FEVER AND SICKNESS BEHAVIOUR DURING SIMULATED *MYCOPLASMA* INFECTION IN RATS

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy

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DECLARATION

I, Tanya Swanepoel, declare that the work contained in this thesis 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 Doctor of Philosophy in the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg.

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RESEARCH OUTPUTS

Published papers

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International conference presentations

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Local conference presentations

Swanepoel T., Harden L.M., Harvey B.H., Roth J. and Mitchell D. A new Gram-positive pyrogen.
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Swanepoel T., Harden L.M., Harvey B.H., Laburn H.P. and Mitchell D. Dissociation of learning and memory from other sickness behaviours following simulated, acute *Mycoplasma* infection in rats.
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ABSTRACT

The acute phase response is implemented by infected hosts in response to exposure to pathogens, including bacteria and viruses. Acute phase responses comprise physiological and behavioural changes, such as fever and a range of “sickness behaviours”, including lethargy and anorexia as well as impairment in learning and memory. Similar to other sickness behaviours, the effect of infection on learning and memory processes has been attributed to the release of pro-inflammatory cytokines, including interleukin-1β (IL-1β) and interleukin-6 (IL-6). However, the exact role of IL-1β and IL-6 in mediating infection-induced cognitive impairment is not clear. Unlike fever, anorexia and lethargy, which may benefit an infected host, the physiological benefit of cognitive impairment during illness is doubtful.

To initiate an acute phase response experimentally, moieties of typical bacteria (Gram-negative and Gram-positive) and viruses frequently are employed. Moieties from the atypical *Mycoplasmas* seldom have been used. Consequently, there is a dearth of information on the physiological mechanisms that underlie acute phase responses following *Mycoplasma* infection, despite the prevalence of the disease in the general population. *Mycoplasma pneumoniae* frequently causes community-acquired pneumonia, which may have serious extra-pulmonary complications, including cognitive deficits. Therefore, I investigated fever and sickness behaviours as well as cytokine responses in simulated, atypical *Mycoplasma* infection.

I implemented an animal model of simulated *Mycoplasma* infection and characterised fever and sickness behaviours, including lethargy and anorexia as well as impairment in learning and memory during acute and recurrent acute simulated infection. I also characterized the response in the periphery and in the brain of individual pro-inflammatory cytokines, IL-1β and IL-6, to administration of fibroblast-stimulating lipopeptide-1 (FSL-1), which simulates

Mycoplasma infection. Using rats, I recorded fever and lethargy with biotelemetry and assessed effects of simulated *Mycoplasma* infection on learning and memory using a Morris Water Maze. In addition, I examined the histology of tissue from the hippocampus, a key brain area involved in spatial learning and memory, to assess residual effects of simulated *Mycoplasma* infection on learning and memory.

I showed that bolus administration of a pyrogenic moiety from *Mycoplasma*, fibroblast-stimulating lipopeptide-1 (FSL-1), dose-dependently induced fever, lethargy, anorexia and body mass stunting in rats. However, FSL-1 administration did not induce concomitant impairment in spatial learning and memory. Importantly, at the time of testing in the Maze, I found the concentrations of IL-1β to be up-regulated in both the hypothalamus and the hippocampus, while the concentrations of IL-6 were unaffected. I also showed that recurrent acute injections of FSL-1, at 10 d intervals, induced recurrent fevers, lethargy and anorexia without the development of pyrogenic tolerance to any of the sickness responses measured. However, there was no residual body mass stunting in rats and also no growth retardation, despite the recurrent simulated infection. Equally importantly, there were neither lasting detrimental effects on spatial learning and memory nor any residual histological damage to the hippocampus of rats.

My findings in simulated *Mycoplasma* infection are important, firstly because *Mycoplasma* infection is prevalent in both developing and developed countries and frequently causes outbreaks, and secondly because *Mycoplasma* infection affects children and adolescents of school-going age. My findings also are encouraging: although lasting detrimental effects, including impairment in learning and memory as well as body mass stunting may occur in other infections, these appear not to be inevitable outcomes in *Mycoplasma* infection.

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 2007/72/04, AESC 2009/43/04, AESC 2010/34/04.

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Tanya

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LIST OF ABBREVIATIONS

BBB	blood-brain barrier
CD36	Cluster of Differentiation 36
CFU	Colony forming units
CINC	cytokine-induced neutrophil chemoattractant-1
CNS	central nervous system
CpG DNA	cystein-phosphodiester-guanine deoxyribonucleic acid
CVOs	circumventricular organs
dsRNA	double-stranded ribonucleic acid
E. coli	*Escherichia coli*
EP_3	Prostaglandin E receptor 3
FSL-1	Fibroblast-stimulating lipopeptide-1
G+ bacteria	Gram-positive bacteria
G- bacteria	Gram-negative bacteria
H3N2	Influenza A virus subtype H3N2
i.a.	intra-arterial
i.c.v.	intracerebroventricular
IFN-α	interferon alpha

i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
IL-1β	interleukin-1 beta
IL-1ra	interleukin-1 receptor antagonist
IL-6	interleukin-6
IL-10	interleukin-10
IRAK	interleukin one receptor-associated kinase
LPS	lipopolysaccharide
MALP-2	macrophage-activating lipopeptide-2
MDP	muramyl dipeptide
M. fermentans	*Mycoplasma fermentans*
M. pneumoniae	*Mycoplasma pneumoniae*
M. salivarium	*Mycoplasma salivarium*
MyD88	Myeloid differentiation factor 88
NF_KB	nuclear factor kappa-B
PAMPs	pathogen-associated molecular patterns
PRR	pattern recognition receptors
PGs	prostaglandins

PGE2	prostaglandin E2
poly I:C	polyinosinic:polycyidylic acid
SCID	severe combined immune deficient
SEA	Staphylococcus enterotoxin A
S. aureus	*Staphylococcus aureus*
s.c.	subcutaneous
S. pneumoniae	*Streptococcus pneumoniae*
S. typhosa	*Salmonella typhosa*
TLRs	Toll-like receptors
TLR 4	Toll-like receptor four
TLR 2/6	Toll-like receptor two/six
TNF-α	tumour necrosis factor-alpha
TRAF-6	tumour necrosis factor receptor-associated factor-6
TRI	temperature response index

PREFACE

In the context of the field of 'host-pathogen interaction', approaches into the pathology of various infections have elucidated new pathways that we can explore to better understand and treat debilitating illness-induced symptoms of the host. Fever and 'sickness behaviours,' a well-coordinated set of behavioural responses, accompany most if not all types of infection and may compromise the sick individual's quality of life. In spite of the prevalence, sickness behaviour in *Mycoplasma* infections is under-researched, particularly the sickness behaviour of impairment in learning and memory. In addition to being a causative agent for community-acquired pneumonia, the atypical bacterium, *Mycoplasma pneumoniae*, has been linked to various life-threatening, extra-pulmonary infections and memory deficits. Infections with *M. pneumoniae* often present asymptomatically giving the infection the nickname of "walking pneumonia". However, the general perception that *Mycoplasma* infection is self-limiting is not true. The possibility of detrimental consequences, including residual impairment in learning and memory, often is overlooked.

Studying the effects of *Mycoplasma*-induced activation of the innate immune system experimentally has been hampered by a lack of an adequate experimental model that does not involve the live pathogen. Recently, it was discovered that a pyrogenic moiety from *Mycoplasma salivarium*, namely fibroblast-stimulating lipopeptide-1 (FSL-1), induces fever and sickness behaviour in rats. However, there has been no investigation of the effects of FSL-1, which simulates *Mycoplasma* infection, on the sickness behaviour of impaired learning and memory. There also has been no investigation into the long-term consequences of simulated *Mycopolasm*a infection, including residual detrimental effects on learning and memory, residual histological changes in the brain or residual physical impairment.

Therefore, the aim of my thesis was to investigate systematically both physiological and behavioural responses, including fever, lethargy, growth and anorexia as well as impairment in learning and memory, in acute (**Chapter 3**) and recurrent (**Chapter 5**) simulated *Mycoplasm*a infection in rats. In order to achieve these aims, I had to acquire the techniques for administering pathogen-associated molecular patterns (PAMPs) and for measuring fever, anorexia and lethargy in rats (**Chapter 2**), which already were established in the laboratory in which I conducted my research (University of the Witwatersrand). However, no one in the laboratory previously had worked with the PAMP that I intended to administer to rats, namely FSL-1. I had to determine the most suitable drug concentrations, dosages and intervention periods for FSL-1 in order to establish a model suitable for recurrent acute infections without the development of pyrogenic tolerance (**Chapter 5**). Only one other study in the laboratory previously had attempted simulation of recurrent infection and had analysed the results from simulated recurrent infections (**Chapter 5**).

Finally and most importantly, no one in the laboratory in which I conducted my research had implemented successfully a Morris Water Maze behavioural model, or indeed any other measure of learning and memory. The Morris Water Maze, which assesses spatial learning and memory in rodents, was a critical component for projects during my PhD (**Chapter 2, 3 and 5**). I also applied skills and experience that I have gained during research for my Masters degree (North-West University, NWU), such as excising certain areas of the rat brain. For studies during my PhD I measured brain and plasma concentrations of the pro-inflammatory cytokines, interleukin-1β (IL-1β) and interleukin-6 (IL-6) (**Chapter 4**) and also investigated histological pathology of certain regions of the brain (**Chapter 5**).

AUTHOR'S CONTRIBUTIONS

Contributions of each co-author to the published or submitted papers contained in this thesis are listed below:

Chapter 3 and 4:

Swanepoel T., Harvey B.H., Harden L.M., Laburn H.P. and Mitchell D. (2011). Dissociation between learning and memory impairment and other sickness behaviours during simulated *Mycoplasma* infection in rats. *Brain, Behavior, and Immunity* **25:** 1607-1616.

I set up collaboration between the University of the Witwatersrand and North-West University (NWU, Potchefstroom Campus). The experimental design then was formulated from discussions with my supervisors, Duncan Mitchell and Brian Harvey (NWU) as well as Lois Harden. I set up a Morris Water Maze for measuring learning and memory in animal models and carried out all behavioural data collection. I employed a new experimental pyrogen, which had not been used previously at the University of the Witwatersrand, and injected all animals myself. I carried out all experimental preparations, data collection, brain tissue and blood collection as well as cytokine assays. I interpreted the results and performed the statistical analyses with guidance from my supervisors as well as Lois Harden and Helen Laburn. I drafted the manuscript and all authors edited the manuscript.

Chapter 5:

Swanepoel T., Harvey B.H., Harden L.M., Laburn H.P. and Mitchell D. (2011). Simulated recurrent *Mycoplasma* infection in rats induces recurrent sickness responses without residual impairment in spatial learning and memory. *Physiology and Behavior* **105:** 800-808.

The experimental design was formulated from discussions with my supervisors, Duncan Mitchell and Brian Harvey (NWU) as well as Lois Harden. I carried out all experimental preparations, injections of experimental pyrogen to animals, data collection and brain tissue collection. I interpreted the results and performed the statistical analyses with guidance from my supervisors as well as Lois Harden and Helen Laburn. I drafted the manuscript and all authors edited the manuscript.

CHAPTER 1

INTRODUCTION

During infective illness the infected individual responds by implementing the "acute phase response" through activation of the innate immune system. The acute phase response comprises a series of physiological and behavioural changes hypothesized to accelerate recovery of the individual, to prevent ongoing damage from infecting pathogens (see Baumann and Gauldie, 1994; Johnson, 2002). Physiological changes include elevation of body temperature, including fever, and a suite of non-specific, non-thermal behavioural changes, collectively known as "sickness behaviour" (Hart, 1988). These changes are thought to be well organized, adaptive responses that are considered critical to host survival (Hart, 1988, 1991; Romanovsky and Szekely, 1998; Soszynski, 2003; Adelman and Martin, 2009). However, scientists have challenged the idea that fever is always beneficial to the host (e.g. Kluger, 1986), and beneficial only to the host (e.g. Ewald, 1980).

Sickness behaviours are not reflexive sickness-induced reactions or passive responses to illness, nor are they signs of debilitation; rather, sickness behaviours are thought to be active, defensive responses that reflect an evolved adaptive state of altered motivation that enables the sick individual to counteract the infection (Aubert *et al.*, 1995; Maier and Watkins, 1998; Konsman *et al.*, 2000; Dantzer, 2001a). Together with fever, sickness behaviours, including anorexia, lethargy, fatigue, malaise, hypersomnia, hyperalgesia and disturbed mood constitute the clinical manifestations of the acute phase response (Hart, 1988; Konsman *et al.*, 2002; Johnson, 2002). In recent years cognitive alterations, which include learning and memory disturbances, also have become recognised as members of the sickness behaviour syndrome, both clinically and experimentally (Capuron *et al.*, 1999; Dantzer, 2001a; Reichenberg *et al.*, 2001; Cunningham and Sanderson, 2008). But, is there really any adaptive value to being cognitively impaired during illness? It may well be that there is no adaptive purpose.

Clinical studies and animal models of infection have demonstrated that the physiological mechanisms underlying fever and sickness behaviours are similar, if not identical, in humans and laboratory animals. Therefore, investigations into sickness behaviour use animal models to mimic the human condition. To avoid ethical issues arising from administering live pathogenic organisms to humans and laboratory animals, pyrogenic moieties of the organisms, e.g., killed organisms or their cell wall components are used during simulated infection to stimulate the innate immune system in the same way as it would be in the actual infection. For example, the glycolipid pyrogenic moiety extracted from cell membranes of Gram-negative bacteria, lipopolysaccharide (LPS), is used widely to simulate Gram-negative infection. So too is polyinosinic:polycytidylic acid (poly I:C) used to mimic the double-stranded RNA of viruses, and peptidoglycans (e.g. muramyl dipeptide; MDP) to mimic Gram-positive bacteria. In experimental animals, fever and sickness behaviour, particularly learning and memory impairment have been studied extensively in infections relating to typical bacteria, i.e. Gram-positive and Gram-negative bacteria (Wellmer *et al.*, 2000; Loeffler *et al.*, 2001; Irazuzta *et al.*, 2001; Bifrare *et al.*, 2003; Hoffmann *et al.*, 2007; Cunningham and Sanderson, 2008), and also in infections relating to viruses (Kent *et al.*, 2007; Self *et al.*, 2009; Okun *et al.*, 2010; Dilger and Johnson, 2010). Aside from these three well established animal models of infection, sickness behaviours must be characterized in various other pathogens. Comparatively little is known about fever and sickness behaviours resulting from 'atypical' bacteria, including the *Mycoplasmas*, despite the prevalence of these pathogens.

The *Mycoplasmas* are causative agents for potentially serious respiratory diseases in humans and other animals and affect people of all ages, including otherwise healthy children between the ages of 5-15 years. Community acquired pneumonia, one of the conditions caused by the *Mycoplasmas,* occurs in a recurrent fashion. Pneumonia still is one of the

main causes of death among children, especially in developing countries (McIntosh, 2002; Waites and Talkington, 2004; Sinaniotis and Sinaniotis, 2005). In humans, pneumonia caused by the *Mycoplasmas* is characterized by headache, malaise, non-productive cough, a low-grade fever, and sometimes wheezing (Ruuskanen and Mertsola, 1999; McIntosh, 2002), but some patients do develop severe life-threatening pneumonia (e.g. Miyashita *et al.*, 2007). In animal models, intracerebroventricular (i.c.v.) administration of heat-inactivated *Mycoplasma fermentans* (*M. fermentans*) to rodents induced significant elevations in body temperature, decreased locomotor and exploratory activity, and suppressed the consumption of food intake (Yirmiya *et al.*, 1997). Apart from the sickness behaviours of lethargy and anorexia, very little is known about another component of sickness behaviour, namely learning and memory impairment, following simulated *Mycoplasma* infection.

This chapter reviews literature on animal models of sickness behaviours induced by simulated bacterial infections, other than *Mycoplasma* infection, and highlights the physiological consequences of such infections. This chapter also raises the questions relating to sickness behaviour in simulated *Mycoplasma* infection that will be addressed in my thesis. Because the febrile response to simulated, systemic bacterial infection is thoroughly characterised and because fever is an inevitable accompaniment of the acute phase response, the background of this thesis will focus mainly on sickness behaviours, and not necessarily on fever.

1.1. INFECTION, PATHOGEN RECOGNITION AND INNATE IMMUNITY

Stimulation of the host's immune system in response to systemic infection, inflammation and injury, usually is initiated in the periphery. However, many of the sickness responses are mediated by the brain and not by peripheral organs. It is hypothesized that during illness the

brain and the immune system establish a bi-directional communication network: the immune system is responsible for providing the brain with information about processes occurring in the periphery, and the brain, in turn, influences and controls activity of peripheral organs and cells of the immune system (see Blalock *et al.*, 1985; Maier and Watkins, 1998). Activation of the host's innate immune system, i.e. detection of pathogens, relies upon recognition of conserved motifs unique to pathogens, known as pathogen-associated molecular patterns (PAMPs). PAMPs are essential for the metabolism and survival of pathogens and they are produced only by pathogens and not by host cells. In this way the host's immune system can distinguish between self and non-self.

PAMPs reflect intrinsic properties of pathogens of a given class (Medzhitov and Janeway, 1997) and act as ligands for pattern recognition receptors (PPR) on immune cells in the host, including the Toll-like receptor family. To date, ten variants of human TLRs (TLR1-TLR10) have been identified (see Figure 1.1), which mediate recognition of a range of pathogens (for reviews see Janssens and Beyaert, 2003; Casanova *et al.*, 2011). TLRs are believed to function as dimers and most TLRs appear to function as homodimers, except TLR1 and TLR6, which form heterodimers with TLR2 (Figure 1.1, Takeuchi *et al.*, 1999; Ozinsky *et al.*, 2000; Takeda *et al.*, 2002).

In the host, interaction between Toll-like receptors (TLRs) and PAMPs initiates the innate immune response to the invading pathogen (Janeway and Medzhitov, 2002; Kapetanovic and Cavaillon, 2007), which leads to activation of intracellular signal transduction pathways and the formation of circulating pro-inflammatory cytokines (Akira *et al.*, 2001; Imler and Hoffmann, 2001; Cartmell and Mitchell, 2005).

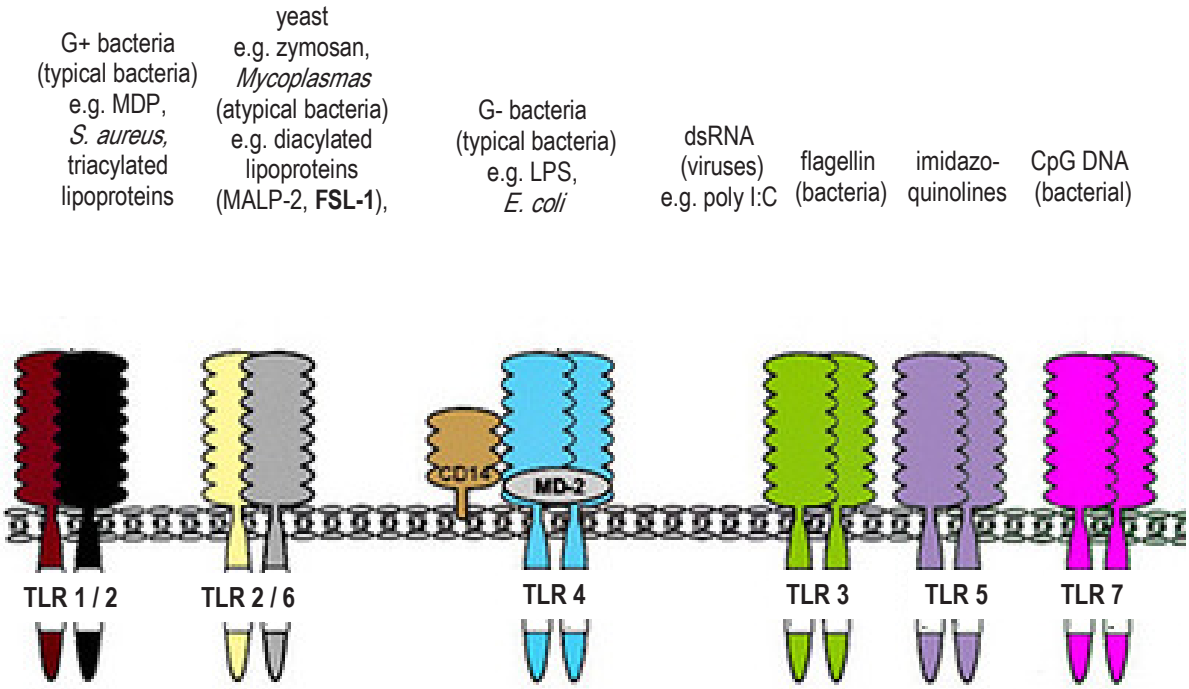

Figure 1.1. The human Toll-like receptor family with its specific PAMPs (adapted from Janssens and Beyaert, 2003). G+, Gram-positive bacteria; MDP, muramyl dipeptide; *S. aureus, Staphylococcus aureus*; MALP-2, macrophage-activating lipopeptide-2; FSL-1, fibroblast-stimulating lipopeptide-1; G- bacteria, Gram-negative bacteria; LPS, lipopolysaccharide; *E.coli, Escherichia coli*; dsRNA, double-stranded ribonucleic acid; poly I:C, polyinosinic:polycyidylic acid; CpG DNA, cystein-phosphodiester-guanine deoxyribonucleic acid; CD14, Cluster of Differentiation 14; MD-2, myeloid differentiation; TLR, toll-like receptor.

Bacterial PAMPs include peptidoglycans and lipoteichoic acid from Gram-positive bacteria as well as endotoxin (a mixture of pure LPS and other proteins, Hitchcock *et al.*, 1986) from the outer membrane of Gram-negative bacteria (see Akira *et al.*, 2006). Unlike typical Gram-negative and Gram-positive bacteria, the *Mycoplasmas* do not possess a cell wall and therefore are often referred to as 'atypical' bacteria. Thus, the *Mycoplasmas* do not possess bacterial modulins such as LPS, lipoteichoic acids or peptidoglycans. However, there is evidence to show that membrane-bound lipoproteins present in cell membranes of all *Mycoplasma* species are capable of activating monocytes, macrophages, lymphocytes or fibroblasts that are cytokine-producing cells (Razin *et al.*, 1998; Shibata *et al.*, 2000).

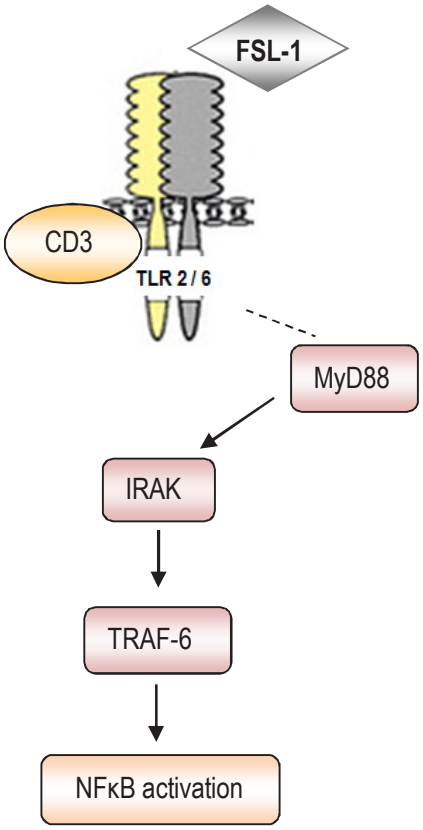

Figure 1.2. Signaling pathways employed by diacylated lipoproteins e.g. fibroblast-stimulating lipopeptide-1 (FSL-1) (adapted from Mitchell *et al.*, 2007). FSL-1, fibroblast-stimulating lipopeptide-1; CD36, Cluster of Differentiation 36; TLR 2/6, Toll-like receptor 2/6; MyD88, Myeloid differentiation factor 88; IRAK, interleukin one receptor-associated kinase; TRAF-6, tumour necrosis factor receptor-associated factor-6; NFκB, nuclear factor kappa-B.

The diacylated lipoprotein, fibroblast-stimulating lipopeptide-1 (FSL-1), which represents the NH_2-terminal sequence of the 44 kDa lipoprotein (LP44) of *Mycoplasma salivarium* (Shibata *et al.*, 2000; Nakamura *et al.*, 2002; Okusawa *et al.*, 2004), has been identified as a potent immunostimulatory compound (Okusawa *et al.*, 2004; Hübschle *et al.*, 2006; Kiura *et al.*, 2006; Greis *et al.*, 2007) signaling through the TLR2 and TLR6 (TLR2/6) heterodimer to

stimulate cells implicated in innate immunity (Figure 1.2, Hübschle *et al.*, 2006; Greis *et al.*, 2007).

Similar to other TLRs, the TLR2/6 heterodimer also signals through the myeloid differentiation factor 88 (MyD88)-dependent pathway (Wesche *et al.*, 1997; Takeuchi *et al.*, 2000). Upon activation with one of its agonists, namely FSL-1, TLR2/6 assembles with CD36, a class B pattern recognition/scavenger receptor involved in inflammation and immunity (Triantafilou *et al.*, 2006; Silverstein *et al.*, 2010). The resulting TLR2/6-CD36 complex is essential for triggering the inflammatory response and leads to signal transduction via receptor associated proteins, including MyD88, IL-1 receptor-associated kinase (IRAK) and TNF receptor-associated factor-6 (TRAF-6) (Hoebe *et al.*, 2005). Downstream signalling cascades ultimately lead to the activation of the transcription factor nuclear factor-κB (NF-κB) (for review see Aderem and Ulevitch, 2000) (see Figure 1.2). NF-κB in turn, promotes transcription and expression of immune response genes, including genes for inflammatory mediators and pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) (for reviews see Miyamoto and Verma, 1995; Rasmussen *et al.*, 2009). Apart from their critical role in innate immunity, TLRs have been implicated in mediating neural plasticity under physiological conditions in various brain regions, including the hippocampus (reviewed in Okun *et al*., 2011) and therefore may have a role in hippocampal-dependent learning and memory (see Okun *et al.*, 2010).

1.2. FEVER AND SICKNESS BEHAVIOURS

In human health care, surprisingly little attention has been given to the fact that, in addition to becoming febrile infected hosts develop sickness behaviours. It is the sickness behaviour,

rather than the fever, which compromises quality of life. Because sickness behaviours often outlast the fever (Pereira and Begum, 1987; Hübschle *et al.*, 2006) it may well be that the non-thermal sickness behaviours, rather than the hyperpyrexia, are responsible for long-term consequences. The sickness behaviours relevant to my studies include anorexia and lethargy as well as impairment in learning and memory. These three phenomena, all of which incur considerable costs, may have deleterious long-term sequelae, especially in children: anorexia because of growth retardation, lethargy because of loss of motivation for life-sustaining processes or other fitness-enhancing activities, and impairment of learning and memory because of subsequent cognitive retardation. Because exposure to infectious agents during childhood is inevitable (Levy *et al.*, 1999), those patients experiencing recurrent bouts of infections are more likely to suffer from long-term consequences. A high prevalence of recurrent childhood infections and recurrent fever accompanied by sickness behaviours can hamper seriously the overall development of children (Martorell *et al.*, 1975; Rowland *et al.*, 1988). Stunting has been reported in children (Cole and Parkin, 1977; Pereira and Begum, 1987; Black, 1991; Stephensen, 1999), but it is not the only long-term consequence of recurrent infection. Residual impairment in learning and memory was shown in animal models of acute (Wellmer *et al*., 2000), acute repeated or chronic simulated bacterial infection (Aubert *et al.*, 1995; Hauss-Wegrzyniak *et al.*, 1998; Hauss-Wegrzyniak *et al.*, 1999; Sparkman *et al.*, 2005c). To date, studies have not investigated the consequences after apparent recovery from recurrent acute infections, where the host recovers between infections.

In addition, early immune activation, such as occurs during prenatal or neonatal infection, affects not only the febrile response, but also the neuroendocrine, neurochemical and behavioural responses later in life (Hornig *et al.*, 1999; Boisse *et al.*, 2004; Spencer *et al.*, 2005; Samuelsson *et al.*, 2006). For example, neonatal rats exposed to 100 $\mu g.kg^{-1}$ LPS

showed significant increases in anxiety and fear processes when they were tested eight weeks later (Spencer *et al.*, 2005). Similarly, prenatal exposure of rats to intraperitoneal (i.p.) injections of IL-6, administered three times at two-day intervals, resulted in impairment of spatial learning when the rats where tested at 20 weeks of age (Samuelsson *et al.*, 2006).

During infection fever is initiated by specific pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6 (Luheshi and Rothwell, 1996). Interaction between cytokines plays a pivotal role in causing not only the febrile response, but also sickness behaviours (for reviews see Dantzer, 2001a, 2004). In the CNS, cytokines (and their receptors) are expressed constitutively under both normal and pathological states. However, CNS concentrations of pro-inflammatory and anti-inflammatory cytokines may increase greatly, when, after appropriate stimulation, these cytokines are induced peripherally, for instance during fever induction (for review see Szelényi, 2001). Although some cytokines are involved in the pathology accompanying infection and inflammation (i.e. pro-inflammatory cytokines), others also seem to have a crucial role in host's defence against disease (i.e. anti-inflammatory cytokines) (see Oppenheim, 2001) and as therapeutic agents to suppress inflammatory processes (e.g. Henderson and Poole, 1994).

In the brain, pro-inflammatory cytokines induce the production of prostaglandins (e.g. Feldberg and Saxena, 1975a, b), including prostaglandin E_2 (PGE_2), which then stimulates the neural pathways that raise body temperature (for reviews see Kluger, 1991; Saper and Breder, 1994; Roth *et al.*, 2006; Hopkins, 2007). However, Gram-positive pathogens and their cell wall components can bypass the cytokine network and still activate the key intermediate, PGE_2, which causes fever (see Mitchell and Laburn, 1997). Apart from its pyrogenic action, PGE_2 also is critically involved in brain-mediated mechanisms underlying sickness behaviour (Johnson and von Borell, 1994; Teeling *et al.*, 2007; Kent *et al.*, 2007). It

is generally believed that sickness behaviours, which are confined to the period during and soon after infection, enable the host to conserve energy needed for the increased metabolic costs of fever and to direct the energy reserves to immune responses, in an effort to increase clearance of the invading pathogen and to fight infection. However, it has been hypothesized that host sickness behaviour, somehow, could benefit the invading pathogen (Poulin, 1994, 1995). It is therefore possible that the host's cytokine network, which protects the host against the consequences of invading pathogens by inducing fever and sickness behaviours, can be manipulated by the pathogen so as to benefit itself (see Henderson *et al.*, 1996).

1.2.1. Pro-inflammatory cytokines as mediators of fever and sickness behaviours

The role of pro-inflammatory cytokines in mediating sickness responses, including fever, lethargy and anorexia in humans, was confirmed when cytokines were administered to treat cancer patients and patients with chronic viral infections, who then developed typical clinical features of illness (e.g. fever, lethargy, anorexia) (Renault and Hoofnagle, 1989; Dinarello, 1997). Experimental animal studies then followed and confirmed the role of pro-inflammatory cytokines, including IL-1β and IL-6, in mediating fever (Saper and Breder, 1992; Luheshi and Rothwell, 1996; Nilsberth *et al.*, 2009) and a suite of sickness behaviours (for review see Dantzer and Kelley, 2007).

Although IL-1 consists of two isoforms, IL-1α and IL-1β (Dinarello, 1991), there appears to be no difference between the biological actions of these two cytokines (Dinarello, 2005). While IL-1α mainly remains in the cell, IL-1β is secreted to play a role in host defense responses to infection or injury, including fever (for review see Dinarello, 1996) and sickness behaviours (e.g., Rothwell and Hopkins, 1995). IL-1β is considered the most potent endogenous pyrogen whether injected either systemically or into the brain (Dascombe *et al.*,

1989; Rothwell *et al.*, 1996; Dinarello, 1996; Miller *et al.*, 1997a; Miller *et al.*, 1997b). In the brain IL-1β is expressed at low concentrations, but IL-1β concentrations may increase massively during pathological conditions, such as during local, systemic of CNS insults (e.g., Szelényi, 2001). Brain IL-1β interacts with its receptor, IL-1R, which is expressed on cells (e.g., microglia and astrocytes) in the hypothalamus and the hippocampus (Loddick *et al*., 1998; Conti *et al*., 2008). Because of its abundant expression in these two areas of the brain, IL-1β has a critical role in not only fever, but also brain-controlled sickness behaviours, including lethargy, anorexia and impairment in learning/memory. Numerous studies have confirmed the role of central IL-1β in mediating fever (for review see Leon, 2002), lethargy (e.g., Harden *et al*., 2008), anorexia (e.g., Kent *et al.*, 1994; Layé *et al.*, 2000) as well as cognitive impairment (for review see Goshen and Yirmiya, 2007).

Similar to IL-1β, brain IL-6 also interacts with its receptor, IL-6R which is also is expressed on cells in the hypothalamus and the hippocampus (Loddick *et al*., 1998; Conti *et al*., 2008). It has been hypothesized that brain IL-6 acts synergistically with IL-1β in the brain to induce fever and sickness behaviour (Cartmell *et al*., 2000; Harden *et al.,* 2008). The contribution of IL-6 (whether released peripherally or centrally) in mediating fever (e.g. LeMay *et al.*, 1990; Cartmell *et al.*, 2000; Harden *et al.*, 2006), lethargy and anorexia (e.g.Hübschle *et al.*, 2006; Harden *et al.*, 2006; Greis *et al.*, 2009) as well as impaired learning and memory (Goshen and Yirmiya, 2007; Dugan *et al.*, 2009) also is well established. However, a recent study suggested that the role of IL-6 in mediating LPS-induced lethargy and anorexia appears to less important than its crucial role in mediating LPS-induced fever (Harden *et al*., 2011). Moreover, the role of IL-6 in mediating the sickness behaviour of impaired learning and memory also seems to be complex (for review see Goshen and Yirmiya, 2007). Nevertheless, because of their prominent and well established roles in fever and sickness

behaviours, measurement of IL-1β and IL-6 in the circulation as well as in the brain became part of my investigations (see **Chapter 4**).

Pro-inflammatory cytokines are produced by peripheral immune cells, e.g. macrophages (e.g. see Maier and Watkins, 1998) and by cells in the central nervous system (CNS), e.g. microglia, astrocytes, neurones and endothelial cells (e.g. see Hopkins and Rothwell, 1995; Szelényi, 2001). Systemic exposure to exogenous pathogens induces the synthesis and release of pro-inflammatory cytokines, including IL-1β and IL-6, and other soluble mediators from activated immune cells at the site of infection/inflammation. Upon interaction with their specific receptors in the brain cytokines induce *de novo* synthesis of secondary pro-inflammatory cytokines or other mediators, including PGE_2.

