STUDIES ON CYTOKINES AS MEDIATORS OF FEVER AND SICKNESS BEHAVIOUR

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DECLARATION

This thesis is submitted in the optional format, approved by the Faculty of Health Sciences, of published work with encompassing introduction and conclusion.

I declare that the work contained in this thesis is my own, except where acknowledged as otherwise. The work herein has not been submitted for a degree at any other university.

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Signed on the _____ day of _____, 2008

OUTPUTS EMANATING FROM WORK CONTAINED IN THIS THESIS

Published papers

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ABSTRACT

The presence of endotoxin in animals and humans triggers a sequence of acute phase responses, which include the synthesis and release of pro-inflammatory cytokines from immune cells, followed by the development of various symptoms of sickness including fever and an array of behavioural responses, commonly referred to as sickness behaviours. Most experimental investigations examining the mechanisms mediating fever and sickness behaviour responses have used purified lipopolysaccharide (LPS), the glycolipid pyrogenic moiety of the Gram-negative bacterial membrane, to trigger the innate immune system. Results obtained from studies using specific antagonists to block the action of cytokines synthesized following systemic administration of LPS, have uncovered important roles for pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, tumour necrosis factor-alpha (TNF-α) and leptin, in mediating fever. Although it has been shown that administration of pro-inflammatory cytokines can induce sickness behaviour in experimental animals, no clear role has been identified for these cytokines as endogenous mediators of sickness behaviours induced following LPS administration.

Using rats as experimental animals and endogenous cytokine antagonism, I therefore investigated whether endogenously released IL-1β, IL-6, TNF-α and leptin are physiologically active not only in the generation of fever, but also in the generation of two specific sickness behaviours, lethargy and anorexia, induced by subcutaneous (s.c.) administration of LPS. Lethargy, anorexia and fever were measured as changes in voluntary wheel-running, food intake and body temperature respectively. I antagonized

the biological action of these cytokines in the periphery following s.c. administration of LPS by injecting rats intraperitoneally (i.p.) with specific anti-rat sera to one of the following: TNF-α, IL-1β, IL-6 or leptin. Peripherally-released leptin appeared to be an important mediator of both fever and anorexia, as the presence of leptin antibodies in the circulation abolished both the anorexia and fever induced by s.c. administration of LPS. In contrast though, whereas the presence of IL-6 antibodies in the circulation abolished the LPS-induced fever, suppression of voluntary activity was reversed by the presence of IL-6 antibodies only to the extent of 27%, and appetite also was not returned to normal levels in the presence of IL-6 antibodies. Thus, IL-6 may be an essential component of LPS-induced fever, but an additional factor or factors, possibly working in parallel with IL-6, may be required to mediate the lethargy and anorexia induced by s.c. administration of LPS. Injecting rats i.p. with TNF-α antiserum or IL-1β antiserum had no effect on LPS-induced lethargy and LPS-anorexia, indicating that if these cytokines are working with peripherally-released IL-6 to induce sickness behaviour, it is likely due to their synthesis in the brain.

Injecting species-homologous rat IL-1β and IL-6 into the brains of conscious rats, I aimed to identify whether either of these two cytokines can act within the brain to induce lethargy and anorexia in the absence of an infection. Intracerebroventricular (i.c.v.) administration of either IL-1β or IL-6 before the night-time active period decreased voluntary activity in the rats in a dose-dependent fashion, whereas only IL-1β decreased food intake and body mass of the rats. It is possible therefore, that increased levels of IL-1β in the brain may be working in parallel with IL-6 released in the periphery to induce lethargy and anorexia following s.c. administration of LPS.

Thus I antagonized the biological action of these cytokines endogenously by administering species-specific antiserum to IL-6 (IL-6AS) i.p., and a caspase-1 inhibitor, which prevents the cleavage of pro-IL-1β to biologically active IL-1β, i.c.v. and monitored the symptoms of sickness induced by LPS until they ceased, so as to determine the cytokine involvement not only in the induction of these responses, but also in the resolution of these responses. Pre-treating rats with either IL-6AS i.p. or a caspase-1 inhibitor i.c.v. attenuated the magnitude and the duration of the anorexia and lethargy induced by LPS administration. LPS-induced fever was completely abolished in rats pre-treated i.p. with IL-6AS, while it was only partially attenuated in rats pre-treated i.c.v. with a caspase-1 inhibitor.

In conclusion, there appears to be some distinct differences in the cytokine-mechanisms regulating fever and sickness behaviours induced by LPS. Identifying the physiological mechanisms mediating fever and sickness behaviours during illness may provide clinicians with more insight into managing not only the thermal, but also the non-thermal responses to infections, responses which may become detrimental to the host if they continue for a prolonged period. My observation that reducing either IL-6 in the circulation or IL-1β in the brain significantly enhances the resolution of anorexia and lethargy, but does not completely prevent them from occurring, appears to indicate that while individual cytokines are possible targets for therapies aimed at alleviating these sickness responses in patients with bacterial infections, to abolish them multiple cytokines may need to be targeted.

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LIST OF ABBREVIATIONS

BBB	blood-brain barrier
COX	cyclooxygenase
CRF	corticotrophin releasing factor
CVOs	circumventricular organs
db/db mice	leptin receptor-deficient mice
gp130	glycoprotein 130
i.a.	intra-arterial
i.c.v.	intracerebroventricular
i.m.	intramuscular
i.p.	intraperitoneal
i.po.	intrapouch
i.v.	intravenous
IL-1α	interleukin-one alpha
IL-1β	interleukin-one beta
IL-1β knockout mice	mice with a null mutation in the interleukin-one beta gene
IL-1ra	interleukin-one receptor antagonist
IL-1RI	interleukin-one receptor type one
IL-1RII	interleukin-one receptor type two
IL-6	interleukin-six
IL-6 knockout mice	mice with a null mutation in the interleukin-six gene
IL-6R	interleukin-six receptor

sIL-6R	soluble interleukin-six receptor
IL-6AS	interleukin-six antiserum
LHA	lateral hypothalamic area
LPS	lipopolysaccharide
LBP	LPS-binding protein
mRNA	messenger ribonucleic acid
NPY	neuropeptide Y
ob/ob mice	leptin deficient mice
ObRb	the long isoform of the leptin receptor
PAMPs	pathogen-associated molecular patterns
PGs	prostaglandins
PGE_2	prostaglandin E_2
5-HT	serotonin
TLR	Toll-like receptors
TNF-α	tumour necrosis factor-alpha
TNFRI	tumour necrosis factor-alpha receptor type one
TNFRII	tumour necrosis factor-alpha receptor type two
s.c.	subcutaneous
VMN	hypothalamic ventromedial nucleus
Zucker *fa/fa* rats	rats with defective receptor-mediated transport and intracellular signalling of leptin

PREFACE

Having cared for a sick child or animal caregivers recognize that most often it is the dramatic changes in behaviour that provide signs that their child or pet is ill. Sick humans and animals often are “feverish”; they do not feel like eating food, have an increased sensitivity to pain and lose interest in their physical and social environments. They feel fatigued and are reluctant to engage in their normal daily activities. Their sleep is also often fragmented and they develop a depressed mood. Each of these sickness symptoms negatively impact on the sick individuals’ quality of life and are extremely uncomfortable, which is often what necessitates medical intervention.

The discovery that these non-specific behavioural symptoms are not only particular to patients with a “common cold” or flu, but in fact also appear to occur in patients with some of the most harmful, costly and debilitating diseases currently experienced in the Western World: coronary heart disease, cancer, obesity, type II diabetes and neurodegenerative disorders associated with aging, has enhanced the effort of basic scientists and clinicians to identify the pathophysiological mechanisms causing these behavioural symptoms, with the view to developing treatment strategies aimed specifically at managing them.

A major breakthrough in identifying the possible mechanisms involved in mediating illness-induced behavioural symptoms arose serendipitously from clinical studies in which proteins important for immune function, known as pro-inflammatory cytokines,

were used in the treatment of the immunosuppression present in cancer patients. Injecting cancer patients with these cytokines produced a suite of sickness responses, including fever, fatigue, malaise, headaches, anorexia and depression, similar to those noted during infection. From these clinical studies showing that pro-inflammatory cytokines, molecules produced by immune cells of the host in response to a variety of disease-causing pathogens, can induce dramatic changes in behaviour resembling those seen during infection, the question has arisen as to whether these proteins are likely mediators of sickness responses. The use of agents that specifically block the synthesis of cytokines in animal models of simulated Gram-negative bacterial infection has successfully been used to identify the involvement of cytokines in mediating fever induced by a variety of disease-causing pathogens. In contrast to the important role established for cytokines as endogenous mediators of fever, no clear picture has emerged as yet regarding their involvement as endogenous mediators of anorexia and lethargy, two brain-controlled sickness behaviours, also known to be induced during an infection.

The main aim of this thesis was therefore to systematically investigate the involvement of four principal cytokines, TNF-α, IL-1β, IL-6 and leptin in mediating not only fever, but two specific behavioural symptoms of sickness, namely lethargy and anorexia, in an animal model of simulated Gram-negative bacterial infection. As sickness and recovery processes are ultimately governed/controlled by the brain, a secondary aim of this thesis was to investigate the action of cytokines within the brain, on voluntary activity and food intake in the absence of a simulated infection.

In **Chapter 1** I review the studies which have thus far examined the contribution of the four principal pro-inflammatory cytokines, IL-1β, IL-6, TNF-α and leptin, in mediating fever and sickness behaviours, specifically anorexia and lethargy, induced by peripheral administration of LPS. **Chapters 2-4** contain publications that form the main body of the thesis. In **Chapter 2** I administered species-and cytokine-specific antisera to examine whether peripherally-released cytokines, IL-1β, IL-6, TNF-α and leptin are physiologically active in the generation of the lethargy, anorexia and fever induced by subcutaneous administration of LPS in rats. To discern direct effects in the brain of IL-6 and IL-1β in mediating sickness behaviours, I injected species-homologous rat IL-6 and IL-1β directly into the brains of conscious rats and examined the dose-response effects on their voluntary activity, food intake and body temperature in **Chapter 3**. **Chapter 3** also looks at whether IL-6 and IL-1β act synergistically within the brain to mediate changes in voluntary activity, food intake and body temperature. In **Chapter 4** I investigate the effects of inhibiting the action of peripherally-released IL-6 and IL-1β in the brain on lethargy, anorexia and fever induced by subcutaneous administration of LPS. In **Chapter 5**, the experimental results presented in the thesis are summarized, unresolved issues are discussed, and conclusions and recommendations are given.

As part of the Declaration, contributions of co-authors to each of the published studies are detailed below:

Chapter 2:

Harden LM, du Plessis I, Poole S, Laburn HP. 2006. Interleukin-6 and leptin mediate lipopolysaccharide-induced fever and sickness behavior. *Physiology & Behavior* 89:146 55.

The idea and experimental design was formulated through discussions with Helen Laburn and Irné du Plessis. I carried out the experimental procedures and analyzed the data. Helen Laburn and Irné du Plessis edited the manuscript. Stephen Poole provided the antisera as well as technical advice on their use.

Chapter 3:

Harden LM, du Plessis I, Poole S, Laburn HP. 2008. Interleukin (IL)-6 and IL-1β act synergistically within the brain to induce sickness behavior and fever in rats. *Brain, Behavior & Immunity* 22:838-849.

The idea and experimental design was formulated through discussions with Helen Laburn and Irné du Plessis. I carried out all of the experimental procedures and analyzed the data. Helen Laburn and Irné du Plessis edited the manuscript. Stephen Poole provided the rat recombinant IL-1β and IL-6 as well as technical advice on their use.

Chapter 4:

Harden LM, du Plessis I, Roth J, Poole S, Laburn HP. 2008. Endogenous antagonism of biologically active IL-6 in the circulation or IL-1β in the brain enhances the resolution of anorexia, lethargy and fever induced by bacterial mimetics. Submitted to *Physiology & Behavior*.

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As supervisors of the candidate, we confirm that she acted as principal investigator on all three studies.

