to rats induced a dose-dependent decrease in both food intake and body mass (see Figure 3.3A and Figure 3.3B respectively); body mass remained stunted for at least 4 days. Body mass stunting is a phenomenon which, to my knowledge, has not previously been demonstrated after the central administration of LPS.

To assess memory in rats I chose to use the fear conditioning task because it has been used extensively in various laboratories to test for memory impairment in rodents (Avital *et al.*, 2003, Barrientos *et al.*, 2004, Goshen *et al.*, 2007, Hein *et al.*, 2007, Pugh *et al.*, 1998, Terrando *et al.*, 2010). In addition, fear conditioning allows for the testing of two different types of memory, specifically hippocampal-dependent and amygdala-dependent memory, which enables a direct comparison between the two different types of memory. Furthermore, the fear conditioning task only requires one period of learning as opposed to other memory paradigms e.g. the Morris water maze which has several learning trials (Morris, 1981). Fear conditioning only requires the administration of a substance once (either before or after learning) however, because the Morris water maze has multiple learning trials it may require multiple administrations of a substance and because I chose to administer substances via the cisterna magna in my study, due to ethical considerations I could only inject my rats once.

Memory is a multi-stage process which includes: acquisition, consolidation, storage and finally retrieval of the stored memory (Abel and Lattal, 2001). In the context of the fear conditioning task; memory acquisition is the stage at which rats learn to make an association between the context, the tone and the foot shock experienced in the context chamber (Abel and Lattal, 2001). Memory consolidation occurs once the animal is returned to its home cage and can take place anywhere between a few minutes to 24 h (Barrientos *et al.*, 2002), it is the process in which the early memory becomes cemented into a fixed state (Abel and Lattal, 2001). Once memory consolidation occurs the fixed memory is then stored in the hippocampus as a long-term memory (Abel and Lattal, 2001, Rudy *et al.*, 2004). Memory retrieval occurs when the rat is returned to the context or auditory chamber for testing and the fear of the context; fear of the tone associations are tested respectively (Abel and Lattal, 2001). It can become complicated to isolate the different stages of memory, particularly when trying to determine which stage has been affected by a substance, this is because often more than one stage could potentially be effected. Therefore the timing of substance administration (either before or after conditioning/learning) is a very important factor to consider. If a substance is administered before conditioning/learning, it has the potential to effect memory acquisition (learning) and memory consolidation (Cunningham and Sanderson, 2008, Thomson and Sutherland, 2006). If a substance is administered after learning has taken place it has the ability to effect memory consolidation (Cunningham and Sanderson, 2008, Thomson and Sutherland, 2006).

When a substance is administered before learning takes place it may result in an abnormal sensory experience during learning (Rudy *et al.*, 2004). During fear conditioning, memory acquisition relies on the formation of a contextual representation which involves binding together the individual features of the context to form a unified neural ensemble (Rudy *et al.*, 2004). However if the rat has an abnormal sensory experience in the context chamber it will form an inadequate or incomplete contextual representation (Rudy *et al.*, 2004), which will result in memory impairment. However

impairment of memory acquisition is not necessarily due to a loss in memory function but is rather due to a performance deficit in forming a contextual representation (Rudy *et al.*, 2004).

Furthermore, when a substance is administered either before or after learning takes place it has the potential to effect memory consolidation. Due to the variability in the period during which memory consolidation occurs it is the stage of memory formation most vulnerable to disruption (Abel and Lattal, 2001). Memory consolidation is hypothesized to rely predominantly on neural plasticity initiated in the hippocampus (Goshen and Yirmiya, 2006, Huang and Sheng, 2010, Yirmiya and Goshen, 2011), which involves the formation, degradation and change of neural pathways and synapses (Yirmiya and Goshen, 2011). The most important type of neural plasticity involved in memory consolidation is long-term potentiation (LTP) (Goshen and Yirmiya, 2006, Huang and Sheng, 2010, Yirmiya and Goshen, 2011), which is a persistent increase in synaptic efficacy following high frequency stimulation of a synapse, it results in long-lasting signal transmission between two neurons (Huang and Sheng, 2010). The administration of an intervention either before or after learning has the ability to impair neural plasticity and LTP, thereby impairing memory consolidation (Goshen and Yirmiya, 2006; Huang and Sheng, 2010; Pugh *et al.*, 2001; Yirmiya and Goshen, 2011). Once memory consolidation is impaired the contextual representation formed during acquisition is no longer available, resulting in weak fear conditioning which will manifest itself as a decrease in freezing during the context test (Rudy *et al.*, 2004).