When an exogenous pathogen gains access to the brain (e.g. in encephalitis or meningitis) it is possible that the pathogen itself could induce the synthesis and release of brain-derived cytokines and so induce fever, in this way by-passing peripherally released cytokines. However, experimental studies that do not support this sequence of events have shown that the febrile response to intrathecal administration of LPS has a delayed onset compared with the febrile response to i.p. administration of LPS (reviewed in Coceani and Akarsu, 1998). Thus, in CNS infection communication across the blood-brain barrier (BBB) between peripheral and central mediators (e.g. cytokines) may not be critical for the development of fever, unless the long delay in the onset of the febrile response implies that there indeed is communication. Without the synthesis and release of cytokines either peripherally, centrally or both, communication between the immune system and the brain, and therefore the development of acute phase responses during infection, may not be possible.

Evidence that blood-borne cytokines could affect aspects of the CNS, including the induction of brain-mediated fever and sickness behaviours, originated from experimental studies in which cytokine neutralizing antibodies, administered peripherally, were found to inhibit fever and sickness behaviours (Kent *et al.*, 1992; Roth *et al.*, 1997b; Cartmell *et al.*, 2000; Roth and De Souza, 2001; Harden *et al.*, 2010). When peripheral cytokines are released into the circulation they may either act as humoral mediators between the periphery and the brain or interact with their specific receptors on glial cells in the circumventricular organs (CVOs) (see Schobitz *et al.*, 1994). CVOs are regions in the brain where the BBB is 'leaky' (Blatteis *et al.*, 1983; Blatteis, 1992). Based on the results of studies conducted over the last two decades, the following four specialized mechanisms that allow blood-borne cytokines access to the brain have been proposed (for reviews see Dantzer, 1994; Quan and Banks, 2007): (1) active and saturable (slow) transport into the brain via carrier-mediated transport mechanisms; (2) entering the brain via CVOs; (3) secretions of secondary mediators (e.g. nitric oxide and PGE_2) from endothelial- and perivascular cells in the BBB; (4) rapid signaling via the neural route, e.g., via the vagus nerve, which may activate specific neural pathways in the brain and also stimulate microglia in the brain to produce cytokines (see Roth *et al*., 2006). However, the role of the vagus in mediating fever and sickness behaviours is limited to a certain dose of LPS (Romanovsky *et al.*, 1997) administered peripherally (Bluthé *et al.*, 1996; Goldbach *et al.*, 1997). Thus, the BBB plays a dynamic role in the communication of peptides, including cytokines, between the periphery and the CNS (Banks *et al.*, 1995).

1.2.2. Lethargy, anorexia and impaired learning and memory as sickness behaviours

The sickness behaviours typical of sick humans and other animals include lethargy, anorexia, social withdrawal, hyperalgesia, increased sleepiness and impaired cognitive

function (Kent *et al.*, 1992; Dantzer, 2001a, b). My study engages three of those behaviours, namely lethargy, anorexia and impaired learning and memory.

1.2.2.1. Lethargy

Infected humans often feel fatigued, listless and drowsy. Similarly, infected animals usually show signs of fatigue. These signs and feelings result in lethargy, a lack of motivation to engage in physical activity and exercise. The limited muscular activity may enable the body to save energy needed for the metabolic costs of fever. Conversely, the fatigue and limited physical activity also may result as a consequence of using the energy to generate fever. However, the energetic consequences of the lethargy of sickness behaviour, if there are any, remain unclear.

Impairment of physical activity has been reported in hosts affected with PAMPs from different classes of pathogens, real or simulated. Experimentally, administration of moieties from Gram-negative bacteria, e.g. *Escherichia coli* or LPS, Gram-positive bacteria, e.g. *Staphylococcus aureus* or MDP, viruses, e.g. influenza virus (H3N2) or poly I:C, the yeast *Saccharomyces cerevisiae*, e.g. zymosan, and also from the *Mycoplasmas*, e.g. macrophage-activating lipopeptide-2 (MALP-2) or FSL-1, have all induced lethargy in rodents (Katafuchi *et al.*, 2003; Campisi *et al.*, 2003; du Plessis *et al.*, 2005; Hübschle *et al.*, 2006, 2007; Jhaveri *et al.*, 2007; Hopwood *et al.*, 2009).

Lethargy can be measured experimentally by measuring different types of activity during infection, real or simulated. For example, changes in spontaneous locomotor activity of

animals in their cages can be measured with activity-sensitive radiotransponders or radiotransmitters that are implanted intra-abdominally (e.g. Kozak *et al.*, 1994; Hübschle *et al.*, 2006; Hopwood *et al.*, 2009), or with activity monitors in an automated open-field (e.g. Engeland *et al.*, 2003). These two techniques are used as indices of day-to-day "house-keeping" activity of animals. In addition, physical exercise measured as wheel running (e.g. in rats) is measured as an index of "voluntary" activity (e.g. Harden *et al.*, 2006, 2008; 2010). In LPS-induced lethargy in rats, suppression in voluntary exercise (measured as wheel-running) was a more prominent feature of illness than was depression in normal locomotor activity (measured as cage activity) (Hopwood *et al.*, 2009). LPS-induced lethargy also appears to be a more sensitive predictor of illness than is LPS-induced anorexia or fever (Skinner *et al.*, 2009).

1.2.2.2. Anorexia

Another sickness behaviour that forms part of the host's acute phase response is loss of appetite, manifested as anorexia (for review see Asarian and Langhans, 2010). As with lethargy, a variety of pathogens or cell wall components of pathogens can cause anorexia. The well known anorexigenic agents include microbial moieties, such as LPS (Sachot *et al.*, 2004; Hopwood *et al.*, 2009; Skinner *et al.*, 2009) and MDP (Langhans *et al.*, 1991; Plata-Salamán and Borkoski, 1993). Hormones and cytokines, including the pro-inflammatory cytokine leptin (Grunfeld *et al.*, 1996; Faggioni *et al.*, 1997; Sachot *et al.*, 2004) that regulate feeding behaviour (Friedman and Halaas, 1998), also cause anorexia. Therefore, anorexia of infection is not PAMP-specific and may be caused by a range of pathogens.

During acute and chronic illness, anorexia occurs as a clinical manifestation that can be beneficial or detrimental depending on the time and duration of the illness (for review see

Plata-Salamán, 1996). For example, in an acute infection "anorexia of infection" (Cole and Parkin, 1977) or "infection-induced malnutrition" (Beisel, 1995) may occur in the absence of fever (McCarthy *et al.*, 1985; O'Reilly *et al.*, 1988) and may act as part of an active defence strategy with a positive survival value (Murray and Murray, 1979; Hart, 1988; Exton, 1997). However, if the disease occurs recurrently or becomes chronic, the long-term reduction in food intake may delay recovery and contribute to weight loss, muscle atrophy and fatigue (Plata-Salamán, 1996; Ravasco *et al.*, 2004), resulting in a metabolic syndrome often referred to as "cachexia" (see Evans *et al.*, 2008). Cachexia usually occurs in concert with anorexia (Bosaeus *et al.*, 2001).

Different magnitudes and durations of anorexia may be induced experimentally by using different PAMPs. Anorexia can easily be measured using well-established experimental techniques that analyze and quantify the animal's feeding behaviour. In rodents, feeding behaviour can be measured as changes in food intake per day. For example, the amount of food consumed usually is quantified by subtracting the amount of food remaining in the cage from a weighed amount of food measured at the same time on the previous day (e.g. Harden *et al.*, 2006, 2008; Harden *et al.*, 2010). Others have used biotelemetry and specially-equipped cages with food supply dishes placed on balances to continuously measure food intake in rats (Hübschle *et al.*, 2006, 2007).

1.2.2.3. Motivation vs. capacity in anorexia and lethargy

In the sickness behaviour of lethargy there appears to be a clear distinction between motivation and capacity for activity (Maier and Watkins, 1998; Larson and Dunn, 2001; Rachal Pugh *et al.*, 2001). In experimental animals the lethargy of sickness behaviours appears to be activity-specific. For example, the optional physical activity measured as

voluntary wheel running was abolished almost completely in rats after a low-dose bolus injection of LPS, while routine daily ("house-keeping") activity measured as cage activity, continued (Hopwood *et al.*, 2009; Skinner *et al.*, 2009). Therefore, what appears to be impaired is the will or motivation to be active and not the capacity to be active (Hopwood *et al.*, 2009; Skinner *et al.*, 2009). Consonant with that theme, anorexic animals (i.e. animals with a loss of appetite) can eat; they just do not want to eat. In LPS-induced anorexia in rats, it was the will or motivation to eat that was impaired and not the capacity to eat (Hopwood *et al.*, 2009; Skinner *et al.*, 2009).

However, motivation is not always impaired in sick animals. During illness, sickness behaviours observed in sick animals may reflect motivational changes rather than a result of depressed or impaired ability for ongoing activities. The phenomenon was demonstrated by Miller (1964) when he showed that the effects of endotoxin on the behaviour of rats were influenced by the outcome of a particular behaviour (Miller, 1964). After receiving an injection of endotoxin, rats increased bar pressing so as to terminate the rotation of a drum that is considered a mild aversive stimulus, whereas rats decreased bar pressing when this action would have resulted in termination of a more pleasant stimulus, e.g., food reward (Miller, 1964). However, based on Miller's (1964) findings it is hypothesized that when the consequence of behavioural depression is harmful or unpleasant the behaviour is less likely to be disrupted by infection (Aubert *et al.*, 1997a, b, 1999; Larson *et al.*, 2001). Thus, sickness behaviour may occur differently on each exposure of the host to an infectious insult and also may depend on the context in which it occurs. For example, whether capacity or motivation or both are affected in sick animals that find themselves in life-threatening situations is still to be determined. One example of such a life-threatening situation is activity in a Morris Water Maze (Morris, 1984) in which a rat needs to swim and find a platform so as to escape from the water. A rat with cytokine-induced lethargy may still swim sufficiently,

because survival depends on it. The Morris Water Maze behavioural paradigm frequently is used to assess another aspect of sickness behaviour, namely learning and memory impairment.

1.2.2.4. Impaired learning and memory

Individuals who have suffered infections as infants appear to be at higher risk of developing cognitive disabilities (Dammann *et al.*, 2002; Stoll *et al.*, 2004). In addition, acute starvation in school children adversely affected cognitive processes, including attention and memory, especially in those children from low-income families who were nutritionally at risk (Pollitt *et al.*, 1998). Emerging evidence shows that exposure to infectious agents during the perinatal period may alter immune responses, including the febrile response, in adulthood and also may increase disease susceptibility and vulnerability to cognitive disorders later in life (Boisse *et al.*, 2004; Ellis *et al.*, 2005, 2006; Bilbo and Schwarz, 2009; Bilbo *et al.*, 2010). A recent study proposed that variation in the intensity of infectious diseases may determine worldwide distribution of cognitive ability (Eppig *et al.*, 2010).

Memory does not exist as a single entity (see Squire and Zola, 1996). Several kinds of memory, including declarative/explicit (cognitive) and non-declarative/implicit (non-cognitive) memory rely on different memory systems in the brain for normal memory functioning. Memory systems include, among others, the hippocampal formation in the medial temporal lobe (e.g. Morris *et al.*, 1982), basal ganglia (e.g. Hikosaka *et al.*, 1998) and neocortex (e.g. prefrontal cortex and temporal cortex) (see Wiltgen *et al.*, 2004). In my studies I investigated declarative memory, particularly memory for spatial layouts that is dependent on the plasticity and integrity of the hippocampus (e.g. Morris *et al.*, 1982).

To investigate the involvement of the hippocampus in processing spatial information and spatial memory, many experimental studies have been conducted in primates. However, the results remain contradictory when compared to the results of similar studies in humans (see Nadel, 1991). Rodents, on the other hand, are used frequently to study spatial learning and memory during infection (Cunningham and Sanderson, 2008) and the studies strongly support a role for the hippocampus in rodent memory for spatial layouts (Morris *et al.*, 1982; Whishaw, 1998). I used rats as an animal model, not only to investigate the effects of simulated infection on aspects of hippocampal structure and function (**Chapter 5**), but also to study spatial learning and memory (i.e. declarative memory) in simulated infection (**Chapters 3 and 5**).

The use and implementation of animal models greatly has improved investigations into cognitive ability, including learning and memory impairment, among those suffering from infection. Initial studies on spatial memory in rodents, as reviewed in a classic paper by Tolman (1948), laid the foundation for general research in the field of cognition (Tolman, 1948). Since then, experimental investigations using various behavioural paradigms to assess spatial learning and memory have been conducted successfully in rodents. Animal models that specifically assess declarative, spatial memory, include the Morris Water Maze (D'Hooge and De Deyn, 2001; Cunningham and Sanderson, 2008), the Y-maze (Sanderson *et al.*, 2009) and various radial arm mazes (Semmler *et al.*, 2007; Chen *et al.*, 2008; Sanderson *et al.*, 2009; Dilger and Johnson, 2010). Whereas each of these behavioural models has contributed to our current understanding of spatial learning and memory during experimental infection, the Morris Water Maze (Morris, 1981) is most widely used (see Cunningham and Sanderson, 2008). Interestingly, Brynskikh and colleagues (2008) showed impairment in learning of SCID (severe combined immune deficient) mice that are depleted

from T-cells (i.e. cells of the adaptive immune system), when learning was assessed in four different learning tasks, including a Morris Water Maze. More detail about the Morris Water Maze will be discussed in **Chapter 2**.

There are two types of declarative memory, namely episodic memory (i.e. to learn new events or skills) and semantic memory (i.e. to learn new facts). Because declarative memory comprises of conscious memories that are intentionally recollected (Cohen and Squire, 1980), specific tests for declarative memory assess the recall or recognition of memories from prior places, lists, shapes etc. (see Squire and Zola, 1996; Gabrieli, 1998). That is what the Morris Water Maze (Morris, 1984) measures: it is a behavioural assay that requires 'visuo-spatial' navigation of rodents swimming in a pool to learn and recall the spatial localization of visible cues in a room. In this way the Morris Water Maze measures 'spatial' learning and memory (see **Chapter 3** and **Chapter 5**). My studies did not extend to the investigation of other learning and memory types, such as non-declarative memory.

Thus far, most studies investigating impaired learning and memory as a component of sickness behaviour following simulated infections, have involved administering LPS to rodents (reviewed in Cunningham and Sanderson, 2008). Several other studies also have established the effects of simulated Gram-positive bacterial infections (Wellmer *et al.*, 2000; Gerber *et al.*, 2004; Barichello *et al.*, 2009; Woodruff *et al.*, 2010) as well as viral infections (Kent *et al.*, 2007; Into *et al.*, 2010; Okun *et al.*, 2010; Dilger and Johnson, 2010) on learning and memory. As yet, very little is known about the effects of *Mycoplasma* infection on learning and memory. There has been one case report of pervasive changes in memory in a 7-year-old girl suffering from possible *M. pneumoniae* encephalitis (Benjamin *et al.*, 2007). Irrespective of the pathogen causing the cognitive alterations, it is likely that the disturbances of memory and learning observed in humans and other animals (e.g., Bohr *et al.*, 1984;

Capuron *et al*., 1999; Wellmer *et al*., 2000) result from the actions of blood-borne and brain-derived pro-inflammatory cytokines (Reichenberg *et al.*, 2001; Goshen and Yirmiya, 2007) and the brain-derived prostaglandins induced by the cytokines (Hein *et al.*, 2007; Kent *et al.*, 2007; Ishida *et al.*, 2007). Administration of cytokines themselves, including IL-1β, IL-6, IL-2, TNF-α and interferon alpha (IFN-α) to patients (Denicoff *et al.*, 1987; Valentine *et al.*, 1998) and experimental animals (Goshen and Yirmiya, 2007; Dugan *et al.*, 2009; Machado *et al.*, 2010) induce memory disturbances. It is therefore possible that cytokines may underlie the long-term changes in cognitive ability later in life as a consequence of infection early in life (see Bilbo and Schwarz, 2009).

1.2.2.5. Spatial learning and memory following simulated systemic infection

Even though PAMPs from many different classes of pathogens have been used in experiments to study the effect of systemic bacterial and viral infections on learning and memory processes, literature on the effect of systemic *Mycoplasma* pathogens or their PAMPs on learning and memory is scant. Table 1 reviews the research findings of investigations on spatial learning and memory, specifically using rodents in a Morris Water Maze after receiving an acute, systemic (i.p.) immune challenge.

The Morris Water Maze may be applied in various ways that differ in duration and design requirements of the task. Although studies employing the Maze have found impairment in either learning or memory or both, several studies have not (see Table 1). For example, administration of the same PAMP (e.g. IL-1β) via the same route (i.p.) and in the same species (i.e. mice) shows different effects on learning and memory (Gibertini, 1996, 1998). Similarly, learning and memory of rodents may be affected differentially after administration of different strains of live Gram-negative bacteria, e.g., *E.coli* or *L. pneumophila* (Gibertini *et*

al., 1995; Barrientos *et al*., 2006). Furthermore, administration of LPS seems to induce impairment in spatial learning without affecting spatial memory (Arai *et al.*, 2001; Sparkman *et al.*, 2005a, b). Some, but not all of the contradictory and inconsistent results (reviewed in Cunningham and Sanderson, 2008) may be related to Maze conditions (refer to section 2.5 in **Chapter 2**).

Table 1.1. Studies investigating spatial learning and memory in a Morris Water Maze after an acute, i.p. immune challenge

PAMP	Dose	Species	Effect on learning	Effect on memory	Reference
Gram-negative PAMP					
Legionella pneumophila (live bacterium)	8 x 10^6 bacterial units per mouse	Mouse	Impairment (hidden platform) No impairment (visible platform)	Impairment (hidden platform) No impairment (visible platform)	Gibertini *et al*., 1995
LPS	400-800 µg.kg^{-1}	Mouse	Impairment	No impairment	Arai *et al*., 2001
LPS	100 µg.kg^{-1}	Rat	Impairment	Impairment	Shaw *et al*., 2001
LPS	250 µg.kg^{-1}	Mouse	Impairment	No impairment	Sparkman *et al*., 2005a, b
E.coli (live bacterium)	1 x 10^{10} CFU.ml^{-1}	Rats	No impairment	No impairment (Short-term memory) Impairment (Long-term memory)	Barrientos *et al*., 2006
LPS	250 µg.kg^{-1}	Mouse	—	Impairment	Lee *et al*., 2008
LPS	10 µg.kg^{-1}	Mouse	—	Impairment (working memory)	Richwine *et al*., 2009
LPS	100 µg.kg^{-1}	Mouse	—	Impairment (working memory	Zhang *et al*., 2009
LPS	1.25 mg.kg^{-1}	Mouse	—	No impairment	Huang *et al*., 2010

PAMP	Dose	Species	Effect on learning	Effect on memory	Reference
Gram-positive PAMP					
SEA	5 µg per mouse	Mouse	No impairment	No impairment	Woodruff *et al*., 2010
Viral PAMP					Ito *et al*., 2010
Poly I:C	20 $mg.kg^{-1}$	Mouse	Impairment (platform position changed) No impairment (platform position fixed)	Impairment (working memory) No impairment (reference memory)	
Pro-inflammatory cytokines					
IL-1β	100 ng per mouse	Mouse	— —	No impairment (reference memory) Impairment (working memory)	Gibertini, 1996
IL-1β	100 ng per mouse	Mouse	No impairment (spaced protocol) (cold water)	—	Gibertini, 1998

PAMP	Dose	Species	Effect on learning	Effect on memory	Reference
IL-1β	100 ng per mouse	Mouse	Impairment (massed protocol) (warm water)	—	Gibertini, 1998
IL-1β	1 mg per mouse	Mouse	Facilitation	—	Gibertini, 1998
IL-1β	2 $\mu g.kg^{-1}$	Rat	No impairment	No impairment	Thomson and Sutherland, 2006

— not tested in the study

E.coli (*Escherichia coli*)

SEA (Staphylococcus enterotoxin A)

Poly I:C (polyinosinic:polycyidylic acid)

CFU (colony forming units)

Although many studies have reported on the detrimental effects of infection on learning and/or memory, emerging evidence from investigations in rodents shows no impairment in learning and/or memory following i.p. injection of IL-1β (Thomson and Sutherland, 2006) and more recently, following i.p. injection of LPS (Huang *et al.*, 2010) or i.p. injection of Staphylococcal enterotoxin A (Woodruff *et al.*, 2010). Importantly, a recent clinical study showed that i.v. administration of LPS to human volunteers does not impair memory (Grigoleit *et al.*, 2010). So, impairment in learning and memory does not seem to be an inevitable consequence of infection.

1.2.3. Brain regions involved in fever, lethargy, anorexia and learning and memory

Several parts of the brain are involved in temperature regulation, energy metabolism and food intake, but the hypothalamus is crucial for these functions (Lepkovsky, 1973; Plata-Salamán, 1998; Morrison *et al.*, 2008). The region in the hypothalamus critical for food intake and energy metabolism is the arcuate nucleus (Elmquist *et al.*, 1999). However, the febrile response is induced by the action of PGE_2 on its specific receptor, EP_3, in the thermogenic region of the hypothalamus, the median pre-optic nucleus, an area crucially involved in raising the thermoregulatory set point (Ushikubi *et al.*, 1998; Lazarus *et al.*, 2007).

The hypothalamus also is important in coordination of cardiovascular function and respiration (e.g. Yeh *et al.*, 1997) without which physical activity is not possible. Together with the hypothalamus, several other parts of the brain are involved in the control of physical activity. The term ‘motor cortex’ is used to describe several cerebral cortical regions that are crucial for motor function. These regions include, among others, the prefrontal lobe that initiates activity in response to the environment and that is responsible for memory of motor activities, the parietal lobe that is responsible for goal-directed voluntary movements, the

primary motor cortex, the supplementary motor area and the premotor area. The cerebellum is responsible for executing and coordinating voluntary movements and muscle tone (for review see Cheney, 1985). Thus, if the functions of these brain regions are compromised during illness, the infected individual will feel fatigued and lethargic, and therefore will be reluctant to engage in physical activity.

Although physical activity (i.e. swimming) is required in the Morris Water Maze, the ability to learn to navigate accurately in the Maze depends on the plasticity and integrity of the hippocampus (for review see Whishaw, 1998). The hippocampus is situated in the medial temporal lobe of the brain (for review see Sejnowski, 2007), and supports a "spatial map" function (O'Keefe and Nadel, 1978). Studies in hippocampal-lesioned animals have confirmed a role for the hippocampus in spatial learning, as well as in spatial reference- and working memory (Olton *et al.*, 1978; Sutherland *et al.*, 1982; Morris *et al.*, 1982; Gage and Robertson, 1985). Similarly, humans with hippocampal damage displayed severe spatial memory impairment (Astur *et al.*, 2002; Goodrich-Hunsaker *et al.*, 2010).

In the event of solving spatial navigational tasks the hippocampus may interact with the parietal cortex (for review see Nitz, 2009). Despite the crucial involvement of the hippocampus in declarative memory, including spatial learning / memory, the hippocampus also has a role in non-spatial types of memory (Brasted *et al.*, 2003; Broadbent *et al.*, 2010). Brain regions adjacent to the hippocampal formation are required for non-declarative learning and memory that are independent of the hippocampus. For example, the basal ganglia and the cerebellum seem to play a significant role in non-declarative, sensorimotor skill learning (Willingham *et al.*, 1996). The basal ganglia also may have a role in visuo-spatial abilities (e.g. Christensen *et al.*, 1992). Thus, different types of memory processes depend on different memory systems in different brain regions.

1.3. SIMULATING INFECTION EXPERIMENTALLY

During infection, real or simulated, activated tissue macrophages and blood monocytes release a broad spectrum of pro-inflammatory cytokines that mediate the febrile response through the induction of PGE_2 and also induce the release of acute phase plasma proteins by the liver. Although fever is an integral part of the host's defense against invading pathogens, fever and sickness behaviours are characteristic of clinical infections. Therefore, fever and sickness behaviours would be expected acute phase responses in simulated, experimental infections. In the following sections of my thesis I will discuss mainly sickness behaviours as part of the acute phase response during simulated infections. I will deal, in part, with fevers, which is not the main focus of my thesis.

1.3.1. Acute challenge

Pathogenic moieties (or PAMPs) from various typical bacteria, e.g. LPS and MDP, atypical bacteria, e.g. FSL-1 or MALP-2 or viruses e.g. poly I:C have been used experimentally to simulate the acute phase response as it would be in the actual infection. A single, acute exposure to an infective pathogen does occur clinically, such as during an infected cut or a cat bite, and does activate the host's acute phase response. However, such events generally are rare in the clinical context of infections. In spite of its clinical rarity, it is that kind of infection that is simulated most frequently in laboratory investigations of the acute phase response. "Acute" childhood infections, such as chicken pox or mumps (both viral infections) as well as diphtheria and ear infections (both bacterial infections), are not really acute infections because the pathogen usually is present in the host for many days. These infections are not simulated accurately by a bolus administration of a PAMP, although a bolus administration of a PAMP does stimulate the innate immune system. For example, a

bolus administration of particularly LPS, mimicking infection caused by Gram-negative bacteria, has been used extensively to study the acute phase responses, including fever, anorexia and lethargy (Hopwood *et al.*, 2009; Skinner *et al.*, 2009; Asarian and Langhans, 2010; Harden *et al.*, 2010).

A bolus administration of LPS at doses ranging from 10 to 250 $\mu g.kg^{-1}$ in rats, whether administered subcutaneously (s.c.) (Skinner *et al.*, 2009) or i.p. (Hopwood *et al.*, 2009) induced dose-dependent lethargy. Intraperitoneal administration in rats of LPS at the highest dose (250 $\mu g.kg^{-1}$) depressed cage activity by almost 60 % and voluntary exercise by nearly 100 % when measured within 24 h (Hopwood *et al.*, 2009). Similarly, i.p. administration of the viral mimetic, poly I:C, at doses between 3 000 - 4 000 $\mu g.kg^{-1}$ in rats, depressed cage activity by up to 50 % and voluntary exercise by almost 90 % (Hopwood *et al.*, 2009). Thus, during a simulated infectious challenge voluntary exercise appeared to be affected more than cage activity, regardless of the pyrogen, i.e. LPS vs. poly I:C, or dose administered (Hopwood *et al.*, 2009). Bolus administration of Gram-positive bacteria or their PAMPs also induces experimental lethargy. Night-time i.p. administration of heat-killed cell walls (2.5 x 10^9) of *S. aureus* depressed cage activity in rats significantly over the first night after the injection (Luker *et al.*, 2000). Even the non-bacterial agent, zymosan induced lethargy in rats over the first and third nights after injection when administered i.p. at a dose of 10 $mg.kg^{-1}$ (Hübschle *et al.*, 2007). Zymosan often is used in animal models of human multiple organ dysfunction syndrome (MODS), in which systemic inflammation or septic shock is studied in relation to organ damage and failure (see Volman et *al.*, 2005).

Important for my study, bolus administration of moieties from *Mycoplasma* induced lethargy that lasted for longer than one day. Intraperitoneal administration of lipopeptides from *Mycoplasma*, including FSL-1 (from *M. salivarium*) or MALP-2 (from *M. fermentans*) at doses

of 100 - 1 000 μg.kg^{-1} in rats, induced lethargy (measured as cage activity) over the first night after the injection, while the lethargy induced by MALP-2 at a dose of 100 μg.kg^{-1} lasted into the third night (Hübschle *et al.*, 2006). Intracerebroventricular (i.c.v.) administration of heat-inactivated *M. fermentans*, at doses of 5.1 - 36.0 μg.kg^{-1} in rats, also induced lethargy (measured as cage activity and as social exploration in an open field) over the first 24 h following the injection (Yirmiya *et al.*, 1997).

PAMP-induced anorexia, like lethargy, seems to develop in a dose-dependent fashion. Bolus administration of moieties from infective organisms typically causes anorexia within 24 h following pyrogen administration, with the magnitude of LPS-induced anorexia in rats and mice depending on the dose of LPS administered (Langhans *et al.*, 1989; Kozak *et al.*, 1994). Food intake in rats was reduced by almost 50 % over the first 24 h following bolus s.c. or i.p administration of LPS at a dose of 250 μg.kg^{-1} (Hopwood *et al.*, 2009; Skinner *et al.,* 2009). Similarly, bolus administration of LPS has reduced food intake in other animal species, including mice and sheep (Murray and Murray, 1979; Baile *et al.*, 1981).The viral mimetic poly I:C, at a bolus dose of 4 000 μg.kg^{-1} but not 3 000 μg.kg^{-1}, in rats, induced anorexia to a similar extent as that induced by LPS at a dose of 250 μg.kg^{-1} (Hopwood *et al*., 2009). Anorexia induced by bolus administration of moieties from Gram-positive bacteria was observed in rats at 3 h after i.p. administration of 2 mg.kg^{-1} MDP (Porter *et al.*, 1998) and at 6 h after i.p. administration of 0.8 mg.kg^{-1} MDP (Langhans *et al.*, 1991). In guinea pigs, bolus intramuscular (i.m.) administration of 50 μg.kg^{-1} MDP resulted in significant anorexia within 24 h after injection (Madu *et al.*, 2007). Furthermore, bolus i.p. administration of 50 mg.kg^{-1} zymosan in mice induced anorexia for up to 4 h after injection, but the food intake was restored after 24 h (Naoi *et al.*, 2006).

Particularly relevant to my thesis is anorexia induced by *Mycoplasma* or its moieties. Bolus i.p. administration of 100 or 1 000 μg.kg^{-1} FSL-1 or MALP-2 induced anorexia over three nights after injections (Hübschle *et al.*, 2006) and i.c.v. administration of heat-inactivated *M. fermentans* (at doses between 5.1 - 36.0 μg.kg^{-1}) caused a significant suppression in food intake over the first 24 h following the simulated infection in rats (Yirmiya *et al.*, 1997).

While experimental anorexia and lethargy are well-described and consistent sickness behaviours in simulated acute infection, studies investigating another component of sickness behaviour, namely impaired learning and memory, thus far have rendered contradictory results. Lipopolysaccharide, which has been used extensively to investigate the effects of Gram-negative infection on learning and memory, has induced impairment in learning and/or memory in rats and mice when tested within 24 h after bolus i.p. administration at various doses (Aubert *et al.*, 1995; Pugh *et al.*, 1998; Thomson and Sutherland, 2005; Sanderson *et al.*, 2009; Terrando *et al.*, 2010; but also see Table 1). Adult rats infected as neonates with *E. coli* (1 x 10^6 colony forming units) had memory impairment after they received a bolus I.P injection of LPS (25 μg.kg^{-1}), either 24 h before or immediately after context exposure in the context pre-exposure facilitation paradigm (Bilbo *et al.*, 2005a, b, 2006). Studies in animal models of acute bacterial meningitis, induced by *Streptococcus pneumoniae*, have reported learning and memory impairments in mice and rats that had survived meningitis (Wellmer *et al.*, 2000; Irazuzta *et al.*, 2001). Bolus administration of the viral mimetic, poly I:C, induced learning and memory impairments in different animal species, including rats, mice, chickens and pigs (Ozawa *et al.*, 2006; Kent *et al.*, 2007; Ito *et al.*, 2010; Dilger and Johnson, 2010). Together with reports of impairment in learning and/or memory following bolus administration of bacterial moieties, neuronal damage in the hippocampus (i.e., lesions or loss of neurons) also has been reported (Wellmer *et al.*, 2000; Leib *et al.*, 2003; Semmler *et al.*, 2007; Hoffmann *et al.*, 2007; Cunningham and Sanderson, 2008).

Bolus injections of moieties from infective organisms are capable of inducing not only short-term, but also long-term impairments in learning and/or memory. Semmler and colleagues (2007) reported long-term memory impairment when rats were tested in a radial maze and in the open field test, three months after a single i.p. injection of LPS at a dose of 10 $mg.kg^{-1}$ (Semmler *et al*., 2007). Neonatal rats that received an i.p. injection of LPS at a dose of 500 $\mu g.kg^{-1}$ had impaired memory when tested 70 days later in an object recognition task (Jenkins *et al.*, 2009), while i.p. administration of *E.coli* induced both retrograde and anterograde amnesia in old, but not young rats, as measured in the Morris Water Maze and other behavioural tasks (Barrientos *et al.*, 2006). In simulated acute bacterial meningitis in mice, induced by bolus administration of *S. pneumoniae* into the right forebrain, long-term spatial learning and memory impairments were evident 180 days later, as measured in a Morris Water Maze (Wellmer *et al*., 2000). Thus, even when fever and sickness behaviour has resolved following acute administration of a moiety from infective organisms, the challenged animal may harbour residual impairment to learning and memory.

In spite of the body of evidence demonstrating impaired learning and memory, recent pre-clinical and clinical studies have contradicted the findings of impairment in learning/memory in response to bolus administration of bacterial moieties (Cunningham and Sanderson, 2008; Huang *et al*., 2010; Woodruff *et al*., 2010; Grigoleit *et al*., 2010). In their review, Cunningham and colleagues (2008) concluded that, in the case of hippocampal-dependent spatial learning and memory, the effects of bolus administration of LPS are not obvious (Cunningham and Sanderson, 2008). Performance deficits, rather than true learning and/or memory deficits frequently have been reported in various behavioural models, including the Morris Water Maze (Sparkman *et al*., 2005a), the delayed matching-to-sample conditional discrimination task and a Y-maze (Gahtan and Overmier, 2001). A recent study in mice confirmed the lack of effect on memory of bolus i.p. administration of 1.25 $mg.kg^{-1}$ LPS

(Huang *et al*., 2010), which is in contrast to results of other studies in mice, given LPS at much lower doses (10 - 250 μg.kg^{-1}) (Sparkman *et al*., 2006; Lee *et al*., 2008; Zhang *et al*., 2009; Richwine *et al*., 2009). Importantly, Huang and co-workers (2010) showed that in spite of other sickness behaviours and a significant increase in concentrations of the pro-inflammatory cytokine, IL-1β, in the hippocampus of mice, bolus administration of LPS did not cause impairment in spatial memory retrieval when tested in a Morris Water Maze 4 h after injection (Huang *et al*., 2010). Additionally, acute i.p. administration of Gram-positive bacterial superantigen, Staphylococcal enterotoxin A (5 μg / animal) did not induce impairment in learning or memory in mice when tested in a Morris Water Maze 2 h after injections (Woodruff *et al*., 2010). In a human model of endotoxaemia, i.v. administration of LPS at a dose of 0.4 ng.kg^{-1} (a dose that is considered to induce low-grade inflammation in the host) did not impair memory functions or executive functions, as measured with various neuropsychological tests, despite causing fever and increases in concentrations of plasma cytokines and other hormones (Grigoleit *et al*., 2010), an observation that contradicted previous findings that showed impairments in declarative memory after subjects had received i.v. LPS at doses between 0.2 - 0.8 ng.kg^{-1} (Reichenberg *et al*., 2001; Krabbe *et al*., 2005). The discrepancy might be explained by the different doses of LPS (0.2 vs. 0.4 vs. 0.8 ng.kg^{-1}), or by the different source of the LPS, i.e., *E. coli* vs. *Salmonella abortus equi* (Grigoleit *et al*., 2010). However, variation in bacterial species could not explain the discrepancies found in experimental animals, since most animal studies have used LPS from *E. coli*.