_____ _____

Helen Laburn Date

_____ _____

Irné du Plessis Date

CHAPTER 1

INTRODUCTION

Pathogenic microorganisms which succeed in overcoming the physical barriers to access the human body trigger a set of immune, physiological, metabolic and behavioural responses in the host, known collectively as the acute phase response (Dantzer, 2004). Of these acute phase responses, fever is the most commonly recognized and it is identified by physicians and patients alike as an elevation of body temperature. Not only will most, if not all patients have high body temperatures during infectious and inflammatory illness, but so too will they experience a cluster of non-specific behavioural changes such as lethargy, depression, increased sleepiness and pain, and suppression of appetite (Hart, 1988; Dantzer, 2001; Johnson, 2002). These behavioural changes, collectively referred to as "sickness behaviour" are now well-established responses to infection and together with fever constitute a highly organized and evolved strategy used by the host to fight infection (Hart, 1988).

Part of the innate immune system's response to infection includes the production of pro-inflammatory cytokines, the involvement of which in the pathogenesis of fever was identified in the late 1970's (Dinarello *et al.*, 1977; Dinarello, 1999). The pro-inflammatory cytokines thought to have significant roles in fever include tumour necrosis factor (TNF), interleukin-1 (IL-1) and interleukin-6 (IL-6), synthesized by monocytes/macrophages and lymphocytes (Luheshi & Rothwell, 1996). Approximately ten years after a role for cytokines in fever was uncovered, evidence began to emerge that cytokines also may be involved in the induction of the sickness behaviours accompanying fever. The evidence came from clinical trials in which purified or recombinant cytokines were used in the treatment of patients with cancer and chronic viral infections such as

hepatitis B and C. Administering cytokines to these patients produced a number of side-effects which included lethargy, weakness, malaise, sleep changes as well as fever (Renault & Hoofnagle, 1989; Dinarello, 1997). It quickly became apparent to clinicians and researchers that these effects observed with systemic administration of cytokines were akin to those experienced by sick animals and humans. Since these observations obtained from clinical studies, experimental administration of pro-inflammatory cytokines to animals has confirmed that most components of sickness behaviour can indeed be induced by pro-inflammatory cytokines, in particular IL-1 (for review see Dantzer *et al.*, 1998; Dantzer, 2004).

Although exogenous administration of cytokines in animals and humans induced sickness behaviour, there nevertheless remained doubt as to whether cytokines synthesized endogenously during infection mediated sickness behaviour. It was proposed that the injection of recombinant cytokines may be at pharmacological doses, inducing effects which are not necessarily representative of the true physiological effects occurring in an infected individual. Moreover, during an actual or simulated infection not only one, but a number of different cytokines are synthesized and these cytokines can affect the synthesis and secretion of each other (Cartmell & Mitchell, 2005). Thus it became clear that an understanding of the cytokine mechanisms mediating fever and sickness behaviour during an infection would need investigations into the contribution of each individual cytokine in mediating sickness responses.

The development of techniques which allowed specific antagonism of individual cytokines, such as receptor antagonists, neutralizing antibodies against cytokines and their receptor molecules, and gene knockout technology, assisted researchers in undertaking investigations into the respective roles of cytokines. These techniques were combined with an established animal model of simulated infection induced by a powerful activator of the innate immune system, lipopolysaccharide (LPS), the glycolipid pyrogenic moiety of the Gram-negative bacterial membrane, injected into various animal species via various routes (Ulevitch & Tobias, 1995).

This introductory chapter summarizes the current views on how the response of the innate immune system to administration of LPS leads to the synthesis of pro-inflammatory cytokines, and it reviews the literature on pro-inflammatory cytokines as mediators of fever and sickness behaviours, specifically anorexia and lethargy, induced by LPS. The questions to be addressed in this thesis also are raised.

1.1 Bacterial-host interactions

A substantial portion of our knowledge on host defence responses and inflammatory mediators during an infectious episode has been derived from studies investigating host responses to LPS administration (Heumann & Roger, 2002). The interest in this molecule and the mechanism of its biological action have significant clinical application, as Gram-negative sepsis in humans is caused by Enterobacteriaceae such as *Escherichia coli* and *Klebsiella* species (Bochud & Calandra, 2003). LPS is composed of two

chemically dissimilar structural regions: the hydrophilic repeating polysaccharides of the core and the O-antigen structures, and a hydrophobic domain known as lipid A. Lipid A is the biologically active moiety of LPS (Heumann & Roger, 2002). Of the different components (LPS, peptidoglycan, porins, pipoproteins, lipopeptides, lipid A-associated proteins, pili, flagellin, DNA (CpG motifs) and endotoxins) of Gram-negative bacteria known to induce inflammation, LPS is proposed to be the most potent (Heumann & Roger, 2002).

1.1.1 Detection of Gram-negative bacteria through cellular receptors

Once bacterial components have entered the host, the innate immune system recognizes the presence of a given pathogen by the so-called pathogen-associated molecular patterns (PAMPs) present on microbes, but not expressed by the host (Janeway & Medzhitov, 2002; Kapetanovic & Cavaillon, 2007). PAMPs are specific, structurally conserved components of certain broad groups of microorganisms (Romanovsky *et al.*, 2006). Classical bacterial PAMPs include LPS of Gram-negative bacteria and lipoteichoic acid or peptidoglycans from Gram-positive bacteria. Detection of PAMPs present on microbes is mediated by pattern recognition receptors expressed on the surface of innate immune cells of the host (Kapetanovic & Cavaillon, 2007). The pattern recognition receptors for PAMPs are germ line encoded receptors known as Toll-like receptors (TLR), which are transmembrane proteins that relay PAMPs-induced signals across the cell-surface membrane (Kapetanovic & Cavaillon, 2007).

There is substantial evidence to indicate that LPS acts via the TLR-4 receptor subtype of the TLR family (Chow *et al.*, 1999). However, for LPS in plasma to confirm responsiveness via its TLR, its lipid A domain has to bind at least two non-signalling host accessory proteins: the constitutive serum protein LPS-binding protein (LBP), and soluble or membrane-bound CD14 (see below) (Cartmell & Mitchell, 2005). LBP dissociates LPS aggregates to form LPS/LBP complexes which are transferred to CD14. The primary role of LBP is therefore to function as a lipid-transfer protein, increasing the rate at which LPS interacts with soluble or membrane bound CD14 (Pugin *et al.*, 1993). CD14 exists as a protein anchored in the outer leaflet of the plasma membrane (mCD14) present on monocytic cells (CD14-positive cells). It also exists in the form of a soluble plasma protein (sCD14) that attaches LPS to CD14-negative cells, such as endothelial cells present in blood and fluids (Heumann & Roger, 2002). CD14 cannot induce activation without a transmembrane signal transducing co-receptor, identified as TLR-4 for LPS (Kapetanovic & Cavaillon, 2007). The membrane-bound CD14 which binds LPS, conveys it to MD-2, a key protein needed for the TLR-4 dependent intracellular signalling (Kapetanovic & Cavaillon, 2007). As Figure 1 illustrates LPS therefore initiates its effects through a heteromeric receptor complex containing CD14, together with the transmembrane protein TLR-4 (Medzhitov & Janeway, 1997), and at least one other protein, MD-2, which is essential to confer LPS responsiveness via its TLR (Shimazu *et al.*, 1999). The importance of TLR-4 in mediating the immune response to LPS has been confirmed *in vivo* with the finding that TLR-4-deficient mice do not respond to LPS (Hoshino *et al.*, 1999). While TLR-4 appears to be the primary mechanism mediating LPS recognition, other receptors such as CD11/CD18 beta-2

integrin and cell-surface proteins known as scavenger receptors may also be involved (Romanovsky *et al.*, 2005).

Once LPS binds to TLR-4 present on the surface of leucocytes, this leads to signal transduction via receptor associated proteins, IL-1 receptor-associated kinase, myeloid differentiation factor 88 (MyD88) and TNF receptor-associated factor (TRAF6), resulting in the activation of transcription factors (nuclear factor-κB and activator protein 1) (Cartmell & Mitchell, 2005). Although TLR-4 signalling can also occur through a MyD88 independent pathway, the MyD88 pathway depicted in Figure 1 is essential for the inflammatory response mediated by LPS. The transcription factors control the expression of immune response genes, which ultimately leads to the synthesis and release of cytokines (Heumann & Roger, 2002; Cartmell & Mitchell, 2005; Kapetanovic & Cavaillon, 2007).

The focus of the discussions of this review will be on the involvement of four principal cytokines (TNF-α, IL-1β, IL-6 and leptin) as endogenous mediators of fever, anorexia and lethargy induced by LPS.

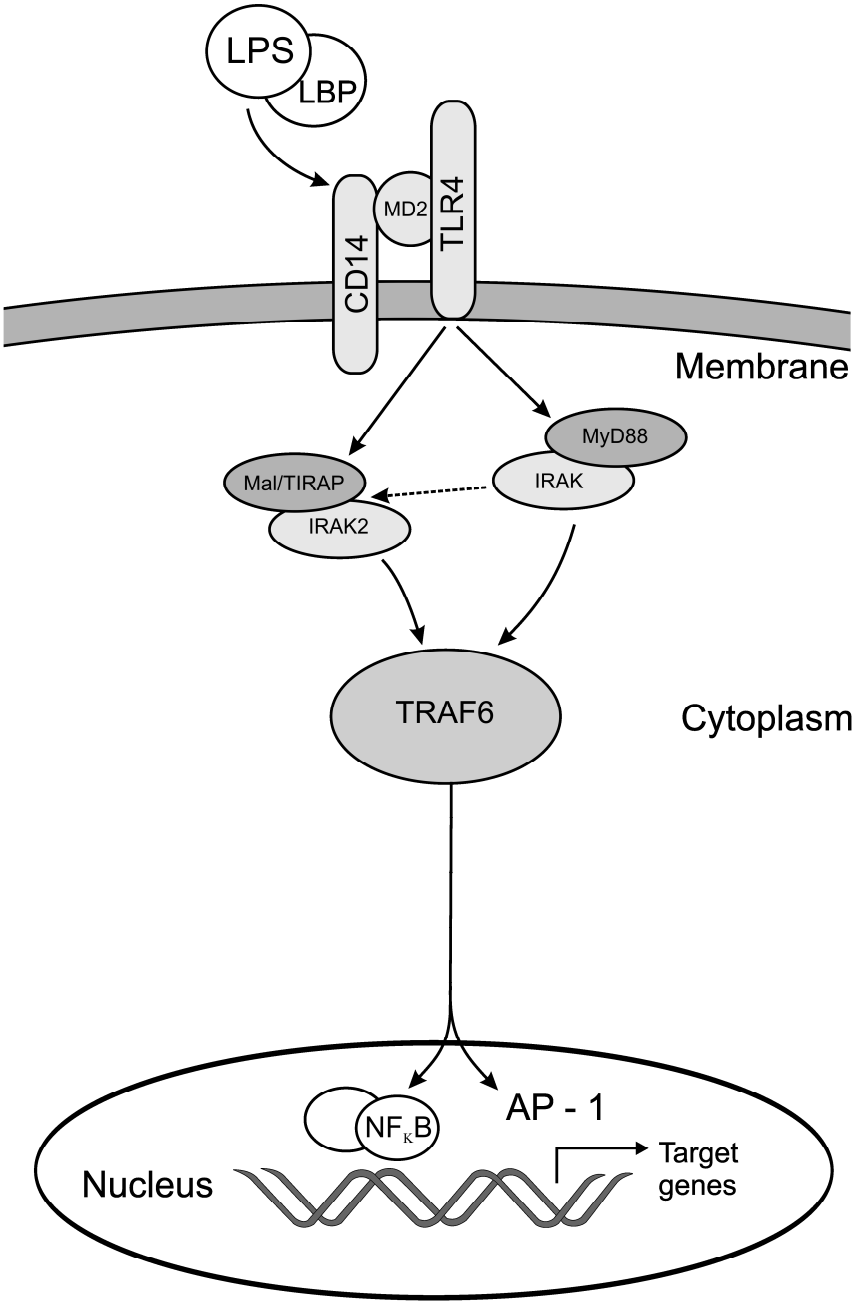

Figure 1. Lipopolysaccharide aggregates are dissociated by LBP to form LPS/LBP complexes which are transferred to CD14. A small cysteine rich secreted glycoprotein, MD-2, is essential for TLR-4 signalling, which leads to signal transduction via receptor associated proteins, IRAK, MyD88 and TRAF6, resulting in the activation of transcription factors (NF-κB and AP-1). The transcription factors control the expression of immune response genes, which ultimately leads to the synthesis and release of cytokines. Abbreviations: Lipopolysaccharide (LPS), LPS-binding protein (LBP), Toll-like receptor 4 (TLR4), IL-1 receptor-associated kinase (IRAK), myeloid differentiation factor 88 (MyD88), TNF receptor-associated factor 6 (TRAF 6), nuclear factor-κB (NF-κB), and activator protein 1 (AP-1) (modified from Cartmell & Mitchell, 2005).