Interestingly despite the strong link between LPS and central infections (*E. coli* has been shown to induce bacterial meningitis and brain abscesses), my study was the first to examine the effect of centrally administered LPS on hippocampal-dependent memory using fear conditioning. My results showed that during conditioning all of the groups were active and explored the chamber approximately 95 – 100 % of the time (see Figure 3.4A) which indicates that none of the rats were

sick or lethargic during conditioning (Barrientos *et al.*, 2012). Although rats assigned to receive 100 µg of LPS froze significantly more during conditioning than the other groups the difference was small and may be attributed to the small sample size and high inter-individual variability however, it is not considered to be biologically significant. Similarly the elevated freezing during the pre-tone period for rats that received 100 µg of LPS does not indicate that rats were sick or lethargic as all the sickness responses had resolved prior to memory testing, the difference is also likely due to the small sample size and high inter-individual variability. Contrary to what I expected based on the findings from peripheral LPS studies (Pugh *et al.*, 1998, Terrando *et al.*, 2010) and despite the potent effect that the intra-cisterna magna injection of LPS had on body temperature, activity, food intake and body mass; LPS had no effect on hippocampal-dependent memory in my study, regardless of the dose administered (see Figure 3.4B). Thus these findings collectively indicate that there appears to be a dissociation between memory impairment and the other sickness responses.

The possible reasons as to why I did not see memory impairment in my study are several fold. Firstly it may be related to the doses of LPS used (10 and 100 µg), previously a study examined the dose-effect of peripherally administered LPS on memory in rats and determined that the low (0.5 mg/kg) and high dose (2 mg/kg) of LPS had no effect on either hippocampal or amygdala-dependent memory, however the moderate dose (1 mg/kg) caused hippocampal-dependent memory impairment (Pugh *et al.*, 1998), therefore peripherally administered LPS appears to act on memory in an U-shaped dose response curve (Pugh *et al.*, 1998). Hence it is possible centrally administered LPS also acts on memory in a U-shaped dose-response curve and therefore the two doses of LPS I administered to my rats may have been too high, in terms of a central infection, to cause memory impairment. In order to verify whether centrally administered LPS acts on memory in a U-shaped dose-response curve, similarly to peripherally administered LPS (Pugh *et al.*, 1998), it would be useful to administer several doses of LPS via the cisterna magna and examine their effects on

memory using fear conditioning. Secondly, because memory consolidation may occur within a highly variable period of time (Abel and Lattal, 2001, Barrientos *et al.*, 2002, Rudy *et al.*, 2004) and even though LPS may have induced an increase in cytokine concentrations within the brain for a protracted period of time, in my study the onset of cytokine elevation may have occurred after memory consolidation had taken place. Thirdly, key proteins known to impair memory consolidation, such as IL-1β, may not have been raised enough at the point at which memory consolidation occurred.

Although I did not measure cytokine concentrations in the brain after I administered LPS via the cisterna magna to rats; previous research in which LPS was administered peripherally to rats showed that there is a relationship between fever and hypothermia with serum and brain concentrations of TNF-α and IL-1β (Blanque *et al.*, 1996, Deak *et al.*, 2005, Skelly *et al.*, 2013). Following a peripheral LPS injection TNF-α serum concentrations increased with the onset of hypothermia and decreased rapidly whereas there was a delayed onset of serum IL-1β elevation (Blanque *et al.*, 1996). In addition, a study in which peripherally administered LPS resulted in hypothermia in mice showed that 2 h after the administration of LPS there was greater hypothalamic mRNA transcription of TNF-α compared to hypothalamic mRNA transcription of IL-1β (Skelly *et al.*, 2013). Furthermore a study in which the peripheral administration of LPS induced a fever showed that brain IL-1β concentrations remained elevated in the brain for a similar period of time that body temperature remained elevated after LPS injection (Deak *et al.*, 2005). Therefore I can speculate in relation to the abdominal temperature data obtained for the rats that received LPS in my study (see Figure 3.1) that brain TNF-α concentrations were originally elevated with the onset of hypothermia at approximately 1-2 h after LPS injection; followed by a delayed increase in IL-1β at approximately 4 h following administration of the low dose (10 µg) of LPS and 18 h for the high dose (100 µg) of LPS. TNF-α has not been shown to play a consistent role in memory impairment

(Goshen and Yirmiya, 2006) and based upon my speculation if TNF-α was raised at the point which memory consolidation took place, it may be the reason that I did not see memory impairment in my study following the intra-cisterna magna administration of LPS to rats. In order to verify this speculation it is important to measure brain TNF-α and IL-1β concentrations at several time points following the intra-cisterna magna injection of LPS.