Thus, the association between acute immune activation and deficits in learning and memory still is unclear both in humans and in other animals. In particular, the effect of acute, simulated systemic *Mycoplasma* infection on learning and memory still is unknown.

1.3.2. Chronic challenge

Various experimental methods have been attempted to induce sustained fevers in an effort to mimic clinical fevers such as occur during a chronic infection. Ways of doing so include repetitive (e.g. daily) administration of a PAMP, with each new challenge being administered before the host recovers from the previous challenge, or continuous infusion (s.c. or i.v.) of a PAMP over days (e.g. O'Reilly *et al*., 1988; du Plessis *et al*., 2005). However, reproduction or simulation in laboratory animals of those long-lasting, sustained fevers, such as experienced clinically e.g. in typhoid fever (e.g. see Hook and Jones, 1966) has not yet been successful, because of fatalities or the development of pyrogenic tolerance. Greisman and colleagues (1961) showed that i.v. administration of 0.5 µg endotoxin from the Gram-negative bacterium, *Salmonella typhosa*, successfully reproduced the clinical symptoms of typhoid fever in healthy volunteers (Greisman *et al*., 1961). However, subsequent experimental studies in humans conducted in the same laboratory confirmed the development of tolerance to the pyrogenic property of *S. typhosa*, *Pseudomonas* and *E. coli* endotoxin when administered *(*i.v.) at daily intervals (e.g. Greisman *et al*., 1968).

Initially the phenomenon of 'tolerance' was described as a pathophysiological adaptation of the host to protect against uncontrolled inflammation, the latter occurring when cells of the immune system were unable to control the inflammatory reaction elicited by endotoxin or other immune stimuli (Zeisberger and Roth, 1998; Biswas and Lopez-Collazo, 2009). When a host is exposed to a certain PAMP, immune cells (e.g. monocytes and macrophages) may not respond to subsequent exposure to the PAMP and therefore may become 'tolerant'. In the host, the result of the phenomenon of 'tolerance' is a diminished acute phase response to subsequent PAMP exposure.

Tolerance to PAMPs from Gram-negative bacteria, e.g. endotoxin or LPS, has been observed in various species of animals, including rats (He *et al.*, 1992; Mekaouche *et al.*, 1996), guinea pigs, (Roth *et al.*, 1994) and rabbits (Goelst and Laburn, 1991; Wakabayashi *et al.*, 1994). Although tolerance to the febrile response was first described in animals after repetitive exposure to LPS, subsequent research has shown that tolerance to the febrile response is not specific to the actions of endotoxin or LPS (for reviews see West and Heagy, 2002; Biswas and Lopez-Collazo, 2009). Tolerance has been reported with repeated administration, or continuous infusion, of moieties from Gram-positive bacteria, viruses, as well as for pro-inflammatory cytokines (Soszynski *et al.*, 1991; Yamashiro *et al.*, 1993; Goldbach *et al.*, 1996; Roth *et al.*, 1997b; Ferreira *et al.*, 2001). For example, studies in guinea pigs have shown different febrile responses to repeated administration (at 3-day intervals) of PAMPs from various bacteria: five i.m. or i.p. injections of either LPS (5 $\mu g.kg^{-1}$; Gram-negative PAMP), MDP (100 $\mu g.kg^{-1}$; Gram-positive PAMP) or FSL-1 (100 $\mu g.kg^{-1}$; *Mycoplasma* PAMP) resulted in tolerance to the febrile response (Roth *et al.*,1994, Roth and Zeisberger, 1995; Roth *et al.*, 1997b; Greiss *et al.*, 2009). However, whereas tolerance is well described for Gram-negative PAMP administration (e.g. Mathison *et al.*, 1990; Zuckerman and Evans, 1992; Nakamori *et al.*, 1995; Roth *et al.*, 1997a), it is less understood for Gram-positive PAMPs. Tolerance does not occur consistently after administration of Gram-positive PAMPs (Goelst and Laburn, 1991; Roth *et al.*, 1997b). Whereas five daily i.v. injections of LPS (0.1 $\mu g.kg^{-1}$) in rabbits resulted in a significant attenuated febrile response on the second and subsequent days, administration of two doses of *S. aureus* cell walls (1 x 10^7 or 5 x 10^7 cells) over the same time period induced unattenuated fevers over five days in other rabbits (Goelst and Laburn, 1991). Similarly, Mphahlele and co-workers produced sustained fevers in goats via continuous (6 days) i.v. infusion with *S. aureus* (2 x 10^{11} cell walls) and showed a possibility of febrile tolerance developing only at the final stage of infusion (Mphahlele *et al.*, 2004). Thus, with repeated injections of LPS, but not *S. aureus*

cell walls, the magnitude and the duration (i.e. fever index) of the febrile response were reduced significantly over time, indicating the development of tolerance to fever in response to LPS, but not to *S. aureus* cell walls (Goelst and Laburn, 1991; Mphahlele *et al*., 2004). However, studies indeed have shown tolerance to PAMPs from Gram-positive bacteria, including MDP, when administered 5 times (spaced three days apart) at a dose of 100 $\mu g.kg^{-1}$ to guinea pigs, rather than injecting the pyrogen daily (e.g., Roth *et al*., 1997b).

It is worth mentioning though that, in tolerant animals, the acute phase responses, including fever, anorexia, lethargy and impaired learning and memory, may each disappear separately (i.e. some disappear more quickly than others) (e.g. O'Reilly *et al*., 1988), or they all may disappear in parallel (du Plessis *et al*., 2005). For example, anorexia was observed in rats following the first i.p. injection of LPS (100 $\mu g.kg^{-1}$), but tolerance developed to the anorexic effect of LPS after the second and third subsequent injections (Langhans *et al*., 1991; Riediger *et al.*, 2010). However, when MDP was administered i.p. to rats at a dose of 1.6 $mg.kg^{-1}$ on four consecutive days, it induced a significant reduction in food intake on each of the four days of administration (Langhans *et al*., 1991). Therefore, compared with the tolerance that develops to the anorectic effect of LPS, tolerance does not seem to develop to the anorectic effect induced by MDP (Langhans *et al*., 1991).

Not only repeated administration, but also long-term infusion of PAMPs, can result in tolerance to the acute phase response and therefore fails to simulate chronic fevers. Studies in rats, using implanted osmotic pumps that allow infusion (i.v. or s.c.) of LPS, reported fevers only at 6 h and 30 h over a 54 h infusion period (Lang and Spitzer, 1987) and only for two days over a 7-day infusion period (O'Reilly *et al*., 1988). The latter study also showed no tolerance to the anorexia induced by LPS, as evidenced by significant depressed food intake over the entire infusion period of 7-days (O'Reilly *et al*., 1988). A more recent study

confirmed tolerance in rats to fever and sickness behaviours induced by both Gram-negative and Gram-positive pyrogens, using continuous s.c. infusion of LPS or MDP, at a rate of 2 $\mu g.kg^{-1}.min^{-1}$ over seven days: LPS infusion induced sustained fevers for only three of the seven days and on only one day during the MDP infusion (du Plessis *et al*., 2005). The authors reported tolerance to two of the sickness behaviours, namely lethargy and anorexia. The body mass of rats during infusion of either LPS or MDP was reduced significantly on the fourth day during the 7-day pyrogen infusion but the rate of weight gain was stabilized again one day after cessation of infusion (du Plessis *et al*., 2005). However, over the 7-day infusion period lethargy was evident for up to two of the seven days in response to LPS infusion, but only for one day of the infusion in response to MDP (du Plessis *et al*., 2005). The decline in lethargy occurred in parallel with the decline in fever (du Plessis *et al*., 2005).

Despite the development of tolerance that might occur, continuous infusion of pyrogens in animals or humans is used to induce chronic inflammatory responses, e.g. sepsis (Vayssettes-Courchay *et al.*, 2002; Lynn *et al.*, 2003), septic shock (Herrera-Gutierrez *et al.*, 2011) and neuroinflammation (Min *et al.*, 2009). Chronic central neuroinflammation induced by continuous LPS infusion into the brain often is used as an animal model to simulate the neurobiology underlying Alzheimer's disease (e.g. Hauss-Wegrzyniak *et al.*, 1998a). Animal studies also have employed the technique of i.c.v. infusion of LPS (and other pyrogens) to investigate impairment in learning and memory as a component of sickness behaviour. For example, i.c.v. infusion with LPS (0.25 $\mu g.h^{-1}$) for 28 days in rats, induced spatial learning and memory impairment, as measured in a Morris Water Maze after 28 days (Cui *et al*., 2008). The impairment in learning and memory possibly could be attributed to the extensive hippocampal inflammation that followed LPS infusion (Cui *et al*., 2008). Similarly, i.c.v. infusion in rats over a period of 37 days resulted in a significant increase in the activation of microglial cells in the hippocampus, which may have contributed to the impairment in spatial

learning and memory observed another 37 days later in a Morris Water Maze (Hauss-Wegrzyniak *et al.*, 2000). So, following repeated injections, or chronic infusion with LPS, tolerance does not seem to develop to the sickness behaviour of impaired learning and memory, which is contrary to the tolerance that develops to lethargy and anorexia. It could be hypothesized that individuals with chronic Gram-negative bacterial infection might be at risk for chronic impaired learning and memory, which could result in long-term cognitive retardation. However, there was no impairment in learning the hidden platform task of the Morris Water Maze over four days of training, following repeated (four consecutive days) i.p. administration in rats of the Gram-positive superantigen, Staphylococcus enterotoxin A (SEA) (Woodruff *et al*,. 2010). Interestingly, when the rats that had received four i.p. injections of SEA were retested one month later, and without further injections, the rats showed improved learning (Woodruff *et al*., 2010).

1.3.3. Recurrent acute challenge

In contrast to the way in which chronic infections have been simulated experimentally, recurrent, acute infections are simulated by administering a series of PAMPs as spaced injections, in such a way that each new challenge is administered after the host has recovered from the acute phase responses caused by the previous injection. Simulating recurrent acute infections is important because systemic infections, whether bacterial or viral, often occur in a recurrent, acute fashion, especially during childhood. Typical recurrent acute infections include recurrent gastroenteritis, recurrent malaria, recurrent viral or bacterial respiratory diseases (e.g. asthma, pneumonia), and others. These recurrent infections, including community-acquired pneumonia that commonly is caused by *Mycoplasma* may have a devastating impact on the developing nervous system of children, especially in developing countries where resources for detecting such infections are likely to

be limited. In spite of their prevalence and importance, recurrent acute infections seldom are investigated in the laboratory as recurrent phenomena.

Nevertheless, some models of recurrent fevers exist. When administered five times, i.m. at three day intervals, the viral mimetic poly I:C (500 $\mu g.kg^{-1}$) induced recurrent fevers in guinea pigs that started three hours after each of the injections and continued for about 20 h (Voss *et al*., 2006). Although the magnitude of the febrile response was highest after the initial poly I:C injection, the shape and duration of the fevers in response to subsequent injections of poly I:C were similar, without progressive reduction in the fever response to the second, third, fourth and fifth injections (Voss *et al*., 2006). Similarly, when MDP (50 $\mu g.kg^{-1}$) was administered at 4-5 day intervals, there was no development of tolerance to the pyrogenic effect of MDP after eight recurrent injections in guinea pigs (Madu *et al*., 2007). More relevant to my study, five repeated administrations of a pyrogenic moiety from *Mycoplasma*, FSL-1, at a dose of 100 $\mu g.kg^{-1}$ induced recurrent fevers in guinea pigs when administered i.a. at three-day intervals (Greiss *et al*., 2009).

Although the fever response during simulated recurrent acute infections (i.e. spaced injections, not daily injections) seems to be better characterized, very little is known about the sickness behaviours during recurrent acute infections. However, growth failure has been reported in growing guinea pigs during simulated recurrent acute Gram-positive bacterial infection (Madu *et al*., 2007). When guinea pigs were injected i.m. with 50 $\mu g.kg^{-1}$ MDP, eight times at 4-5-day intervals, each recurrent injection elicited decreased food intake as well as fever (Madu *et al*., 2007). Moreover, Langhans and colleagues (1991) showed that repeated i.p. administration at two-day intervals of 0.6 $mg.kg^{-1}$ MDP, but not of 100 $\mu g.kg^{-1}$ LPS, resulted in significantly reduced food intake of rats after each of the injections (Langhans *et al*., 1991). In a recent Alzheimer's dementia mouse-model, transgenic Tg2576 mice were

infected four times (i.p.) with 2.5 x 10^5 colony forming units of *S. pneumonia*, spaced 30 days apart (i.e. recovery between injections) (Ebert *et al.*, 2010). Surprisingly, when learning and memory were tested in the infected rats on a weekly basis in a Morris Water Maze there was no impairment in spatial learning or memory during the course of infections. Therefore, recurrent systemic infections did not aggravate the course of experimental Alzheimer's dementia in rats (Ebert *et al*., 2010). Whether there also is a lack of impairment in learning and memory during recurrent acute infections in otherwise healthy adult rats (as opposed to rats with Alzheimer's dementia), still is unknown.

1.4. RATIONALE FOR CURRENT STUDY

In the African context, pulmonary disease still is the leading cause of paediatric morbidity and mortality, and frequently is caused by 'atypical' bacteria such as the *Mycoplasmas* (e.g. Black, 2006, 2008). *Mycoplasma pneumoniae* (*M. pneumoniae*) is a cause of community-acquired pneumonia, which in turn is a major cause of recurrent infection in school children and adolescents (Waites and Talkington, 2004; Black, 2008). Evidence also has implicated *M. pneumoniae* in chronic inflammatory conditions, such as asthma (e.g. Sutherland and Martin, 2007). Infections with *M. pneumoniae* may involve both the upper and the lower respiratory tract and occur both endemically and epidemically wordwide. For example, a recent study in HIV-infected hospitalized children in India showed a 32.2% prevalence of *M. pneumoniae* infection among children with respiratory tract infections (Nadagir *et al*., 2011). An epidemic of *M. pneumoniae* infections were reported in Denmark in 2010 and a similar increase in *M. pneumoniae* infections, with the identification of two novel strains of *M. pneumoniae*, was reported in England and Wales over the same period (Chalker *et al*., 2011). In many other countries, including South Africa, the prevalence and burden of disease caused by the *Mycoplasmas* still are undefined and need to be explored (Black,

2006, 2008), particularly in high-risk populations such as those in schools, hospitals and in the military where a large number of people reside in close proximity.

Moreover, *M. pneumoniae* has severe extra-pulmonary manifestations, including arthritis (e.g. Cedillo *et al.*, 1992; Oen *et al.*, 1995), meningitis, encephalitis and other life-threatening neurologic complications (for reviews see McIntosh, 2002; Narita, 2009). Very recently, another strain of mycoplasma, namely *Mycoplasma salivarium*, was isolated in cerebral abscesses from two adult patients (Ørsted *et al*., 2011). Thus, the general perception that infection, caused by *Mycoplasma* species, is self-limiting and confined to the respiratory system is incorrect. Although our understanding of mycoplasma organisms' cell biology, host interactions, disease transmission and treatment strategies have improved, very little is known about the sickness responses experienced by the host during mycoplasma infection.

1.4.1. Pyrogenic moieties of the *Mycoplasmas*

The *Mycoplasmas* can be distinguished phenotypically from conventional bacteria, because they lack a peptidoglycan cell wall (for review see Dybvig and Voelker, 1996). Because of this, the *Mycoplasmas* often are referred to as 'atypical' bacteria. Although membrane-bound lipoproteins in the *Mycoplasmas* are capable of activating cytokine-producing cells of the innate immune system (for review see Razin *et al.*, 1998), the lack of an experimental moiety for simulating *Mycoplasma* infections has restricted advances in studying *Mycoplasma*-induced acute phase responses. More than two decades ago it was demonstrated that moieties of the *Mycoplasmas* that are devoid of LPS can modulate several components of the immune system (Rauth and Praz, 1989). When it was realised that some effects of the *Mycoplasmas* are mediated by the central nervous system (e.g. Brenner *et al.*, 1994), and that i.c.v. administration of heat-inactivated *M. fermentans* in rats activated the

hypothalamus-pituitary adrenal axis (HPA) (Weidenfeld *et al.*, 1995), further explorative studies were quick to follow.

Shortly after *M. fermentans* was found in the brains of AIDS patients (Blanchard and Montagnier, 1994), the behavioural responses to *Mycoplasma* infection in rats were investigated (Yirmiya *et al*., 1997). The study showed that i.c.v. administration of heat-inactivated *M. fermentans* in rats induced fever, suppressed the consumption of food intake, decreased locomotion and social exploration, and reduced body weight (Yirmiya *et al*., 1997). After the discovery and identification of *Mycoplasma*-specific PAMPs, including macrophage-activating lipopetide-2 (MALP-2, Mühlradt and Frisch, 1994; Mühlradt *et al.*, 1997) and fibroblast-stimulating lipopeptide-1 (FSL-1, Shibata *et al*., 2000; Nakamura *et al*., 2002; Okusawa *et al*., 2004), the question arose as to how the *Mycoplasmas* interact with host cells (Rottem, 2003). Later studies in rats and guinea pigs characterized the pyrogenic properties of synthetic diacylated lipopeptides from the *Mycoplasmas*, including FSL-1 from *M. salivarium* and MALP-2 from *M. fermentans* (Hübschle *et al*., 2006; Greis *et al*., 2007). Simulated, systemic infection induced by i.p. administration of either FSL-1 or MALP-2 induced fever and sickness behaviours in rats via activation of the TLR2/6 heterodimer (Hübschle *et al*., 2006; Greis *et al*., 2007).

Therefore, using otherwise healthy rats as a rodent model, and FSL-1 as a pyrogenic moiety of *Mycoplasma* to simulate infection, I set out to investigate the autonomic and behavioural consequences of simulated acute, and recurrent acute, *Mycoplasma* infection. My main focus was to assess the degree to which *Mycoplasma* infection affects growth and physical activity in rats and compromises the capacity to learn and to remember, which represents a completely novel study that to date has not been undertaken.

I have addressed the following questions:

Does the sickness behaviour outlast the fever, as it does for other bacterial infections (Pereira and Begum, 1987; Hübschle *et al*., 2006)? Does the expected anorexia lead to temporary weight loss with catch-up, or permanent stunting (Madu *et al*., 2007)? Particularly relevant to my study, is there impairment of learning and memory during the infection, and, perhaps more perniciously, are there long-term cognitive deficits and/or residual histological damage to the hippocampus (required for learning and memory) after resolution of recurrent acute infections? What are the roles of the pro-inflammatory cytokines, IL-1β and IL-6, in simulated *Mycoplasma* infection? Is there differential production of these two cytokines in brain regions known to be important in mediating fever and sickness behaviour, namely the hypothalamus and the hippocampus?

Understanding the consequences of FSL-1 injections and clarifying the roles of pro-inflammatory cytokines in the plasma and in the brain would allow me to contribute to a better understanding of the pathways involved in *Mycoplasma*-induced fever and sickness behaviour, particularly with regard to learning and memory processes. Although previous studies already have characterized some aspects of the acute phase response to systemic administration of FSL-1 in rats, my aim was not only to confirm these earlier findings but to extend those findings by improving our understanding of the effects of FSL-1 on learning and memory processes. I also investigated other aspects of the acute phase response, including fever, lethargy and anorexia during simulated acute *Mycoplasma* infection, and for the first time, during simulated recurrent acute *Mycoplasma* infection.

1.5. THESIS AIMS

The aim of my thesis was to study autonomic and behavioural consequences of acute, and recurrent acute activation of the immune system in rats, following systemic administration of FSL-1, an agent which simulates *Mycoplasma* infection.

Chapter 1 reviews current literature on bacterial infections, other than *Mycoplasma* infection that thus far has contributed much to our understanding of sickness responses, including fever, lethargy, anorexia and particularly impaired learning and memory. **Chapter 2** contains common methodologies used in my thesis. In **Chapter 3** I have investigated fever and sickness behaviours, specifically anorexia, lethargy and learning and memory processes following simulated, acute *Mycoplasma* infection. In **Chapter 4** I sought to examine the contribution of plasma and brain pro-inflammatory cytokines, IL-1β and IL-6, in mediating *Mycoplasma*-induced fever and sickness behaviours following simulated acute *Mycoplasma* infection. In **Chapter 5** I have studied evidence for a lasting effect on learning and memory as well as changes in histology of the hippocampus following simulated recurrent acute *Mycoplasma* infection. Finally, **Chapter 6** expresses the implications of my results and provides future prospects that could be considered to take this work further.

CHAPTER 2

COMMON METHODOLOGIES

2.1. HOUSING AND HANDLING OF ANIMALS

Male Sprague-Dawley rats were bred in the Central Animal Service (CAS) of the University of the Witwatersrand. During my studies, the rats were housed individually in cages in a specially-equipped, temperature-controlled room (22.5 ± 0.5 ºC), on a 12:12 hour light:dark cycle with lights on at 07:00 local time. The room also contained a Morris Water Maze (see section 2.5.1, Figure 2.1). In all experiments I used male rats to avoid the influence of cyclic changes in female steroid hormones (Owen, 1975) that may affect hippocampal-dependent task performance, hippocampal anatomy, and hippocampal cell function (Warren *et al.*, 1995; Warren and Juraska, 1997). All rats were handled daily and had access to food and water *ad libitum*. All cages were cleaned twice weekly and the clinical status of rats was monitored and recorded daily, either by myself or by staff of the CAS.

2.2. GENERATING FEVER AND SICKNESS BEHAVIOUR

The diacylated synthetic lipoprotein, fibroblast-stimulating lipopeptide-1 (FSL-1), which represents the NH_2-terminal sequence of the 44 kDa lipoprotein LP44 of *M. salivarium* (Shibata *et al*., 2000; Nakamura *et al*., 2002; Okusawa *et al*., 2004) was used as a putative exogenous PAMP. FSL-1 was purchased as a lyophilized mixture of RR and RS stereo-isomers (EMC microcollections, Tübingen, Germany; Product L7000) and the lyophilized powder was reconstituted with sterile, pyrogen-free phosphate-buffered saline (PBS) to a concentration of 1 $mg.ml^{-1}$. All injections were administered intraperitoneally (i.p.) at 16:00, i.e. 3 h before lights-off. A dose-response curve for FSL-1, at doses of 10, 100 and 1 000 $\mu g.kg^{-1}$ has been established in rats (Hübschle *et al*., 2006). I therefore decided to use doses of 500 and 1 000 $\mu g.kg^{-1}$ in my studies.

2.3. MEASURING FEVER AND LETHARGY

Body core temperature and cage activity of rats were measured continuously by remote biotelemetry using radiotransponders that were pre-calibrated individually by the manufacturer (Mini Mitter, St. Paul, MN, USA) to a calibrated accuracy of 0.1 ºC.

Rats had the radiotransponders (G2 E-Mitters, Mini Mitter, St. Paul, MN, USA) implanted intra-abdominally under general anaesthesia induced by intramuscular injection of a combination of 100 $mg.kg^{-1}$ ketamine hydrochloride (Anaket-V, Bayer, South Africa) and 5 $mg.kg^{-1}$ xylazine (Chanazine, Bayer, South Africa). Once the rats were anaesthetised, the surgical area was shaved and cleaned with chlorhexidine gluconate. A 20 mm incision was made through the skin and muscle, into the peritoneal cavity. The transponder (size ~ 15.5 mm x 6.5 mm; mass ~ 1.1 g) was inserted into the abdomen and sutured to the abdominal wall. The wound then was sutured and treated with a topical antiseptic (Necrospray®) and the animals were allowed seven days to recover from surgery in a warm recovery room. A receiver plate (ER-4000, Mini Mitter, Sunriver, OR, USA; size ~ 0.56 m x 0.29 m x 0.07 m) placed under each rat's cage monitored the physical activity of rats in their cages based on detection of transponder movement. Transponder output frequency and transponder position were displayed on a computer screen at 5 min intervals as temperature and activity counts. E-mitters have been used previously for studying acute phase responses to various pathogens and in various species (Garami *et al.*, 2011; Coon *et al.*, 2011).

2.4. MEASURING ANOREXIA AND GROWTH

Food containers were filled daily with 200 g of the pelleted rat chow (Epol, Johannesburg, South Africa). Food intake was measured at 16:00 by weighing the remaining pellets in the

food container as well as food fragments on the cage floor. However, food powder on the cage floor was ignored. Food powder generally weighs less than a gram and has been reported to be similar amongst rats (Mueller *et al.*, 1997). Body mass of the rats was weighed with a bench top-loading scale (Diamond®) to an accuracy of 1 g and recorded daily at 16:00.

2.5. MEASURING LEARNING AND MEMORY

2.5.1. The Morris Water Maze

The Morris Water Maze was described and developed 30 years ago by Richard Morris (Morris, 1981, 1982, 1984), and has been employed as a research tool for studying mainly learning and memory (Brandeis *et al.*, 1989). Today the Morris Water Maze still is a popular test for studying the neurobiology and neuropharmacology of spatial learning and memory in rodents (see D'Hooge and De Deyn, 2001; Terry, 2009). Since its development the Water Maze also has been used for evaluating the effects of aging, experimental brain lesions and drug effects in rodents (Barnes, 1988; D'Hooge and De Deyn, 2001) as well as in stroke research (for review see DeVries *et al.*, 2001). Several animal studies have confirmed the successful use of the Maze in investigations of neurodegenerative and neuropsychiatric illnesses where cognition is impaired, including animal models of Alzheimers disease (e.g. Nitta *et al.*, 1994; Deshmukh *et al.*, 2009) and posttraumatic stress disorder (Harvey *et al.*, 2003). Because of the reliability and robustness of the Water Maze, it has been adapted for use in humans by employing life-size mazes (Wertlieb and Rose, 1979; Bohbot *et al.*, 1998; 2002) and virtual water mazes (three-dimentional pool) (e.g. Astur *et al.*, 1998).

The Morris Water Maze is based upon a reward principle: although rats can swim, they do not like water and they want to escape from it. Escaping from water as a technique to motivate learning was used long before the development of the Morris Water Maze (Glaser, 1910; Wever, 1932; Waller *et al.*, 1960). Indeed, some stress paradigms are designed closely around the typical swimming-learning protocol used in the Morris Water Maze in which the stressor (swimming in the pool) promotes resilience due to adaptive learning (learning to escape the water) (e.g., Brown et al., 2001; Wegener *et al*., 2010). Using the escape technique in a Water Maze allows motivation for escape behaviour without the animals being exposed to electric shock, extensive pre-training, or food deprivation. The Maze measures the ability of rodents to learn and remember the spatial location of visual cues in a room in order to find and escape onto a submerged platform located in a pool of water (Figure 2.1). Having to swim provides the motivation for optimal learning as well as for remembering the task.

Learning is assessed mainly by the degree to which the rats show a decrease in swimming latency as a function of repeated trials. This approach is based on the idea that animals will attempt to develop an optimal strategy to explore their environment and escape from the water. The “Cued test” (see Morris, 1984), where the platform is visible and raised above the water, can help to determine if any differences in a particular rat’s performance are due to motivational/emotional or sensorimotor abnormalities as opposed to learning and memory impairment. The Cued test therefore assesses learning and memory processes that are independent of the hippocampus (Cunningham and Sanderson, 2008; Sanderson *et al.*, 2009). Further confirmation that the location of the platform has been learned is that, if the platform is removed, i.e. the “Probe trial” (see Morris, 1982), rats will spend more time searching the area near the former location of the platform. Thus rats will show a spatial bias for that area. Measures for spatial bias during a Probe trial include, for example, the percent

time spent in the correct (i.e. target) quadrant or zone in which the platform was located, percent time spent in the target quadrant/zone vs. the other three quadrant/zones, platform crossings as well as the time, distance and speed travelled to the former platform location (see Maei *et al.*, 2009). Successful navigation to the submerged platform in order to escape the water requires acquisition, processing, consolidation, retaining and retrieval of the spatial localization of visual cues (see Poucet *et al.*, 2000), and depends on the integrity of the hippocampus (Sutherland *et al.*, 1982; Sutherland and Rudy, 1987; Jarrard, 1993).

My Morris Water Maze was developed based on the method of Hamlyn and colleagues (2009) with minor amendments (Hamlyn *et al*., 2009). The Maze consisted of a circular black-walled pool (0.5 m high and 1.75 m in diameter), divided into four virtual quadrants and filled with water (25-26 ºC) to a depth of 350 mm. An adjustable black platform (100 mm in diameter) was placed within the pool, either 10 mm below the water surface, rendering it accessible but invisible, or 15 mm above the surface (for the Cued test), so visible to the rats when swimming (see Figure 2.1). The water was rendered opaque with potassium permanganate (which I also used as a disinfectant) and stirred between trials to eliminate possible olfactory cues. A white noise generator (ANY-maze; Stoelting, IL, USA) excluded search strategies based on auditory cues. Four stationary poster boards, supporting different geometrical black shapes against a white background, surrounded the pool and served as extra-maze visual spatial cues. I concealed myself behind the poster boards while the rats were swimming. The length of time that a rat remained in the water in each swim was limited to 60s.

The pool was illuminated by indirect light to reduce light reflections from the water surface. A camera (Canon Digital Video Camcorder, Model DM-MV550i E) situated above the pool, and a video tracking system (ANY-maze; Stoelting, IL, USA) recorded swimming activities.

Figure 2.1 Illustration of the experimental set-up of a water maze task (drawn by Roy Hollowday, University of the Witwatersrand).

2.5.2. Advantages and limitations of a Morris Water Maze

The Morris Water Maze has many advantages over other available behavioural models that assess cognitive processes. An important advantage is that the Maze tests a high level of cognitive function: behavioural responses in the Maze are not merely reflex responses, which could be the case in, e.g. electric shock paradigms. Immersion into water, though unpleasant, also may not be as aversive as food deprivation or electric shock paradigms, which apply negative reinforcement. Other advantages of the Maze include a short training period (without pre-training) with a modest number of animals as well as operation of video tracking systems, reducing distraction of animals by the experimenter and identifying confounding factors such as motor, visual and motivational deficits. The Morris Water Maze also is a very versatile paradigm in which spatial and non-spatial learning, re-learning as well as reference and working memory processes can be assessed (see Terry, 2000).

However, as with all other animal behavioural models the Morris Water Maze also has limitations, especially when used to assess sick animals (see Cunningham & Sanderson, 2008). Hypothermia and hyperthermia as well as sickness behaviours, such as lethargy, affect performance in the Maze, thus potentially confounding assessment of cognition (Ahlers and Riccio, 1987; Sparkman *et al*., 2005a, b; Cunningham and Sanderson, 2008; Sanderson *et al*., 2009). Because the Maze procedure has to be run by hand, it could be more tedious than other paradigms that use fully automated equipment. Technical and procedural variables, e,g., dimensions of the pool, immersion into water and water temperature may cause endocrine responses to stress, such as the release of glucocorticoid (e.g. Sandi, 1998), which could skew the results. Other confounding factors, such as visual acuity, motivation, stress and anxiety also need thorough consideration when using the Morris Water Maze as a cognitive tool (see Sharma, 2009). Moreover, whereas the Maze

measures declarative, spatial learning and memory it does not measure non-declarative, non-spatial types of memory. Thus, measures of Maze performance in experimental animals (e.g. rodents) are not direct measures of human cognitive function. Although the underlying functional mechanisms of the brain are shared across most mammalian species, one has to acknowledge the differences in complexity between human and rodent behaviour. Direct extrapolation of results in rodent studies to humans is therefore ill advised (see Lindner, 1997).

2.5.3. Validation of my use of a Morris Water Maze

Animal models of human conditions should be reliable and well validated in terms of predictive, construct and face validity (see van der Staay, 2006). Predictive validity refers to the ability of the animal model to accurately predict the behaviour and events it is supposed to model as well as the response of the animal to treatment as used in the human condition. Construct validity requires that the animal model be based on the same physiological and neurobiological mechanisms as the human condition, and refers to the accuracy with which a test measures what it is supposed to measure. Face validity implies that an animal model, at least, should be similar to the human condition that it is mimicking with respect to aetiology, symptomatology and treatment (van der Staay, 2006). The Morris Water Maze indeed has predictive, construct and face validity (see Terry, 2009): pharmacological studies in the Maze have shown that neurotransmitter systems, including glutamate, dopamine and GABA that are involved in mediating spatial learning and memory are most critical for adequate performance in the Maze (McNamara and Skelton, 1993). Importantly, the Maze has been used in humans, albeit as a “Virtual Morris Water Maze” (e.g. Astur *et al*., 1998, 2002, 2004; Newhouse *et al.*, 2007), and has proven highly sensitive for assessing various

human conditions, including cognitive decline in children and adults (Overman *et al.*, 1996; Lindner, 1997).

I validated my use of the Morris Water Maze technique by means of a pharmacological challenge with scopolamine, a known amnesic drug. Scopolamine, a centrally acting anti-cholinergic (anti-muscarinic) agent routinely is used to induce amnesia in laboratory animals (Diez-Ariza *et al*., 2003; Janas *et al*., 2005; Choi *et al*., 2006) and humans (Antonova *et al.*, 2010). Previous studies also have used scopolamine to induce memory impairment in rats before testing them in a Morris Water Maze (Ormerod and Beninger, 2002; Takahata *et al.*, 2005; Hirst *et al.*, 2006). None of the studies has reported long-term debilitating effects (motivation, motor activity, sensory processing) on the rats' performance following administration of scopolamine at various doses. Consequently, I used scopolamine as a positive control in an experimental model for assessing spatial learning and memory deficits in a Morris Water Maze. On the basis of pilot studies, I injected rats i.p. with 0.8 $mg.kg^{-1}$ scopolamine (Sigma-Aldrich, St. Louis, MO, USA), a dose sufficient to induce amnesia. To prove validity of my use of the Maze, I would expect scopolamine to impair performance in the Maze, i.e. induce impairment in learning and memory.

2.5.4. Maze protocol

Before the start of each of the experiments, the rats were exposed to an initial habituation trial in the Morris Water Maze from which the platform had been removed (see Morris, 1981), during which they were allowed to swim freely for 120 s to explore the pool and its surroundings. The rats then were subjected to a single 60 s Cued test with the platform visible and placed in the centre of the pool to confirm motivation, vision and physical ability of rats. Thereafter the rats underwent a set of training sessions (one per day) over four days

(i.e. acquisition phase) intended for them to learn the position of a submerged platform in the Water Maze. One training session consisted of four learning trials per day (e.g. four attempts to search and swim to the submerged platform), with 90 s inter-trial intervals. Each rat was placed on the submerged platform for 10 s to orientate before the start of the initial trial on each day. Data recording started when the rat was placed into the water, tail first and with its head facing the wall of the pool. The quadrant in which the rats were released was changed between trials; using different starting points prevents the animal from using unintended response strategies such as swimming in the same direction. The rat was allowed 60 s to find the submerged platform. If the rat did not find the platform, it was guided gently to the platform. Once on the platform, rats were allowed to remain there for an additional 10 s. The rat then was removed from the platform and the next rat started its training trial.