1.1.2 From LPS-induced synthesis to detection of cytokines through cellular receptors

The experimental model of systemic infection, that is intravenous (i.v.) or intraperitoneal (i.p.) administration of LPS to laboratory animals, has been by far the most popular model used to investigate fever and sickness behaviour responses. The rationale for administering LPS systemically is that it mimics responses induced by bacterial septicaemia, in which the exogenous pyrogen is present in the circulation. Injection of LPS into a pre-formed subcutaneous airpouch is used as an experimental model of sterile localized infection. In this experimental model the exogenous pyrogen remains at the site of administration, and so does not enter the circulation. In response to i.v. or i.p. administration of LPS, TNF-α appears first in the circulation, followed by trace amounts of IL-1β and large amounts of IL-6 (Givalois *et al.*, 1994). Injection of LPS into a preformed subcutaneous airpouch induces a significant elevation in the concentration of TNF-α, IL-1β and IL-6 (in that sequence) at the site of infection (that is within the pouch), but IL-6 is the only cytokine detected in the circulation (Miller *et al.*, 1997b; Cartmell *et al.*, 2000). Thus the pattern of cytokines induced in the circulation during experimental models of systemic and localized infection appears to differ.

Not only have pro-inflammatory cytokines been detected in the circulation following systemic administration of LPS, but they also appear in the brain (Rivest *et al.*, 2000). It has been suggested that these cytokines in the brain could be derived from the periphery or they could be synthesized *de novo* within the brain following the systemic immune challenge. Potential sources of cytokine synthesis in the brain include microglia,

astrocytes, neurones and endothelial cells (Rothwell *et al.*, 1996). Whether in the periphery or the brain, cytokines produce their selective biological effects by binding to specific membrane-bound receptors, thereby triggering a cascade of events, leading to signal transduction which promotes transcription and upregulation of expression of specific target genes, for example those for the synthesis of cyclooxygenase (COX) (a detailed description of the signal-transduction pathways for IL-1, TNF and IL-6, is provided in the review by Cartmell & Mitchell, 2005).

The IL-1 cytokine family consists of two agonists, IL-1α and IL-1β, and a highly selective endogenous IL-1 receptor antagonist (IL-1ra) (Dinarello, 1991). *In vivo* all three cytokines are synthesized initially as precursors, of which pro-IL-1α and pro-IL-1ra are biologically active. Pro-IL-1β is inactive as it lacks a signalling peptide, and therefore remains inside the cell in which it was synthesized (Thornberry *et al.*, 1992; Burns *et al.*, 2003). To be biologically active, pro-IL-1β requires proteolytic cleavage by an enzyme known as caspase-1 or interleukin-1-converting-enzyme to active mature IL-1β, which is then secreted from the cell (Thornberry *et al.*, 1992; Fantuzzi & Dinarello, 1999; Burns *et al.*, 2003). There are no differences between the biological actions of IL-1β and IL-1α (Dinarello, 2005b). However, IL-1α is thought to remain mainly cell-associated, while IL-1β is secreted and therefore, more likely to play a role in the physiological responses such as fever and sickness behaviour (Dinarello, 2005b). IL-1α, IL-1β and IL-1ra all bind the same IL-1 receptors on target cells: type I (IL-1rI) and type II (IL-1rII) IL-1 receptors (Dinarello, 1991; Conti *et al.*, 2004). IL-1 exerts its biological effects through initial interaction with the IL-1rI receptor to form a functional signalling

receptor complex (Sims *et al.*, 1993; Lang *et al.*, 1998). The IL-1rII receptor acts as a "decoy" molecule binding IL-1 with high affinity yet it fails to activate the signalling cascade (Lang *et al.*, 1998).

Like IL-1, TNF occurs in two forms, α and β (Cartmell & Mitchell, 2005). Of the two different forms, TNF-α is proposed to be primarily involved in the physiological responses of fever and sickness behaviour. There are two transmembrane signalling receptors, type I (TNFRI, p55) and type II (TNFRII, p75), by which TNF mediates its pleiotropic effects (Bazzoni & Beutler, 1996). A third soluble receptor also exists that acts endogenously as an antagonist of TNF activity (Himmler *et al.*, 1990).

The biological effects of IL-6 are mediated by a specific receptor complex that consists of two functionally different subunits: a specific ligand-binding receptor (IL-6R), not capable of transducing activity; and a non-ligand binding signal transducing glycoprotein (gp 130) (Cartmell & Mitchell, 2005). Together IL-6R and gp 130 form a high-affinity IL-6 binding site that triggers specific transduction signals (Kishimoto *et al.*, 1995). Soluble forms of the ligand-binding IL-6 receptor subunit have also been discovered (Rivest *et al.*, 2000; Roth *et al.*, 2004b). These soluble IL-6 receptors can bind IL-6 and associate with cellular gp130 to initiate signal transduction (Roth *et al.*, 2004b).

Leptin is a 16kDA pleitrophic protein encoded by the *ob* gene, which belongs to the long-chain helical cytokine family that also includes IL-6 and is produced mainly by white adipose cells (Zhang *et al.*, 1994). The biological effects of leptin are induced by

signalling via the long isoform of the leptin receptor, ObRb (Bates *et al.*, 2003; Bates *et al.*, 2004). The ObRb receptor has signalling activities similar to those of the IL-6 type cytokine receptors (Vaisse *et al.*, 1996).

Receptors for IL-1, IL-6, TNF and leptin have been identified on peripheral cells and they have been localized in the brain of rodents (Hopkins & Rothwell, 1995; Zabeau *et al.*, 2003). Having discussed the interactions between LPS and immune cells, which lead to the synthesis of pro-inflammatory cytokines in response to systemic administration of LPS, the subsequent sections summarize the findings obtained from studies using genetic ablation or physiological antagonism of a particular cytokine or its receptor to investigate the action of specific cytokines, released in the periphery and the brain, in mediating the sickness responses of fever, anorexia and lethargy induced by peripheral administration of LPS. These findings also are compared with those obtained from the more commonly used pharmacological approaches of cytokine administration.

1.2 Cytokines as mediators of fever induced by LPS

It is generally believed that the febrile response is mostly beneficial to the host acutely, as an increase in body temperature has been shown to potentiate specific immunological responses and inhibit the growth of at least some viral and bacterial pathogens (Hart, 1988). The increase of body core temperature that occurs during a fever appears to be due to a regulated elevation of the temperature set point resulting from changes in the firing rates of neurones localized in the preoptic area of the hypothalamus (Conti *et al.*,

2004). It has been suggested that during fever, pyrogenic molecules decrease firing rates of warm-sensitive neurones, and increase firing rates of cold-sensitive neurones (as a consequence of synaptic inhibition), thereby suppressing heat-loss responses and enhancing heat production and heat-retention responses (Mackowiak & Boulant, 1996). These changes in firing rates have been reported in studies using *ex vivo* protocols, which included administration of recombinant TNF-α, IL-1β and IL-6 to hypothalamic tissue slices, and are similar to the responses observed in conscious febrile animals (Shibata & Blatteis, 1991). Thus it has been proposed that pro-inflammatory cytokines are the likely pyrogenic molecules acting within the hypothalamus to increase the temperature set-point during fever, which results in the relative changes in firing rates of thermo-sensitive neurones (Hori *et al.*, 1988; Nakashima *et al.*, 1989; Nakashima *et al.*, 1991; Shibata & Blatteis, 1991; Xin & Blatteis, 1992).

1.2.1 Interleukin-1β

IL-1β was the first cytokine considered to be an endogenous pyrogen. The initial evidence was based on the findings that peripheral and central injections of recombinant IL-1β into various laboratory animals induces fever (Dascombe *et al.*, 1989; Murakami *et al.*, 1990; Cao *et al.*, 2001). Although IL-1β may be a potent pyrogenic cytokine when injected systemically (Dascombe *et al.*, 1989), there remains uncertainty concerning its involvement as an endogenous pyrogen mediating fever induced by systemic administration of LPS. The uncertainty surrounding IL-1β as an endogenous pyrogen has arisen because of the poor correlation noted between the fever response and plasma IL-1β

concentrations (Kluger, 1991), the repeated failure of many studies to detect biologically active IL-1β in plasma during simulated infections (Hopkins & Humphreys, 1989, 1990) and the contradictory findings obtained from studies in which IL-1β antagonism was used to investigate the involvement of IL-1β in LPS-induced fevers. In support of IL-1β acting as an endogenous pyrogen, are the reports that fever in response to peripheral LPS administration is inhibited by: (i) peripherally administered IL-1β antiserum or IL-1ra (Long *et al.*, 1990b; Smith & Kluger, 1992; Luheshi *et al.*, 1996; Miller *et al.*, 1997a) and (ii) centrally administered IL-1β antiserum or IL-1ra (Klir *et al.*, 1994; Luheshi *et al.*, 1996; Miller *et al.*, 1997a; Cartmell *et al.*, 1999). Reports not in favour of IL-1β acting as an endogenous pyrogen are the findings that IL-1β knockout mice, mice which are deficient in IL-1β production, respond with only a slightly reduced (Kozak *et al.*, 1995b) or even enhanced (Alheim *et al.*, 1997) fever following LPS administration, and mice deficient in the IL-1 type I receptor respond with virtually the same fevers as wild-type mice (Leon *et al.*, 1996; Labow *et al.*, 1997). Therefore, IL-1β appears to play a role, though perhaps not an essential role in fever induced by systemic and local administration of LPS.

1.2.2 Interleukin-6

In contrast to the minor role proposed for IL-1β in mediating LPS-induced fever, the role of IL-6 appears to be a major one. The first pieces of evidence in support of IL-6 as an endogenous pyrogen in both humans and experimental animals came from the observations of excellent correlations between the magnitude and duration of the fever

response and plasma IL-6 concentrations (Nijsten *et al.*, 1987; LeMay *et al.*, 1990). Moreover, injecting recombinant IL-6 into the brains of rats (LeMay *et al.*, 1990; Dinarello *et al.*, 1991) and systemically in rabbits (Helle *et al.*, 1988) induced fever. Since these early findings, a critical role for endogenous IL-6 in LPS-induced fever has been confirmed by the finding that IL-6 knockout mice, mice carrying a null mutation in the IL-6 gene and therefore defective IL-6 production, do not develop a fever when injected with a low dose of LPS (50 μg kg^{-1}) (Chai *et al.*, 1996; Kozak *et al.*, 1998). Having established that IL-6 is indeed a critical endogenous pyrogen, the question arose as to whether the fever response induced by systemic and local administration of LPS required the central (brain-based) or peripheral pool of IL-6. The finding that central and not peripheral administration of IL-6 induced fever in wild-type mice, lead to the speculation that it is the action of IL-6 within the brain which is important for fever. However, more recently the studies of Cartmell *et al*., (2000) and Rummel *et al*., (2006) have shown that neutralizing the action of IL-6 in the periphery using a species-specific IL-6 antiserum, completely prevented fever from occurring following localized administration of LPS. Thus there is direct evidence that not only centrally released, but also peripherally-released IL-6 can act as an essential signal to the brain to induce fever.