4.2. Interleukin-1β induced sickness responses

Numerous studies have determined that the peripheral and central administration of LPS results in the *de novo* synthesis and release of a host of pro-inflammatory cytokines (e.g. IL-1α, IL-1β, IL-6, IL-8 and TNF-α) in the brain which work additively and synergistically in order to induce sickness responses (Conti *et al.*, 2004, Leon, 2004). The three pro-inflammatory cytokines predominantly associated with LPS-induced sickness responses are IL-1β, IL-6 and TNF-α (Conti *et al.*, 2004, Skelly *et al.*, 2013). Although it would have been preferable to examine and compare the effects of all of the three major cytokines implicated in LPS-induced sickness responses, based on time constraints I chose one – namely IL-1β.

I chose to administer IL-1β instead of IL-6 and TNF-α because the intracerebroventricular administration of IL-1β consistently induces fever (Anforth *et al.*, 1998, Harden *et al.*, 2008), anorexia (Plata-Salamán, 1994) and lethargy (Harden *et al.*, 2008, Plata-Salamán, 1994), whereas the intracerebroventicular administration of IL-6 consistently induces fever (Harden *et al.*, 2008) but not other sickness behaviours such as anorexia (Harden *et al.*, 2008). In addition although the intracerebroventicular administration of TNF-α consistently induces anorexia, depending on the dose of TNF-α (Plata-Salamán *et al.*, 1988) it may either induce hypothermia or a fever (Leon, 2004). Moreover, studies which examined the relative potency of IL-1β, IL-6 and TNF-α by

comparing the magnitude of sickness responses induced by the individual cytokines administered at the same dose determined that in central infections IL-1β is the most potent inducer of fever, lethargy and anorexia (Harden *et al.*, 2008, Sonti *et al.*, 1996). Furthermore, IL-1β is also the pro-inflammatory cytokine that has been implicated most often in neurodegenerative diseases, as well as memory impairment associated with central infections (Cunningham *et al.*, 2005, Goshen and Yirmiya, 2006, Rothwell and Luheshi, 2000), whereas the central administration of IL-6 and TNF-α have not consistently been shown to have an effect on memory (Goshen and Yirmiya, 2006).

Although I am the first researcher to my knowledge to have examined the effects of IL-1β (100 ng) administered via the cisterna magna on body temperature in rats, my findings are in line with previous research which demonstrated the pyrogenic properties of intracerebroventricularly administered IL-1β to rodents (Anforth *et al.*, 1998, Busbridge *et al.*, 1989, Cao *et al.*, 2001, Harden *et al.*, 2008, Kent *et al.*, 1992, Li *et al.*, 2001, Montkowski *et al.*, 1997, Oitzl *et al.*, 1993). I demonstrated that IL-1β administered via the cisterna magna is potently pyrogenic and resulted in the development of a fever which lasted for up to 48 h (see Figure 3.5). Furthermore, I demonstrated that the intra-cisterna magna administration of IL-1β (100 ng) to rats resulted in lethargy which was maintained for up to 2 nights after injection (see Figure 3.6) as well as a decrease in food intake and body mass, which are indicators of anorexia, and which were maintained for 2 and 3 days after injection respectively (see Figure 3.7). My findings that IL-1β is a potent promoter of lethargy and anorexia further confirmed and supported the results in the literature which have shown that intracerebroventricularly administered IL-1β causes a decrease in activity (Bluthé *et al.*, 2000, Montkowski *et al.*, 1997, Plata-Salamán, 1994), food intake and body mass (Bluthé *et al.*, 1995, Bluthé *et al.*, 2000, Harden *et al.*, 2008, Kent *et al.*, 1992, Kent *et al.*, 1994, Montkowski *et al.*, 1997, Sonti *et al.*, 1996). Collectively, central IL-1β appears to be a potent mediator of sickness responses, regardless of the route by which it is administered centrally.