The day after the last training trial on day 4, rats were assessed on their ability to remember the Maze task in a single 30 s Probe trial (platform removed), as described in section 2.5.1.

CHAPTER 3

SICKNESS BEHAVIOURS DURING SIMULATED, ACUTE *MYCOPLASMA* INFECTION

Data presented in this chapter have been published:

Swanepoel T., Harvey BH., Harden LM., Laburn HP. and Mitchell D. 2011. Dissociation between learning and memory impairment and other sickness behaviours during simulated *Mycoplasma* infection in rats. *Brain, Behavior, and Immunity* **25:** 1607-1616.

3.1 ABSTRACT

To investigate fever and sickness behaviours, specifically, lethargy and anorexia, as well as potential consequences for learning and memory following simulated, acute *Mycoplasma* infection, I have simulated the effects of *Mycoplasma* infection, in rats, by administering fibroblast-stimulating lipopepide-1 (FSL-1), a pyrogenic moiety of *Mycoplasma salivarium*. I measured the effects on body temperature, cage activity, food intake, and on spatial learning and memory in a Morris Water Maze. Male Sprague-Dawley rats had radiotransponders implanted to measure abdominal temperature and cage activity. After recovery, rats were assigned randomly to receive intraperitoneal (i.p.) injections of FSL-1 (500 or 1000 $\mu g.kg^{-1}$ in 1 $ml.kg^{-1}$ phosphate-buffered saline; PBS) or vehicle (PBS, 1 $ml.kg^{-1}$). Body mass and food intake were measured daily. Training in the Maze commenced eighteen hours after injections and continued daily for four days. Spatial memory was assessed on the fifth day. FSL-1 administration induced a dose-dependent fever (~ 1 ºC) for two days, lethargy (~ 78 %) for four days, anorexia (~ 65 %) for three days and body mass stunting (~ 6 %) for at least four days. Eighteen hours after FSL-1 administration, when rats were febrile, lethargic and anorexic, learning in the Maze was unaffected. There also was no memory impairment. My results support emerging evidence that impaired learning and memory is not inevitable during simulated infection.

3.2 INTRODUCTION

Impaired learning and memory has become entrenched as a member of the sickness behaviours, the suite of behaviours associated with the acute phase response. Its membership is supported by observations of sickness-induced cognitive dysfunction in acute experimental bacterial infections (as reviewed by, but also for contradictory evidence see, Cunningham and Sanderson, 2008) and, to a lesser extent, viral infections (Kent *et al*., 2007; Dilger and Johnson, 2010). However, there is emerging evidence that learning and memory is not always impaired when sickness behaviour is induced (Thomson and Sutherland, 2006; Cunningham and Sanderson, 2008; Huang *et al*., 2010; Woodruff *et al*., 2010; Grigoleit *et al*., 2010). Knowing whether or not impaired learning and memory is inevitable during the response to infection is crucial for assessing the cognitive consequences of infection in patients, especially in children in their phase of rapid learning. If cognition is impaired by acute infection, then the patients most likely to be compromised are those who are victims of recurrent acute infection.

Community-acquired pneumonia occurs in a recurrent fashion, commonly in children and adolescents (for review see McIntosh, 2002). One causative agent of this illness, *Mycoplasma pneumoniae*, has severe extra-pulmonary manifestations, including life-threatening neurologic complications (for review see Narita, 2009). A case study of a 7-year-old girl suffering from possible *M. pneumoniae* encephalitis reported evidence of pervasive changes in memory (Benjamin *et al*., 2007). Investigations into learning and memory impairment as a sickness behaviour have been undertaken for the behaviour following acute bacterial infections. However, despite the prevalence of the illness, there have been no systematic investigations of learning and memory following *Mycoplasma* infections. This is in

spite of intracerebroventricular (i.c.v.) administration of heat-inactivated *Mycoplasma fermentans* to rodents, inducing other sickness behaviours (Yirmiya *et al*., 1997, 1999).

Although killed pathogens are used experimentally to generate sickness behaviours, pyrogenic moieties of pathogens, extracted from the cell walls of bacteria (e.g. lipopolysaccharide; LPS from Gram-negative bacteria) or mimicking the double-stranded RNA of viruses (polyinosinic:polycytidylic acid; poly I:C), are used more frequently to stimulate the innate immune system, as it would be in the actual infection. Although the *Mycoplasmas* can be distinguished phenotypically from conventional bacteria by the lack of a cell wall (for review see Dybvig and Voelker, 1996), membrane-bound lipoproteins in the *Mycoplasmas* are capable of activating cytokine-producing cells of the innate immune system (for review see Razin *et al.*, 1998). The *Mycoplasmas*, though, activate different Toll-like receptors than do other pathogens (Takeuchi and Akira, 2001). One of the lipoproteins, fibroblast-stimulating lipopeptide-1 (FSL-1), the diacylated lipopeptide from *M. salivarium*, induces sickness behaviour when administered to rats (Hübschle *et al*., 2006). Whether FSL-1 administration induces learning and memory impairment has not been investigated.

I therefore investigated the effects of FSL-1 administration in rats on changes in spatial learning and memory performance, as well as on fever, lethargy and anorexia. I used the Morris Water Maze (see Morris, 1984), a standard tool for investigating spatial learning and memory performance in animals (for review see D'Hooge and De Deyn, 2001) and humans (virtual Morris Water Maze) (Aguirre *et al*., 1996; Astur *et al*., 2004; Goodrich-Hunsaker *et al*., 2010).

3.3 MATERIALS AND METHODS

(Also refer to **Chapter 2**, i.e. 'Common methodology')

3.3.1. Animals

Fifty seven male Sprague-Dawley rats with an average body mass of 381 ± 29 g (mean ± SD) on injection day were used, with no significant differences in the average body mass of the treatment groups.

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 (clearance certificates AESC 2009/43/04. and AESC 2010/34/04).

3.3.2. Pyrogen administration

The lyophilized powder of FSL-1 was reconstituted with sterile, pyrogen-free phosphate-buffered saline (PBS) and injected i.p. at a dose of 500 or 1000 $\mu g.kg^{-1}$, at 1 $ml.kg^{-1}$ volume. The dosage of FSL-1 was established after pilot studies showed that doses of 500 $\mu g.kg^{-1}$ and 1000 $\mu g.kg^{-1}$ induced pronounced dose-dependent fever and sickness behaviours within 6 h of administration. In these pilot studies, I administered an even higher dose of FSL-1 (2000 $\mu g.kg^{-1}$) but the ensuing fever and sickness behaviours did not appear to differ from that following administration of 1000 $\mu g.kg^{-1}$ (data not shown). To reduce the risk of toxic responses, and to reduce cost, I did not follow up the 2000 $\mu g.kg^{-1}$ dose systematically. I injected the rats at 16:00 so that the peak in fever would coincide with the dark phase of the 24 h day, when rats are most active.

3.3.3. Body temperature and cage activity

I recorded body core temperature and cage activity of rats continuously by remote biotelemetry as discussed in detail in section 2.2 of **Chapter 2**. Although I measured 24 h activity, which includes day time and night time activity, for the purpose of this study I chose to analyze only night time cage activity when rats are most active.

3.3.4. Food intake and body mass

Food intake and body mass of the rats, before and after intervention, were recorded daily at 16:00 (refer to section 2.4 of **Chapter 2**).

3.3.5. Learning and memory: Morris Water Maze

The Morris Water Maze apparatus and the protocol I used are discussed in detail in section 2.5 of **Chapter 2**.

Spatial learning and memory in the Morris Water Maze were measured as described previously (Hamlyn *et al.*, 2009), with minor modifications. Technical and procedural variables need thorough consideration when one uses the Morris Water Maze as a cognitive tool (see Sharma, 2009), especially when studying learning and memory in immune-challenged animals (reviewed in Cunningham and Sanderson, 2008). Therefore, I paid particular attention to exclude or reduce potential confounders which I could manage, such as water temperature, room temperature, dimensions of the pool, gender, housing and hormonal status of the rats. I also handled the rats regularly in an attempt to reduce stress levels. Furthermore, because I did not want to confound measurement of fever and activity,

which are most pronounced during the nocturnal period or rats, I did not immerse the rats over that time and chose to start the training in the Water Maze 18 h after FSL-1 administration.

I measured the rats' swim speed, latency to platform, distance travelled and the time spent in the target zone of the maze. I validated the use of the Morris Water Maze by injecting rats i.p. with 0.8 $mg.kg^{-1}$ scopolamine (Sigma-Aldrich, St. Louis, MO, USA), an agent which induces amnesia in laboratory animals (Diez-Ariza *et al*., 2003; Janas *et al*., 2005; Choi *et al*., 2006) and humans (Antonova *et al*., 2010).

3.3.6. Experimental procedure

The rats were assigned randomly to four experimental groups. The rats in two groups received either 500 $\mu g.kg^{-1}$ FSL-1 (n = 8) in PBS or 1000 $\mu g.kg^{-1}$ FSL-1 (n = 10) in PBS. The rats of the other two groups received phosphate-buffered saline (PBS, 1 $ml.kg^{-1}$; n = 10/group), and served as controls; PBS and FSL-1, at one of the two doses, were administered randomly.

As described in **Chapter 2** (section 2.5.4) the rats were first exposed to an initial habituation trial followed by a Cued test in the Morris Water Maze. The rats then received their injection of FSL-1 (at one of the two doses) or PBS on the same day that the Cued test was performed, but at 16:00.

The next day (about 16 h after the injections), the rats were tested again in a Cued test (60 s) to determine the effect, if any, of FSL-1 on motivation, vision and motor function. Training in the Morris Water Maze (i.e. acquisition phase) then commenced about 2 h later (i.e. 18 h

after the injections). I chose to wait 18 h before training the rats based on data from prior pilot studies, which showed that the acute fever have resolved by then; hyperthermia itself induces anterograde amnesia in rats (Ahlers and Riccio, 1987). For four consecutive days, rats were given four 60 s trials per day, in which they attempted to find the submerged platform from different randomly-chosen starting quadrants.

A single 30 s Probe trial, in which the platform was removed from the water, was conducted on the fifth day, to assess recall of spatial reference memory (recalling phase). Over the 5 d Morris Water Maze procedure, body core temperature and cage activity were measured continuously with biotelemetry and food intake and body mass were measure daily.

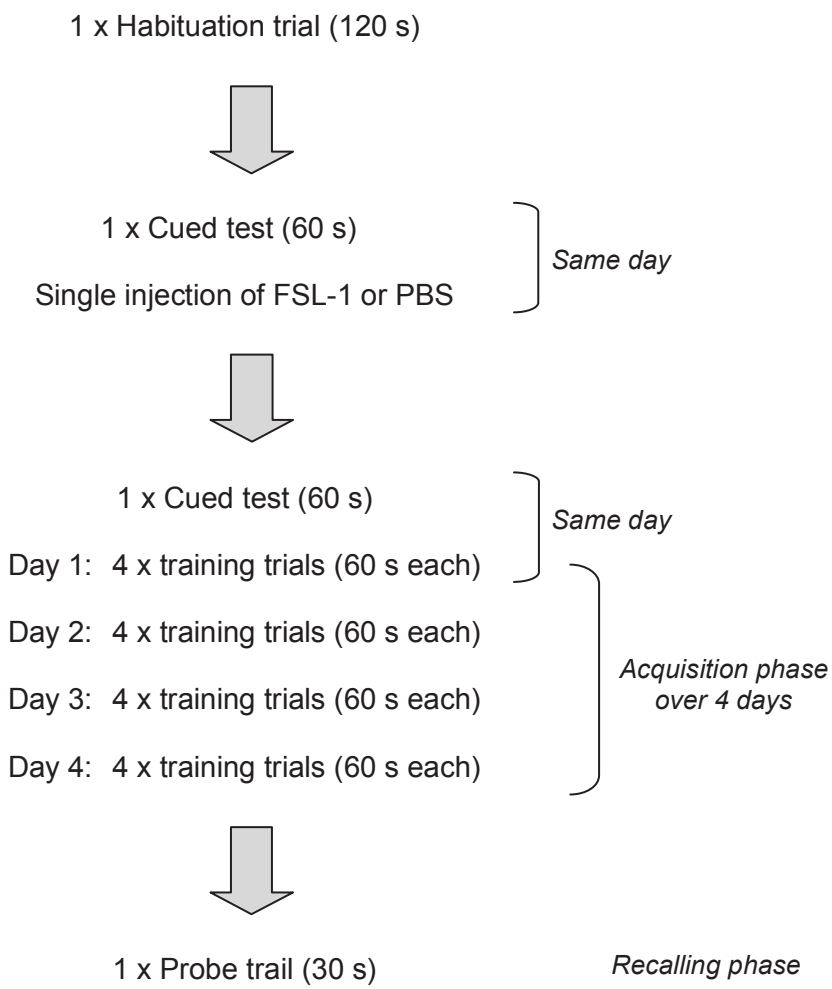

Figure 3.1. Schematic representation of the experimental procedure of training and testing rats in a Morris Water Maze, after receiving a single injection of 500 or 1000 μg.kg^{-1} FSL-1 or 1ml.kg^{-1} PBS.

3.3.7. Data analyses

There were no differences between the two groups of rats receiving PBS, so I subsequently combined data from rats given PBS to form one control group (n = 20). For statistical purposes I used the thermal response index (TRI, ˚C.h) as an integrated measure of fever duration and magnitude; TRIs were calculated separately over four successive 12 h periods on the day of injection starting at 19:00. To take account of circadian rhythms in body temperature, TRIs were calculated as the time integrals of the differences between the abdominal temperature of each rat and the abdominal temperature of that rat at the same time, averaged for the three days before the day of injection, when the rats were undisturbed in their home cages. TRIs were analyzed by one-way analysis of variance (ANOVA) with *post hoc* tests when ANOVA showed significance.

Night-time cage activity (19:00-07:00) was expressed as percent change from the mean activity measured for the same time period over the four days before injection. Food intake was expressed as grams of food consumed in 24 h per 100 g of body mass. Twenty-four hour change in body mass was determined by subtracting the body mass measured on each day after the injection from the average body mass measured for three days before injection, and expressed as change in body mass (g) per 100 g of rat body mass. Changes in activity, body mass and food intake were compared by means of two-way repeated measures ANOVA (intervention, time, interaction) with *post hoc* tests when ANOVA showed significance.

For each of the training days in the Morris Water Maze the mean speed, latency and distance to reaching the submerged platform over all four trials on that day, were determined for each rat, and then averaged across the group of rats. Changes in speed, latency and

distance measured during the four-day acquisition phase in the Morris Water Maze were compared by means of two-way repeated measures ANOVA (intervention, time, interaction) with *post hoc* tests when ANOVA showed significance. A one-way ANOVA was performed for indices measured during the Cued tests and during the Probe trial in the Morris Water Maze.

Data are expressed as mean ± SD and a statistical significance was accepted for $P < 0.05$.

3.4 RESULTS

3.4.1. Body temperature and cage activity

Abdominal temperatures of rats receiving FSL-1 started to exceed significantly those of rats receiving PBS about 4 h after injection (Fig. 3.2). On average the body temperature of rats receiving FSL-1 peaked approximately 6 h after the injections at 39 ºC, a degree higher than that of rats receiving PBS. On the night immediately after the injections the mean 12 h thermal response indices (TRI) (Fig. 3.3) for rats receiving 500 or 1 000 $\mu g.kg^{-1}$ FSL-1 were not significantly different from each other ($P > 0.05$), but were significantly greater than the mean 12 h TRI for rats receiving PBS ($P < 0.05$), (F (2,37) = 54.78, $P < 0.0001$). On the following day the mean 12 h TRI (Fig. 3.3) for rats receiving 500 or 1 000 $\mu g.kg^{-1}$ FSL-1 were significantly different from each other ($P < 0.05$), and both significantly greater than the mean 12 h TRI for rats receiving PBS ($P < 0.05$), (F (2,37) = 62.84, $P < 0.0001$) . On the second night the mean 12 h TRI for rats receiving 1 000 $\mu g.kg^{-1}$ FSL-1 was significantly greater than the mean 12 h TRI for rats receiving 500 $\mu g.kg^{-1}$ FSL-1 ($P < 0.05$) and for rats receiving PBS ($P < 0.05$), (F (2,37) = 5.14, $P = 0.01$). Similarly, on the second day the mean 12 h TRI for rats receiving 1 000 $\mu g.kg^{-1}$ FSL-1 was significantly greater than the mean 12 h TRI for rats receiving 500 $\mu g.kg^{-1}$ FSL-1 ($P < 0.05$) and for rats receiving PBS ($P < 0.05$), (F (2,37) = 31.67, $P < 0.0001$), (Fig. 3.3). By the third night and day after injection, the temperatures of rats that had received FSL-1 were not different to those of rats that had received PBS.

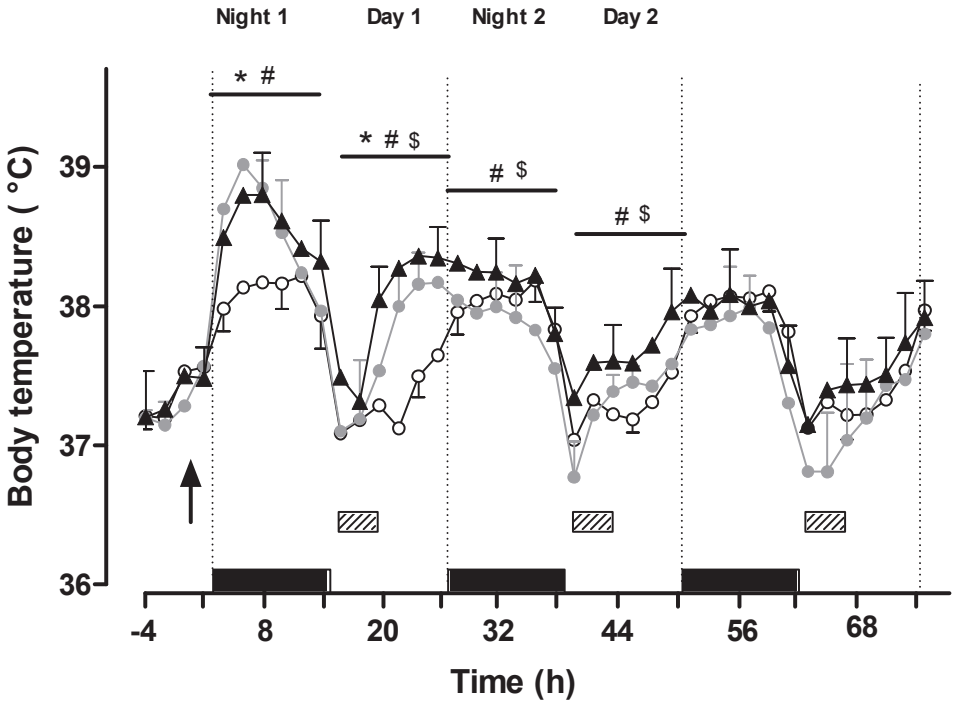

Figure 3.2. Abdominal temperatures (mean ± SD) of rats over 72 h after receiving a single i.p. injection of 1 ml.kg^{-1} PBS (○; n = 20) or FSL-1 at doses of 500 µg.kg^{-1} in 1 ml.kg^{-1} PBS (●; n = 8) or 1 000 µg.kg^{-1} in 1 ml.kg^{-1} PBS (▲; n = 10). Five-minute recordings of each rat's temperature were averaged over 2 h intervals and then averaged for the group. The arrow indicates time of injection (16:00) and the black bars indicate lights off (19:00-07:00). The hatched bars indicate when rats were swimming in the Morris Water Maze. Significant differences: * PBS vs. 500 µg.kg^{-1}; $^{\#}$ PBS vs. 1 000 µg.kg^{-1}; $^{\$}$ 500 µg.kg^{-1} FSL-1 vs. 1 000 µg.kg^{-1} FSL-1, during 12 h periods as shown.

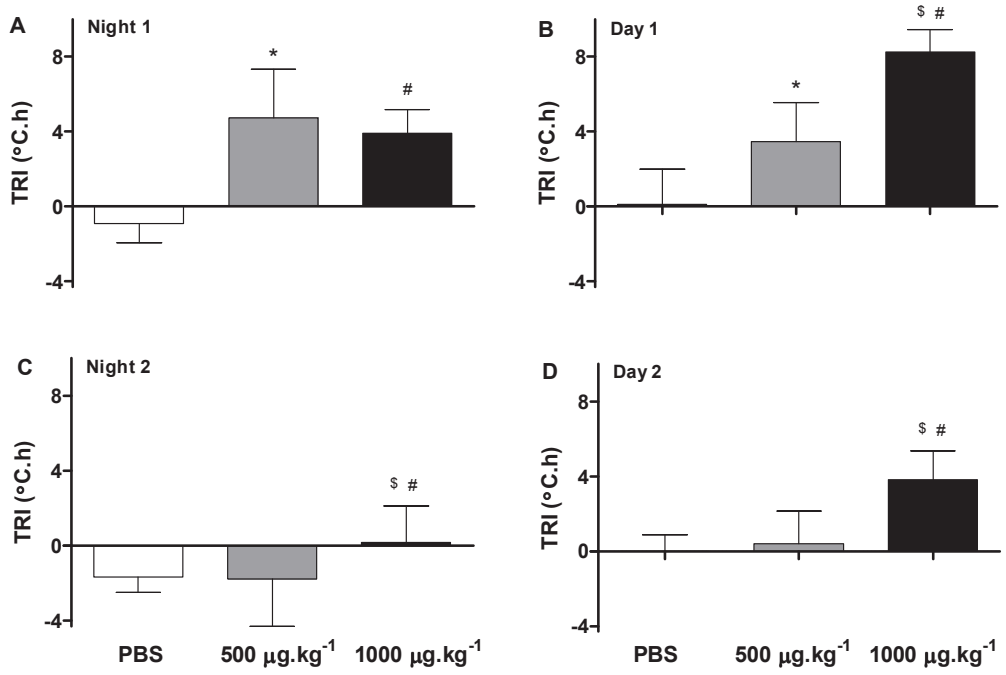

Figure 3.3. Twelve hour night-time and day-time thermal response indices (TRIs) (mean ± SD) of rats over 48 h after receiving a single i.p. injection of 1 ml.kg^{-1} PBS (n = 20) or FSL-1 at doses of 500 µg.kg^{-1} in 1 ml.kg-1 PBS (n = 8) or 1 000 µg.kg^{-1} in 1 ml.kg-1 PBS (n = 10). Significant differences: * PBS vs. 500 µg.kg^{-1}; $^{\#}$ PBS vs. 1 000 µg.kg^{-1}; $^{\$}$ 500 µg.kg^{-1} FSL-1 vs. 1 000 µg.kg^{-1} FSL-1.

Figure 3.4 shows the change in nocturnal cage activity of rats after receiving an i.p. injection of 500 or 1 000 μg.kg^{-1} FSL-1, or PBS. There was a significant statistical interaction between intervention and time (F (6,105) = 97.62, P < 0.0001). Cage activity was depressed significantly over the first night, by nearly 80 %, following an injection of FSL-1 at doses of both 500 μg.kg^{-1} (P < 0.0002) and 1 000 μg.kg^{-1} (P < 0.0002), compared to activity after PBS injection. From the second night, the magnitude of the decrease in cage activity of rats receiving FSL-1 was dependent on the dose of FSL-1 injected, although still significant for both doses, compared to PBS (P < 0.0002). Rats treated with 1 000 μg.kg^{-1} FSL-1, but not 500 μg.kg^{-1} FSL-1, still had a significant decrease in the cage activity on the third (P < 0.0002) and fourth (P < 0.04) days after the injection, compared to rats treated with PBS.

3.4.2. Food intake and body mass

On average, rats consumed 9.7 ± 0.8 g of food, per 100 g of body mass per day, for the four days before receiving injections (Fig. 3.5A). Two-way repeated measures ANOVA of food intake following injections showed a significant interaction between intervention and time (F (6,105) = 32.50, P < 0.0001). Over the first day after injections, rats receiving FSL-1 at both doses consumed significantly less food than did rats receiving PBS (P < 0.0002). How long food intake was suppressed depended on the dose of FSL-1 injected. FSL-1 at a dose of 500 μg.kg^{-1} suppressed food intake significantly for two days (P < 0.0004) whereas a dose of 1 000 μg.kg^{-1} FSL-1 suppressed food intake significantly for three days (P < 0.0002). Food intake returned to pre-injection value by the fourth day after injection of FSL-1 at 1 000 μg.kg^{-1}.

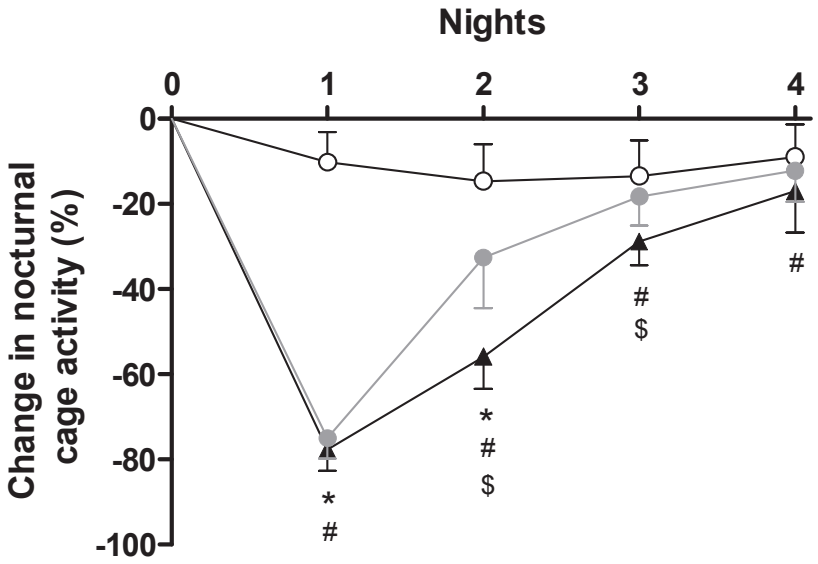

Figure 3.4. Night-time (19:00-07:00) cage activity (mean ± SD) of rats after receiving one i.p. injection of 500 µg.kg^{-1} FSL-1 in 1 ml.kg^{-1} PBS (●; n = 8), 1 000 µg.kg^{-1} FSL-1 in 1 ml.kg^{-1} PBS (▲; n = 10), or 1 ml.kg^{-1} PBS (○; n = 20). Activity was expressed as percent change from the mean activity measured over the same time period for the four previous days, with -100 % representing a complete cessation of cage activity. Significant differences: * PBS vs. 500 µg.kg^{-1}; $^{\#}$ PBS vs. 1 000 µg.kg^{-1}; $^{\$}$ 500 µg.kg^{-1} FSL-1 vs. 1 000 µg.kg^{-1} FSL-1.

Figure 3.5B shows the daily change in body mass of rats receiving i.p. injections of PBS or FSL-1. Two-way repeated measures ANOVA of change in body mass following injections showed a significant interaction between intervention and time (F (6,105) = 12.24, P < 0.0001). The body mass of rats receiving 500 or 1 000 μg.kg^{-1} FSL-1 decreased equally on the first day after the injections, compared to the body mass change of rats receiving PBS (P < 0.0002). From the second day, the magnitude of the decrease in body mass was dependent on the dose of FSL-1 administered. Although the decreases following injections of both doses of FSL-1 were significantly different from the decrease following injection of PBS (P < 0.0002), injection of the 1 000 μg.kg^{-1} dose resulted in a significantly greater decrease than did injection of the 500 μg.kg^{-1} dose (P < 0.0002). The body mass of rats receiving 500 μg.kg^{-1} FSL-1 stabilized a day after injection, while that of the rats receiving 1000 μg.kg^{-1} FSL-1 stabilized only two days after injection. However, injection of both doses of FSL-1 resulted in stunting, at least for the four-day period of observation, because body mass remained significantly below pre-injection mass, and below the body mass of the rats that had received PBS.

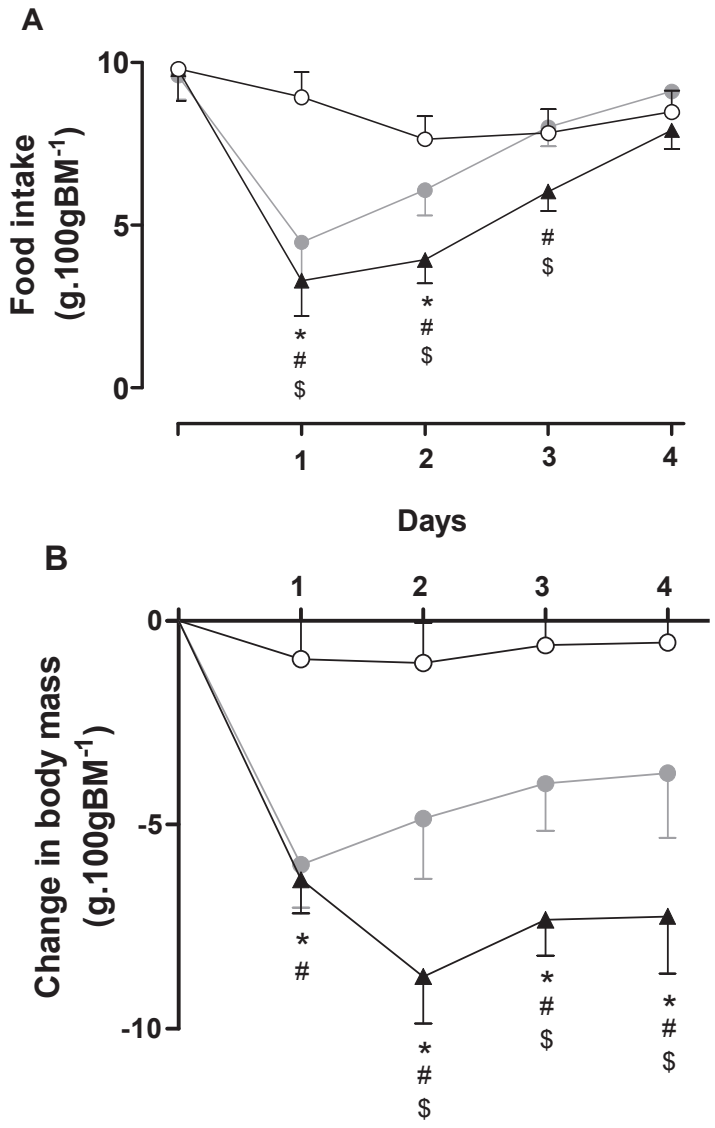

Figure 3.5. Food intake (mean ± SD) (A) and change in body mass (mean ± SD) (B) of rats after one i.p. injection of 500 µg.kg^{-1} FSL-1 in 1 ml.kg^{-1} PBS (●; n = 8), 1 000 µg.kg^{-1} FSL-1 in 1 ml.kg^{-1} PBS (▲; n = 10), or 1 ml.kg^{-1} PBS (○; n = 20). Food intake was expressed as grams of food consumed in 24 h per 100 g of body mass (BM) on that day. Change in body mass was measured over successive 24 h intervals starting at 16:00 and was expressed as grams of BM per 100 g of BM on that day. Significant differences: * PBS vs. 500 µg.kg^{-1}; $^{\#}$ PBS vs. 1 000 µg.kg^{-1}; $^{\$}$ 500 µg.kg^{-1} FSL-1 vs. 1000 µg.kg^{-1} FSL-1.

3.4.3. Learning and memory: Morris Water Maze

Figures 3.6B and 3.6F validate my use of the Morris Water Maze, and confirm the retarding effect of scopolamine on learning in rats (Fig. 3.6F). In the Cued test (visible platform) there were no significant differences between the rats receiving scopolamine and those receiving PBS with respect to their latency to locate the platform (t (28) = 0.78, P = 0.44; unpaired t-test; Fig. 3.4B). This result was confirmed using swim speed (t (28) = 0,69, p = 0.49; unpaired t-test; data not shown) and distance to platform (t (28) = 0.02, P = 0.99; unpaired t-test; data not shown). Over the four training days after i.p. injection of 0.8 mg.kg^{-1} scopolamine or 1 ml.kg^{-1} PBS (Fig. 3.6F), the latency to reaching the submerged platform decreased significantly for both groups of rats (time effect: F (3,84) = 41.11, P < 0.0001). However, the latency was significantly longer in the rats that had received scopolamine (intervention effect: (F (1,28) = 89.85, P < 0.0001), but there was no significant interaction between intervention and time (F (3,84) = 1.79, p = 0.15).

Figure 3.6 also shows the effect of an i.p. injection of FSL-1 or PBS on learning and memory in rats, as measured by performance in the Morris Water Maze. When rats were given a Cued test (visible platform) before interventions and training in the maze, there were no significant differences between those receiving FSL-1 and those receiving PBS, in any of the indices measured, including latency to platform, speed and distance to platform (data not shown). However, when rats were given the Cued test 16 h after injections and before they began training in the maze, the swim speed (Fig. 3.6C) of rats receiving 1 000 µg.kg^{-1} FSL-1 was significantly slower than was the swim speed of rats receiving 500 µg.kg^{-1} FSL-1 and PBS (F (2,34) = 9.62, P = 0.0005). Despite the decrease in swim speed, neither the latency to the visible platform (Fig. 3.6A, F (2,34) = 1.71, P = 0.19), nor the distance to the visible platform (Fig. 3.6D, F (2,34) = 2.48, P = 0.09) was significantly different between the groups.