1.2.3 Tumour-necrosis factor

The body temperature effects of IL-1β and IL-6 always are those of an increase in body temperature. TNF-α on the other hand, has been reported to have both body temperature raising (pyrogenic) and body temperature lowering (antipyretic) actions. In favour of a

pyrogenic action for peripherally-released TNF-α are reports that LPS-induced fever is attenuated in: (i) rabbits treated with monoclonal TNF-α antibodies (Kawasaki *et al.*, 1989), (ii) rolipram, a type-IV phosphodiesterase inhibitor that inhibits the production of TNF-α (Mabika & Laburn, 1999) and (iii) guinea-pigs treated with a TNF-binding protein known to be a potent inhibitor of TNF-α (Roth *et al.*, 1998). In contrast an antipyretic action of peripherally-released TNF-α is supported by reports that: (i) neutralizing the peripheral action of endogenously produced TNF-α using antiserum against TNF or soluble TNF receptors, enhanced the fever induced by LPS administration in rats (Long *et al.*, 1990a; Klir *et al.*, 1995), (ii) systemic administration of a low dose of TNF-α (which on its own has no effect on body temperature) attenuated LPS-induced fever in rats (Long *et al.*, 1992; Klir *et al.*, 1995; Kozak *et al.*, 1995a) and (iii) mice lacking both the TNF p55 and p75 receptors developed larger fevers in response to LPS administration, than did wild-type mice (Leon *et al.*, 1997).

It has been proposed that the pyrogenic or antipyretic effects of TNF-α may depend on the species of experimental animal used, as closer examination of the results presented above show that TNF-α appears to be a pyrogenic molecule in rabbits and guinea-pigs, but not in rats and mice. Moreover, the different actions of TNF-α in the fever pathway also may be related to the dose of the fever-inducing agent used, as injection of a low dose (50 μg kg^{-1} i.p.) of LPS produced similar fever responses between TNF p55/p75 knockout mice and wild-type mice, while injection of a higher dose of LPS (2.5 mg kg^{-1} i.p.) resulted in larger fevers in TNF p55/p75 knockout mice (Leon *et al.*, 1997). Injecting rats with high, septic-like doses of LPS (2.5 mg kg^{-1}) produces significantly

greater concentrations of TNF-α in serum compared to that measured when injecting lower doses of LPS (Long *et al.*, 1990a; Sharma *et al.*, 1992). It is possible therefore, that feedback mechanisms exist which are activated by the concentration of the cytokine itself, whereby moderate increases in circulating TNF-α act to increase body temperature, while greater concentrations of circulating TNF-α act as a signal to reduce body temperature (Kluger *et al.*, 1995; Conti *et al.*, 2004).

1.2.4 Leptin

Following reports that circulating concentrations of leptin increase acutely with LPS administration (Sarraf *et al.*, 1997; Faggioni *et al.*, 1998; Finck *et al.*, 1998) and in a similar fashion to known pyrogenic cytokines such as IL-6, a number of investigations examining the role of leptin in the fever pathway were undertaken. Initial evidence in support for leptin as an endogenous pyrogen was based on the findings that the febrile response to systemic administration of LPS was largely attenuated in Zucker *fa/fa* rats, rats in which the receptor-mediated transport and intracellular signalling of leptin are defective (Rosenthal *et al.*, 1996). Moreover, peripheral and central injection of recombinant leptin into rats induced fever (Luheshi *et al.*, 1999).

Studies undertaken subsequently have revealed that these early findings supporting the involvement of leptin in the fever pathway may however, be more complicated to interpret than was initially thought, as the febrile response of Zucker *fa/fa* rats varied depending on the ambient temperature at which the studies were conducted (Ivanov &

Romanovsky, 2002; Steiner *et al.*, 2004). Injecting these mutant rats systemically with a low dose of LPS resulted in a polyphasic fever in a thermally neutral environment, but an attenuated fever in a cool environment (Ivanov & Romanovsky, 2002; Steiner *et al.*, 2004). It is known that in a cold environment rats depend on brown fat thermogenesis not only to regulate body temperature normally but also to produce a febrile response (Ivanov & Romanovsky, 2002). Zucker *fa/fa* rats are not capable of activating brown fat thermogenesis however, as their brown fat is morphologically and functionally defective (Seydoux *et al.*, 1990). The attenuated fever response observed in Zucker *fa/fa* rats housed in a cool environment, may therefore be due to a lack of thermogenesis, rather than the absence of leptin signalling *per se*. That these mutant rats responded with a polyphasic fever similar to that noted in non-mutant rats in a thermally neutral environment, suggested that leptin signalling is not required in the febrile response. These negative findings should be viewed with caution however; studies have shown that the *fatty* mutation in Zucker rats may in fact permit normal functioning of the leptin receptor in at least some experimental paradigms (Wang *et al.*, 1998; Ivanov & Romanovsky, 2002).

More substantial evidence in support of leptin as an endogenous pyrogen has emerged with the use of antibodies to antagonize circulating leptin following peripheral administration of LPS; LPS-induced fever was significantly attenuated (Sachot *et al.*, 2004). Under some experimental conditions peripherally-released leptin therefore does appear to contribute to mediating the fever response induced by systemic administration of LPS.

1.2.5 The relative contributions of cytokines released in the periphery and the brain in mediating LPS-induced fever

Table 1 summarizes the research findings discussed in the sections above where the roles of cytokines released in the periphery and the brain in mediating fever induced by systemic and local administration of various doses of LPS were investigated using techniques which allowed selective inhibition or blockade of cytokine actions or effects. To account for the involvement of cytokines in mediating fever being dependent on the magnitude of the fever response, the studies included in Table 1 administered LPS over a broad range of doses (10 μg kg^{-1} - 2500 μg kg^{-1}). In these studies the relative contribution of a particular cytokine is rated by whether the fever response is abolished, attenuated or not affected (-) when the cytokine effect is absent. Clearly the findings of no effect, attenuation or abolition obtained from studies using techniques which allowed selective inhibition or blockade of cytokine actions or effects need to be interpreted in light of the fact that the results may depend on whether the dose of the antagonist used completely neutralized the action of a given cytokine within the specific compartment (peripheral or central) into which it is injected. Of the findings presented in Table 1 the most prominent are the complete abolition of the fever response when the effect of IL-6 is absent. The creditability of these findings are strengthened by the observations that: (i) the use of two different techniques to block the action of IL-6, genetic ablation and immunological antagonism, yielded similar finding, and (ii) the study of Cartmell *et al.*, (2000) reported undetectable concentrations of IL-6 in the plasma of rats that received LPS and the IL-6 antiserum.

Table 1. The effect of antagonizing the action of cytokines released in the periphery and the brain on fever induced by LPS

	Fever	LPS dose and route	Animal species	References
Interleukin-6				
Peripheral administration of:				
IL-6 antibodies	Abolished	100 μg kg^{-1}, i.po.	Rats	Cartmell *et al.*, 2000 Rummel *et al.*, 2006
Central administration of:				
IL-6 antibodies	Attenuated	1000 μg kg^{-1}, i.p.	Rats	Rothwell *et al.*, 1991
IL-6 knockout mice	Abolished	50 μg kg^{-1}, i.p.	Mice	Chai *et al.*, 1996
IL-6 knockout mice	—	2500 μg kg^{-1}, i.p.	Mice	Kozak *et al.*, 1998
Interleukin-1β				
Peripheral administration of:				
IL-1ra	Attenuated	100 μg kg^{-1}, i.p.	Rats	Luheshi *et al.*, 1996
IL-1β antibodies	Attenuated	10 μg kg^{-1}, i.p.	Rats	Long *et al.*, 1990b
Central administration of:				
IL-1ra	Attenuated	100 μg kg^{-1}, i.p.	Rats	Luheshi *et al.*, 1996
IL-1ra	Attenuated	100 μg kg^{-1}, i.po.	Rats	Cartmell *et al.*, 1999
IL-1β antibodies	Attenuated	50 μg kg^{-1}, i.p.	Rats	Klir *et al.*, 1994
IL-1β knockout mice	Attenuated	2500 μg kg^{-1}, i.p.	Mice	Kozak *et al.*, 1995b
IL-1 type I knockout mice	—	50 μg kg^{-1}, i.p.	Mice	Leon *et al.*, 1996
IL-1 type I knockout mice	—	2500 μg kg^{-1}, i.p.	Mice	Leon *et al.*, 1996

Table 1. continued

	Fever	LPS dose and route	Animal species	References
Tumour necrosis factor-α				
Peripheral administration of:				
TNF-α antibodies	Attenuated	0.625 μg kg^{-1}, i.v.	Rabbits	Kawasaki *et al.*, 1989
TNF-binding protein	Attenuated	10 μg kg^{-1}, i.a.	Guinea pigs	Roth *et al.*, 1998
TNF-receptor antagonist	—	20 μg kg^{-1}, i.m.	Guinea pigs	Roth *et al.*, 1997
TNF-α antibodies	Enhanced	10 μg kg^{-1}, i.p.	Rats	Long *et al.*, 1990a
TNF soluble receptors	Enhanced	50 μg kg^{-1}, i.p.	Rats	Klir *et al.*, 1995
TNF double-receptor knockout mice	—	50 μg kg^{-1}, i.p.	Mice	Leon *et al.*, 1997
Leptin				
Peripheral administration of:				
Leptin antibodies	Attenuated	100 μg kg^{-1}, i.p.	Rats	Sachot *et al.*, 2004
Leptin receptor-deficient rats (Koletsky rats)	—	10 μg kg^{-1}, i.v.	Rats	Steiner *et al.*, 2004
Leptin receptor-deficient rats (Koletsky rats)	—	100 μg kg^{-1}, i.v.	Rats	Steiner *et al.*, 2004
Zucker (*fa/fa*) rats	—	10 μg kg^{-1}, i.v.	Rats	Ivanov & Romanovsky, 2002

i.po. = intrapouch, i.p. = intraperitoneal, i.v. = intravenous, i.a. = intra-arterial, i.m. = intramuscular
— = no effect
Studies of the present thesis are excluded from this table

The attenuated responses noted when the effect of IL-1β was absent appeared also to accurately reflect the contribution of IL-1β in mediating LPS-induced fever, as most of the studies reported that increasing the dose of the cytokine antagonizing agent did not produce a greater attenuation of the LPS-induced fever (Luheshi *et al.*, 1996; Cartmell *et al.*, 1999). The role of TNF-α in mediating LPS-induced fever appears more complex and less clear than that of IL-6 and IL-1β, as findings obtained from studies in which complete neutralization of TNF-α was confirmed, found attenuated (Roth *et al.*, 1998) or enhanced (Long *et al.*, 1990a) fevers. Not only is there uncertainty concerning the role of TNF-α in mediating LPS-induced fever but so too for leptin. While peripheral administration of leptin antibodies attenuated LPS-induced fever no effect was noted in leptin receptor-deficient rats (Steiner *et al.*, 2004) and Zucker rats (Ivanov & Romanovsky, 2002).

From the findings presented in Table 1 it therefore appears that while other cytokines released in the periphery and the brain may play contributory roles, endogenous circulating IL-6 is likely to be the major endogenous pyrogen mediating fever in response to systemic and localized infection induced by LPS (50 and 100 μg kg^{-1}). Irrespective of the relative roles played by the various endogenous cytokines in fever, the question arises as to whether cytokines are the final mediators of fever and if not which other molecules are the most likely candidates for this function.

1.2.6 Brain mediators of cytokine effects on body temperature

Exposure of the host to an exogenous pyrogen such as LPS not only results in the release of pro-inflammatory cytokines, but also results in the release of a number of enzymes that catalyse the formation of small signalling molecules such as prostaglandin E_2 (PGE_2). The formation of PGE_2 depends on the activity of the cyclooxygenase (COX) enzyme, which exists in two isoforms, the constitutively expressed COX-1 and the inducible form COX-2 (Ivanov & Romanovsky, 2004). The synthesis of PGE_2 via the induction of COX-2 is a key event in the fever response, as it has been shown that mice which lack PGE_2 synthesizing enzymes do not develop a fever in response to LPS administration (Li *et al.*, 1999; Engblom *et al.*, 2003). It is likely that endogenous cytokines induce fever following peripheral administration of LPS via the generation of these PGE_2 synthesizing enzymes, as neutralization of endogenous circulating IL-6 also attenuates COX-2 upregulation in the cerebral microvasculature of rats (Rummel *et al.*, 2006) and the changes in firing rates of thermoregulatory neurones induced with administration IL-1β and IL-6 to tissue slices obtained from guinea pigs and rats, is prevented by concurrent administration of COX inhibitors (Hori *et al.*, 1988; Xin & Blatteis, 1992).