In order to assess memory rats were assigned to receive IL-1β (100 ng) either 2 h *before* conditioning or immediately *after* conditioning, they were then tested for fear of the context and fear of the tone 7 days later. Rats that received IL-1β (100 ng) immediately *after* conditioning (learning) were not under the influence of IL-1β during conditioning; hence my results showed that these rats explored the context chamber with equal interest to rats that received BSA and froze for ~ 1 % (see Figure 3.8A). Although the relationship between IL-1β and memory is complex, the literature suggests that centrally administered IL-1β (either via the third cerebral ventricle or via the hippocampus), immediately *after* conditioning, is consistently either beneficial or deleterious to hippocampal-dependent memory (Barrientos *et al.*, 2002, Barrientos *et al.*, 2004, Goshen *et al.*, 2007, Hein *et al.*, 2007, Pugh *et al.*, 1999). Interestingly my study showed that the intra-cisterna magna administration of 100 ng of IL-1β immediately *after* conditioning had no impact on hippocampal-dependent memory (see Figure 3.9 B). Similar findings for memory function have not previously been reported when using the fear conditioning task which is surprising because IL-1β has been hypothesized to act on memory in an inverted U-shaped pattern which suggests that basal levels of IL-1β in the brain are necessary for memory formation to take place but any deviation either by causing over or under expression of IL-1β will be detrimental to memory consolidation (Goshen *et al.*, 2007, Goshen and Yirmiya, 2006). My results further add to the complexity of the relationship between IL-1β and memory and may be due to several reasons. Firstly a study which peripherally administered LPS to rats determined that LPS acted on hippocampal-dependent memory in a U-shaped dose response, that is to say that a mid range dose of LPS caused hippocampal-dependent memory impairment but a low and high dose of LPS had no effect on memory (Pugh *et al.*, 1998)., therefore it is a possibility that IL-1β may also act in a U-shaped dose-response on memory. As evidence for the potential U-shaped dose-response of IL-1β on memory, studies which centrally administered (via the third cerebral ventricle or via the hippocampus) 10 ng

of IL-1β to rodents have consistently demonstrated that IL-1β causes memory impairment at this dose (Barrientos *et al.*, 2004, Goshen *et al.*, 2007, Hein *et al.*, 2007, Pugh *et al.*, 1999). However, the dose I administered to rats (100 ng) is 10 times greater than that used in previous studies and showed no memory impairment. Furthermore a study in which peripherally administered *E. coli* caused hippocampal-dependent memory impairment in the fear conditioning task showed that hippocampal IL-1β levels were raised to 1 x 10^{-5} pg/mg and 1.2 x 10^{-5} pg/mg at 2 and 4 h after injection respectively (Barrientos *et al.*, 2009) whereas after the central administration of IL-1β I obtained IL-1β concentrations of 15 pg/mg and 22 pg/mg at 2 and 4 h after IL-1β injection in the hippocampus respectively. In order to determine if IL-1β does act on memory in a U-shaped dose-response it is important to test the effect that several doses of IL-1β have on hippocampal-dependent memory. Secondly, all of the studies which showed hippocampal-dependent memory impairment administered IL-1β intracerebroventricularly or intrahippocampally (Barrientos *et al.*, 2004, Goshen *et al.*, 2007, Hein *et al.*, 2007, Pugh *et al.*, 1999), whereas I administered IL-1β via the cisterna magna. A study in which the tracer inulin was injected into the cisterna magna or the third cerebral ventricle of rats noted that there were subtle differences in the early distribution of the tracer within the brain depending on the site of injection (Proescholdt *et al.*, 2000), therefore it may be possible that the different routes of injection affect memory differently. In order to determine if there is a difference between the effect each route has on memory, injections via the cisterna magna, third cerebral ventricle and hippocampus should be compared using the same dose of IL-1β.

My results showed that rats that received 100 ng of IL-1β 2 h *before* they were conditioned froze more during conditioning (learning) than rats that received IL-1β immediately *after* conditioning and rats that received BSA (see Figure 3.8A). Rats that received IL-1β 2 h *before* being conditioned froze approximately 13 % of the time they were in the context chamber, in addition they were febrile (see Figure 3.5) and had elevated brain IL-1β concentrations (see Figure 3.9) during

conditioning (learning), therefore the increased freezing time during conditioning may be indicative that rats were also lethargic. Furthermore, rats that were conditioned 2 h *after* receiving IL-1β showed both hippocampal and amygdala-dependent memory impairment (see Figure 3.8 B and C respectively).