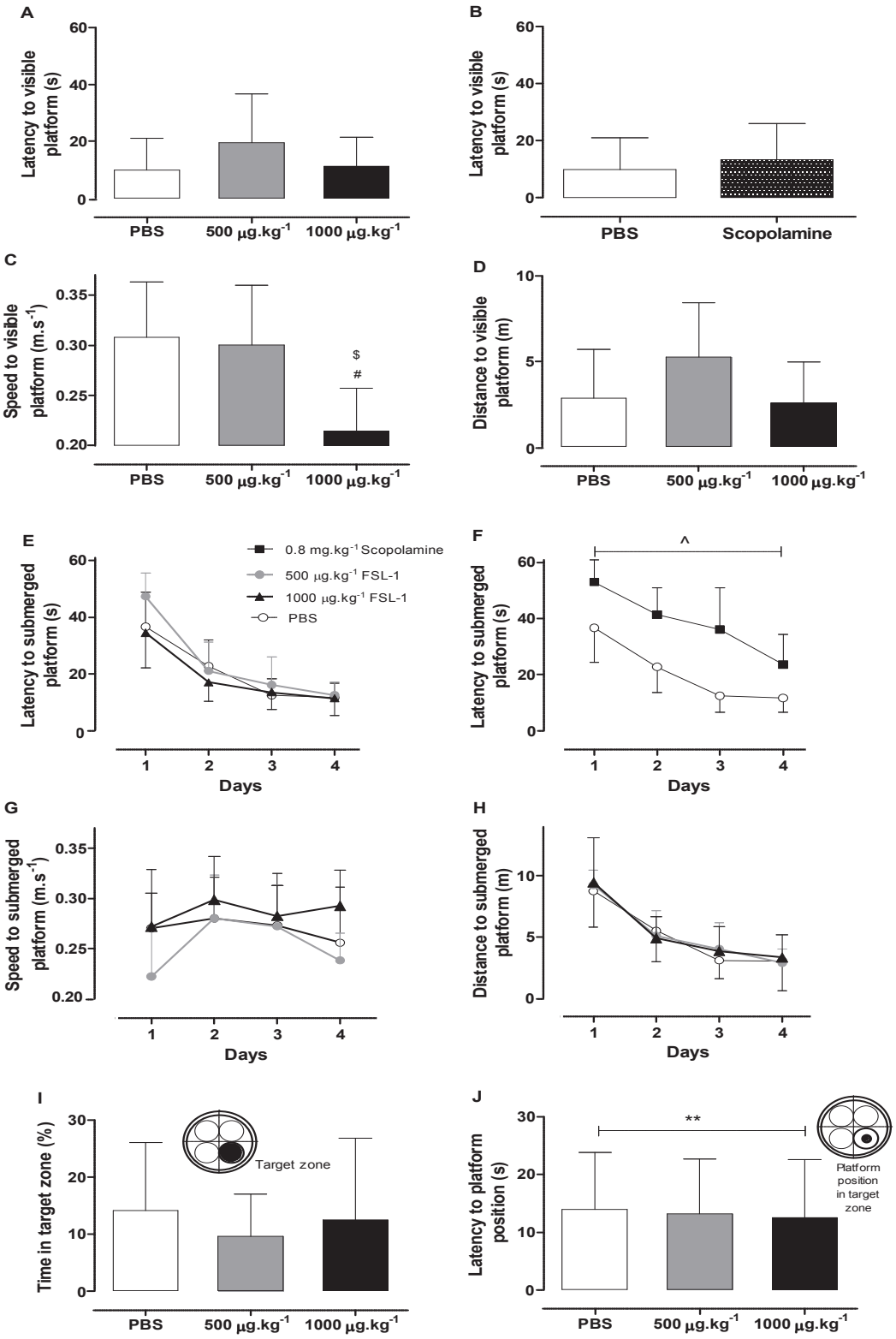

Figure 3.6. Performance (means ± SD) of rats as measured in a Morris Water Maze. Rats receiving one i.p. injection on Day 0 of 500 µg.kg^{-1} FSL-1 in 1 ml.kg^{-1} PBS (n = 8), 1000

µg.kg^{-1} FSL-1 in 1 ml.kg^{-1} PBS (n = 9), or 1 ml.kg^{-1} PBS (n = 20) were given a single attempt to find the visible platform in the Cued test during which latency (A), swimming speed (C) and distance (D) to the visible platform were measured. During the acquisition phase 18 h post injection, rats were given four trials per day over four days where latency (E), swimming speed (G) and distance (H) to the submerged platform were measured; mean values for the four trials are presented. Reference memory was tested on the fifth day post injection with a single Probe trial (no platform), during which percentage time in the target zone (I) and latency to locate the original position of the platform (J) were measured. Rats receiving i.p. injections of 0.8 mg.kg-1 scopolamine (n = 10) showed retarded learning over four days with a submerged platform (F), but unaffected performance with a visible platform in the Cued test (B), compared with rats receiving 1 ml.kg-1 PBS (n = 20). Significant differences: $^{\#}$ PBS vs. 1000 µg.kg^{-1} FSL-1; $^{\$}$ 500 µg.kg^{-1} FSL-1 vs. 1 000 µg.kg^{-1} FSL-1;
$^{\wedge}$ Scopolamine vs. PBS; ** PBS, 500 µg.kg^{-1} FSL-1, 1 000 µg.kg^{-1} FSL-1 vs. 30 s.

Over the four training days the latency to reaching the submerged platform (Fig. 3.6E) decreased significantly for all three groups of rats (time effect: F (3,102) = 78.35, P < 0.0001), but there was no significant intervention effect (F (2,34) = 2.34, P = 0.11) or interaction (F (6,102) = 1.74, P = 0.12). Similarly, over the four training days the distance to the submerged platform (Fig. 3.6H) decreased significantly for all three groups of rats (time effect: F (3,102) = 47.57, P < 0.0001), but there was no significant intervention effect (F (2,34) = 0.20, P = 0.82) or interaction (F (6,102) = 0.38, P = 0.89). For swim speed there was a significant time effect (F (3,102) = 3.27, P = 0.02) over the four training days (Fig 3.6G), but there was no significant intervention effect (F (2,34) = 3.18, P = 0.054) or interaction (F (6,102) = 1.13, P = 0.35). When the rats were tested in the Probe trial (platform removed) on the fifth day, neither the percentage time spent in the target zone of the water maze (Fig. 3.6I, F (2,34) = 0.43, P = 0.65) nor the latency to reach the platform position (Fig. 3.6 J, F (2,36) = 0.0632, P = 0.94) differed between the groups of rats. There also were no significant differences in the swim speed, distance to the former platform position, or virtual platform crossings between the groups of rats that had received PBS and FSL-1 (data not shown). Moreover, for all groups, the mean latency to the former platform position was significantly shorter than 30s (see section 3.4) (PBS, t (19) = 7.23, p < 0.0001; 500 µg.kg^{-1} FSL-1, t (7) = 5.0, P = 0.0016; 1 000 µg.kg^{-1} FSL-1, t (8) = 5.17, P = 0.0009; one-sample t-test).

3.5 DISCUSSION

To my knowledge, I am the first to assess potential deficits in spatial learning and memory, as a component of sickness behaviour, following simulated *Mycoplasma* infection in rats. I showed that parenteral administration of a single bolus of FSL-1, the pyrogenic moiety of *M. salivarium*, generated fever for up to 48 h, and profound sickness behaviour, in a dose-dependent manner. It caused anorexia for three days and lethargy for four days, as well as body mass stunting for at least four days. The lethargy was sufficiently intense so as almost to abolish cage activity on the night after FSL-1 administration. However, FSL-1 administration did not cause impairment of spatial learning in rats, as measured in the Morris Water Maze 18 h after the initial FSL-1 injection, a time at which they still were febrile, lethargic and anorexic. Thus, FSL-1 induced profound sickness behaviour but spared spatial learning and memory.

Although the rats were febrile, lethargic and anorexic during most of the training days in the Morris Water Maze (see Fig. 3.2), their spatial learning appeared not to be impaired as they did learn: the time taken for the rats to learn the location of the submerged platform reduced progressively over the four-day training period with the rate of reduction (Fig. 3.6E) unaffected by earlier FSL-1 administration. The absence of an effect of FSL-1 in the Morris Water Maze was not the result of failure of the test procedure, because scopolamine administration, a challenge used to validate the procedure, induced the expected impairments in spatial learning (Fig. 3.6F). Moreover, the rats did remember (as assessed in the Probe trial on the fifth day) despite being stunted as a consequence of sickness: earlier FSL-1 administration did not increase the latency exhibited by trained rats to find the former location of the platform after the platform had been removed (Fig. 3.6J). Thus hippocampal-

dependent learning and memory functions, as tested at the time points I used, appear to be spared during the acute phase response induced by simulated *Mycoplasma* infection.

A test procedure that studies a behavioural endpoint that is dependent on locomotion, such as the Morris Water Maze, may deliver spurious results if the rats are locomotor impaired. I therefore measured cage activity not only as an index of the sickness behaviour of lethargy, but, equally importantly, to assess whether any diminished performance in the Morris Water Maze could be attributed to lethargy rather than a decline in cognitive function. After FSL-1 administration, the rats were lethargic, at least for voluntary activity; they virtually had abandoned their typical nocturnal cage activity on the night immediately after the FSL-1 administration and reduced activity for a few nights thereafter (Fig. 3.4). However, lethargy did not affect their swimming performance during the four-day acquisition phase in the maze (Fig. 3.6 E,G,H). At the time at which I tested spatial memory rather than learning, cage activity had returned to normal, so lethargy was not a potential confounding factor then. The Cued test component of the Morris Water Maze procedure, in which the escape platform is visible to the rats, distinguishes impairments in motivation and sensorimotor functions from impairments in learning and memory. Following administration of my highest dose of FSL-1, the rats initially swam more slowly in the Cued test (Fig. 3.6C), but not sufficiently to affect the latency (Fig. 3.6A) or the distance (Fig. 3.6D) required to reach the visible platform, which were not altered significantly. Thus, even though they had abandoned much of their spontaneous cage activity, the rats swam competently in the Morris Water Maze during their simulated *Mycoplasma* infection, so no deconvolution of the effects of FSL-1 on locomotor performance and learning was necessary.

My results relate to the consequences of parenteral administration of a single bolus of FSL-1 in rats, at doses up to 1 000 $\mu g.kg^{-1}$. It is possible that repeated i.p. injections of FSL-1

(Greis *et al*., 2007), or intracerebroventricular (i.c.v.) injections of FSL-1 (Abe *et al.*, 2010), as is the case with LPS, could induce a more profound inflammatory response in the brain with accompanying hippocampal malfunction and cognitive impairment. Also, my conclusion that learning and memory was spared during the fever and sickness behaviour, which followed FSL-1 administration, has to be confined to the components of the process of spatial learning and memory that are tested by the Morris Water Maze, as I implemented it. For example, while I assessed spatial reference memory in the Maze, I did not assess spatial working memory (see Lacosta *et al.*, 1999; Sparkman *et al*., 2006; Richwine *et al*., 2009). Furthermore, the Probe trial was conducted on the fifth day after FSL-1 administration, a time at which the rats were stunted but no longer febrile, lethargic or anorexic. Therefore, if I had detected impairment in spatial reference memory, that impairment would have been a residual effect of the simulated acute infection, rather than dependent on concurrent fever, lethargy and anorexia. Finally, it is possible that FSL-1, like LPS, could adversely affect cognition in a hippocampus-independent task, e.g. the Y-maze task (see Sanderson *et al*., 2009), or in a task that is not physically demanding (e.g. Barnes-maze) and not as stressful as the Morris Water Maze (Harrison *et al*., 2009).

Though I believe that I am the first to assess potential deficits in learning and memory during simulated *Mycoplasma* infection, I am not the first to explore fever and sickness behaviour during such simulated infection. Hübschle and colleagues (2006) showed that FSL-1, administered intraperitoneally at doses of 100 and 1 000 $\mu g.kg^{-1}$, dose-dependently induced fever, lethargy, anorexia and adipsia in rats; that team also confirmed the pyrogenic properties of the FSL-1 in guinea pigs (Greis *et al*., 2007, 2009). My data, derived from a different strain of rat (Sprague-Dawley vs. Wistar) generally are in line with the findings of Hübschle *et al*. (2006), although I did not observe consistent hypothermia preceding fever (see Fig. 3.2) which Hübschle *et al*. (2006) observed when administering FSL-1 at a dose of

1 000 μg.kg^{-1}. In my hands, administration of both doses of FSL-1 resulted in significant body mass stunting for at least four days, a phenomenon not reported previously for FSL-1 challenge, though mass of rats may recover later. I also did not find learning impairment despite the prevailing fever; hyperthermia itself induced anterograde amnesia in rats (Ahlers and Riccio, 1987).

The dissociation between impairment of learning and memory and other sickness behaviours, which I have shown parallels emerging evidence in rodents that shows no impairment in learning and/or memory following i.p. injection of IL-1β (Thomson and Sutherland, 2006) and, more recently, i.p. injection of LPS (Huang *et al*., 2010) or i.p. injection of Staphylococcal enterotoxin A (Woodruff *et al*., 2010). Acute administration of LPS at a dose of 1 mg.kg^{-1} in mice also did not induce hippocampal neuronal damage (Chung *et al*., 2010), and a sub-pyrogenic dose of LPS failed to impair spatial working and reference memory in hippocampal-dependent spatial tasks (Sanderson *et al*., 2009). Importantly, a recent clinical study also has shown that i.v. administration of LPS to human volunteers does not impair memory (Grigoleit *et al*., 2010).

However, the dissociation that I and others have observed is not a consistent finding of all investigations of sickness behaviour. In experimental animals, learning and memory impairment indeed has been reported during Gram-positive bacterial infections (live *Streptococcus pneumoniae*) (Wellmer *et al*., 2000; Gerber *et al*., 2004; Barichello *et al*., 2009), and viral infections (poly I:C) (Kent *et al*., 2007; Dilger and Johnson, 2010; Okun *et al*., 2010). However, the majority of studies reporting that learning and memory is impaired have employed Gram-negative bacterial infections (LPS or live *Escherichia coli*) and various behavioural tasks including the Morris Water Maze (as reviewed in Cunningham and Sanderson, 2008), the Y-maze and the T-maze (Sanderson *et al*., 2009), contextual fear

conditioning (Pugh *et al*., 1998; Bilbo *et al*., 2006, 2008; Barrientos *et al*., 2006, 2009), context pre-exposure (Bilbo *et al*., 2005a, b, 2007) radial mazes (Semmler *et al*., 2007; Chen *et al*., 2008; Sanderson *et al*., 2009) and autoshaping (Aubert *et al*., 1995). In the Maze though, results obtained with LPS, at various doses, seem to be inconsistent for reasons unrelated to Maze technicalities. So, the effects of LPS on hippocampal-dependent spatial learning and memory still are inconclusive (Cunningham and Sanderson, 2008). It remains to be investigated why experimental infection, real or simulated, results in impaired learning and memory in some animal studies, but not in other animal studies, my study included, and not in human volunteers.

In the case of my study, it could be that there is something different about simulated *Mycoplasma* infection. Different bacterial and viral pathogens may activate the innate immune system in different ways via recognition by pathogen-specific members of the Toll-like receptor (TLR) family (Janssens and Beyaert, 2003). For example, membrane components from *Mycoplasma* are recognized by heterodimers formed from both TLR-2 and TLR-6 (Takeuchi *et al*., 2001). In the host, activation of a specific TLR, or combination of TLRs (e.g. TLR2/6) initiates a cascade of intracellular events that results in synthesis and release of a variety of pro- and anti-inflammatory cytokines which can be specific for those TLRs (for review see Takeuchi and Akira, 2010). *In vitro* studies have shown that FSL-1, acting presumably via the TLR 2/6 heterodimer, can induce IL-1β and IL-6 production (Shibata *et al*., 2000; Nakamura *et al*., 2002; Into *et al*., 2002; Okusawa *et al*., 2004; Senn, 2006; Rose *et al*., 2009), as do the pyrogenic moieties of other pathogens (Cartmell and Mitchell, 2005). So if the acute phase responses to FSL-1 administration depend on it being a TLR2/6 agonist, those responses are likely to involve cytokines including IL-1β and IL-6. This latter association is considered in Chapter 4. However, the situation is complicated by ambiguities about how pro-inflammatory cytokines affect learning and memory (reviewed in

Goshen and Yirmiya, 2007) and more specifically, how IL-1β affects learning and memory processes (Goshen *et al*., 2007; Huang and Sheng, 2010).

Apart from any consequences that my study might have for my understanding of the role of impaired learning and memory in sickness behaviour, I believe I have made an important contribution to understanding sickness behaviour responses during an acute infectious episode. Although the rats had abandoned almost all of their spontaneous cage activity following administration of the higher dose of FSL-1, they swam competently, albeit slower, during the Cued test. Thus, the lethargy of sickness behaviour appears to be activity-specific: what appeared to be impaired was the will or motivation to be active (i.e. in their cages), not the capacity to be active (i.e. in the Morris Water Maze). Consonant with that theme, in LPS-induced anorexia in rats, it is the will to eat that is impaired and not the capacity to eat (Hopwood *et al*., 2009; Skinner *et al*., 2009). Similarly, the optional activity of voluntary wheel running was abolished almost completely in rats after a low-dose bolus injection of LPS, while routine daily ("house-keeping") activity measured as cage activity, continued (Hopwood *et al*., 2009; Skinner *et al*., 2009). Because activity in the Morris Water Maze is not optional, and survival of the rats depends on escaping from the water, I hypothesize that the rats invoked their capacity for activity, unimpaired by concurrent sickness behaviour. I believe that in sickness behaviour, the distinction between motivation and capacity (a distinction also reported in reviews by Maier and Watkins, 1998; Rachal-Pugh *et al*., 2001; Larson and Dunn, 2001), has important clinical implications, and deserves more research.

Returning to the primary goal of my study, which was to assess learning and memory in the full context of the acute phase response, I found no impairment in spatial learning and memory, as assessed by a Morris Water Maze during simulated, acute *Mycoplasma*

infection in rats. My results show, for the first time, a dissociation of spatial learning and memory processes from other sickness behaviours following systemic administration of FSL-1, a pyrogenic moiety of *Mycoplasma* and support an emerging body of evidence that, contrary to the usual view, impaired learning and memory is not an inevitable consequence of exposure to pyrogenic pathogens.

CHAPTER 4

CYTOKINES AS MEDIATORS OF SIMULATED, *MYCOPLASMA*-INDUCED SICKNESS BEHAVIOUR

Part of the data presented in this chapter has been published:

Swanepoel T., Harvey BH., Harden LM., Laburn HP. and Mitchell D. 2011. Dissociation between learning and memory impairment and other sickness behaviours during simulated *Mycoplasma* infection in rats. *Brain, Behavior, and Immunity* **25:** 1607-1616.

4.1. ABSTRACT

Pro-inflammatory cytokines, including interleukin (IL)-6, are key mediators of fever and sickness behaviours induced by fibroblast-stimulating lipopeptide-1 (FSL-1), a pyrogenic moiety of *Mycoplasma*. Whether the pro-inflammatory cytokine, IL-1β, in the plasma, or both IL-1β and IL-6 in the brain, is involved in mediating sickness responses in rats after administration of FSL-1, is unknown. Moreover, the potential role for IL-1β and IL-6 in learning and memory processes, following FSL-1 administration in rats, also is not known. Male Sprague-Dawley rats had radiotransponders implanted to measure body core temperature. After recovery, rats randomly received a single I.P administration of FSL-1 at a dose of 1 000 $\mu g.kg^{-1}$, or 1 $ml.kg^{-1}$ vehicle (phosphate-buffered saline, PBS). Plasma and brain concentrations of IL-1β and IL-6 were measured at 3 h and 18 h after injections. Concentrations of IL-1β were elevated significantly in the plasma and brain at 3 h, and to a similar extent in both the hippocampus and hypothalamus at 18 h, after FSL-1 administration. However, concentrations of IL-6 were markedly increased in the plasma at 3 h after FSL-1 administration, but not at 3 h or 18 h in either of the brain regions. During simulated *Mycoplasma* infection in rats plasma and brain IL-1β, but not IL-6, may mediate fever, lethargy and anorexia, but not learning and memory impairment. However, if IL-6 and IL-1β act synergistically the presence of IL-6 in the brain potentially may have facilitated the action of brain IL-1β.

4.2. INTRODUCTION

That cytokines play a pivotal role in communication between the periphery and the brain during bacterial or viral infection is well established (Maier and Watkins, 1998; Larsun and Dunn, 2001; Kelley *et al.*, 2003; Dantzer, 2004). Cytokines act as messengers for communication between immune cells, the endocrine system and the central nervous system (e.g. see Maier and Watkins, 1998) and help orchestrate the physiological and behavioural adjustments that occur in the host during sickness. During inflammation and infection cytokines are synthesized both peripherally and centrally, and can affect the synthesis and secretion of each other (Cartmell and Mitchell, 2005). The pro-inflammatory cytokines IL-1β and IL-6 have significant roles in sickness responses, including fever, anorexia, lethargy and impaired learning and memory, induced by a Gram-negative pyrogenic moiety, namely lipopolysaccharide (LPS) (Luheshi and Rothwell, 1996; Dantzer *et al*., 2004; Harden *et al*., 2008, 2010; Goshen and Yirmiya, 2007). Moreover, serum concentrations of IL-1β and IL-6 were elevated significantly in patients with community-acquired pneumonia that was caused by *Mycoplasma pneumoniae* (Lieberman *et al.*, 1997; Hsieh *et al.*, 2001).

Membrane components of the *Mycoplasmas*, including fibroblast-stimulating lipopeptide-1 (FSL-1), are capable of inducing *in vitro* and *in vivo* production of the pro-inflammatory cytokines IL-1β, IL-6 and tumor-necrosis factor alpha (TNF-α) (Mühlradt and Schade, 1991; Yirmiya *et al*., 1999; Shibata *et al*., 2000; Nakamura *et al*., 2002; Into *et al*., 2002; Okusawa *et al*., 2004; Hübschle *et al*., 2006). For example, systemic (intraperitoneal, i.p.) administration in rats of FSL-1, extracted from *Mycoplasma salivarium*, induced acute phase responses including fever, lethargy, anorexia and adipsia, accompanied by elevated concentrations of pro-inflammatory cytokines IL-6 and TNF-α in the plasma (Hübschle *et al*.,

2006). Whether plasma IL-1β also has a role to play in FSL-1 induced sickness responses in rats, including fever, lethargy and anorexia, is unknown. Moreover, i.c.v. administration of heat-inactivated *Mycoplasma fermentans* in rats induced the expression of TNF-α and IL-1β in various brain regions, including the hippocampus and the hypothalamus, which was associated with anorexia and suppressed social behaviour (Yirmiya *et al*., 1999). However, Yirmiya's team did not measure brain concentrations of IL-6. Contributions of IL-1β and IL-6, in the brain, in mediating FSL-1 induced sickness responses in rats, are unknown.

I have shown that administration of FSL-1, which simulates the acute phase responses to *Mycoplasma* infection, dose-dependently induced fever, lethargy and anorexia (see **Chapter 3**), and therefore confirm the findings of Hübschle and colleagues (2006). In addition, I also found body mass stunting in rats, following i.p. administration of FSL-1, but did not find impairment in spatial learning and memory (see **Chapter 3**). The dissociation of learning and memory impairment, from fever, lethargy, anorexia and stunting following acute administration of FSL-1 that I have shown, could be explained by differential production of, or sensitivity to, pro-inflammatory cytokines in those brain areas required for learning and memory, including the hippocampus. Spatial learning and memory, in humans and other animals, depend on the integrity and plasticity of the hippocampus (e.g. Morris, 1982; Squire, 1992). However, the hypothalamus mediates fever and anorexia (Lepkovsky, 1973; Morrison *et al.*, 2008) and also is important in mediating lethargy, indirectly. The hypothalamus has a crucial role in controlling cardiovascular and respiratory processes (Yeh *et al.*, 1997), without which physical activity is not possible.

To investigate the possible role for IL-1β and brain IL-6 in mediating FSL-1 induced fever, lethargy and anorexia, and to investigate any link between effects on learning and memory

and the presence of IL-1β and IL-6 in the brain, I measured concentrations of these two pro-inflammatory cytokines in the plasma and brain following acute FSL-1 administration in rat.

4.3. MATERIALS AND METHODS

(Also refer to **Chapter 2**, i.e. 'Common methodology')

4.3.1. Animals

Twenty four male Sprague-Dawley rats with an average body mass of 335 ± 18 g (mean ± SD) on injection day were used, with no significant differences in the average body mass of the treatment groups.

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 (clearance certificate AESC 2010/34/04).

4.3.2. Pyrogen administration

The lyophilized powder of fibroblast-stimulating lipopeptide-1 (FSL-1) was reconstituted with sterile, pyrogen-free phosphate-buffered saline (PBS) (refer to section 2.2 in **Chapter 2**). Rats received a single i.p. injection of FSL-1 (n = 15) at a dose of 1 000 $\mu g.kg^{-1}$, at 1 $ml.kg^{-1}$ volume, or 1 $ml.kg^{-1}$ vehicle (PBS, n = 9). I injected the rats at 16:00 so that the peak in fever would coincide with the dark phase of the 24 h day, when rats are most active.

4.3.3. Body temperature

I recorded body core temperature of rats continuously by remote biotelemetry as discussed in detail in section 2.3 of **Chapter 2**.

4.3.4. Food intake and body mass

Food intake and body mass of rats, before and after intervention, were recorded daily at 16:00 (refer to section 2.4 in **Chapter 2**).

4.3.5. Cytokine concentrations

I measured concentrations of IL-1β and IL-6 in the plasma, as well as in the hypothalamus and hippocampus of rats after administration of FSL-1 or PBS. Brain tissue of rats destined for cytokine analysis was collected in different rats, 3 h and 18 h after administration of FSL-1 or PBS. I chose the 3 h time point because the only previous study of FSL-1 administration to rats had shown that plasma cytokine concentrations were increased 3 h after acute FSL-1 administration (Hübschle *et al*., 2006). I chose the 18 h time point because that coincided with the time after FSL-1 administration at which performance was assessed in a Morris Water Maze (refer to **Chapter 3**).

At the designated time after FSL-1 administration, rats were anaesthetized deeply with an i.p. injection of 1 ml sodium pentobarbital (Euthapent, 200 $mg.ml^{-1}$; Kyron Laboratories (Pty) Ltd., South Africa). Blood was collected via cardiac puncture into sterile tubes containing EDTA, and centrifuged at 5 300 g at 4 ºC for 10 min. Anaesthetized rats then were perfused transcardially for 2 min with about 250 ml of ice-cold sterile saline (0.9 % NaCl) via the left

ventricle. Tissue from the hypothalamus and from the hippocampus was dissected out rapidly on an ice-chilled plate, placed separately in micro-centrifuge tubes, snap-frozen in liquid nitrogen and stored in a – 80 ºC freezer until enzyme-linked immunosorbent assay (ELISA) was performed.

For these assays, tissue was dissociated mechanically with an ultrasonic cell disrupter (Microson XL 2000, Newton, CT, USA), in cold Iscove's culture medium (0.25 ml) containing 5% fetal calf serum and a cocktail enzyme inhibitor (100 mM amino-*n*-caproic acid, 10 mM EDTA, 5-benzamidine-HCL, and 0.2 mM phenylmethyl sulfonyl fluoride). Sonicated samples were centrifuged at 10 000 g at 4 °C for 10 min. Supernatants were removed and kept at 4 °C. Bradford protein assays (Bradford, 1976) were performed to determine total protein concentrations in sonicated samples. Concentrations of IL-1β and IL-6 protein were determined with commercially-available rat IL-1β and IL-6 ELISA kits (R&D Systems, Minneapolis, MN, USA), in duplicate, according to the manufacturer's instructions, and normalized to total protein (pg.mg total protein^{-1}). The detection limit for the IL-1β assay was 5 pg.ml^{-1} and 21 pg.ml^{-1} for the IL-6 assay.

4.3.6. Data analysis

The cytokine concentrations were not normally distributed so I log-transformed them for statistical analysis. I then compared plasma IL-1β and IL-6 concentrations separately, over the two time points (3 h vs. 18 h) in response to intervention with two-way ANOVA and *post hoc* tests when ANOVA showed significance, for rats given FSL-1 or PBS. Brain concentrations of IL-1β and IL-6 were compared separately, over the two brain sites (hypothalamus and hippocampus) in response to intervention with two-way ANOVA and *post hoc* tests when ANOVA showed significance.

4.4. RESULTS

Mean abdominal temperatures (data not shown) before rats were euthanized did not differ from those shown in Fig. 3.2 in **Chapter 3**. For plasma IL-1β concentrations (Fig. 4.1) a two-way ANOVA showed a significant intervention (FSL-1 vs. PBS) effect (F (1,20) = 27.30, P < 0.0001), a significant time effect (F (1,20) = 11.87, P = 0.003) and a significant interaction (F (1,20) = 9.44, P = 0.006). IL-1β concentrations were elevated significantly at 3 h in rats that had received FSL-1 compared to 18 h after injection (significant interaction) and compared to rats that had received PBS (P < 0.001). Regrettably, the plasma concentrations of IL-6 (Fig. 4.2) exceeded the ceiling of the assay of 2 000 pg.ml^{-1} at 3 h, so I could not validly analyze 3 h plasma IL-6 concentrations statistically. However, there was no doubt that plasma IL-6 was raised markedly 3 h after FSL-1 injection.

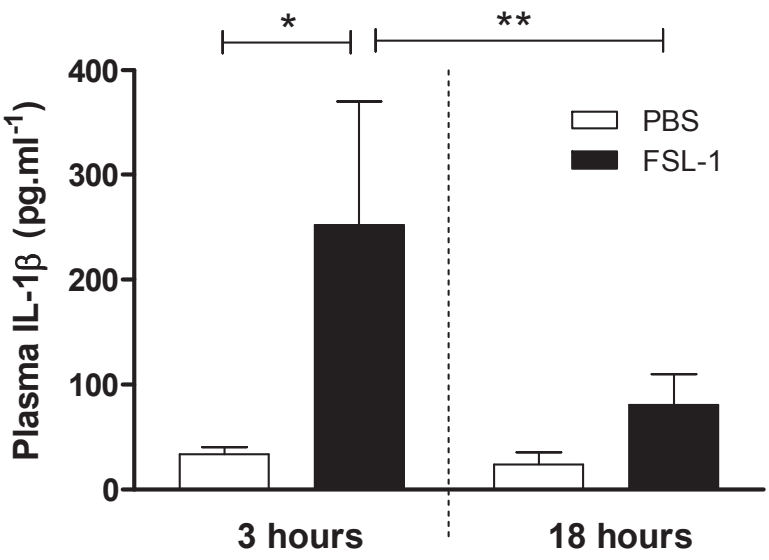

Figure 4.1. Plasma IL-1β concentrations (means ± SD) in rats at 3 h and 18 h after receiving a single i.p. injection of 1 000 μg.kg^{-1} FSL-1 in 1 ml.kg^{-1} PBS (n = 6 at 3 h; n = 9 at 18 h), or 1 ml.kg^{-1} PBS (n = 4 at 3 h; n = 5 at 18 h). Significant differences based on log-transformed data: * 3 h [IL-1β]: FSL-1 vs. PBS; : ** 3 h [IL-1β] FSL-1 vs. 18 h [IL-1β] FSL-1.

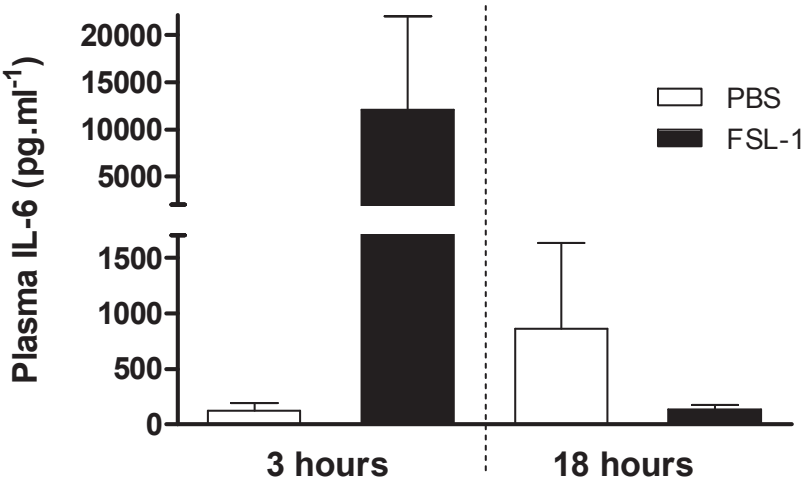

Figure 4.2. Plasma IL-6 concentrations (means ± SD) in rats at 3 h and 18 h after receiving a single i.p. injection of 1 000 μg.kg^{-1} FSL-1 in 1 ml.kg^{-1} PBS (n = 6 at 3 h; n = 9 at 18 h), or 1 ml.kg^{-1} PBS (n = 4 at 3 h; n = 5 at 18 h).

Figure 4.3 shows brain IL-1β concentrations three hours and eighteen hours after injections of FSL-1 or PBS. Three hours after injections there was a significant intervention effect (F (1,16) = 7.31, P = 0.02), but there were no significant differences between brain sites (F (1,16) = 1.48, P = 0.24) and no interaction (F (1,16) = 1.09, P = 0.31). At this time point (3 h), the concentration of IL-1β in rats that had received FSL-1 was elevated significantly in the hypothalamus (P < 0.05). Eighteen hours after injections there was a significant intervention effect (F (1,24) = 18.73, P = 0.0002), but there were no significant differences between brain sites (F (1,24) = 1.79, P = 0.19) and no interaction (F (1,24) = 0.73, P = 0.40). At this time point (18 h), when other rats were learning in a Maze (see **Chapter 3**), rats that had received FSL-1 had significantly elevated IL-1β concentrations in the hippocampus (P < 0.05) and in the hypothalamus (P < 0.01), compared to those rats that had received PBS. However, concentrations of IL-1β in the hippocampus and hypothalamus were not significantly different.

For brain IL-6 concentrations (Fig. 4.4) at three hours after injections there was no significant intervention effect (F (1,16) = 2.67, P = 0.12), or brain site effect (F (1,16) = 1.28, P = 0.27) or interaction (F (1,16) = 0.62, P = 0.44). At three hours, the concentrations of IL-6 were not significantly different in the hippocampus (P = 0.56) nor in the hypothalamus (P = 0.11) for rats that had received either FSL-1 or PBS. Similarly, at eighteen hours after injections, there was no significant intervention effect (F (1,24) = 1.78, P = 0.19), or brain site effect (F (1,24) = 0.30, P = 0.59) or interaction (F (1,24) = 0.40, P = 0.53). At eighteen hours, the concentrations of IL-6 were not significantly different in the hippocampus (P = 0.18) nor in the hypothalamus (P = 0.63) for rats that had received either FSL-1 or PBS.