1.3 Cytokines as mediators of the anorexia induced by LPS

Loss of appetite and the concomitant decrease in food intake is frequently observed in sick humans and animals, and along with fever forms the most common sign of infection observed in both the clinical and experimental situation (Plata-Salaman, 1996a). The

anorexia experienced during illness appears to be a robust phenomenon that occurs throughout a broad variety of species, as well as in response to various different infectious agents. It is a valuable short-term response aimed at supporting the immunological response of the host to eliminate the pathogen (Murray & Murray, 1979; Hart, 1988; Exton, 1997) and it does not appear to be a consequence of fever, as it can occur in the absence of fever (McCarthy *et al.*, 1985; O'Reilly *et al.*, 1988).

It is generally believed that illness-related anorexia results from the modulation of normal homeostatic processes that regulate feeding in healthy individuals (Langhans, 2007). As such it has been proposed that the loss of appetite observed during infection must eventually result from signalling to a final pathway for appetite control that resides within the brain (Plata-Salaman, 1998a). Injection of cytokines into the hypothalamus and cerebral ventricles of rats has been shown to inhibit feeding (Kent *et al.*, 1994; Sonti *et al.*, 1996). It is likely therefore that cytokines could act as these signalling molecules within the brain to reduce food intake during infection. A common and related effect of infection and cytokine administration is a decrease in body mass (Bluthé *et al.*, 1992b; Bluthé *et al.*, 1994a; Leon *et al.*, 1996), which at least acutely, is thought to reflect the decreased food intake.

1.3.1 Interleukin-1β

Reports from numerous studies have compellingly demonstrated that IL-1β administration is particularly potent at reducing appetite in humans and experimental

animals (Plata-Salaman & Ffrench-Mullen, 1992; Langhans *et al.*, 1993; Plata-Salaman, 1996b; Sonti *et al.*, 1996; Montkowski *et al.*, 1997; Pecchi *et al.*, 2006). The loss of appetite is observed when IL-1β is injected into the periphery or the brain of experimental animals, indicating that IL-1β can act from both sites to induce anorexia (Kent *et al.*, 1996). The anorexigenic responses induced with the administration of IL-1β are similar to those seen following LPS administration (Plata-Salaman *et al.*, 1988). Subsequent studies have shown however, that the anorexigenic responses observed with administration of IL-1β do not appear to reflect the pathophysiological mechanism by which systemic administration of LPS induces anorexia. Administration of IL-1ra peripherally or centrally, failed to attenuate anorexia induced by systemic administration of LPS in rats (Kent *et al.*, 1992b). Moreover, mice genetically deficient in IL-1β or the IL-1 type I receptor demonstrated similar reductions in food intake and body mass following systemic administration of LPS as those of wild-type mice (Kozak *et al.*, 1995b; Leon *et al.*, 1996). Although IL-1β may be able to induce anorexia when injected, it appears to be of little importance in terms of mediating the decrease in food intake observed with systemic administration of LPS.

There have been a few notable exceptions though, in which studies have identified some involvement of either peripherally-released or centrally released IL-1β in mediating the depressing effects of LPS on food intake and body mass. Central administration of IL-1ra attenuated the reduction in food intake induced by systemic administration of LPS in mice (Layé *et al.*, 2000), peripheral administration of IL-1ra attenuated the reduction in body mass induced by systemic administration of LPS in rats (Bluthé *et al.*, 1992b) and

the appetite suppressive effects of centrally, but not systemically, administered LPS were attenuated in IL-1β-converting enzyme-deficient mice, mice which do not produce active IL-1β (Burgess *et al.*, 1998).

Taken together, these findings are therefore not compatible with an exclusive role of endogenous IL-1β in mediating the effects of bacterial products on food intake and body mass. The most likely explanation for the negative findings showing no or little involvement of IL-1β in mediating LPS-induced anorexia in the gene knockout mice studies (deficient IL-1β, IL-1 type I and IL-1β-converting enzyme) and the IL-1ra studies, is that one or several other pro-inflammatory cytokines compensate for the absence of IL-1β. An obvious candidate cytokine to compensate for the lack of IL-1β is TNF-α, as synergistic effects between IL-1β and TNF-α have been noted in inducing anorexia and decreasing body mass in rats and mice (Bluthé *et al.*, 1994a; Sonti *et al.*, 1996).

1.3.2 Tumour-necrosis factor

Although it has been shown that TNF-α can act directly within the brain to suppress food intake and decrease body mass in rats (Bluthé *et al.*, 1994a; Sonti *et al.*, 1996; Palin *et al.*, 2007), as with IL-1β, the evidence in support of an exclusive role for TNF-α in mediating anorexia induced by LPS is not convincing and moreover also contradictory. TNF double-receptor knockout mice responded with a similar reduction in food intake and body mass as wild-type mice to systemic administration of LPS (Leon *et al.*, 1997), whereas rats treated with specific TNF-α antagonists responded with attenuated anorectic

responses to systemic administration of LPS (Sharma *et al.*, 1992; Porter *et al.*, 2000; Töllner *et al.*, 2000).

Closer examination of the results from these studies reporting an attenuated anorectic response revealed two important points, which provide some insight into understanding the role of TNF-α in LPS-induced anorexia. Firstly, it appears that the degree of attenuation depends on the dose of LPS administered, as treating rats with the nonspecific phosphodiesterase inhibitor pentoxifylline, which inhibits the production of TNF-α *in vivo* (Porter *et al.*, 2000), completely abolished the anorectic effect of i.p. injected LPS (100 μg kg^{-1}), but only attenuated the anorectic effect induced by a higher dose of i.p. injected LPS (250 μg kg^{-1}) (Porter *et al.*, 2000). Secondly, following treatment with a TNF-binding protein, the attenuation of the anorexia and body mass loss was predominantly noted during days two and three after the LPS injection (Töllner *et al.*, 2000). Similar findings of a gradual attenuation of the decrease in food intake and body mass have also been reported in rats pre-treated with TNF-α monoclonal antibodies (Sharma *et al.*, 1992). The attenuated responses noted in these two studies occurred however, during a period when TNF-α would not have been detectable in plasma (Kozak *et al.*, 1997b). Moreover, neutralizing the biological activity of TNF-α did not facilitate complete recovery of LPS-induced anorexia, as the food intake and body mass of rats injected with LPS and the TNF- binding protein never reached the values of the control rats injected with saline. It is likely that peripherally-released TNF-α is therefore exerting its effect by inducing secondary endogenous mediators, which then participate in this

anorectic response. One likely mediator is leptin, a hormone known to regulate feeding behaviour (Friedman & Halaas, 1998).

1.3.3 Leptin

Leptin, the product of the *ob* gene has been shown to be an important central nervous system signalling factor for the control of energy balance and body adiposity (Friedman & Halaas, 1998). The concentration of leptin in the circulation primarily indicates the production of leptin by white adipocytes, and reflects both long-term (days) and short-term (hours) changes in leptin production (Steiner & Romanovsky, 2007). Changes in fat storage regulate long-term changes in leptin production and influences the quantity of food consumed relative to the amount of energy expended (Friedman & Halaas, 1998). The role of leptin and its receptors in the homeostasis of body mass is demonstrated by the observations that rodents that have mutations in either their signalling receptors (i.e. *db/db* mice or *fa/fa* Zucker rats) or in leptin production (i.e. *ob/ob* mice) are extremely obese (Campfield *et al.*, 1995; Halaas *et al.*, 1995). Short-term changes in the concentration of leptin in the circulation can occur independently of body fat mass or loss (Steiner & Romanovsky, 2007). Leptin production is decreased a few hours after the onset of fasting, and during conditions of negative energy balance acts as an anti-starvation signal to suppress energy expenditure and stimulate appetite (Steiner & Romanovsky, 2007).

The observation that daily i.p. administration of leptin decreases food intake and body mass in both obese and non-obese mice (Halaas *et al.*, 1995; Pelleymounter *et al.*, 1995), together with the finding that LPS and pro-inflammatory cytokines increase the production of leptin in adipose tissue, prompted investigations into the possible role of leptin in mediating the appetite-and body mass-reducing effects of LPS (Grunfeld *et al.*, 1996; Faggioni *et al.*, 1997; Finck *et al.*, 1998). These studies showed that the increase in leptin in the circulation and leptin mRNA in adipose tissue following LPS administration correlates with the decrease in food intake (Grunfeld *et al.*, 1996), and leptin receptor-deficient (*db/db*) mice are partially resistant to LPS-induced anorexia (Faggioni *et al.*, 1997). In support of the findings noted in leptin receptor-deficient mice (*db/db*) suggesting some involvement of leptin in mediating LPS-induced anorexia, others found that neutralizing the biological activity of leptin in the circulation significantly attenuated the LPS-induced decrease in food intake and body mass in rats (Sachot *et al.*, 2004). Not only did reducing the biological activity of leptin in the circulation prevent LPS-induced anorexia, but it also attenuated the up-regulation of IL-1β in the hypothalamus of the rats (Sachot *et al.*, 2004). It is possible then that leptin could be acting in the hypothalamus to induce anorexia by increasing IL-1β there (Layé *et al.*, 2000; Sachot *et al.*, 2004).

As noted with the studies investigating the role of leptin in mediating LPS-induced fever, the findings for LPS-induced anorexia in animals with genetic defects in the leptin system are not always in full agreement with those obtained from studies in which leptin antiserum was administered. Leptin deficient (*ob/ob*) mice and obese (*fa/fa*) Zucker rats do not exhibit attenuated anorectic responses following LPS administration (Faggioni *et*

al., 1997; Lugarini *et al.*, 2005). It is unclear why discrepancies have been reported on leptin's role in LPS-induced anorexia. It has been proposed that humoral mediators, other than leptin such as IL-1β and TNF-α (Sachot *et al.*, 2004), may compensate for the absence of leptin in animals with genetic defects in the leptin system. Moreover, due to the importance of food intake for survival, the control of food intake is not solely dependent on a single pathway, but rather is regulated by different pathways involving mediators other than leptin, such as cholecystokinin, insulin and glucagon. It is possible therefore that the absence of the leptin-mediated pathway occurring early on in development of mutant animals may merely result in other pathways involved in food intake homeostasis compensating. These alternative pathways could be activated during pathological conditions like LPS stimulation, leading to the observed anorexia in *db/db* mice and *fa/fa* Zucker rats. In general, while leptin may not be a necessary prerequisite or the final mediator of the anorectic response to systemic administration of LPS, it nonetheless appears to contribute to the response under some experimental conditions.

1.3.4 Interleukin-6

Of the pro-inflammatory cytokines investigated thus far as mediators of LPS-induced anorexia, IL-6 has emerged as the least likely cytokine to be involved. It can decrease food intake in rats when injected peripherally or centrally, but less in comparison to the effects noted when other cytokines are administered (Schöbitz *et al.*, 1995; Plata-Salaman, 1996b; McCarthy, 2000a). Moreover, IL-6 knockout mice responded with the same decrease in food intake as that noted for wild-type mice following systemic

administration of LPS (Fattori *et al.*, 1994; Swiergiel & Dunn, 2006). Interestingly though, it appears that peripherally-released IL-6 is involved in mediating the loss of body mass induced by systemic administration of LPS in rats (Strassmann *et al.*, 1993; Bluthé *et al.*, 2000b). Whether the involvement of IL-6 in mediating the LPS-induced decrease in body mass reported in the studies by Strassman *et al.*, (1993) and Bluthé *et al.*, (2000b) was related to changes in food intake is uncertain, as food intake was not measured in these two studies. Body mass loss is a complex variable however, that is dependent not only on food intake, but also on excretion of faeces and urine, and increased metabolism (Lennie, 1998). It has been observed that patients infused with IL-6 exhibit a number of the metabolic changes found in catabolic states (Stouthard *et al.*, 1995) and rats injected with IL-6 have reduced gastric emptying (McCarthy, 2000b). IL-6 may therefore contribute to the cachexia associated with chronic diseases, in which a significant part of the severe weight loss is due to the metabolic changes occurring during the illness (Strassmann & Kambayashi, 1995; Plata-Salaman, 1996a; Baltgalvis *et al.*, 2008).