Rats that received IL-1β 2 hours *before* being conditioned were febrile and had elevated brain IL-1β concentrations during conditioning and for several hours afterward (~ 8 h) therefore rats were under the influence of IL-1β during and after learning which gives an indication that IL-1β may have affected either memory acquisition or memory consolidation. During memory acquisition rats were under the influence of IL-1β which may have resulted in an abnormal sensory experience during conditioning (learning) and therefore may have caused the formation of an incomplete contextual representation (Rudy *et al.*, 2004) which will have resulted in both hippocampal and amygdala-dependent memory loss. In order to verify if memory acquisition was indeed affected it would be useful to test memory retention for the context and the tone before memory consolidation has taken place ~ 1 h after conditioning (Barrientos *et al.*, 2006) and if rats show memory impairment it can be confirmed that IL-1β administered 2 h *before* conditioning affected memory acquisition. Memory consolidation occurs in the hippocampal formation and the central administration of IL-1β has consistently been demonstrated to impair hippocampal-dependent memory through the impairment of LTP and neural plasticity (Goshen and Yirmiya, 2006, Huang and Sheng, 2010, Yirmiya and Goshen, 2011). If damage occurs to the hippocampus after the administration of a substance it will result in impaired hippocampal-dependent memory whereas amygdala dependent memory remains intact and unaffected (Phelps, 2004, Rudy *et al.*, 2004, Sanders *et al.*, 2003). Throughout the literature when memory consolidation is affected by IL-1β; memory impairment in the fear conditioning task is limited to hippocampal-dependent memory (Barrientos *et al.*, 2002, Barrientos *et al.*, 2004, Pugh *et al.*, 1998, Pugh *et al.*, 1999). However, I showed that the administration of IL-

1β 2 h *before* conditioning impaired both hippocampal and amygdala-dependent memory, therefore my results are inconsistent with the literature and consequently it is unlikely that memory consolidation was affected in my study.

4.2.1. Brain IL-1β concentrations

In view of the lack of information regarding sickness responses following the intra-cisterna magna injection of IL-1β I set out to further investigate these relationships by measuring the concentration of IL-1β in the hippocampus and hypothalamus at several time points after intra-cisterna magna injection of IL-1β. I chose to investigate the concentrations of IL-1β in the hypothalamus because the thermoregulatory centre of the brain is hypothesized to be located in the pre-optic nucleus of the hypothalamus (Conti *et al.*, 2004) moreover, the hypothalamus is also hypothesized to be the primary site for regulation of feeding (Plata-Salamán, 1998). Furthermore, I chose to investigate the concentration of IL-1β in the hippocampus because the hippocampus is the predominant brain structure involved in declarative or hippocampal-dependent memory (Phelps, 2004).

Although not significantly different across time points, BSA induced the presence of IL-1β in both the hippocampus and the hypothalamus (see Figure 3.9), this is not surprising as it has been reported that BSA can induce an inflammatory response when administered to rats (Jankovic *et al.*, 1962). Compared to rats that received BSA, rats that received IL-1β (100 ng) had significantly elevated IL-1β concentrations at 1, 4 and 8 h after injection in both the hypothalamus and the hippocampus (see Figure 3.9). Anomalously, at 2 h hippocampal and hypothalamic IL-1β concentrations were similar for rats that received either IL-1β or BSA, which most likely is due to a small sample size of rats that received BSA as well as high inter-individual variability. Nonetheless, there was sustained elevation in IL-1β concentrations (in both the hypothalamus (15 – 28 pg/mg) and hippocampus (15-

24 pg/mg)) from 1 - 8 h after injection within the group of rats that received IL-1β. The elevated concentrations of IL-1β in the hypothalamus at 1 h corresponded to the onset of the febrile response, moreover the return of IL-1β concentrations to baseline at 24 h corresponded to the resolution of fever which collectively gives an indication that IL-1β plays a role in the mediation of fever. In addition, by 24 h after injection rats were lethargic and showing signs of anorexia (decrease in food intake and body mass) which gives an indication that IL-1β may play a role in the initiation of lethargy and anorexia. Moreover, rats that received IL-1β 2 h *before* conditioning had elevated levels of IL-1β in the hippocampus during learning which resulted in memory impairment. Therefore, it appears that if learning occurs during a time in which IL-1β is elevated within the brain (~ 15 pg/mg) it could result in memory impairment.