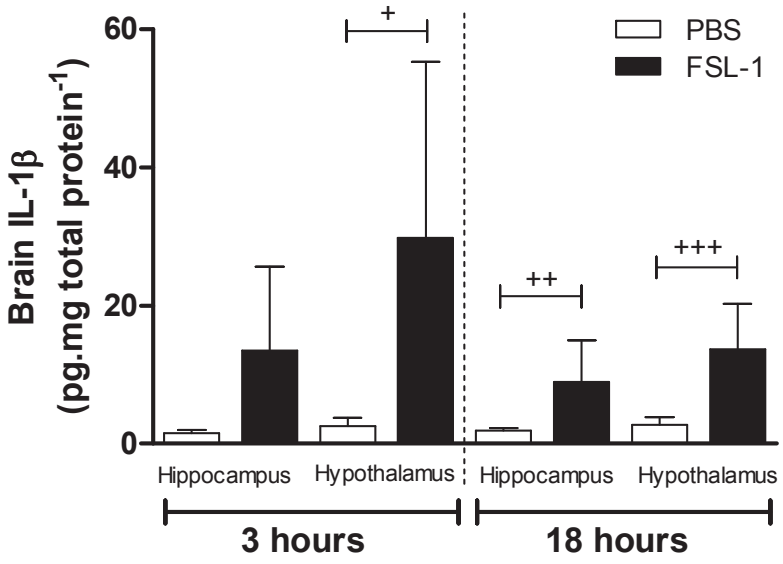

Figure 4.3. Brain IL-1β concentrations (means ± SD) in rats at 3 h and 18 h after receiving a single i.p. injection of 1 000 µg.kg^{-1} FSL-1 in 1 ml.kg^{-1} PBS (n = 6/brain site at 3 h; n = 9/brain site at 18 h), or 1 ml.kg^{-1} PBS (n = 4/brain site at 3 h; n = 5/ brain site at 18 h). Significant differences based on log-transformed data: $^{+}$ 3 h [Hypothalamic IL-1β]: FSL-1 vs. PBS; $^{++}$ 18 h [Hippocampal IL-1β]: FSL-1 vs. PBS; $^{+++}$ 18 h [Hypothalamic IL-1β]: FSL-1 vs. PBS.

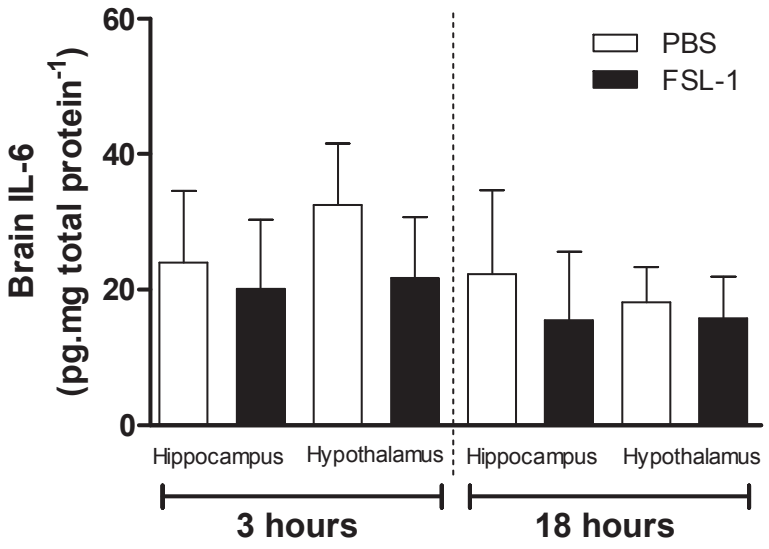

Figure 4.4. Brain IL-6 concentrations (means ± SD) in rats at 3 h and 18 h after receiving a single i.p. injection of 1 000 µg.kg^{-1} FSL-1 (n = 6/brain site at 3 h; n = 9/brain site at 18 h) in 1 ml.kg^{-1} PBS, or 1 ml.kg^{-1} PBS (n = 4/brain site at 3 h; n = 5/ brain site at 18 h). There were no significant differences in either of the brain sites at either time point, after administration of either FSL-1 or PBS.

4.5. DISCUSSION

Pro-inflammatory cytokines, in particular IL-1β and IL-6, appear to be key mediators of fever and sickness behaviours, including lethargy, anorexia and impaired learning and memory (Kelley *et al*., 2003; Dantzer, 2001; Goshen and Yirmiya, 2007). Therefore, I measured concentrations of both IL-1β and IL-6 in the plasma as well as in brain regions known to be important in mediating fever and sickness behaviour, namely the hypothalamus and the hippocampus. My study is the first, I believe, to have measured cytokine concentrations in the brains of rats following administration of FSL-1, which simulates *Mycoplasma* infection. Concentrations of IL-1β and IL-6 in plasma and brain tissue were measured in a sub-sample of rats 3 h after FSL-1 administration, an established time of release of plasma pro-inflammatory cytokines (Hübschle *et al.,* 2006), and in another sub-sample of rats 18 h after FSL-1 administration, a time at which I tested other rats for spatial learning and memory in a Morris Water Maze (**see Chapter 3**).

I found that at 3 h, but not at 18 h, after FSL-1 administration, the concentration of IL-1β was elevated significantly in the plasma (Fig. 4.1). IL-6 concentrations in the plasma were elevated massively, 3 h following administration of FSL-1 (Fig. 4.2), but unfortunately, the data could not be analysed statistically. Thus, 3 h after administration of FSL-1, plasma concentrations of both IL-1β and IL-6 may have a role in the initiation of the febrile response induced by FSL-1 (see section 3.4.1 and Fig. 3.2, **Chapter 3**). In addition, at the same time point (i.e. 3 h) the concentrations of IL-1β, but not IL-6, were elevated significantly in the hypothalamus (Fig. 4.3; Fig. 4.4), thereby allowing a possible involvement for brain concentrations of IL-1β in the initiation of the febrile response induced by FSL-1. In the brain, IL-6 was present, but not up-regulated at either brain site, 3 h after FSL-1 administration, compared to after PBS administration (Fig. 4.4). That IL-6 was not elevated in either the

hippocampus or the hypothalamus indicates that there was neither significant transport of circulating IL-6 into those brain sites, nor *de novo* synthesis of IL-6 in the brain. Three hours after FSL-1 administration may be too early for brain IL-6 concentration to be affected.

Eighteen hours after FSL-1 administration neither the concentration of IL-1β nor that of IL-6 was elevated significantly in the plasma, but the rats still had fever, lethargy and anorexia. Therefore, at 18 h peripheral concentrations of neither IL-1β nor IL-6 could play a role in maintaining FSL-1 induced fever and sickness behaviours. If IL-1β or IL-6 has any role at 18 h after FSL-1 administration it may be a role in the brain. Eighteen hours after FSL-1 administration the concentration of IL-1β (although no longer at its peak), but not IL-6, was elevated significantly to a similar extent in both the hippocampus and the hypothalamus (Fig. 4.3; Fig. 4.4). In my investigations of sickness behavior, 18 h after FSL-1 administration rats actively were learning in a Morris Water Maze (see section 3.4.3 and Fig. 3.6, **Chapter 3**), while at the same time being febrile, lethargic and anorexic. Thus, the concentration of IL-1β in the hypothalamus that was sufficient to accompany sickness responses mediated by the hypothalamus, in this case fever, lethargy and anorexia, did not impair hippocampal function underlying spatial learning. The absence of elevated concentrations of IL-6 in the brain at 18 h after FSL-1 administration (Fig. 4.4) does not prove that IL-6 had no role in mediating the fever, lethargy and anorexia that was evident in the rats at that time. At 18 h, IL-6 was present in the brain (Fig. 4.4) and it is possible that brain IL-6 concentrations may have risen temporarily between 3 h and 18 h after administration of FSL-1. There also is the possibility that IL-1β and IL-6 act synergistically in the brain and that the action of IL-1β may depend on IL-6 being present.

I did not measure concentrations of the pro-inflammatory cytokine, TNF-α, following administration of FSL-1 as others have (Hübschle *et al*., 2006). It is possible that

concentrations of TNF-α in the brain may have a role in mediating fever, lethargy and anorexia induced by FSL-1. Other pro-inflammatory cytokines, namely leptin and cytokine-induced neutrophil chemoattractant-1 (CINC-1) that are known to be involved in fever (Harden *et al.,* 2006; Soares *et al*., 2008; Kamerman *et al*., 2011) also may be involved in fever induced by FSL-1 administration. Moreover, there may be a role for anti-inflammatory cytokines, such as interleukin-10 (IL-10) and IL-1 receptor antagonist (IL-1ra), in modulating the febrile response (e.g. Cartmell *et al*., 2001 Ledeboer *et al*., 2002) to FSL-1 administration. It also may be the case that one or more of the above-mentioned cytokines has a different concentration in the hippocampus and hypothalamus.

For cytokines to be able to modulate fever and sickness behaviours, they have to be able to interact with the brain via humoral or neural pathways (e.g. see Dantzer *et al.*, 2000; Roth *et al*., 2006). Cytokines in the brain, including IL-1β and IL-6, may interact with their receptors (IL-1R and IL-6R) which are expressed abundantly on cells (e.g. astrocytes and microglia) in brain regions including the hippocampus (e.g. granule cells) and the hypothalamus (Loddick *et al.*, 1998; Conti *et al.*, 2008). The hypothalamus controls body temperature and eating behaviour (Lepkovsky, 1973; Morrison *et al*., 2008), but also is important in coordination of cardiovascular function and respiration (e.g. Yeh *et al*., 1997) without which physical ability is not possible. Parenteral administration in rats of FSL-1 increased concentration of IL-1β in the hypothalamus (~ 13 pg.mg protein^{-1}, Fig. 4.3), a concentration which is characteristic of hypothalamic IL-1β concentrations in other simulated infections (Rachal Pugh *et al.*, 2001; Richwine *et al.*, 2008; Richwine *et al.*, 2009; Barrientos *et al.*, 2009; Terrando *et al.*, 2010; Dilger and Johnson, 2010; Harden *et al.*, 2010; Huang *et al*., 2010). My finding also parallels a previous study in rats that showed increased concentrations of IL-1β in several brain regions, including the hypothalamus, following i.c.v. administration of heat-inactivated *M. fermentans* (Yirmiya *et al*., 1999). However, my study is the first to show that in simulated

systemic *Mycoplasma* infection, induced by i.p. administration of FSL-1, IL-1β in the hypothalamus may have a role in mediating fever, lethargy and anorexia (see **Chapter 3**).

Like some using LPS (Richwine *et al*., 2008), I did not find an increase in brain IL-6 concentrations (Fig. 4.4). However, increased brain IL-6 concentrations have been associated with fever and sickness behaviour during simulated Gram-negative bacterial infection (Nilsberth *et al.*, 2009; Richwine *et al.*, 2009; Terrando *et al.*, 2010; Frank *et al.*, 2010; Teeling *et al.*, 2010). The actions of IL-6 to causing an increase in body temperature, perhaps also to cause sickness behaviour may depend on IL-6 being present in the brain concurrently, if IL-6 acts synergistically with IL-1β within the brain to induce fever and sickness behaviour in rats (Cartmell *et al*., 2000; Harden *et al*., 2008). Central, or peripheral, co-administration of non-pyrogenic doses of IL-1β and IL-6 induced fever in rats, but when injected alone, neither cytokine had any effect on body temperature (Cartmell *et al*., 2000; Harden *et al*., 2008). So, because IL-6 in the brain seems to act in concert with IL-1β, I hypothesize that the presence of IL-6 in the brain may have facilitated the action of IL-1β, even though brain IL-6 concentration itself did not change.

It is also possible that IL-6 may affect some, but not all brain functions: administration (i.p.) in mice of biologically active IL-6, at doses between 0.031-2.0 μg per animal, reduced the scopolamine-induced amnesia without affecting scopolamine-induced hyperactivity (Bianchi *et al.*, 1997). That IL-6 may have a role in central modifications that occur following peripheral immune activation was confirmed in a study that reported peripheral endogenous IL-6 to be a primary mediator of fever and sickness behaviour induced by s.c. administration of LPS (Harden *et al*., 2006). Similar to the increased plasma concentrations of IL-6 I have observed following i.p. administration of FSL-1, Hübschle and colleagues (2006) previously have reported elevated plasma concentrations of IL-6 in rats, which was associated with the

fever induced by i.p. administration of 100 μg.kg^{-1} FSL-1 (Hübschle *et al*., 2006). Intraperitoneal or i.a. administration of FSL-1 (100 or 1000 μg.kg^{-1}) in guinea pigs also induced a marked increase in plasma concentrations of IL-6, which coincided with the observed fever (Greis *et al*., 2007). Anomalously, after intratracheal inoculation of *M. hyopneumoniae* (strain P5722–3, 1 x 10^{10} units.L^{-1}) in growing pigs IL-6 was undetectable in the plasma of infected pigs (Escobar *et al.*, 2004). In spite of this anomalous result, systemic administration of most PAMPs generally induces a substantial rise in plasma concentrations of IL-6, which may be associated with the fever and sickness behaviours following administration of that PAMP, even though brain concentrations of IL-6 may not be elevated concurrently. Circulating concentrations of IL-1β were undetectable in pigs infected with *M. hyopneumoniae* (strain P5722–3, 1 x 10^{10} units.L^{-1}), so my finding of significant elevations in concentrations of IL-1β in the plasma 3 h after administration of FSL-1, which simulates *Mycoplasma* infection, is novel.

The role of IL-1β, but also IL-6, has been investigated extensively in another aspect of sickness behaviour, namely impaired learning and memory (see Goshen and Yirmiya, 2007), but the results remain controversial. However, most of the studies have shown that elevated concentrations of either IL-6, or IL-1β, are detrimental for spatial learning and memory (reviewed in Goshen and Yirmiya, 2007). The hippocampus is crucial for spatial learning and memory processes (Morris *et al*., 1982; Squire, 1992). Interestingly, i.c.v. administration of IL-1β, but not IL-6, into the hippocampus of experimental animals induced impairment in spatial learning and memory (Oitzl *et al*., 1993; Pugh *et al*., 1999; Barrientos *et al*., 2002). The lack of impairment in learning and memory after administration of IL-6 was confirmed in IL-6 knockout mice in which an improvement in learning and memory was observed (Balschun *et al*., 2004). Improved learning and memory in knockout mice may imply that endogenous IL-6 indeed does impair learning and memory, even if additional IL-6 does not

worsen the impairment. So, the role of IL-6 in learning and memory seems to be complex. That I found IL-6 to be present in the brain, including the hippocampus, without having an effect (detrimental or beneficial) on spatial learning and memory (**Chapter 3**), is in line with studies that also reported no effects after i.p. administration of IL-6 (Bianchi *et al*., 1997, 1998; Brennan *et al*., 2004). Moreover, I did not find impairment in spatial learning of rats despite concentrations of IL-1β being elevated significantly in the hippocampus (~ 9 pg.mg protein^{-1}) at 18 h after FSL-1 administration. My finding is consistent with a recent study that showed no impairment in spatial memory of rats despite significant elevations of IL-1β in the hippocampus (~ 7 pg.mg protein^{-1}), 4 h after i.p. administration of 1.25 mg.kg^{-1} LPS (Huang *et al*., 2010). IL-1β seems to be the cytokine induced most consistently in the brain following immune activation (Abraham and Williams, 2003). Therefore, numerous studies have investigated the effect of administration of IL-1β itself on cognition and have shown elevated concentrations of this cytokine to be detrimental for learning and memory, whether administered i.c.v., i.p. or intra-hippocampal (reviewed in Goshen and Yirmiya, 2007). Despite these reports, there remains uncertainty concerning the specific involvement of IL-1β, produced endogenously in the hippocampus during simulated infection, on spatial learning and memory. An inverted U-shaped dose-response relationship has been proposed for IL-1β and memory functioning (Goshen *et al.*, 2007). According to this model, basal or slight elevations in IL-1β concentrations, specifically in the hippocampus, improve and promote aspects of spatial memory whereas any deviation from the physiological range, either by excessive elevation in IL-1β concentrations or by blocking IL-1β signalling, impair hippocampal-dependent memory and neural plasticity (see Goshen and Yirmiya, 2007). Thus, the role of both IL-1β and IL-6 in hippocampal-dependent learning and memory processes, during simulated infection, still is inconclusive. Further research is needed not only to elaborate why simulated infection sometimes affects learning and memory and

sometimes not, but also to elucidate the role of pro-inflammatory cytokines when learning and memory are impaired.

In conclusion, I have confirmed that in experimental animals the acute phase responses to i.p. administration of FSL-1, including fever, lethargy and anorexia are associated with elevated plasma and brain concentrations of the cytokines IL-1β and IL-6. Because concentrations of IL-1β were elevated to a similar extend in both the hippocampus and the hypothalamus 18 h following administration of FSL-1, the lack of impairment in spatial learning and memory that I have found at a similar time when rats actively were learning in a Maze (see **Chapter 3**) cannot be explained by differential production of pro-inflammatory cytokines in the brain. I support the emerging evidence that learning and memory not always are impaired when there is, at the same time, a significant increase in concentration of IL-1β, specifically in the hippocampus. A fundamental relationship between elevated concentrations of pro-inflammatory cytokines in the hippocampus and impairment in learning and memory has not been established yet.

CHAPTER 5

SICKNESS BEHAVIOURS DURING SIMULATED

RECURRENT ACUTE *MYCOPLASMA* INFECTION

Data presented in this chapter have been published

Swanepoel T., Harvey BH., Harden LM., Laburn HP. and Mitchell D.
Simulated systemic recurrent *Mycoplasma* infection in rats induces recurrent sickness responses without residual impairment in spatial learning and memory.
Physiology and Behavior **105:** 800-808.

5.1 ABSTRACT

In spite of their prevalence and importance clinically, recurrent acute infections seldom have been investigated in the laboratory. I set out to measure fever and sickness behaviour in simulated recurrent *Mycoplasma* infection; *Mycoplasma* is a common cause of recurrent acute infection in humans. Specifically, the aim was to investigate residual detrimental cognitive effects, such as impaired learning and memory, after apparent recovery from a series of simulated recurrent infections. Male Sprague-Dawley rats had radiotransponders implanted to measure abdominal temperature and cage activity. After recovery, rats received three intraperitoneal (i.p.) injections, 10 days apart, of either fibroblast-stimulating lipopeptide-1 (FLS-1), a pyrogenic moiety of *Mycoplasma salivarium* at a dose of 500 $\mu g.kg^{-1}$ in 1 $ml.kg^{-1}$ phosphate-buffered saline (PBS), or vehicle (PBS, $1ml.kg^{-1}$). Body mass and food intake were measured daily. For measurement of learning and memory, training in a Morris Water Maze commenced 10 days after the last of the three successive injections and continued daily for four days. Spatial memory was assessed on the following day. Hippocampal tissue of rats was collected on the day of the last exposure to the Maze. Recurrent FSL-1 administration induced recurrent fevers (~ 1 ºC) for about 9 hours, recurrent lethargy (~ 40-60 %) for one day, recurrent anorexia (~ 16-30 %) for one day and recurrent reductions in the rate of mass gain (~ 112 %) for one day, but the stunting resolved by the subsequent injection. Recurrent FSL-1 administration did not result in tolerance to fever, lethargy or anorexia. There was no residual histological damage to the hippocampus and no residual detrimental effect in learning or memory in rats. Though we cannot extrapolate our results directly to humans, our results imply that children who have recovered from recurrent acute *Mycoplasma* infection may not be at high risk of stunting or impaired spatial learning and memory.

5.2 INTRODUCTION

Particularly in resource-poor countries, exposure to infectious agents during childhood is inevitable. Recurrent childhood infections and recurrent fever, accompanied by a suite of brain-controlled behavioural changes (sickness behaviours), can hamper seriously the growth and overall development of children (Martorell *et al*., 1975; Rowland *et al*., 1988). *Mycoplasma* is a common causative agent for recurrent community-acquired pneumonia and affects children, adolescents and adults (see McIntosh, 2002). Whereas many adults with *Mycoplasma pneumoniae* present as asymptomatic (giving the infection the nickname of "walking pneumonia"), the same is not true for infected children (for reviews see Waites and Talkington, 2004; Vervloet *et al.*, 2007). For example, a follow-up case report of an adolescent patient with *M. pneumoniae* showed impaired executive functioning and memory deficits, as long-term sequelae (Termine *et al.*, 2005). Thus, a potential problem for developing countries, which needs to be addressed, is whether growth and capacity to learn in children is compromised by a recurrent acute infection, such as *M. pneumoniae*, even when the children appear well after such an infection.

Studying the effects of recurrent, *Mycoplasma*-induced, acute activation of the innate immune system, especially on cognition and growth in children, has been hampered by a lack of an adequate animal model that does not involve a live pathogen. The opportunity for such a study has been realised by the recent availability of a synthetic lipopeptide, fibroblast stimulating lipopeptide-1 (FSL-1), an analogue of a pyrogenic moiety of *Mycoplasma salivarium*. After it was discovered that FLS-1 is capable of stimulating cells implicated in innate immunity (Shibata *et al*., 2000; Nakamura *et al*., 2002; Okusawa *et al*., 2004), subsequent *in vivo* studies showed that acute intraperitoneal administration of FLS-1 induced fever and sickness behaviours in rats and guinea pigs, which was accompanied by

elevations in plasma and brain concentrations of the pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour-necrosis factor alpha (TNF-α) (Hübschle *et al*., 2006; Greis *et al*., 2007; **Chapter 4**). The duration of the sickness behaviours induced by FSL-1 outlasted the febrile response (Hübschle *et al*., 2006; **Chapter 4)**.

Because sickness behaviours may outlast the fever after stimulation of the innate immune system, it is possible that the sickness behaviours, rather than the hyperpyrexia, are better biomarkers of long-term sequelae. For example, "anorexia of infection" (Cole and Parkin, 1977) may act as part of an active defence strategy (Exton, 1997) to conserve energy during acute infections, but, when an infection occurs frequently or becomes chronic, the reduced food intake may contribute to mass loss, muscle atrophy and fatigue (Ravasco *et al.*, 2004; Ravasco *et al.*, 2007). Although the suite of sickness behaviours is complex, those relevant to my study are anorexia, lethargy and impairment in learning and memory. These three sickness behaviours may have deleterious long-term sequelae in children: anorexia because of growth retardation or permanent stunting, lethargy because of loss of motivation for life-sustaining physical activity, and impairment of learning and memory because of subsequent cognitive retardation, the latter likely to have the most impact on the child's welfare.

To discern whether there is any impairment in growth or physical activity, or residual impairment in learning and memory, following recurrent acute activation of the innate immune system, I investigated the effects of recurrent acute FSL-1 administration in growing rats between the ages of 36 and 56 days, which represents the adolescent age group in humans (see Quinn, 2005). The rats received three intraperitoneal injections of the same dose of FSL-1, 10 days apart, which I showed to be long enough apart for the acute effects on innate immunity to have resolved, and for each subsequent response to have the same magnitude. I then tested for residual effects on learning and memory after the rats had

recovered from the effects of the third injection. To do so I used the Morris Water Maze, which is a preferred instrument to assess spatial learning and memory deficits in experimental animals (for review see D'Hooge and De Deyn, 2001) and in humans (i.e. the virtual Morris Water Maze) (Aquirre *et al*., 1996; Astur *et al*., 2004; Kallai *et al*., 2005; Goodrich-Hunsaker *et al*., 2010). In addition, I measured body core temperature, cage activity, food intake and rate of growth throughout the acute stimulations of innate immunity, and in the subsequent period of recovery. Necrotic and apoptotic neuronal cell death of the hippocampal formation has been reported after experimental infusion of lipopolysaccharide (LPS, the pyrogenic moiety of Gram-negative bacteria) (Hauss-Wegrzyniak *et al*., 1998; Lee *et al*., 2008; Cui *et al*., 2008), but also in autopsy and animal models of bacterial meningitis (Leib *et al*., 1996; Zysk *et al*., 1996; Nau *et al*., 1999). Therefore, I also examined the histology of tissue from the hippocampus, a brain area crucial for spatial learning and memory (Morris *et al*., 1982; Squire, 1992), after rats had received three spaced injections of FSL-1 and once the Morris Water Maze procedures were completed.

My aim was not to simulate chronic infections. Rather, I simulated recurrent acute infections (i.e. with recovery between infections) that are typical of *Mycoplasma* infections (e.g. pneumonia). Thus, although residual impairment in learning and memory has been detected in rats following acute, repeated (i.e. consecutive days) or chronic (i.e. infusion) administration of LPS (Aubert *et al*., 1995; Hauss-Wegrzyniak *et al*., 1998, 1999; Sparkman *et al*., 2005b), my study is to the best of my knowledge the first to assess residual deficits in learning and memory in otherwise healthy rats, following recurrent acute stimulation of the innate immune system.

5.3 MATERIALS AND METHODS

(Also refer to **Chapter 2**, i.e. 'Common methodology')

5.3.1. Animals

Thirty three male Sprague-Dawley rats with a body mass of 130 ± 27 g on the day of the first injection were used, with no statistically significant differences in the mean body mass of the treatment groups. On the day of the first injection the rats were about 5 weeks old. When the procedures ended about a month later, the rats weighed 362 ± 26 g. The rats had access to pelleted rat chow and water *ad libitum* and were housed individually in cages in a temperature-controlled room on a 12:12 hour light: dark cycle, with lights on at 07:00 local time (see section 2.1 in **Chapter 2**).

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 (clearance certificate AESC 2007/72/4).

5.3.2. Pyrogen administration

The lyophilized powder of fibroblast-stimulating lipopeptide-1 (FSL-1) was reconstituted with sterile, pyrogen-free phosphate-buffered saline (PBS) to a concentration of 1ml.kg^{-1}. Based on pilot studies, I gave the rats three intraperitoneal (i.p.) injections of FSL-1 at a dose of 500 µg.kg^{-1}, 10 days apart, to simulate recurrent acute infection with *Mycoplasma*. I have shown in **Chapter 3** that a dose of 500 µg.kg^{-1} FSL-1 induced pronounced fever and sickness behaviours in rats within 6 h of administration, while pilot studies showed that tolerance to the pyrogenic effect of FSL-1 did not develop when it was injected at 10 day intervals. I

injected the rats at 16:00 so that the peak in response to FSL-1 would occur during the dark phase of the 24 h day, when rats are most active.

5.3.3. Body temperature and cage activity

I recorded body core temperature and cage activity of rats continuously by remote biotelemetry as discussed in detail in section 2.2 of **Chapter 2**. I analysed nocturnal activity (19:00 - 07:00), which accounts for virtually all of a rat's voluntary locomotor activity and is not confounded by activities such as food replenishment.

5.3.4. Food intake and body mass

Food intake and body mass of the rats, before and after intervention, were recorded daily at 16:00 (refer to section 2.4 of **Chapter 2**).

5.3.5. Learning and memory: Morris Water Maze

The Morris Water Maze apparatus and the protocol I used are discussed in detail in section 2.5 of **Chapter 2**.

Spatial learning and memory in the Morris Water Maze were measured as described previously (see section 2.5 in **Chapter 2**, but also see Fig. 5.1). Because tests in a Morris Water Maze are sensitive to technical and procedural variables, I paid particular attention to exclude or reduce potential confounders that I could manage, such as water temperature, room temperature, dimensions of the pool, gender, housing and hormonal status of the rats. I also handled the rats regularly in an attempt to reduce stress levels.

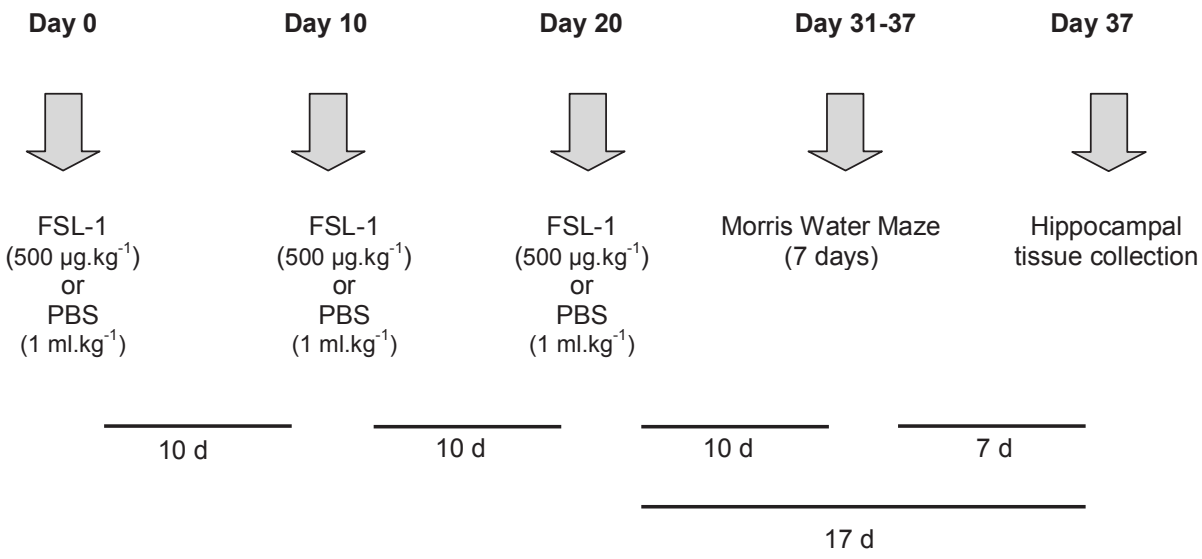

Figure 5.1. Sequence of injections and investigations. Rats received three single injections, at 10 d intervals, of either FSL-1, or PBS, before they were tested in a Morris Water Maze.

I measured the rats' swim speed, latency and distance travelled to the platform, and the time spent in the target zone of the maze. I previously have validated the protocol for use of the Morris Water Maze (see section 2.5.3 in **Chapter 2**, but also **Chapter 3**), by testing rats given scopolamine (Sigma-Aldrich, St. Louis, MO, USA), a drug known to induce amnesia in laboratory animals (Diez-Ariza *et al.*, 2003; Janas *et al.*, 2005; Choi *et al.*, 2006).

5.3.6. Experimental procedures

The rats were assigned randomly to two groups, one group receiving three successive i.p. injections of 1ml.kg^{-1} PBS (n = 16), and the other receiving three successive i.p. injections of 500 µg.kg^{-1} FSL-1 in PBS (n = 17), at 1 ml.kg^{-1} volume. The injections were given at 10 d intervals (Fig. 5.1).

On the eleventh day after the last of the three injections, rats started their training in the Morris Water Maze (see Fig 5.1; procedure described in detail in **Chapter 2**). They were given an initial habituation trial in the Maze (see Morris, 1981), a single 60 s "Cued" test (see Morris, 1984), four days of training (i.e. phase of acquisition of memory) and a single 30 s "Probe" trial. One hour after the "Probe" trial, the rats again were tested in a "Cued" test. Rats then were returned to their home cages, until euthanasia the next day when hippocampal tissue was excised (see section 5.3.7 below). Over the seven days of the Morris Water Maze procedure, I continued to measure body core temperature and cage activity with biotelemetry as well as food intake and body mass (see sections 5.3.3 and 5.3.4 above).

5.3.7. Histopathology

I examined hippocampal tissue in a sub-sample of rats (n = 19) for possible histopathological changes induced by recurrent i.p. injections of either 500 $\mu g.kg^{-1}$ FSL-1 (n = 10) or 1 $ml.kg^{-1}$ PBS (n = 9). Brain tissue of rats destined for histology was collected 17 days after administration of the last of the three injections, i.e. the day of the last exposure to the Morris Water Maze (see Fig. 5.1). Rats were anaesthetized deeply with an i.p. injection of 1 ml sodium pentobarbital (Euthapent, 200 $mg.ml^{-1}$; Kyron Laboratories (Pty) Ltd., South Africa) and were perfused transcardially for 2 min with about 250 ml of ice-cold sterile saline (0.9 % NaCl) via the left ventricle. Hippocampal tissue was dissected out rapidly on an ice-chilled plate and placed in microcentrifuge tubes, to which 0.5 ml RNA*later* Solution (AEC Amersham, (Pty) Ltd., South Africa) was added to preserve the tissue. Post-fixed hippocampal tissue, in the micro-centrifuge tubes, then was snap-frozen in liquid nitrogen and stored at – 80 ºC. For histopathological examination, specimens were cut at 50 µm intervals and stained in cresyl violet. The sections were inspected for necrosis, and cells in the dentate gyrus with morphological changes compatible with apoptosis were counted with a microscope (Olympus BX41) in three visual fields (per rat) at 400x magnification. Apoptosis was scored as follows: 0-5 apoptotic cells = 0; 6-30 apoptotic cells = 1; more than 30 apoptotic cells = 2 (see Pfister *et al.*, 2000). The histopathology was performed by a qualified histopathologist, Bridget Mitchell, who was blind to the experimental interventions.

5.3.8. Data analysis

I used the thermal response index (TRI, ºC.h) as an integrated measure of fever duration and magnitude. To take account of circadian rhythms in body temperature, TRIs were calculated as the time integrals of the differences between the abdominal temperature of

each rat after injection and the abdominal temperature of that rat at the same time of day, averaged for the three days before the day of injection, when the rats were undisturbed in their home cages. TRIs were calculated separately for day 0, day 10 and day 20, over a 12 h period starting at 19:00, i.e. three hours after injections. For statistical analyses, TRIs were compared between groups separately on days 0, 10 and 20 by *t*-tests. Nocturnal cage activity counts detected over 5 min were summed for the same twelve hours following injections. Cage activity was expressed as a percent change from the mean activity measured for the same time period over three nights before the injection. For statistical analyses, percent change in cage activity was compared between the groups over the first five days following each of the injections, separately, by two-way repeated-measures ANOVA.

Food intake was expressed as grams of food consumed in 24 h per 100 g of rat body mass. Twenty-four hour change in body mass (g) of each rat was determined by subtracting the body mass measured at 16:00 from the body mass measured at the same time on the previous day. The rate of mass gain was expressed as change in body mass per 24 h ($g.d^{-1}$). For statistical analyses, changes in food intake as well as changes in body mass were compared between the groups over the first two days following each of the injections, separately, by two-way repeated-measures ANOVA.

For each of the training days in the Morris Water Maze the mean speed, mean distance and latency to reaching the submerged platform over all four trials on that day was determined for each rat and then averaged across the group of rats. Changes in the indices measured during the four-day acquisition phase in the Morris Water Maze were compared between groups and over time, by two-way repeated-measures ANOVA. Indices measured during the Cued tests were analyzed by means of unpaired *t*-tests. The percentage time spent in the

zones of the Morris Water Maze during the Probe trial was analyzed with one-way ANOVA, separately, for rats that had received PBS and for rats that had received FSL-1. Appropriate corrections were applied for multiple comparisons and *post hoc* tests were performed when ANOVA showed significance.

Data are expressed as mean ± SD and a statistical significance was accepted for $P < 0.05$.

5.4 RESULTS

5.4.1. Body temperature and cage activity

Five minute recordings of abdominal temperature responses were averaged over 15 min intervals and plotted as temperature-time curves (Fig. 5.2). Handling and injection caused an immediate, but temporary, hyperthermia of similar magnitude and duration for rats receiving both FSL-1 and PBS injections. Then, about 3 h after each of the three successive injections, on day 0, day 10 or day 20, abdominal temperatures of rats that had received FSL-1 started to exceed those of rats that had received PBS. On average the body temperature of rats that had received FSL-1 peaked approximately 6 h after the injections, at about 38.8 ºC, a degree higher than that of rats that had received PBS. Twelve hour TRIs (19:00-07:00) calculated for rats that had received FSL-1 injections showed significant differences to the TRIs calculated for rats that received PBS (Fig. 5.3) on day 0 (t (31) = 6.79, $P < 0.0001$), day 10 (t (31) = 4.96, $P < 0.0001$), and day 20 (t (31) = 5.95, $P < 0.0001$). The mean 12 h TRIs on day 0, day 10 and day 20 of rats that had received FSL-1 were not significantly different from each other (F (2,32) = 1.30, P = 0.29, one-way repeated measures ANOVA) (Fig. 5.3).