1.3.5 The relative contributions of cytokines released in the periphery and the brain in mediating LPS-induced anorexia

Table 2 summarizes the research findings discussed in the sections above where the roles of cytokines released in the periphery and the brain in mediating anorexia induced by systemic administration of LPS were investigated using techniques which allowed selective inhibition or blockade of cytokine actions or effects. Studies investigating LPS-

induced anorexia have assessed feeding responses in rats and mice by measuring either the amount of food pellet or sweetened milk consumed (Swiergiel & Dunn, 1999). Although milk and food pellet intake are both measures of feeding which are affected during infection, milk intake has been shown to be less susceptible to disruption by infection than food pellet intake (Swiergiel *et al.*, 1997). The findings reported in this review were therefore restricted to those in which food intake was used as a measure of feeding. The attenuated responses noted when the effect of IL-1β in the brain and TNF-α in the periphery were absent appeared to accurately reflect the contribution of these cytokines in mediating anorexia induced by systemic administration of LPS, as the studies reported that the particular cytokine was not detected in the brain or plasma following administration of the respective cytokine or cytokine receptor antagonist (Sharma *et al.*, 1992; Layé *et al.*, 2000; Töllner *et al.*, 2000). The study of Sachot *et al.*, (2004) in which leptin antiserum was administered peripherally, did not however provide any evidence of the degree of neutralization achieved with the dose of antiserum administered.

Table 2. The effect of antagonizing the action of cytokines released in the periphery and the brain on anorexia induced by LPS

	Anorexia	LPS dose and route	Animal species	References
Interleukin-6				
IL-6 knockout mice	—	1000 μg kg^{-1}, i.p.	Mice	Fattori *et al.*, 1994
IL-6 knockout mice	—	1 μg $mouse^{-1}$, i.p.	Mice	Swiergiel & Dunn, 2006
Interleukin-1β				
Peripheral administration of:				
IL-1ra	—	400 μg kg^{-1}, i.p.	Rats	Kent *et al.*, 1992b
Central administration of:				
IL-1ra	—	400 μg kg^{-1}, i.p.	Rats	Kent *et al.*, 1992b
IL-1ra	Attenuated	5 μg $mouse^{-1}$, i.p.	Mice	Layé *et al.*, 2000
IL-1β knockout mice	—	2500 μg kg^{-1}, i.p.	Mice	Kozak *et al.*, 1995b
IL-1 type I knockout mice	—	2.5 μg $mouse^{-1}$, i.p.	Mice	Bluthé *et al.*, 2000a
IL-1 type I knockout mice	—	50 μg kg^{-1}, i.p.	Mice	Leon *et al.*, 1996
IL-1 type I knockout mice	—	2500 μg kg^{-1}, i.p.	Mice	Leon *et al.*, 1996
IL-1β-converting enzyme-deficient mice	—	10 μg $mouse^{-1}$, i.p.	Mice	Burgess *et al.*, 1998
Tumour necrosis factor-α				
Peripheral administration of:				
TNF-α antibodies	Attenuated	3000 μg kg^{-1}, i.p.	Rats	Sharma *et al.*, 1992
TNF-binding protein	Attenuated	5000 μg kg^{-1}, i.p.	Rats	Töllner *et al.*, 2000
TNF-binding protein	—	1 μg $mouse^{-1}$, i.p.	Mice	Swiergiel & Dunn, 1999
TNF-binding protein + IL-1ra	—	1 μg $mouse^{-1}$, i.p.	Mice	Swiergiel & Dunn, 1999

Table 2. continued

	Anorexia	LPS dose and route	Animal species	References
Central administration of:				
TNF-binding protein	—	1 μg mouse^{-1}, i.p.	Mice	Bluthé *et al.*, 2000a
TNF double-receptor knockout mice	—	50 μg kg^{-1}, i.p.	Mice	Leon *et al.*, 1997
TNF double-receptor knockout mice	—	2500 μg kg^{-1}, i.p.	Mice	Leon *et al.*, 1997
Leptin				
Peripheral administration of:				
Leptin antibodies	Attenuated	100 μg kg^{-1}, i.p.	Rats	Sachot *et al.*, 2004
Leptin-receptor deficient mice (*db/db*)	Attenuated	1 μg mouse^{-1}, i.p.	Mice	Faggioni *et al.*, 1997
Leptin-deficient mice (*ob/ob*)	—	1 μg mouse^{-1}, i.p.	Mice	Faggioni *et al.*, 1997

i.p. = intraperitoneal
— = no effect
Studies of the present thesis are excluded from this table

As such it is possible that the degree of attenuation noted in their study may not represent the complete contribution of peripherally-released leptin in mediating LPS-induced anorexia. The research findings presented in Table 2 appear to reflect the complexity of the interactions between cytokines and/or other molecules in mediating food intake during illness, as anorexia induced by systemic administration of LPS is mostly not affected by the absence of individual cytokines and when it is affected, the response is attenuated only and not abolished.

Whilst important, these studies, unlike those investigating LPS-induced fever, have only used an experimental animal model of systemic infection to investigate the role of cytokines in mediating LPS-induced anorexia. Different patterns of cytokines have been shown to be induced during systemic and localized infection/inflammation (Fattori *et al.*, 1994). Thus the role of cytokines in mediating anorexia may differ depending on the origin of the infection. The involvement of cytokines in mediating anorexia induced during a localized Gram-negative bacterial infection has however, not been investigated.

1.3.6 Brain mediators of cytokine effects on feeding

Cytokines can directly (via neuronal mechanisms) or indirectly (via modulation of brain chemistry) change the activity of hypothalamic neurones involved in the control of food intake (Plata-Salaman & Ffrench-Mullen, 1994; Plata-Salaman, 1996a, 1998b). The neurones are sensitive to changes in the concentration of glucose in the blood (Guyton & Hall, 2000). An increase in the concentration of blood glucose increases the rate of firing

of glucose-sensitive neurones in the satiety centre in the ventromedial nuclei (VMN) of the hypothalamus (Guyton & Hall, 2000). The same increase in blood glucose concentration simultaneously decreases the firing of glucose-sensitive neurones in the hunger centre of the lateral hypothalamic area (LHA) (Guyton & Hall, 2000). These responses predict that inhibition of LHA and activation of VMH will result in anorexia. Injecting rats i.c.v. with IL-1-β or TNF-α specifically suppresses the neuronal activity of the glucose-sensitive neurons in the LHA (Plata-Salaman *et al.*, 1988), whereas IL-1β excites the glucose-sensitive neurones in the VMH (Kuriyama *et al.*, 1990).

The control of feeding also is associated with various neurotransmitter and neuropeptide systems in the hypothalamus. There is substantial data to show that cytokines can modulate hypothalamic chemistry by stimulating the synthesis of mediators such as prostaglandins, and interacting with various neurotransmitters and neuropeptides within the brain. Although the exact contribution of these direct and indirect actions of cytokine mechanisms in mediating the decrease in feeding during infection is yet to be established, some of the proposed interactions are discussed below.

1.3.6.1 Prostaglandins

In rats the anorexia induced by systemic administration of LPS is significantly attenuated with selective pharmacological or genetic blockade of COX-2-generated prostaglandins (Johnson *et al.*, 2002). The partial, rather than complete, attenuation of food intake has lead to the suggestion, that unlike the fever response, prostaglandin-dependent pathways

do not seem to be crucially involved in mediating LPS-induced anorexia. Consistent with this observation in laboratory animals, is the clinical observation that patients with infections/inflammation receiving antipyretic therapy still exhibit anorexia (Plata-Salaman, 1996a). In contrast, selective pharmacological or genetic blockade of COX-2 generated prostaglandins are very effective antagonists of anorexia induced by administration of IL-1β and TNF-α (Bluthé *et al.*, 1992a; Langhans *et al.*, 1993; McCarthy, 2000a; Pecchi *et al.*, 2006; Elander *et al.*, 2007). The differential importance of prostaglandins in mediating LPS versus cytokine induced anorexia, not only identifies distinct mechanisms of action, but also is consistent with the view that endogenous IL-1β and TNF-α are not exclusive mediators of the anorectic effects of LPS (Langhans, 1996).

1.3.6.2 Neuropeptides

The interaction between cytokines and neuropeptides in the regulation of feeding responses to microbial products such as LPS is not yet fully understood, but a number of cytokine-neuropeptide interactions have been proposed (Langhans, 2007). IL-1β has been implicated in interactions with two specific neuropeptides, neuropeptide Y (NPY) and corticotrophin releasing factor (CRF). NPY is a potent orexigenic or feeding-stimulating neuropeptide (Sahu *et al.*, 1988) and it has been suggested that IL-1β can modulate feeding by reducing hypothalamic NPY levels (Langhans & Hrupka, 1999). In support of the involvement of CRF, a known anorexigenic neuropeptide (Uehara *et al.*, 1989), as a cytokine-induced mediator of anorexia during infection, are the findings that hypothalamic CRF mRNA was increased following i.p. injection of IL-1β (Suda *et al.*,

1990) and anorexia induced by IL-1β was attenuated by i.c.v. administration of a CRF antagonist (Uehara *et al.*, 1989).

1.3.6.3 Neurotransmitters

Serotonin and histamine are components of important neurotransmitter systems involved in the control of feeding. Administration of IL-1β, TNF-α and IL-6 have been shown to increase central serotonergic (5-HT) activity known to decrease food intake (Gemma *et al.*, 1991; Zalcman *et al.*, 1994; Clement *et al.*, 1997; Wang & Dunn, 1999) and pretreatment with a highly-specific 5-HT_{2C} receptor antagonist blocked the anorexia induced by both peripheral and central injections of LPS or IL-1β in rats (von Meyenburg *et al.*, 2003a, b; Asarian *et al.*, 2007). Unlike serotonin, the involvement of histamine as a downstream mediator of cytokines in LPS-induced anorexia has not yet been investigated. The findings in rats that changes in neuronal histamine modulate feeding behaviour (Ookuma *et al.*, 1993) and that peripheral administration of IL-1β increases the hypothalamic histamine turnover rate (Kang *et al.*, 1995), has lead some to hypothesize that IL-1β could be inhibiting feeding during infection by increasing the synthesis and release of histamine in the hypothalamus (Plata-Salaman, 1998a).

1.4 Cytokines as mediators of lethargy induced by LPS

Not only may sick individuals experience a fever and have little interest in eating food but they also may feel lethargic. Lethargy is a state of hypoactivity characterized by a

general feeling of fatigue and an unwillingness to perform normal daily routine activities (Hart, 1988). As with the febrile and anorexic responses of the host to infection, it has been proposed that inactivity during infection may be a valuable response, which also is aimed at supporting the immunological response of the host to eliminate the pathogen (Hart, 1988). By engaging in less muscular activity the energy reserves which are needed for the increased metabolic costs of fever are conserved (Hart, 1988). Moreover, by resting and not moving around, less heat is lost via convection, which aids the heat production required for the fever response (Hart, 1988). It is likely that cytokines could mediate the lethargy experienced during infection, as administration of cytokines increases the sensation of fatigue reported by healthy human subjects at rest (Spath-Schwalbe *et al.*, 1998) and reduces general locomotor activity in rats (Schöbitz *et al.*, 1995; Montkowski *et al.*, 1997).