4.3. Future studies

In order to further complete my current study it would be useful to examine the effect of several doses of LPS administered via the cisterna magna on memory (and the other sickness responses) in order to confirm whether centrally administered LPS exerts its effect on hippocampal-dependent memory in a U-shaped dose response, similarly to the U-shaped dose response seen with peripherally administered LPS (Pugh *et al.*, 1998). Moreover, it would be useful to map out the expression of hypothalamic and hippocampal pro-inflammatory cytokines IL-1β and TNF-α concentration at several time points following the intra-cisterna magna administration of LPS in order to investigate the role of these cytokines in LPS-induced hypothermia and fever, as well as to explore why LPS did not induce memory impairment in my study.

My results have demonstrated that memory impairment may appear in conjunction with other sicknesses responses if learning occurs when IL-1β is elevated in the brain. However, because IL-1β

was administered to rats 2 h *before* conditioning IL-1β may have affected either memory acquisition or memory consolidation. In order to determine whether memory acquisition was effected following administration of IL-1β, 2 h *before* conditioning, fear of the context should be tested ~ 1 h after conditioning, before memory consolidation has taken place. Moreover, the dose of IL-1β (100 ng) administered in my study has not previously been studied for its effect on memory, if IL-1β acts on memory in a U-shaped dose response 100 ng of IL-1β may have been too high to affect memory. Therefore it would be interesting to conduct a dose response study using IL-1β administered via the cisterna magna at doses previously seen to effect memory when administered via the third cerebral ventricle (e.g. 1 ng, 2 ng and 10 ng). In addition, the effect of IL-1β administered via the cisterna magna has not previously been examined for its effect on memory. There are differences between the early distribution of substances within the brain when administered via the cisterna magna and third cerebral ventricle (Proescholdt *et al.*, 2000); consequently there may be differences between the routes for their effect on memory. Therefore it would be of interest to compare the effect of one dose of IL-1β, administered via the cisterna magna and the third cerebral ventricle, on memory. My study could also potentially be replicated using a human model of infection by peripherally administering LPS and/or IL-1β to adults at different time points, either before or after a learning task has taken place. The human study would give some insight as to whether the results seen in my study are limited to animal models of infection or whether they may apply to humans as well.

Finally, infection and inflammation are not caused by the isolated increase of one pro-inflammatory cytokine but rather through the collective action of a pro-inflammatory network which includes multiple pro-inflammatory cytokines (e.g. IL-1, IL-6, IL-8, TNF-α) and other downstream mediators (e.g. PGE_2, Interferons) (Donzis and Tronson, 2014). Similarly when IL-1β is administered exogenously to rats it does not act on the brain alone, it exerts its effects on the brain both directly and indirectly via signaling of the pro-inflammatory network (Donzis and Tronson,

2014). Therefore many of the effects caused by the exogenous administration of IL-1β are due to the cumulative effect of many pro-inflammatory cytokines (Donzis and Tronson, 2014). Consequently, given the complexity of the relationship between IL-1β and memory, shifting focus from the individual effects of pro-inflammatory cytokines on memory to the effect of the whole pro-inflammatory cascade on memory by using live organisms or moieties may be more constructive in understanding the impact of immune activation on memory (Donzis and Tronson, 2014).

Chapter 5

Conclusion

My results suggest that a simulated, acute central infection in rats severe enough to induce a host of sickness responses including fever, anorexia and lethargy may occur without incurring any negative cognitive sequelae. The dissociation between memory impairment and other sickness responses in my study parallels emerging evidence in other rodent models of infection that peripherally administered IL-1β (Thomson and Sutherland, 2006) and fibroblast-stimulating lipopeptide-1, the moiety of *Mycoplasma salivarium* (Swanepoel *et al.*, 2011), can induce fever, anorexia and lethargy in the absence of memory impairment. However, as demonstrated by my study the timing of learning relative to inflammation within the brain is very important. If learning occurs during a time at which there is inflammation in the brain and/or when the host is exhibiting signs of sickness such as fever and lethargy it may result in memory impairment.