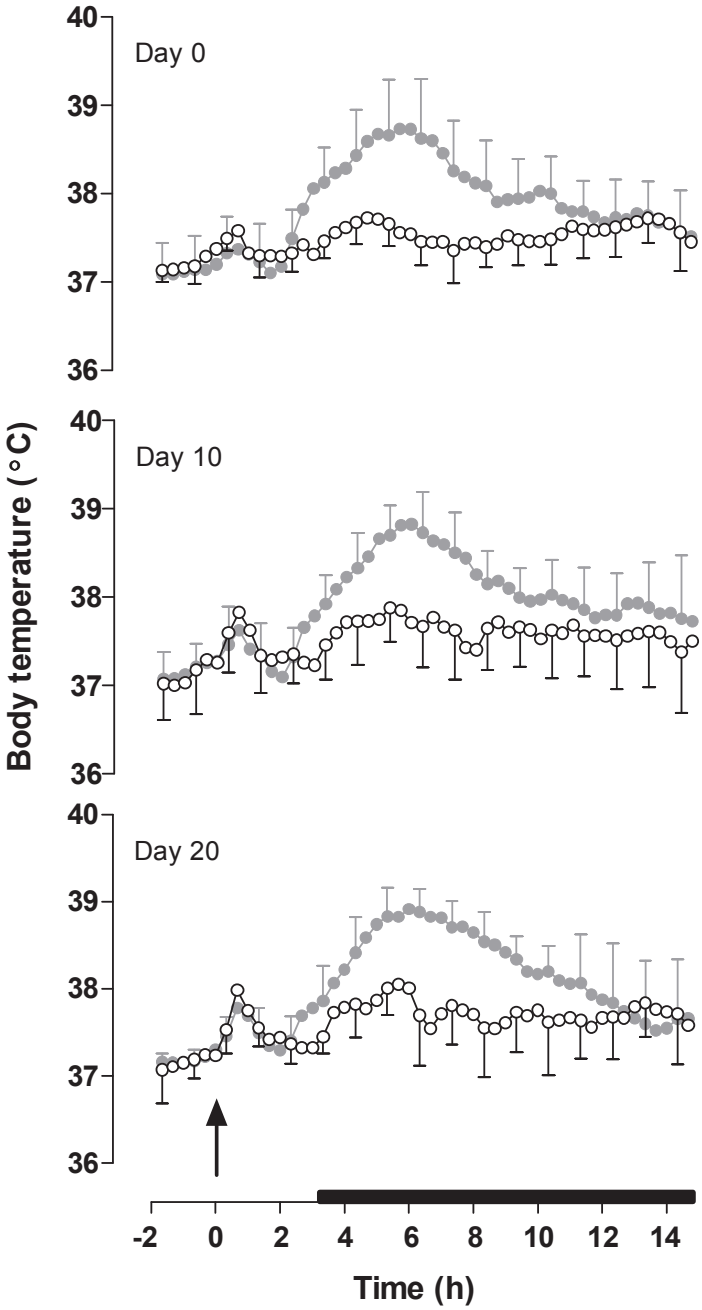

Figure 5.1. Abdominal temperature (mean ± SD) of rats from 2 h before to 15 h after they received three single i.p. injections, spaced 10 days apart, of either 500 μg.kg^{-1} FSL-1 in 1 ml.kg^{-1} PBS (●; n = 17), or 1 ml.kg^{-1} PBS (○; n = 16). The arrow indicates time of injection (16:00) and the black bar indicates lights off (19:00-07:00). See Fig. 5.3 for statistical analyses.

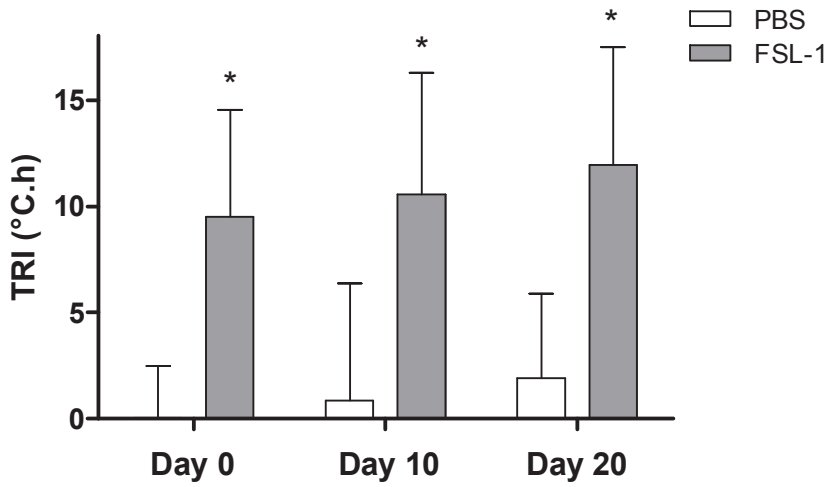

Figure 5.2. Twelve hour night-time (19:00 - 07:00) thermal response indices (TRIs) (mean ± SD) of rats after receiving three i.p. injections, 10d apart, of 1 ml.kg^{-1} PBS (n = 17) or FSL-1 at a dose of 500 µg.kg^{-1} in 1 ml.kg^{-1} PBS (n = 16). Significant differences: * PBS vs. FSL-1.

Figure 5.4 shows the change in nocturnal cage activity of rats after receiving an i.p. injection of either FSL-1 or PBS. On average over the first nights after injection, the activity of rats was depressed by 59 ± 21% after the first injection, by 40 ± 31% after the second injection and by 47 ± 29% after the third injection and those depressions were not significantly different from each other (F (2,32) = 1.63, P = 0.21, one-way repeated measures ANOVA). The main effects of intervention (F (1,31) = 58.99, P < 0.0001), time (F (4,124) = 49.16, P < 0.0001) and interaction (F (4,124) = 41.57, P = 0.27) after the first injection were significant such that the activity of rats was depressed significantly over the first (P < 0.003) and second (P < 0.003) nights after the FSL-1 injection compared to the activity of rats that received PBS. The main effects of intervention (F (1,31) = 13.09, P = 0.001), time (F (4,124) = 18.11, P < 0.0001) and interaction (F (4,124) = 17.51, P < 0.0001) after the second injection were significant such that the activity of rats was depressed significantly only over the first night after the FSL-1 injection compared to activity of rats that received PBS (P < 0.003). Similarly, the main effects of intervention (F (1,31) = 6.24, P = 0.02), time (F (4,124) = 30.61, P < 0.0001) and interaction (F (4,124) = 30.52, P < 0.0001) after the third injection of FSL-1 were significant such that the activity of rats was depressed significantly only over the first night after the injection of FSL-1 compared to activity of rats that received PBS (P < 0.003).

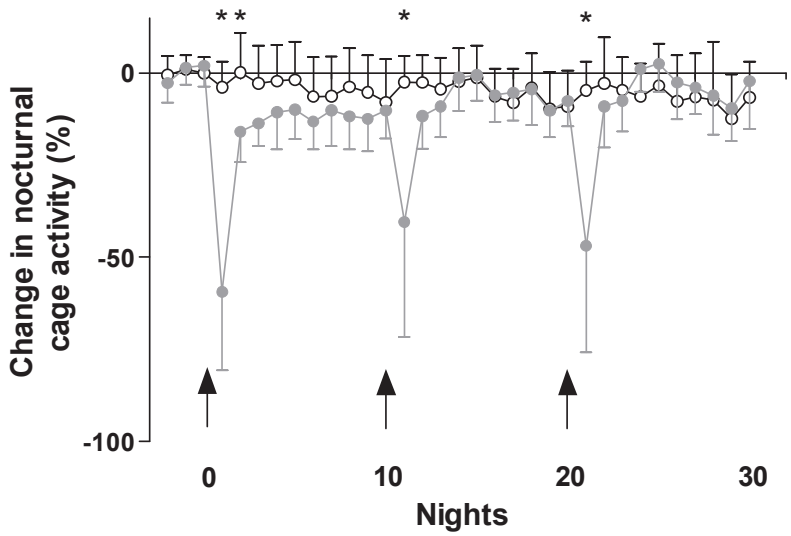

Figure 5.4. Nocturnal (19:00-07:00) cage activity (mean ± SD) of rats after receiving three, spaced, i.p. injections of either 500 μg.kg^{-1} FSL-1 in 1 ml.kg^{-1} PBS (●; n = 17), or ml.kg^{-1} PBS (○; n = 16). Cage activity was expressed as a percent change from the mean activity measured over the same time period for the four days before the first injection. Negative changes represent a reduction in cage activity below pre-injection levels. The arrows indicate the days on which each of the three injections was administered. Significant differences: * FSL-1 vs. PBS; see sections 5.3.3 and 5.3.8 in text for details.

5.4.2. Food intake and body mass

On average, rats consumed 19.7 ± 2.1 g of food per 100 g of body mass over the three days before receiving FSL-1 or PBS injections (Fig. 5.5A). The main effect of intervention (F (1,31) = 12.62, P = 0.001), time (F (1,31) = 18.43, P = 0.0002) and interaction (F (1,31) = 9.36, P = 0.005) after the first injection of FSL-1 were significant such that rats consumed significantly less food over the first day compared to rats that received PBS (P < 0.003). After the second injection, the main effect of intervention (F (1,31) = 3.45, P = 0.07), time (F (1,31) = 0.98, P = 0.33) and interaction (F (1,31) = 6.75, P = 0.01) indicated that there were no significant differences in the amount of food consumed by rats that received either FSL-1 or PBS. After the third injection the main effect of intervention (F (1,31) = 24.86, P < 0.0001), time (F (1,31) = 6.71, P = 0.01) and interaction (F (1,31) = 24.11, P < 0.0001) were significant such that rats consumed significantly less food over the first day immediately after the FSL-1 injection compared to rats that received PBS (P < 0.003). Furthermore, after each of the three FSL-1 injections, the 24 h change in food consumption (per 100 g of BM) of rats was significantly different (F (2,32) = 12.10, P = 0.0001, one-way repeated measures ANOVA): the decrease in food consumption after the first FSL-1 injection was significantly greater than that after the second (P < 0.05) and third (P < 0.05) FSL-1 injections. From the third day after each of the three injections, rats that had received FSL-1 consumed similar amounts of food to the rats that had received PBS.

Figure 5.5B shows the rate of mass gain of rats before and after they had received three i.p. injections of either FSL-1 or PBS, spaced 10 d apart. Before injections, rats grew at similar constant growth rates. However, after the first injection of either FSL-1 or PBS, the main effect of intervention (F (1,31) = 15.91, P = 0.0004), time (F (1,31) = 87.26, P < 0.0001) and interaction (F (1,31) = 44.13, P < 0.0001) were significant such that the rate of mass gain of

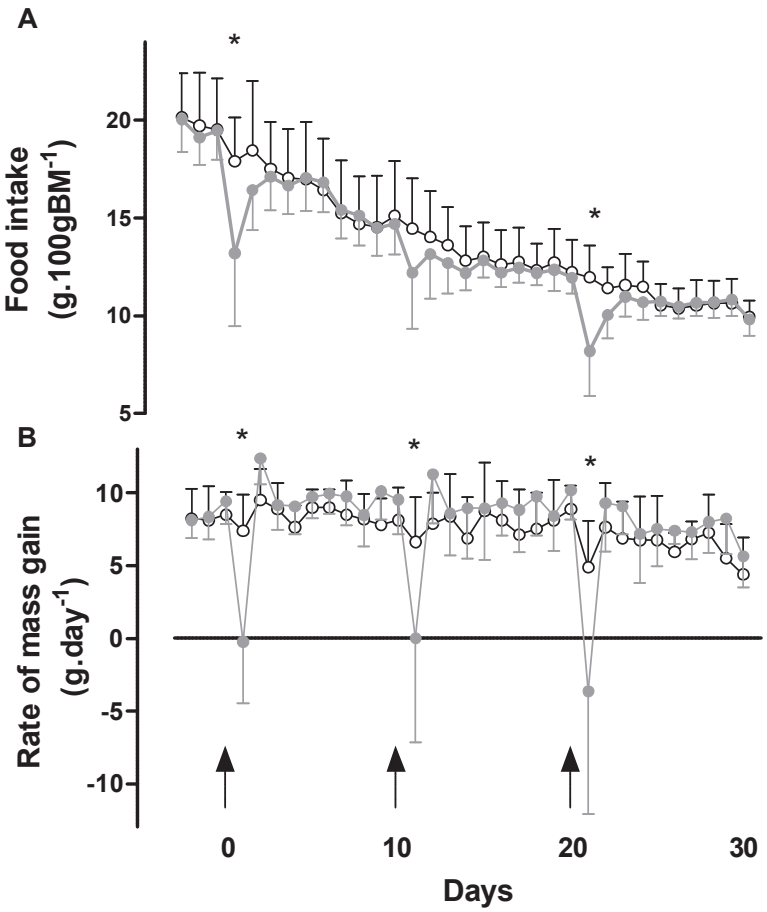

Figure 5.5. Daily (24 h) food intake (mean ± SD) and rate of mass gain (mean ± SD) of rats measured for 10 d after receiving three, spaced, i.p. injections of either 500 µg.kg^{-1} FSL-1 in 1 ml.kg^{-1} PBS (●; n = 17), or 1 ml.kg^{-1} PBS (○; n = 16). Food intake (A) was calculated as grams of food consumed in 24 h per 100 g of body mass on that day. The rate of mass gain (B) was calculated over successive 24 h intervals starting at 16:00, and was expressed as grams of body mass gain, per day. The arrows indicate the days on which each of the three injections was administered. Significant differences: * FSL-1 vs. PBS; see sections 5.3.4 and 5.3.8 in text for details.

rats that had received FSL-1 was reduced over the first day after the injection compared to that of rats that had received PBS (P < 003). Similarly, after the second injection of either FSL-1 or PBS, there was no significant intervention effect (F (1,31) = 3.84, P = 0.06), but the main effect of time (F (1,31) = 23.18, P < 0.0001) and interaction (F (1,31) = 14.86, P < 0.0005) were significant such that the rate of mass gain of rats that had received FSL-1 was reduced significantly over the first day compared to that of rats that had received PBS (P < 0.003). After the third injection of either FSL-1 or PBS the main effect of intervention (F (1,31) = 7.29, P = 0.01), time (F (1,31) = 40.33, P < 0.0001) and interaction (F (1,31) = 17.01, P = 0.0003) were significant such that the rate of mass gain of rats that had received FSL-1 was reduced over the first day compared to that of rats that had received PBS (P < 0.003). Furthermore, after each of the three FSL-1 injections the rate of reductions was not statistically significantly different from each other (F (2,32) = 40.33, P < 0.0001, one way repeated measures ANOVA). From the third day after each of the three injections, rats that had received FSL-1 grew at rates not significantly different to those of the rats that had received PBS.

5.4.3. Learning and memory: Morris Water Maze

Figure 5.6 shows the performance of rats as measured in a Morris Water Maze after completing three i.p. injections at 10 day intervals of either FSL-1 or PBS (see Fig. 5.1). During the Cued test (visible platform), a day before the rats started their 4-day acquisition training, there were no significant differences between the two groups of rats in any of the indices measured, namely latency to finding the platform (Fig. 5.6A; t (31) = 1.72, P = 0.09), distance swum to the platform (Fig. 5.6B; t (31) = 0.75, P = 0.46) and speed of swimming (Fig 5.6C; t (31) = 1.84, P = 0.08).

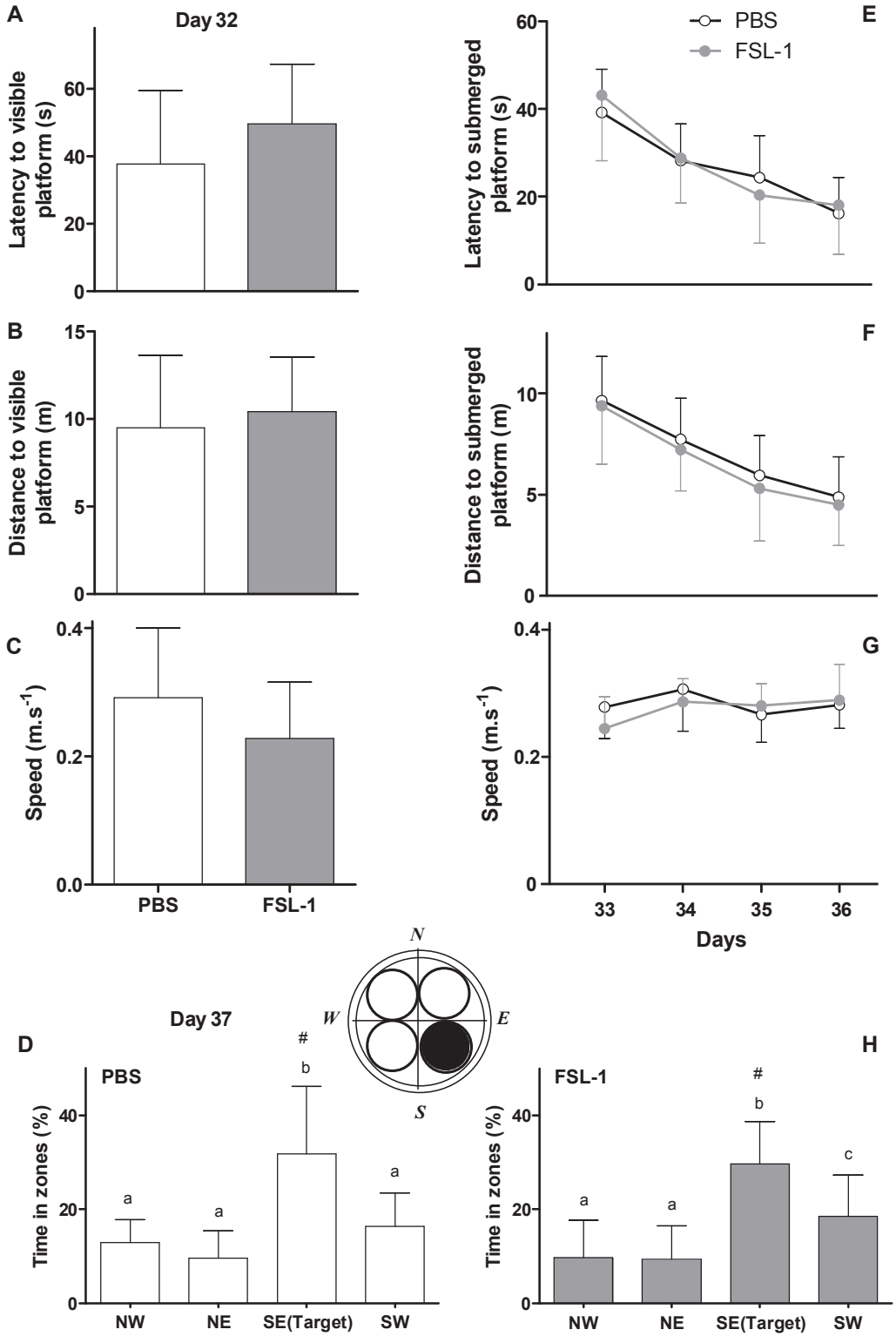

Figure 5.6. Performance of rats during the Cued test, the phase of acquisition of memory and the phase of memory recall, as measured in a Morris Water Maze from 12 d after the

last of the three successive i.p. injections of either 500 μg.kg^{-1} FSL-1 in 1ml.kg^{-1} PBS (●; n = 17), or 1ml.kg^{-1} PBS (○; n = 16). Data are means ± SD. During the Cued test (A-C), which was performed on Day 32, the rats were given one attempt at finding the visible platform during which latency (A), distance swum (B) and swim speed (C) were measured. During the phase of acquisition of memory (E-G), the rats were given four trials per day over four days to find the submerged platform; mean values for the four trials are presented, and latency (E), distance swum (F) and swim speed (G) were measured. The phase of memory recall was performed on Day 37, in which reference memory was tested with a single Probe trial, and the % time spent in each zone of the Maze (D, H) was measured. # indicates significant differences to the other three zones.

When the Cued test was repeated at the end of the "Probe" trial, there again were no significant differences between the two groups of rats in any of those indices (data not shown).

Over the four-day training in the Maze (i.e. the phase of acquisition of memory), there was a significant main effect of time (F (3,93) = 35.15, P < 0.0001) with the latency to reach the submerged platform (Fig. 5.6E) decreasing for both groups of rats, but there was no significant intervention effect (F (1,31) = 0.07, P = 0.79) and no significant interaction (F (3,93) = 0.91, P = 0.44). Similarly, the distance to reach the submerged platform (Fig. 5.6F) decreased significantly for both groups of rats (time effect: F (3,93) = 35.70, P < 0.0001), but there was no significant intervention effect (F (1,31) = 0.91, P = 0.35) and no significant interaction (F (3,93) = 0.05, P = 0.98). Furthermore, for swim speed (Fig. 5.6G) there was a significant time effect over the four days (F (3,93) = 4.04, P = 0.01), but there was no significant intervention effect (F (1,31) = 0.61, P = 0.44) and no significant interaction (F (3,93) = 2.25, P = 0.09).

When the rats were tested in the Probe trial (platform removed) 24 h after the last acquisition trial, there was a significant difference in the percentage time spent in each of the four zones (i.e. NW, NE, SE, SW) for rats that had received PBS (Fig. 5.6D; F (3,60) = 19.52, P < 0.0001) and for rats that had received FSL-1 (Fig. 5.6H, F (3,64) = 22.81, P < 0.0001). Rats in both groups spent significantly more time searching for the platform in the SE target zone (where the platform was originally located) compared to the other three zones (i.e. NW, NE, SW), so both groups of rats successfully could recall the former location of the platform. The mean latency to the platform position (data not shown) was significantly shorter than the cut-off value of 30 s for both rats that had received PBS (t (15) = 5.04, P = 0.0001) and rats that had received FSL-1 (t (15) = 5.04, P < 0.0001). Furthermore, there were no significant

differences in the swim speed, distance swum and latency to finding the former platform position, or platform crossings, between rats that had received PBS or FSL-1 (data not shown).

5.4.4. Histopathology

There was no significant evidence of apoptosis or neurodegeneration (necrosis) in any of the treatment groups. Specifically, histology of the granular cell layer of the dentate gyri (Fig. 5.7) showed no evidence of histopathological changes in cresyl violet brain sections of the hippocampi of rats receiving either PBS (Fig. 5.7A) or FSL-1 (Fig. 5.7B).

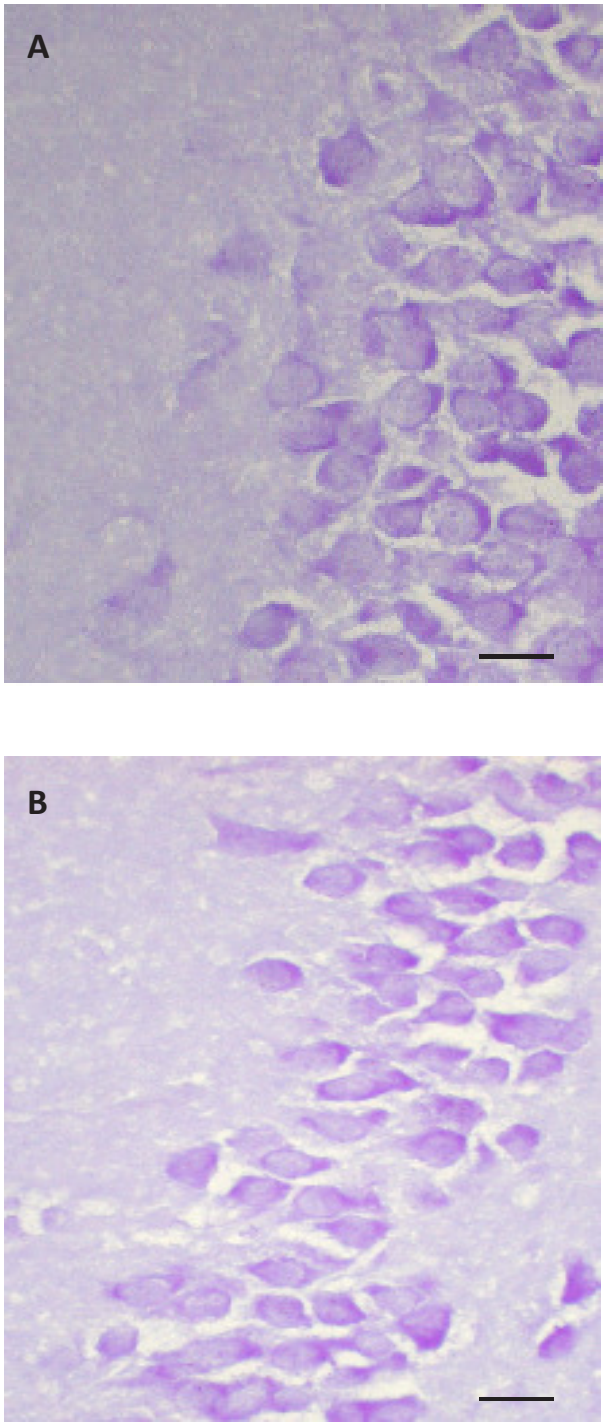

Figure 5.7. Cresyl violet stained sections (400 x magnification; bar = 25 µm) of the granular cell layer of the hippocampal dentate gyrus of rats 17 days after receiving the last of the three successive i.p. injections of either 1 $ml.kg^{-1}$ PBS (n = 9) (A) or 500 $\mu g.kg^{-1}$ FSL-1 in 1 $ml.kg^{-1}$ PBS (n = 10) (B).

5.5 DISCUSSION

There have been many experimental studies of simulated acute fever, and some of chronic fevers, but my study is one of few to investigate the consequences of recurrent, acute simulated infections. I have shown in growing rats that repetitive parenteral administration, at 10 day intervals, of FSL-1, the pyrogenic moiety of *Mycoplasma salivarium*, generated recurrent fevers without the development of tolerance to the pyrogen, and caused recurrent lethargy and anorexia without the development of tolerance to those two sickness behaviours. Importantly, repetitive administration of FSL-1, at the dosage we used, did not leave residual impairment in spatial learning or memory when rats were tested in a Morris Water Maze. In accordance with the lack of effect on spatial learning and memory, histology of the dentate gyri of rats also showed no evidence of apoptotic cell death after the simulated, recurrent acute *Mycoplasma* infection.

In seeking to model a recurrent infection, I set out to simulate repetitive infections at intervals long enough for the acute phase effect of the first injection to have resolved, and achieved this goal. Repetitive administration of FSL-1 generated recurrent fevers of statistically similar magnitude, which started about 3 h after each of the three injections and lasted for about 9 h (Fig. 5.2, 5.3). However, two of the sickness behaviours that I measured, namely lethargy and anorexia, were affected differentially, somewhat, after each of the three injections of FSL-1. The initial magnitudes of the lethargy were the same after each of the three injections, but the lethargy resolved more rapidly after the second and third injections, then being confined to one day (Fig. 5.4). Contrary to what was seen with lethargy, the initial magnitude of anorexia induced by the first injection was significantly different from that after the second and third injection (Fig. 5.5A), perhaps because the rats were younger at the first injection.

Together with the recurrent fever and anorexia, the rats exhibited reduced rate of mass gain after each of the three FSL-1 injections (Fig. 5.5B). Again, the initial reduction in rate of mass gain was statistically similar after the three injections. The recurrent acute reduction in food intake and mass gain following repetitive administration of FSL-1, were superimposed on a progressive reduction in food intake per 100 g of body mass in both groups of rats, as they grew (Fig. 5.5). Their average body mass almost tripled over the duration of the procedure, so absolute food intake increased appreciably. Interestingly, on the second day following each of the three injections of FSL-1 there was a small, albeit insignificant, increase in rate of mass gain relative to that of rats that had received PBS, despite no difference in food intake (Fig. 5.5B), ruling out a rebound hyperphagia. Rather, I speculate that, following the simulated infection, the rats digested and absorbed the food more slowly than did rats receiving PBS, resulting in a slower transit time and delayed gastric emptying and hence, increased residual food or faecal material in the gastrointestinal tract. A reduction in transit time and delayed gastric emptying as well as inhibition of gastric acid and pepsin secretion (e.g. Uehara *et al*., 1990), has been shown previously with i.p. LPS (Langhans *et al*., 1990; Takakura *et al.*, 1997; Liang *et al.*, 2005; Chen *et al.*, 2010). However, the dominant effect of recurrent FSL-1 administration on rate of mass gain remained as a recurrent transient reduction in that rate (Fig. 5.5B).

Having demonstrated that repeated administration of FSL-1, at 10-day intervals, induced recurrent acute fever and sickness behaviour, I then could address my primary questions, namely whether the accumulated effects of these transient insults left any residual impairment in spatial learning and memory; and whether any observed impairments may be associated with apoptosis in the hippocampus, the principle brain region involved in spatial learning and memory. Rats started their training in the maze 10 days after the last injection

of PBS or FSL-1, a time at which the acute effects of the last injection of FSL-1 had resolved. If I had detected impaired learning and memory, such impairments could not have arisen because the agents that induce fever and sickness behaviour (i.e. pro-inflammatory cytokines) still were active. In fact, in the Cued test (Fig. 5.6A-C) motivation and sensorimotor functions of rats were not affected adversely after they had received FSL-1 repetitively, given that the latency and distance travelled to the visible platform, as well as the swim speed of rats were not altered significantly, compared to those that followed PBS administration. Similarly, during task acquisition over four days with a submerged platform (Fig. 5.6E-G), the progressive reduction in the latency and distance to finding the submerged platform was the same for both groups of rats, confirming that earlier recurrent acute administration of FSL-1 had no residual effects on learning. Finally, when the platform was removed during the Probe trial (Fig. 5.6D, H) the rats showed the same, significant, spatial bias for the former location of the platform in the target zone relative to the other zones, regardless of whether they had received prior FSL-1 or PBS administration. Thus, recurrent FSL-1 administration also had no residual effects on spatial memory. Furthermore, histology of sections of the granular layer of the hippocampal dentate gyri (Fig. 5.7A, B) showed no significant evidence of apoptosis in the rats that had received recurrent FSL-1 injections (Fig. 5.7B). Since an intact hippocampus is essential for successful spatial learning and memory (Aguirre *et al*., 1996; D'Hooge and De Deyn, 2001), these findings are congruent with data obtained in the Morris Water Maze. Taken together, the evidence presented here demonstrates that recurrent bouts of immune stimulation by FSL-1, at the dosage I used and allowing for adequate recovery between challenges did not leave residual deleterious effects on spatial learning and memory as assessed in a Morris Water Maze.

The dose of FSL-1 that I employed was effective in inducing unequivocal fever and sickness behaviours, though I cannot exclude the possibility that higher doses of FSL-1, when

administered repetitively, could have induced residual effects on spatial learning and memory. It also is possible that a longer series of recurrent injections of FSL-1, or a different route of administration, e.g. intracerebroventricular (i.c.v.), could induce a more profound brain inflammatory response with accompanying hippocampal cell damage and/or impairment in spatial learning and memory. My results are confined to effects of simulated *Mycoplasma* infection; administration of live organisms (e.g. *M. hyopneumoniae*, see Escobar *et al*., 2004, 2007), mimicking more closely what happens in *Mycoplasma*-induced pneumonia, may have other deleterious effects on cognition that I did not detect. Moreover, the Morris Water Maze, measures only hippocampal-dependent spatial learning and memory. It is possible that I could have detected residual impairment in working rather than reference memory, or in other behavioural tasks such as the T-Maze or the Y-maze (see Sanderson *et al*., 2009), the Barnes-maze (Harrison *et al.*, 2009), the radial arm maze (Semmler *et al*., 2007; Chen *et al*., 2008; Sanderson *et al*., 2009), contextual fear conditioning (e.g. Pugh *et al.*, 1998; Bilbo *et al.*, 2008) or context pre-exposure (Bilbo *et al*., 2005a, b, 2007), some of which measures components of hippocampal-independent learning.

That I found no residual effects on spatial learning and memory was not the consequence of test insensitivity, as I previously have shown impaired learning ability of rats in a Morris Water Maze (**Chapter 3**) after administration of scopolamine, a substance often used to induce amnesia in laboratory animals (e.g. Choi *et al*., 2008). Other animal studies have shown that administration of drugs can lead to "state-dependent" effects on learning (Overton, 1964). For example, when one assesses learning and memory during immune stimulation, the context in which subsequent retrieval of memories is tested should be similar to the context in which learning occurred, to control for contextual differences in training (i.e. learning) and testing (i.e. memory) (for review see Cunningham and Sanderson, 2008). My

study was not compromised by issues of context, because I had ceased all other interventions before testing learning and memory in the Morris Water Maze.

Previous studies, however, have reported residual effects on learning and memory following acute, or chronic, simulated infection. For example, mice that had received an i.p. injection of LPS (250 $\mu g.kg^{-1}$) four hours before being tested had acquisition decrements that persisted up to 10 days, as assessed in a non-spatial, hippocampal-dependent, avoidance conditioning task (Sparkman *et al*., 2005b). When rats were injected with a single i.p. injection of LPS (250 $\mu g.kg^{-1}$) on day two of acquisition training in a non-spatial, hippocampal-independent autoshaping task, the observed learning impairment persisted across 12 days of testing (Aubert *et al*., 1995). Chronic neuro-inflammation, induced by 28 days of LPS infusion into the fourth ventricle of rats, impaired spatial memory in the Morris Water Maze as measured in a hippocampal-dependent task (Cui *et al*., 2008; Min *et al*., 2009) and LPS infusion over 33 days in rats induced microglial activation which correlated with impaired performance in a Morris Water Maze task (Hauss-Wegrzyniak *et al*, 1999). More importantly, LPS infusion for 4 weeks in rats caused residual impairment in spatial working memory when they were tested a week later in a hippocampal-dependent T-maze (Hauss-Wegrzyniak *et al*., 1998), and a similar residual impairment in spatial working memory was observed in rats, when they were tested in a Morris Water Maze 37 days after cessation of LPS infusion over periods of 37 or 74 days (Hauss-Wegrzyniak *et al*., 2000). In my study, the absence of residual detrimental effects on learning and memory as measured in the hippocampal-dependent Morris Water Maze may have resulted from the different pyrogen we administered (FSL-1 vs. LPS), the route of administration (i.p. vs. infusion), or the frequency of pyrogen administration (single injection vs. chronic infusion vs. spaced single injections).