1.4.1 Interleukin-1β and interleukin-6

Although limited, there is some evidence which implicates IL-1β as a possible mediator of lethargy. Central administration of IL-1β in rats decreases locomotor activity (Montkowski *et al.*, 1997) and the time to fatigue during forced treadmill running (Carmichael *et al.*, 2006). In support of IL-6 as a mediator of lethargy are the findings that: (i) central administration of IL-6 decreases locomotor activity in rats (Schöbitz *et al.*, 1995), (ii) administration of human recombinant IL-6 induces a sensation of fatigue in healthy humans at rest (Spath-Schwalbe *et al.*, 1998), (iii) treatment with IL-6 antibodies induces an immediate disappearance of previously debilitating fatigue reported

by patients with multicentric Castleman disease, a disease characterized by a dysregulated overproduction of IL-6 (Nishimoto *et al.*, 2000; Nishimoto *et al.*, 2005) and (iv) fatigue in patients with cancer is positively correlated with circulating levels of IL-6 (Schubert *et al.*, 2007). Despite the above reports from studies showing that IL-1β and IL-6 can induce fatigue and decrease activity levels, there has been no evidence to support the involvement of either of these two cytokines in mediating the lethargy induced in response to microbial products such as LPS. Mice genetically deficient in IL-1β or the IL-1 type I receptor demonstrated similar reductions in locomotor activity following systemic administration of LPS as did wild-type mice (Kozak *et al.*, 1995b; Leon *et al.*, 1996).

1.4.2 Tumour-necrosis factor

In contrast to the lack of evidence in support of the involvement of IL-1β and IL-6 in mediating the lethargy induced in response LPS, there have been some, albeit contradictory, reports suggesting a role for peripherally-released TNF-α in mediating LPS-induced lethargy. Pre-treating rats and mice with TNF-α inactivating agents (antiserum to TNF-α or a TNF soluble receptor) did not prevent the decrease in locomotor activity induced by LPS, but did seem to facilitate a faster recovery to normal activity levels (Kozak *et al.*, 1995a). In contrast, others have found that LPS-induced lethargy is not attenuated in rats treated with a TNF-binding protein (Töllner *et al.*, 2000) or mice which lack functional TNF-α receptors (Leon *et al.*, 1997).

1.4.3 The relative contributions of cytokines released in the periphery and the brain in mediating LPS-induced lethargy

Table 3 is a summary of the research findings discussed in the sections above where the role of cytokines in mediating lethargy induced by systemic administration of LPS were investigated using techniques which allowed selective inhibition or blockade of cytokine actions or effects. These studies investigating LPS-induced lethargy have quantitatively assessed the hypoactivity, characteristic of a state of lethargy, in rats and mice by measuring general locomotor activity of the experimental animal in its home cage. The research findings presented in Table 3 appear to reflect the complexity of the interactions between cytokines and/or other molecules in mediating lethargy during illness, lethargy induced by systemic administration of LPS is mostly not affected by the absence of IL-1β and TNF-α. Whilst important, these studies, as with those investigating LPS-induced anorexia, have only used an experimental animal model of systemic infection to investigate the role of cytokines in mediating LPS-induced lethargy. Moreover, the investigations have not comprehensively investigated all the likely cytokine mediators. IL-6 has been implicated as a putative mediator of lethargy in an animal model of localized inflammation induced by turpentine (Kozak *et al.*, 1997a). What, if any, involvement IL-6 may have in mediating lethargy induced during a localized Gram-negative bacterial infection is unknown.

Table 3. The effect of antagonizing the action of cytokines released in the periphery and the brain on lethargy induced by LPS

	Lethargy	LPS dose and route	Animal species	References
Interleukin-1β				
IL-1β knockout mice	—	2500 μg kg^{-1}, i.p.	Mice	Kozak *et al.*, 1995b
IL-1 type I knockout mice	—	50 μg kg^{-1}, i.p.	Mice	Leon *et al.*, 1996
IL-1 type I knockout mice	—	2500 μg kg^{-1}, i.p.	Mice	Leon *et al.*, 1996
Tumour necrosis factor-α				
Peripheral administration of:				
TNF-α antibodies	Attenuated	2500 μg kg^{-1}, i.p.	Mice	Kozak *et al.*, 1995a
TNF-binding protein	—	5000 μg kg^{-1}, i.p.	Rats	Töllner *et al.*, 2000
TNF-α soluble receptors	Attenuated	2500 μg kg^{-1}, i.p.	Mice	Kozak *et al.*, 1995a
TNF double-receptor knockout mice	—	50 μg kg^{-1}, i.p.	Mice	Leon *et al.*, 1997
TNF double-receptor knockout mice	—	2500 μg kg^{-1}, i.p.	Mice	Leon *et al.*, 1997

i.p. = intraperitoneal
— = no effect
Studies of the present thesis are excluded from this table

1.5 Mechanisms of action by which peripherally-released cytokines signal the brain

From the discussions in the sections above there is significant evidence that cytokines present in the circulation can act as important humoral mediators of the brain-controlled fever mechanism. Figure 2 below shows a schematic presentation of the three proposed humoral mechanisms by which signalling between cytokines present in the circulation and the brain can occur.

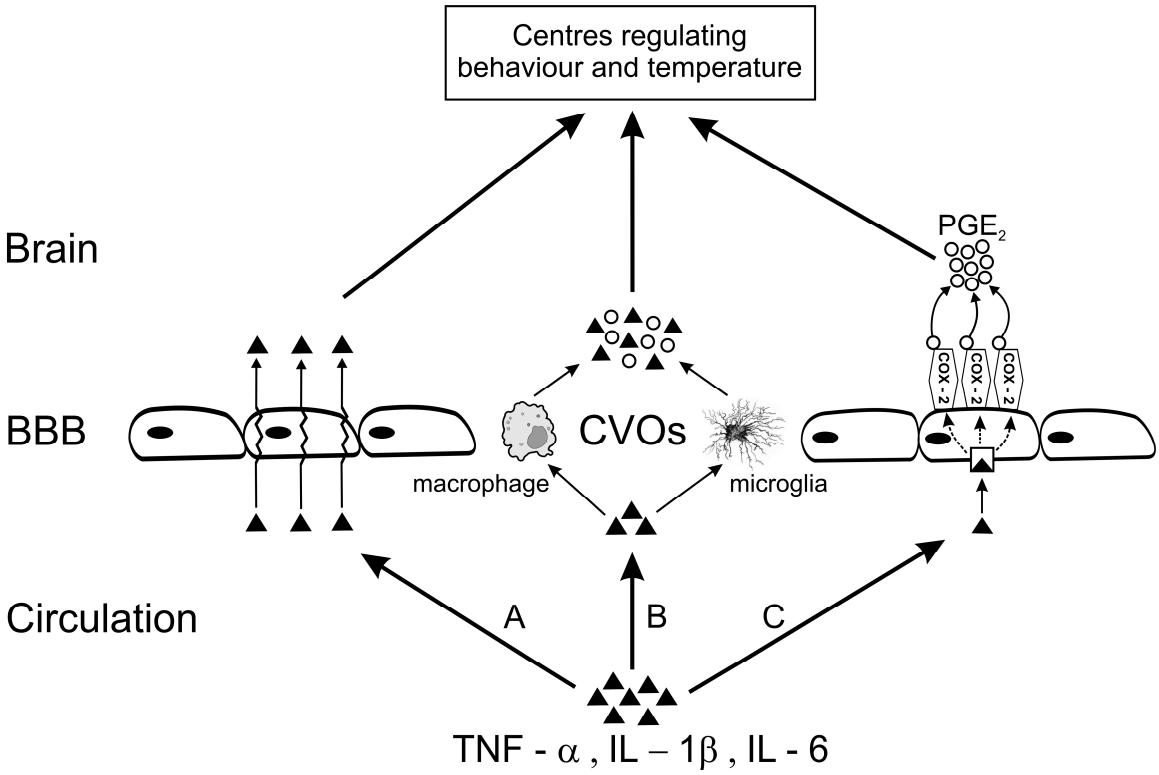

Figure 2. Schematic presentation of the three proposed humoral mechanisms by which circulating cytokines, synthesized in response to peripheral administration of LPS, can signal the brain: (A) carrier-mediated transport across the blood-brain barrier (BBB), (B) access through the circumventricular organs (CVOs), and (C) synthesis of prostaglandin-E_2 (PGE_2) in cells forming the BBB. Abbreviations: pro-inflammatory cytokines (▲), cyclooxygenase-2 (COX-2), cytokine receptor (⛰), PGE_2 (○).

On the one hand, there is experimental evidence which has identified distinct carrier-mediated transport mechanisms to chaperone cytokines, including TNF-α (Osburg *et al.*, 2002), IL-1β (Banks *et al.*, 1989; Banks *et al.*, 1993), IL-6 (Banks *et al.*, 1994) and leptin (Banks & Lebel, 2002), from the blood to the brain. The physiological significance of carrier transport across the blood brain barrier (BBB) has however, been questioned due to the finding that this system transports less than 0.3% of the cytokines present in the blood into the central nervous system (Elmquist *et al.*, 1997; Cartmell & Mitchell, 2005). The reason for this small proportion appears to be because these transport mechanisms are very slow and easily saturable (Conti *et al.*, 2004). The concentrations of TNF-α and IL-1β in the circulation do not usually exceed 100 pg mL^{-1} in patients during a nonfatal infectious episode (Rothwell *et al.*, 1996). In contrast plasma concentrations of IL-6 can often reach 10-20 μg mL^{-1} during fever in experimental animals and in humans (Rothwell *et al.*, 1996). Although it may allow significant quantities of IL-6 to enter the brain, it is unlikely that the carrier-mediated transport of peripherally-released TNF-α and IL-1β across the BBB is the primary mechanism by which these two cytokines signal the brain to evoke fever and sickness behaviour. During chronic infection however, when endogenous cytokines are elevated for extended periods of time in the circulation or when the BBB is breached, direct entry of these circulating cytokines may be of greater physiological importance (Elmquist *et al.*, 1997; Cartmell & Mitchell, 2005).

An alternative humoral pathway is the access of cytokines through the circumventricular organs (CVOs), a specialized neural region along the margins of the ventricular system that has fenestrated capillaries and therefore no blood-brain barrier (BBB). The absence

of a BBB for these CVOs enables cytokines in the circulation to come into contact with the cells of the CVOs (Quan & Banks, 2007). It is possible that cytokines in the circulation may enter the perivascular space passively and interact with specific receptors located on the surface of glial cells, perivascular macrophages or endothelial cells. These cells may produce additional pro-inflammatory cytokines or secondary mediators such as PGE_2, which would act directly or indirectly on neurones that project to other brain structures or disseminate into adjacent brain parenchyma by volume diffusion (Komaki *et al.*, 1992; Roth *et al.*, 2004a; Rummel *et al.*, 2005b). In addition to cytokines in the circulation acting on the CVOs, the observation that endothelial cells in the CVOs also constitutively express CD14 and TLR4 means that it is possible that bacterial fragments themselves can induce the synthesis of pro-inflammatory cytokines at the level of the CVOs (Laflamme & Rivest, 2001).

The two mechanisms discussed thus far, have mainly focused on cytokines present in the circulation having to gain access to the brain tissue itself to be able to signal thermoregulatory neurones. Over the last ten years another mechanism whereby cytokines present in the circulation do not actually have to gain access to the brain tissue to signal thermoregulatory neurones, but rather they can mediate the induction of cytokine synthesis and other signalling molecules within cells of the BBB, which can then penetrate into the brain tissue, has gained prominence (Quan & Banks, 2007). Endothelial cells of cerebral microvasculature constitutively express receptors for TNF-α (Nadeau & Rivest, 1999) and IL-1 (Konsman *et al.*, 2004), with the receptor for IL-6 being expressed under inflammatory conditions (Vallieres & Rivest, 1997). Peripheral

administration of LPS, IL-1β, TNF-α and IL-6 has been shown to induce genomic activation of the brain endothelium leading to the expression of COX-2, which is required for the formation of PGE_2 (Cao *et al.*, 1996; Yamagata *et al.*, 2001; Nadjar *et al.*, 2003; Rummel *et al.*, 2005a; Rummel *et al.*, 2006). The notion that endothelial cells can interact with circulating cytokines to release additional cytokines, or secondary mediators such as PGE_2, therefore not only applies to the sensory CVOs, but the entire brain endothelium (Roth *et al.*, 2006).