No direct extrapolation of the outcome of my study can be made to humans, moreover the results from my study cannot be extrapolated to all central infections. Cognitive damage as a result of central bacterial infections is dually mediated by centrally released pro-inflammatory cytokines and by the direct toxicity of bacterial components on the brain (Nau and Bruck, 2002, Weber and Tuomanen, 2007). Experimental models of bacterial meningitis using live organisms (*E. coli, Streptococcus pneumoniae, Klebsiella pneumoniae*) have consistently been demonstrated to cause cognitive deficits in rats (Barichello *et al.*, 2014a, Barichello *et al.*, 2013, Barichello *et al.*, 2010, Barichello *et al.*, 2014b). As confirmation of the dual mediation of cognitive damage, experimental models of bacterial meningitis in rats have demonstrated that live bacterial organisms result in the expression of pro-inflammatory cytokines throughout the brain including the hippocampus (Barichello *et al.*, 2013) and furthermore that bacterial toxicity results in neuronal apoptosis in the hippocampus (Braun *et al.*, 1999). Both, the expression of pro-inflammatory cytokines and bacterial toxins have previously been associated with impaired learning and cognitive dysfunction (Loeffler *et al.*, 2001). Interestingly, although experimental models of LPS infection have determined that

LPS is associated with cognitive dysfunction (Pugh *et al.*, 1998), as well as the expression of pro-inflammatory cytokines in the brain (Conti *et al.*, 2004), *in vitro* studies examining the toxicity of LPS have determined that LPS is free of the prototypical bacterial toxins which may cause cognitive impairments in central infections (Weber and Tuomanen, 2007). Therefore, although I simulated a central model of infection using both LPS and IL-1β my results cannot be extrapolated to other central bacterial infections because even though LPS and IL-1β both cause the release and synthesis of pro-inflammatory cytokines in the brain they are free from the prototypical bacterial toxins associated with other central bacterial infections.

In conclusion despite having a severe central infection, memory impairment is not a ubiquitous sickness response and may only occur in conjunction with fever, anorexia and lethargy when learning occurs during an immune reaction within the brain.

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ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2012/08/C4

APPLICANT: **Ms T L Baartman**

DEPARTMENT: **Department of Physiology**

PROJECT TITLE: Investigating sickness responses following central administration of different doses of fibroblast-stimulating lipopeptide-1 (FSL-1) to rats

Number and Species

100 Sprague-Dawley male rats (150-200 g)

Approval was given for to the use of animals for the project described above at an AESC meeting held on **27 March 2012**. This approval remains valid until **31 March 2014**.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

Conditions:

- Details on how often and for how long rats are run in the Morris Water Maze are provided to the committee.
- The dosages are motivated (The requested doses are higher than those reported in the referenced paper).
- Competency with injections into the cisterr magna must be demonstrated to Dr Erlwanger before any experimentation is conducted on live rats. 10 rats are provided and may be euthanized for this purpose.
- For live rats, injections into the cistern magna are only performed under anaesthesia.
- Post-surgery observation should continue for a minimum of two hours and should include the use of a score sheet.

Signed: _____ (Chairperson, AESC) Date: 4/5/12

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 4/5/12

cc: Supervisor:
Director: CAS

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ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2012/38/04

APPLICANT: **Ms T L Baartman**

DEPARTMENT: **Department of Physiology**

PROJECT TITLE: **Investigating sickness responses following central administration of different doses of a pyrogenic moiety of *Mycoplasma pneumoniae* (FAM-20) to rats**

Number and Species

102 male Sprague-Dawley rats

Approval was given for to the use of animals for the project described above at an AESC meeting held on **25 September 2012**. This approval remains valid until **30 September 2014**.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

Conditions:

- Score sheet used for monitoring condition of rats

Signed: _____ (Chairperson, AESC) Date: 5/10/12

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 8/10/12

cc: Supervisor:
Director: CAS

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ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2013/28/04

APPLICANT: Ms T Baartman

SCHOOL: Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: Investigating the temporal sequence of IL-1 beta synthesis in the brain during a simulated central infection in rats

Number and Species

160 male Sprague-Dawley rats

Approval was given for to the use of animals for the project described above at an AESC meeting held on 20130528. This approval remains valid until 20150527.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

Signed: _____ (Chairperson, AESC) Date: 5/6/13

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 5/6/13

cc: Supervisor: Dr T Swanepoel
Director: CAS

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