I administered FSL-1 for the first time to rats at an age of about 36 days, and again on 46 and 56 days. The absence of detectable or residual effects of recurrent acute FSL-1 administration to growing rats may have been specific to the stage of development at which I tested for those effects. Rats reach "puberty" at 40 to 60 days of age (Kohn and Clifford, 2002), an age also comparable, on average, to that of an 11-12 year old child (Krisman-Scott *et al.*, 2002; Quinn, 2005). So the stage of development in the rats' lives at which I tested the effects of recurrent FSL-1 administration on spatial learning and memory was equivalent to that of school-aged children during early adolescence. Other studies in rats have shown that neonatal exposure to LPS was associated with dramatic impairment of memory consolidation during development and in adulthood, when rats were exposed to another immune challenge (Bilbo *et al.*, 2005b; Bilbo *et al.*, 2005a; Bilbo *et al.*, 2006). Moreover, neonatal administration of a single dose of LPS to rats resulted in a loss of hippocampal CA1-3 parvalbumin neurons in adulthood (Jenkins *et al.*, 2009). So, recurrent administration of FSL-1 to neonatal rats may well have left residual effects on learning and memory.

There is another life stage at which recurrent FSL-1 administration might leave residual effects on learning and memory, namely old age. Peripheral injection, or chronic infusion of LPS to aged rats, increased the number of activated microglia in the hippocampus, and induced a heightened inflammatory cytokine response in the hippocampus which resulted in atrophy of hippocampal neurons (Hauss-Wegrzyniak *et al.*, 1999; Richwine *et al.*, 2008). Furthermore, systemic infection, which activates microglial cells in the brains of humans (Lemstra *et al.*, 2007) and other animals (Semmler *et al.*, 2005), is thought to worsen the neurological symptoms in patients suffering from neurodegenerative diseases, such as Alzheimer's disease, in which microglia already are activated (Perry *et al.*, 2003; Perry *et al.*, 2007). Animal models of chronic neurodegeneration have shown that subsequent systemic

infection resulted in further activation of microglia, which in turn evoked exaggerated sickness behaviours (Combrinck *et al.*, 2002; Cunningham *et al.*, 2005). Because the hippocampus is one of the brain areas to show damage in neurodegenerative diseases (see (Jellinger, 1998; Jellinger and Bancher, 1998), recurrent systemic infections may aggravate the cognitive decline in patients with Alzheimer's disease (Holmes *et al.*, 2003). So, recurrent administration of FSL-1 to aged rats may have had a different outcome. However, a recent mice model of neurodegenerative disorders showed that recurrent systemic infection with *Streptococcus pneumoniae* did not affect the onset or the course of Alzheimer's disease (Ebert *et al.*, 2010).

Though residual impairment of hippocampal-dependent learning and memory as a consequence of hippocampal damage may occur in neonatal rats, following recurrent injections of FSL-1, they did not occur in my "adolescent" rats. My findings in "adolescent" rats parallel those of Mouihate and Pittman (1998), who found no evidence for apoptotic cell death in several brain areas, including the hippocampus, in adult rats, after repeated (5 consecutive days) i.p. injections of LPS, at a dose that induced sustained fevers for four days (Mouihate and Pittman, 1998). In mice, Terrazzino and colleagues (2002) also found no apoptotic cells in sections of the hippocampus, among other brain areas, two weeks after repeated (4 consecutive days) i.p. injections of LPS (Terrazzino *et al.*, 2002). Although the above two studies also have simulated repeated infections (daily injections, not spaced injections), they did not measure spatial learning and memory, as I did. Tanaka and colleagues (2006), however, did find impaired spatial memory, but did not observe neuronal cell death in adult rats after repeated (5 consecutive days) administration of LPS into the hippocampal CA1 region (Tanaka *et al.*, 2006).

Apart from contributing towards the understanding of impaired learning and memory as a sickness behaviour, I also have contributed new information about tolerance following repeated FSL-1 injections, though the phenomenon relates to a design requirement of my study rather than an outcome. Tolerance, the tachyphylaxis evident in the acute phase response following repeated activation, seems to develop after administration of the pyrogenic moieties of most, if not all, classes of infective organisms (Zeisberger and Roth, 1998; West and Heavy, 2002; Cartmell and Mitchell, 2005; Biswas and Lopez-Collazo, 2009), but at a rate and with a degree of completeness that depends on the number of injections, the interval between them and the route of administration (for example see Mouihate and Pittman, 1998, Voss *et al*., 2006; Greis *et al*., 2009). Five injections of FSL-1, spaced 3 days apart and at a dose of 100 $\mu g.kg^{-1}$, did not induce tolerance when the moiety was injected intra-arterially, but did so when it was injected intraperitoneally (Greis *et al*., 2009). I now have shown that, in growing rats repeated administration of FSL-1 at a dose of 500 $\mu g.kg^{-1}$, spaced 10 d apart, generated recurrent fevers of similar magnitude and duration after each of three injections. Thus, even at the higher dose, there was no pyrogenic tolerance to repeated i.p. FSL-1 when injections were given at 10 day intervals. Also, the magnitude of the anorexia and lethargy that followed i.p. FSL-1 administration was the same after the three repeated injections, though these sickness behaviours lasted longer after the first injection than they did after subsequent injections. Presumably as a result primarily of the anorexia, but perhaps also because of the increased metabolic demand of the fever, the growth rates (Fig. 5.5B) of the rats were reduced by a similar magnitude over the first 24 h after each of the three injections of FSL-1. However, I did not observe sustained stunting, implying that the stunting which lasted for four days following a single injection of FSL-1 at a similar dose (see Fig. 3.5B, **Chapter 3**) was reversed by ten days, presumably by the slight but persistent daily increases in rate of mass gain relative to that following PBS administration (see Fig. 5.5B). Acute intratracheal inoculation with *Mycoplasma*

hyopneumoniae (strain P5722–3, 3 ml of 1 x 10^{10} units.L^{-1}) also had no effect on growth in growing pigs (Escobar *et al*., 2004). However, growth failure did occur in guinea pigs given spaced repeated injections of muramyl dipeptide, a pyrogenic moiety of Gram-positive organisms (Madu *et al*., 2007).

Though residual effects on learning and memory of solitary acute bacterial infections and chronic infections have been studied previously (Aubert *et al*., 1995; Hauss-Wegrzyniak *et al*., 1998, 2000; Sparkman *et al*., 2005b), I believe mine is the first study to measure residual learning and memory after simulated recurrent, acute infections. Also, previous studies have not addressed the issue of whether only learning and memory are affected, or whether any impaired learning and memory represents one component of a suite of residual sickness behaviours. Because I was interested particularly in the residual deficits in learning and memory that might persist, once the rats were apparently well after the effects of the last in their series of simulated infections had resolved, I assessed learning and memory at a time when no residual fever, anorexia or lethargy was present. Moreover, I used FSL-1 and 'adolescent' rats in the current study because I wanted to assess the consequences of recurrent acute stimulation of innate immunity, such as would occur in growing children of school-going age subject to repetitive bouts of e.g., community-acquired pneumonia, commonly caused by *Mycoplasma.* I believe that my model of recurrent acute *Mycoplasma* infection has significant potential to be implemented in many other models of acute infection that occur recurrently. For example, recurrent acute fevers commonly occur in meningitis, malaria and influenza that are caused by other classes of organisms, including Gram-positive bacteria, protozoa and viruses.

In conclusion, though I cannot extrapolate directly the outcome of my study, which employed a pyrogenic moiety of *Mycoplasma* in rats, to the real infection caused by live organisms in

humans, the clinical implications of my study are encouraging. First, I did not find any residual histological damage to the hippocampus nor did I observe lasting detrimental effects on spatial learning and memory. Secondly, I did not observe permanent stunting in my rats. If I had observed these phenomena in the simulated infections there would have been a high likelihood of them occurring, and being aggravated, in the real infections. Ultimately, studies in children need to be undertaken in order to determine whether or not apparently-well children who have recovered from recurrent acute *Mycoplasma* infections have residual deficits in learning and memory.

CHAPTER 6

CONCLUSION

Although *Mycoplasma* primarily causes respiratory disease, *Mycoplasma pneumoniae* has been implicated in life-threatening meningitis as well as memory deficits. Experimentally, very little still is known about *Mycoplasma*-induced sickness behaviour and no one previously has investigated its effect on learning and memory. I have investigated various sickness responses during acute and recurrent acute simulated *Mycoplasma* infection. I also was interested, particularly, in the consequences of recurrent acute infections, which seldom are being simulated in the laboratory. *Mycoplasma* is a common cause of recurrent infections. To avoid safety concerns and ethical issues arising from administration of pathogenic live organisms, such as *Mycoplasma pneumoniae*, to experimental animals (see e.g. Pietsch *et al*., 1994; Wubbel *et al*., 1998; Chu *et al*., 2003) I used a pyrogenic moiety from *M. salivarium*, namely fibroblast-stimulating lipopeptide-1 (FSL-1). Acute administration of FSL-1 in rats induces fever and sickness behaviours (Hübschle *et al*., 2006). I administered FSL-1 both acutely and recurrently to a different strain of rat (Sprague-Dawley vs. Wistar) and at a different dose (500 vs.100 $\mu g.kg^{-1}$) previously used (Hübschle *et al*., 2006). Contrary to the dose of 100 $\mu g.kg^{-1}$, which Hübschle and colleagues (2006) used in Wistar rats, my pilot studies showed that, in my hand, administration of FSL-1 at doses of 500 $\mu g.kg^{-1}$ and higher, but not 100 $\mu g.kg^{-1}$, induced pronounced fever and sickness behaviours (lethargy and anorexia) in Sprague-Dawley rats.

Having established successfully an animal model of simulated acute as well as simulated recurrent *Mycoplasma* infection, I investigated fever, lethargy, growth and anorexia as well as learning and memory processes. After acute, parenteral administration of my higher dose of FSL-1 (i.e. 1 000 $\mu g.kg^{-1}$) the sickness behaviours lasted for longer than that described by Hübschle and colleagues (2006): I observed four days of lethargy, three days of anorexia and at least four days of body mass stunting (**Chapter 3**), a phenomenon not reported by Hübschle and colleagues (2006). In fact, no one previously has observed stunting in

simulated acute *Mycoplasma* infection. I further investigated potential roles for pro-inflammatory cytokines, interleukin-1 (IL-1β) and interleukin-6 (IL-6) in mediating *Mycoplasma*-induced sickness responses by measuring the concentrations of these two cytokines in the hypothalamus, required for eating behaviour and body temperature regulation. In rats, sickness responses induced by FSL-1, including fever, lethargy and anorexia appear to be mediated by peripherally-released pro-inflammatory cytokines, including IL-6 and tumor necrosis factor-α (TNF-α) (Hübschle *et al*., 2006). I have shown, for the first time, increased concentrations in the plasma and brain of another pro-inflammatory cytokine, namely IL-1β (**Chapter 4**), accompanying FSL-1-induced fever, lethargy and anorexia as well as stunting in rats (**Chapter 3**).

I measured fever, lethargy and anorexia over a course of days whilst learning and memory were being assessed. As a test procedure, I chose to use a Morris Water Maze that tests a high component of cognitive function and where the responses are not simply reflexes. In Table 1 (**Chapter 1**) I summarized studies that have measured spatial learning and memory in a Morris Water Maze, after a single i.p. administration of a given moiety / pathogen-associated molecular pattern (PAMP), either from typical bacteria (Gram-positive or Gram-negative) or viruses. Using FSL-1, which simulates *Mycoplasma* infection, my studies extend what already is known about the effects of acute immune activation on learning and memory processes: I have shown, in rats, that a single i.p. administration of FSL-1, at doses of 500 or 1 000 $\mu g.kg^{-1}$, did not induce impairment in spatial learning and memory, as measured in a Water Maze as I have implemented it. Because the learning environment during Morris Water Maze testing can be manipulated in various ways, studies have found contradicting results with other PAMPs on memory processes. For example, following acute administration of a given PAMP, studies have reported impairment in spatial learning and/or memory (i.e. working memory) when the platform within the pool was relocated to a new

position throughout testing (Gibertini, 1996; Richwine *et al.*, 2009; Zhang *et al.*, 2009; Ito *et al.*, 2010). However, when the platform remained in a fixed location in the Maze no impairment in spatial learning or memory (i.e. reference memory) was observed, after acute administration of the same PAMP (Gibertini, 1996; Ito *et al.*, 2010). I also showed that reference memory was unaffected after acute administration of FSL-1 (**Chapter 3**), although I did not measure working memory. Moreover, water temperatures between 18-25 ºC have been used to induce stress conditions as means of interfering with acquisition rates and consolidation processes during memory formation (Gibertini, 1998; Akirav *et al.*, 2001). For example, in a warm-water Maze (23 ºC) IL-1 appears to interfere with learning (Gibertini, 1998), which is contrary to what I have found in my Water Maze in which the water temperature was kept constant between 25-26 ºC (based on the "standard" protocol of Morris, 1981) (**Chapter 3 and 5**). In addition to the manipulation of water temperature, the level of "emotionality" of animals that are tested in the Maze also can be manipulated or altered by using 'massed' or 'spaced' training protocols (Gibertini, 1998). Massed training typically consists of between 3-12 trials on one day, whereas spaced training consists of a limited number of trials (e.g. 4 trials) over a number of days (e.g. 3 days). Spaced training trials are considered to be more effective for the production of robust learning and memory than are massed training trials (Scharf *et al.*, 2002). I therefore used spaced training trials of four trials per day over four days, during the acquisition phase in my Morris Water Maze, to ensure optimal learning and memory (**Chapter 3 and 5**).

Unlike most previous authors I have assessed learning and memory in the full context of the acute phase response, in my case induced by *Mycoplasma* infection. I found that spatial learning and memory were spared at a time when the rats still were febrile, lethargic, anorexic and stunted (**Chapter 3**). I believe that the lack of impairment in learning and memory, and the dissociation from other brain-controlled sickness behaviours, is an

important finding, and therefore I measured concentrations of IL-1β and IL-6 in the hippocampus, required for spatial learning and memory. At the time of active learning in the Maze the rats had significantly elevated concentrations of IL-1β in both the hypothalamus and the hippocampus (see **Chapter 4**). Although increased concentrations of IL-1β in the hippocampus of rats have been associated with impairment in learning and memory (for review see Goshen and Yirmiya, 2007; Barrientos *et al*., 2009), in my study increased concentrations of IL-1β in the hippocampus were not associated with any impairments. Following i.p. administration of FSL-1 in rats, the concentration of IL-1β was slightly higher in the hypothalamus than in the hippocampus, albeit insignificantly (see **Chapter 4**). Thus, following FSL-1 administration to rats, the same concentration of IL-1β in the hypothalamus that seems to mediate fever and lethargy was not sufficient to induce impairment in hippocampal-dependent learning and memory (**Chapter 3**). Further investigations are required using other models of assessing learning and memory, such as the Y-maze and the T-maze, to confirm that learning and memory is spared in the face of other sickness behaviours following systemic administration of FLS-1. Studies also need to investigate whether or not FSL-1 can cross the blood-brain-barrier (BBB), after systemic administration. If FSL-1 crosses the BBB, studies need to characterise the brain areas in which receptors for FSL-1 (i.e. toll-like receptor 2/6; TLR2/6) are present. It is possible that some brain areas, including the hypothalamus, may be more 'sensitive' to the effects of FSL-1, than are other areas like the hippocampus. Ultimately, FSL-1 needs to be injected into the brain, for example using intracerebroventricularly (i.c.v.) injections, to confirm its effect on learning and memory processes.

Following FSL-1 administration to rats, I measured cage activity as an index of lethargy and also to assess whether any diminished performance in the Water Maze could be attributed to lethargy rather than a decline in cognitive function. In my opinion, whether or not impairment

in learning or memory is observed in a Morris Water Maze following simulated, systemic infection depends on the pyrogen and the dose administered, the route of administration, and importantly, the time of testing in the Maze. If infected animals are tested in the Maze before resolution of the acute phase response, it may not be surprising that sickness responses, including lethargy, may affect locomotion or swimming speed and not necessarily learning or memory itself (see Cunningham and Sanderson, 2008). Although other measures of activity exists, I used biotelemetry to monitor physical activity of rats before, during and after they were exposed to training and testing in the Maze. Moreover, the use of biotelemetry also allowed me to monitor continuously body temperature of rats while they were swimming: not only hyperthermia (Ahlers and Riccio, 1987), but also hypothermia may cause anterograde memory loss (Richardson *et al*., 1983). Although hyper- or hypothermia and lethargy did not confound results in my study, I propose that biotelemetry be implemented together with the Morris Water Maze in future experiments, to exclude confounding factors, such as changes in body temperature and lethargy.

Interestingly, I have shown that acute administration of my higher dose of FSL-1 (i.e.1 000 $\mu g.kg^{-1}$) abolished almost all spontaneous cage activity (also referred to as 'house-keeping activity') of rats, yet they swam competently in a Morris Water Maze (**Chapter 3**). I therefore hypothesize that the rats may have invoked both their motivation and capacity for activity that was unaffected by concurrent lethargy. However, a distinction between motivation and capacity in sickness behaviour has been discussed previously (e.g., see Maier and Watkins, 1998; Larson and Dunn, 2001). Recently, the distinction between motivation and capacity for activity was reported in rats that showed impaired motivation for physical exercise (measured as voluntary wheel running), but less impairment in their capacity to be active, e.g. during normal house-keeping activity (measured as cage activity) (Hopwood *et al*., 2009; Skinner *et al*., 2009). Because voluntary physical exercise (e.g. voluntary wheel

running) is optional, it is more likely to be impaired during sickness (Hopwood *et al*., 2009; Skinner *et al*., 2009). However, activity in a Water Maze is not optional and survival of the rats depends on escaping from the water. My observation that the rats swam competently, despite being lethargic, contributes to what already is known about activity during sickness: in a life-and-death juncture, such as occurs when rats are forced to swim (e.g. in a Water Maze) in order to survive, the sickness behaviour of lethargy may be suppressed, because if it was implemented it would have threatened survival. I therefore support previous evidence that the sickness behaviour of lethargy may be activity specific (Hopwood *et al*., 2009; Skinner *et al*., 2009). Moreover, motivation for survival may override many aspects of sickness behaviour and I believe that this phenomenon may have important implications for the behaviour of ill individuals who may find themselves in situations where survival is their only option.

Many attempts to simulate chronic, sustained fevers experimentally have failed. However, I have shown that it is possible to simulate recurrent acute fevers, in my case during simulated recurrent *Mycoplasm*a infection. In spite of their prevalence and importance, recurrent acute infections seldom are investigated in the laboratory. Following repetitive administration to rats of 500 $\mu g.kg^{-1}$ FSL-1, 10 days apart, I observed recurrent fevers, recurrent lethargy and recurrent anorexia, but no body mass stunting (**Chapter 5**). I showed that FSL-1, which simulates *Mycoplasma* infection, is a reliable pyrogen that produces consistent, pronounced, recurrent acute phase responses experimentally in rats, without the development of tolerance to fever, lethargy or anorexia (**Chapter 5**), when it is administered at 10-day intervals. However, I did not observe residual impairment in learning and memory in rats, a finding consistent with recent evidence in mice that had received acute or repeated administration of Staphylococcal enterotoxin A (SEA) (Woodruff *et al*., 2010). My study is the first, I believe, to have assessed residual deficits in learning and memory in otherwise

healthy rats following recurrent acute stimulation of the innate immune system, i.e. spaced injections of a pyrogen with recovery between infections (**Chapter 5**). However, residual impairment has been detected in rats after repeated (i.e. daily injections with no recovery between infections) or chronic administration (i.e. continuous infusion) of lipopolysaccharide (LPS) (Aubert *et al*., 1995; Hauss-Wegrzyniak *et al*., 1998, 1999; Sparkman *et al*., 2005), which is different to my model of recurrent acute administration (i.e. spaced injections with recovery between infections).

In addition to measuring residual learning and memory following simulated recurrent infection I investigated whether any observed impairments could be associated with apoptosis and necrosis in the hippocampus, the principal brain region involved in spatial learning and memory. For the histopathology, I used the technique of cresyl violet staining to identify apoptosis in the dentate gyrus of the hippocampus (see Pfister *et al.,* 2000). However, it is advisable to use other staining methods, including Caspase assays, Annexin V assays, cell permeability assays or DNA fragmentation in conjunction with cresyl violet staining in future experiments. Although I did not observe any residual histological pathology in the hippocampus following simulated recurrent *Mycoplasma* infection (**Chapter 5**), it remains to be confirmed whether *Mycoplasma* infection may affect other aspects of hippocampal-dependent learning and memory, including non-spatial learning and memory. Recurrent, central *Mycoplasma* infections, rather than peripheral *Mycoplasma* infections, also may affect learning and memory processes. Moreover, it is possible that, in *Mycoplasma* infection, the genetic background of the host is an important determinant of whether or not the infected individual will have impairment in learning and memory (see Chu *et al*., 2006). Impairment also may depend on age, the type and severity of infection as well as the duration of the infection. A case study in a 7-year old girl has reported pervasive changes in memory following *M. pneumoniae* encephalitis (Benjamin *et al*., 2007), but

cognitive impairment does not seem to be significant in adults or aged individuals who suffer from *M. pneumoniae* encephalitis (Hokkanen *et al*., 1996a,b). Therefore, if other behavioural paradigms that assess other types of hippocampal-independent non-declarative learning and/or memory are employed in addition to a Morris Water Maze, we ultimately may be able to identify the mechanisms involved in *Mycoplasma*-induced cognitive impairment and whether the impairment is specific to a certain age group or not. Recurrent *Mycoplasma* infection, which affects all age groups, could have long-term detrimental cognitive consequences. Therefore, there could be value in repeating my experimental procedures to simulate *Mycoplasma* infection in very young and very old rats. I believe that my model of recurrent acute infection has significant potential to be implemented in other experimental models of infection, including influenza, malaria and meningitis, which often occur acute recurrently. The potential consequences of different types of infections on various aspects of learning and memory that might persist once the infected host had recovered from recurrent infections require more investigation.

Before any hypotheses are constructed about the consequences of recurrent infection in children, based on the outcome of my studies, the factors influencing acute phase responses in children, rather than rats, will have to be considered. For example, in humans helminthic infections might affect immune reactions to concomitant infections (e.g. tuberculosis) that occur with high frequencies in helminth-endemic areas. In the host, helminthic infections induce immune hypo-responsiveness to subsequent infections, which is characterized by elevated T-helper cell type 2 (Th2) cytokines and IgE production (Bentwich *et al*., 1999; Bundy *et al*., 2000). Studies also have shown that intestinal helminth infections could impair cognitive ability (see Watkins and Pollitt, 1997; Dickson *et al*., 2000a; Dickson *et al*., 2000b).

Although no direct extrapolation of the outcome of my acute, recurrent study in rats can be made to humans, the clinical implications of my study are hopeful in that I did not observe permanent stunting in rats. Equally importantly, I did not find any residual histological damage to the hippocampus of rats nor did I find lasting detrimental effects on spatial learning and memory. Nonetheless, *Mycoplasma* infection still is a major problem especially in developing countries and affects many children. Therefore, studies in children are needed to determine whether or not apparently-well children who have recovered from recurrent acute *Mycoplasma* infections have residual deficits in learning and memory.

Not only are *Mycoplasma* infections prevalent in developing countries, but emerging evidence also has implicated *Mycoplasma pneumoniae* in facilitating allergic airway diseases, such as asthma (Sutherland and Martin, 2007; Schroder *et al*., 2008), with increasing incidence in developed countries (European Community Respiratory Health Survey, 1996). The increased incidence of respiratory tract infections in developed countries presumably is because of lifestyle changes and improved general hygiene in those industrialized countries (ISAAC Steering Committee, 1998), a phenomenon often referred to as the 'hygiene hypothesis' (Strachan, 1989). According to the hygiene hypothesis, reduced exposure to micro-organisms may be associated with increased prevalence of allergic conditions (Strachan, 1989; Strachan *et al*., 1994, 1996, 1997). Thus, early childhood exposure to pathogens, typically within households, may protect against allergic sensitization (see Gangal and Chowgule, 2009). A more recent hypothesis, which parallels the hygiene hypothesis, is the 'parasite-stress hypothesis', which proposes a positive relationship between exposure to infectious disease, albeit at low levels, and high intelligence (see Eppig *et al*., 2010). My observation with regard to sparing of learning and memory during acute and recurrent acute simulated *Mycoplasma* infection not only is encouraging, it also may be in

line with the 'parasite-stress hypothesis', although we should not equate "normal" learning and memory to high intelligence.

FUTURE PERSPECTIVES AND RECOMMENDATIONS

Because of its robustness, I implemented the Morris Water Maze for measuring learning and memory in my studies. The Maze also has been adapted for studies in humans. The use of advanced 3D-computer technology as well as the implementation of virtual water tasks (i.e. the "Virtual Morris Water Maze") allows researchers to test spatial navigation and memory ability in humans under laboratory conditions (Astur *et al.*, 1998, 2001, 2004; Hamilton *et al.*, 2002). The virtual mazes have proven highly sensitive when used in the assessment of cognition in children (Overman *et al*., 1996; Leplow *et al*., 2003), novel treatments for age-related cognitive decline (for review see Lindner, 1997; Driscoll *et al*., 2003), gender differences in cognitive ability and spatial navigation in both adults (Moffat *et al*., 1998; Astur *et al*., 1998; 2004; Mueller *et al*., 2008; Woolley *et al*., 2010) and in children (Newhouse *et al*., 2007), cognitive processes in children with Fetal Alcohol Syndrome (Hamilton *et al*., 2003), cognitive ageing (Newman and Kaszniak, 2000), hippocampal damage or dysfunction (Astur *et al*., 2002) as well as the involvement of the hippocampus in spatial navigation (Aquirre *et al*., 1996; Astur *et al*., 2004; Goodrich-Hunsaker *et al*., 2010). In the clinical setting, the virtual mazes seem to have advantages over the traditional spatial memory paradigms in terms of their flexibility, generalizability and experimental control (see Astur *et al*., 2004), although some still prefer the traditional methods (Grigoleit *et al*., 2010). Although the virtual mazes have not been used in humans to study learning and memory processes as part of infection-induced sickness responses, their use should be considered.

Studies investigating infection-induced learning and memory impairment are crucial, because exposure to pathogens is inevitable. Children, in particular, are at risk of recurrent infections and of developing chronic infectious diseases, which may have detrimental outcomes for cognition. *Mycoplasma*-induced community-acquired pneumonia is prevalent in children in both developing countries as well as in industrialized, developed countries. Although *M. pneumoniae* classically has been considered an extracellular (or membrane-associated) organism, accumulating evidence suggests that this pathogen and other strains of the *Mycoplasmas* may colonize, exist and replicate as intracellular pathogens within human cells (Dallo and Baseman, 2000; Meseguer *et al*., 2003). Thus, the ability of the *Mycoplasmas* to act as intracellular pathogens may contribute to their pathogenicity. Because cytokines appear to mediate the acute phase response to many infections, including *Mycoplasma* infection, studies of cytokine antagonism for therapeutic intervention will be critical. Agents that block the actions of cytokines already are available clinically and may be used in studies of cytokine-mediated fever and sickness behaviours during real, or simulated, *Mycoplasma* infection. Furthermore, using endogenous antagonism of the biological action of cytokines, the actual role of various pro-inflammatory cytokines in mediating *Mycoplasma*-induced fever and sickness behaviours can be confirmed. In simulated acute *Mycoplasma* infection in rats I have shown that increases in concentrations of the pro-inflammatory cytokine, IL-1β, in both the plasma and brain accompany fever, lethargy, anorexia and body mass stunting without affecting learning and memory (**Chapter 3 and 4**).

My finding that learning and memory (as measured with a Morris Water Maze) were spared in the presence of an increased concentration of IL-1β is at odds with the findings of others. Infection-induced over-expression of pro-inflammatory cytokines, including IL-1β in the brain, has been associated strongly with learning and memory impairment (Oitzl *et al*., 1993;

Rachal Pugh *et al*., 1999; Song *et al*., 2004; Song and Horrobin, 2004). Consequently, studies are needed to clarify whether altered concentrations in the CNS of individual cytokines result from a pathological condition, or whether altered expression of cytokines results as a consequence of a disorder. In simulated *Mycoplasma* infection, induced by FSL-1, investigations also are needed to determine what high plasma concentrations of IL-6 achieve if there are no changes in brain concentrations of this cytokine (**Chapter 4**).

Cytokine antagonism also could be used to investigate the roles of pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α, in learning and memory processes. In contrast to fever, lethargy and anorexia that are well established sickness responses to many infections, learning and memory impairment is not an inevitable consequence of infection, as I and others have shown (Thomson and Sutherland, 2006; Huang *et al*., 2010; Woodruff *et al*., 2010; **Chapter 3**). The question remains why the same pyrogenic moiety (e.g., IL-1β or LPS) in the same species sometimes impairs learning and memory in the same task (i.e., Morris Water Maze) and sometimes not (refer to Table 1, **Chapter 1**). Whether the latter phenomenon also is true for other pyrogenic moieties needs to be explored, because during infection brain regions required for fever may be affected differentially from those brain regions required, specifically, for learning and memory.

In situations where learning and memory are critical for survival of the host, sickness behaviours may be suppressed. In **Chapter 3** I have shown that learning and memory, competencies which were crucial for survival of rats in a Morris Water Maze behavioural test, were unaffected in spite of the sickness behaviour of lethargy the rats had experienced. Further studies are needed to investigate the effect of other sickness behaviours, including anorexia, on learning and memory during life-and-death situations. Whether anorexia will be suppressed, in order to enhance survival, has clinical relevance particularly for children in

resource-poor, developing countries who suffer simultaneously from infection and malnutrition. Although nutrition is crucial for mental development (Lynn, 1990, 1993), the nutritional status of children also may affect intelligence in the absence of infection (Barber *et al*., 2005). Therefore, experiments in food-deprived animals are needed to determine whether cognitive function, including learning and memory will be spared if it could increase chances for survival. Similarly, it needs to be investigated whether learning and memory take priority over fever or the sickness behaviour of, for example hyperalgesia, when survival behaviour is employed during illness. These questions can be addressed by injecting species-specific neutralizing antibodies against those cytokines mediating the particular infection. For measuring learning and memory, the Morris Water Maze may be used in conjunction with other models of learning and memory, which do not necessarily depend on physical activity, such as swimming. Sickness behaviours, including lethargy may confound results in those models that rely on physical activity, such as the Morris Water Maze.

Because learning/memory impairment appears to be a common, albeit not ubiquitous, consequence of immune activation, the question remains whether there is an adaptive purpose to illness-induced cognitive impairment? In my view, the type of infection (e.g., bacterial vs. viral vs. fungal), the site of infection (e.g., local vs. systemic vs. central) as well as the severity and duration of the inflammation may be important predictors for illness-induced cognitive impairment. It is possible that a longer-lasting, severe, CNS infection may increase the chances of cognitive impairment, as opposed to a mild, one-day, local infection. The frequency of the infection (i.e., once-off, repeatedly or recurrently) also may be a critical predictor of whether cognitive impairment is likely to occur or not. Chronic or recurrent activation of the immune response may render the brain more susceptible for neurologic sequelae, including cognitive deficits. Other factors, such as old age or health status, i.e., patients with HIV, tuberculosis, brain injury or neurodegenerative diseases (e.g. Alzheimer's

disease) also may predispose patients to cognitive decline during infection. All these factors may clarify, or even contribute, to the existence of illness-induced cognitive impairment, but the adaptive purpose thereof still is incomprehensible.

Moreover, activation of the host's immune system may result in latent vulnerability to subsequent activation and recurrent inflammatory challenges may uncover, or even exacerbate existing clinical symptoms. *Mycoplasma* infection is not the only type of infection that occurs recurrently. The bacterium *Streptococcus pneumoniae* is a common cause, if not the main cause, for recurrent pneumonia throughout infancy and childhood (e.g., see Stein and Cauduro Marostica, 2007). Other recurrent infections, including meningitis, malaria and influenza could have long-term detrimental consequences, such as growth retardation or cognitive retardation after resolution of the recurrent infection. If residual detrimental effects of infection persist, they need to be investigated experimentally. Management requires an understanding of the physiological processes which link the infecting pathogen to the sickness behaviour, particularly so that targets for intervention can be identified. When simulating infections, experimentally, the use of pyrogenic moieties from organisms should be considered instead of using live pathogenic organisms. While I have used FSL-1 derived from *M. salivarium* (which appears to have a lower pyrogenicity compared to *M. pneumoniae*), moieties from other species in the genus, such as MALP-2 from *M. fermentans* (Mühlradt *et al*., 1997) or FAM20 from *M. pneumoniae* (Shimizu *et al*., 2008) also should be considered to simulate recurrent infections caused by the *Mycoplasmas*. So too should moieties form other causative pathogens, particularly pneumolysin from *S. pneumoniae* (e.g., see Hirst *et al*., 2004), be used for future research into simulating infections that occur recurrently.

Finally, in contrast to simulating chronic infections in the laboratory, there appears to be no barrier for simulating recurrent infections experimentally. These studies ultimately will help us elucidate whether recurrent acute infections indeed have lasting detrimental outcomes. Growth retardation, loss of motivation for life-sustaining activities and cognitive retardation may have a devastating impact on the infected individual's welfare.

CHAPTER 7

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APPENDIX

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2007/72/4

APPLICANT: Ms T Swanepoel

SCHOOL: Physiology
DEPARTMENT:
LOCATION: Medical School

PROJECT TITLE: **The effect of recurrent simulated Gram-positive infection on growth and learning and memory**

Number and Species

48 male Sprague dawley rats

Approval was given for to the use of animals for the project described above at an AESC meeting held on 20071127. This approval remains valid until 20091127

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:

Signed: _____ Date: _____30/11/2007_____
(Chairperson, AESC)

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: _____ Date: 30/11/2007
(Registered Veterinarian)

cc: Supervisor:
Director: CAS

Works 2000/lain0015/AESCCert.wps

AESC 3

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2009/43/04

APPLICANT: Ms T Swanepoel

SCHOOL: Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: **The effects of administering different doses of fibroblast stimulating lipopeptide-1 (FSL-1) on learning and memory in rats**

Number and Species

52 Sprague Dawley rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 24.11.2009. This approval remains valid until 24.11.2011

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:

Experiment 3 must be conducted only once the use of 2000 micrograms/kg FSL has been deemed safe in 4 rats followed for 7 days. The AESC is to be informed in writing of the outcome of this "pilot" component of the study

Signed: _____ (Chairperson, AESC) Date: 09/12/2009

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: 10/12/09

cc: Supervisor:
Director: CAS

Works 2000/lain0015/AESCCert.wps

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2010/34/04

APPLICANT: Ms T Swanepoel

SCHOOL: Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: Cytokine expression in the brain following systemic FSL-1 administration

Number and Species

60 male Sprague Dawley rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 29.06.2010. This approval remains valid until 29.06.2012

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:

That the dose of sodium pentobarbital employed to euthanase rats is 100mg per kg.

Signed: _____ Date: 15/07/2010
(Chairperson, AESC)

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: _____ Date: 15/07/2010
(Registered Veterinarian)

cc: Supervisor:
Director: CAS