In addition to the three humoral pathways by which inflammatory challenges in the periphery can communicate with the brain, an alternative rapid signalling neural pathway, possibly involving cutaneous and vagal neural signals, which functions independently of circulating cytokines, has also been proposed (Bluthé *et al.*, 1994b; Blatteis & Sehic, 1997; Ross *et al.*, 2000). The role of the vagus nerve in the transmission of information from the periphery to the brain has been assessed by vagotomy experiments in which the vagus nerve is sectioned under the diaphragm. Subdiaphragmatic vagotomy resulted in an attenuation of the fever response only when LPS was administered at a low dose (Romanovsky *et al.*, 1997) and via the i.p. route (Goldbach *et al.*, 1997). In terms of mediating sickness behaviours, attenuated responses with subdiaphragmatic vagotomy also appeared to be specific to the abdominal cavity (an i.p. injection), because vagotomy did not attenuate the behavioural effects of IL-1β when this cytokine was injected by either the s.c. or i.v. routes (Bluthé *et al.*, 1996). When activated by peripheral cytokines the vagus appears to be able to activate specific neural pathways in the brain which are

involved in fever and sickness behaviour and to stimulate microglia in the brain to produce cytokines (Layé *et al.*, 1995; Bluthé *et al.*, 1996; Roth *et al.*, 2006).

Having established that vagal afferents may be involved in mediating the effects of peripheral cytokines under some experimental conditions, the question arose as to whether this response was specific to afferent neural signals from the viscera. To block the signals of cutaneous afferents induced by LPS injection into an air pouch, an experimental model of localized subcutaneous inflammation, a local anaesthetic was co-administered with the LPS. Using this experimental approach it was found that cutaneous afferents, as with vagal afferents, contribute as a communication pathway to the brain, but again only when low (10 μg kg^{-1}) and not high (100 μg kg^{-1}) doses of LPS are administered (Ross *et al.*, 2000). A possible explanation for the apparent lack of involvement of neural signals when LPS is administered at high doses could be that these doses induce a greater increase in circulating cytokines, known humoral mediators, which can override the lack of a neuronal signal (Roth & De Souza, 2001). Although the relative importance of these different humoral and neural pathways by which peripherally-released cytokines can communicate with the brain remains an area of much debate, it is accepted that cytokines synthesized in the periphery are an important pathway for communication to the brain during a peripheral immune challenge.

1.6 Thesis aims

Having reviewed all the studies investigating the roles of endogenous IL-1β, IL-6, TNF-α and leptin in mediating fever induced by systemic and local administration of LPS in the sections above, it is evident that peripherally-released cytokines, in particular IL-6, are important endogenous mediators in the fever pathway. In contrast, although it has been shown that systemic administration of pyrogenic cytokines can induce anorexia and lethargy in experimental animals, no clear role has been identified for these cytokines as endogenous mediators of anorexia and lethargy induced following systemic and local administration of LPS. Using proven techniques and highly quantifiable measures of each response I therefore set out to try and clarify the role of endogenous pyrogenic cytokines in mediating anorexia and lethargy induced following local subcutaneous (s.c.) administration of LPS. All the measurements were recorded concurrently, as not only did I investigate the contribution of cytokines in mediating each of the individual sickness responses (anorexia, lethargy and fever), but I also investigated if their involvement differed between responses. Core body temperature was measured using temperature-sensitive radiotransmitters which were implanted intra-abdominally and anorexia was assessed by measuring the quantity of food consumed over a specified time period. These are both proven measurements techniques which provide highly quantifiable measures of core body temperature and anorexia. In terms of lethargy, researchers have traditionally measured changes in general locomotor activity of an experimental animal in its home cage to identify whether the animal is lethargic or not. The lethargy accompanying illness, however, not only results in an unwillingness of the individual to

perform normal daily routine activities, but also to engage in physical exercise. When running wheels are attached to the cages of experimental animals such as rats and mice, they willingly engage in spontaneous physical exercise. Challenging mice with *Corynebacterium parvum* or *Brucella abortus* antigen has been shown to reduce voluntary wheel-running (Sheng *et al.*, 1996; Ottenweller *et al.*, 1998; Sheng *et al.*, 2001). Voluntary-wheel running and general locomotor activity (measured as cage activity) are both therefore appropriate measures of lethargy during infection. It has been noted however, that suppression of voluntary exercise is a more sensitive measure of fatigue and lethargy induced by LPS, than is suppression of cage activity. Voluntary-wheel running of rats, for example, is almost completely abolished by a single i.p. injection of LPS (75 μg kg^{-1}) whereas cage activity is only decreased by ~50% (Hopwood and Harden unpublished observations). I therefore chose to use voluntary wheel-running as a highly quantifiable and sensitive measure of lethargy for the studies presented in Chapters 2, 3, and 4.

The findings presented in Tables 1, 2 and 3 from studies investigating the involvement of cytokines in mediating LPS-induced fever and anorexia, highlighted that acute pharmacological or immunological antagonism of cytokines has generally proven to be a more physiological and successful approach to use when investigating the contribution of individual cytokines in mediating specific sickness responses, rather than genetic ablation of a particular cytokine or its receptor. The interpretation of the findings from knockout mice have often proven to be problematic, as for a particular gene knockout mouse to survive to adulthood it is likely that compensatory mechanisms would have had to

develop to enable the mouse to cope with the loss of the deleted cytokine's action. In the studies presented in Chapters 2 and 4 I therefore specifically chose to use pharmacological or immunological antagonism of cytokines to determine the relative involvement of individual cytokines in mediating the sickness responses induced following LPS administration.

1.6.1 Aim 1

Of the pro-inflammatory cytokines released by immune cells following systemic and local administration of LPS, IL-6 has emerged as an important circulating mediator of the fever response (Cartmell *et al.*, 2000). Another cytokine-like hormone, leptin, also apparently plays a role in LPS-induced fever and anorexia in rats (Sachot *et al.*, 2004). However, whether these or other peripherally-released cytokines are involved in the lethargy observed following s.c. administration of LPS remains uncertain. **The aim of the experiments presented in Chapter 2 was therefore to examine whether the LPS-induced release of the cytokines, IL-1β, IL-6, TNF-α and leptin in the periphery is involved in mediating lethargy, anorexia and fever in rats.** To determine the involvement of each of these cytokines I antagonized their action following LPS administration by administering species-specific antisera to each individual cytokine peripherally. Lethargy, anorexia and fever were measured as changes in voluntary wheel-running, food intake and body temperature respectively. I found, as have others, that the LPS-induced fever in rats was abolished if either IL-6 or leptin antisera were administered before LPS. In addition, I showed for the first time that peripherally-

released IL-6 plays a role in the suppression of voluntary activity, as well as in the suppression of food intake induced by s.c. administration of LPS. The voluntary activity and food intake observed after the administration of the IL-6 antiserum was however, unlike the fever response, reduced but not abolished. It would appear therefore, that while peripherally-released IL-6 has a critical role to play in the events regulating fever, an additional factor(s), possibly other cytokines, are working in parallel with IL-6 to regulate the lethargy and anorexia induced by LPS.

1.6.2 Aim 2

As shown in chapter 2 and by others (Kent *et al.*, 1992b; Kozak *et al.*, 1995b; Leon *et al.*, 1996; Leon *et al.*, 1997; Swiergiel & Dunn, 1999; Töllner *et al.*, 2000) antagonizing the action of other peripherally-released cytokines, TNF-α and IL-1β, has no effect on lethargy and anorexia in rats and mice during infection and inflammation. Thus it appeared likely that if other cytokines are working in conjunction with peripherally-released IL-6 to induced lethargy and anorexia it is primarily due to their action within the brain. **The first aim of the experiments presented in Chapter 3 was therefore to determine the direct effects of injecting IL-1β and IL-6 into the brain, on voluntary wheel-running, food intake and body temperature in rats.** The observation that no single cytokine appears to regulate the responses of anorexia and lethargy induced by LPS, has lead to the proposal that interactions between cytokines are required to affect food intake and voluntary activity following LPS administration. In particular, cytokine interactions observed as physiological synergy, in which the effect of a combination of

substances exceeds the effect of the individual constituents, has been demonstrated *in vivo* (Bluthé *et al.*, 1994a; Sonti *et al.*, 1996; Lenczowski *et al.*, 1999). **The second aim of the experiments presented in Chapter 3 was to assess whether IL-6 and IL-1β act synergistically within the brain to mediate changes in voluntary activity, food intake and body temperature.** I injected species-homologous rat IL-6 and IL-1β separately and in combination, into the brains of conscious rats and examined the dose-response effects. I found that i.c.v. administration of species-homologous rat IL-6 and IL-1β on their own before the night-time active period decreases voluntary activity and also increases body temperature in rats in a dose-dependent fashion. While i.c.v. administration of both IL-1β and IL-6 decreased voluntary activity, only IL-1β administration decreased food intake. Moreover, I identified new findings regarding the synergistic relationship between IL-1β and IL-6 in the brain, by showing that central co-administration of doses of IL-1β and IL-6 which, when injected on their own were non-pyrogenic and did not affect food intake and body mass, induced fever and anorexia when they were co-injected centrally. It would appear that the synergistic action of IL-1β and IL-6 in the brain is not inevitable however, as I did not observe a synergistic effect on the suppression of voluntary wheel-running when both cytokines were co-injected.

1.6.3 Aim 3

From the findings obtained in the studies presented in chapters 2 and 3, I hypothesized that endogenous brain IL-1β is the likely central cytokine, working in parallel with IL-6 released in the periphery, to induce anorexia and lethargy following s.c. administration of

LPS. **The aim of the experiments presented in Chapter 4 was therefore to determine the relative contribution of both peripherally-released IL-6 and centrally released IL-1β in mediating the lethargy, anorexia and fever induced by s.c. administration of LPS in rats.** To decrease the biological activity of IL-6 in the circulation and IL-1β in the brain I administered species-specific antiserum to IL-6 i.p. and a caspase-1 inhibitor, shown to prevent the cleavage of pro-IL-β to biologically active IL-1β, i.c.v. I found that both peripherally-released IL-6 and IL-1β in the brain are involved in the induction and maintenance of LPS-induced lethargy and anorexia, and confirmed that circulating IL-6 is the primary endogenous pyrogen mediating LPS-induced fever.

CHAPTER 2

Interleukin-6 and leptin mediate lipopolysaccharide-induced fever and sickness behavior.

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Interleukin-6 and leptin mediate lipopolysaccharide-induced fever and sickness behavior

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Abstract

Pro-inflammatory cytokines, interleukin (IL)-1β, IL-6 and tumor necrosis factor-alpha (TNF-α) synthesized by activated macrophages and monocytes in response to administration of lipopoysaccharide (LPS), are considered important mediators of fever and sickness behavior. We administered rat-specific antisera for TNF-α, IL-1β, IL-6 and leptin, to determine the involvement of peripherally released cytokines in LPS-induced fever and sickness behavior, measured as suppression of voluntary wheel-running and food intake. Male Sprague–Dawley rats (~200 g) selected for their predisposition to spontaneously run on running wheels were anaesthetized with a combination of ketamine hydrochloride (80 mg/kg i.m.) and xylazine (4 mg/kg i.m.) and implanted intra-abdominally with temperature-sensitive radiotelemeters. Rats were injected intraperitoneally with anti-rat sera to one of the following, TNF-α, IL-1β, IL-6 or leptin or with pre-immune sheep serum, followed by a subcutaneous injection of either LPS (250 μg/kg) or sterile saline. Lipopolysaccharide administration induced a ~1.3 (0.2) °C fever lasting ~10 h and reduced voluntary running by 93 (8.6)% and food intake by 51 (21.3)% compared to the saline response (ANOVA, $P<0.05$). Injection of anti-IL-6 serum or anti-leptin serum abolished the LPS-induced fever, anti-TNF-α serum affected only the early phase of fever and anti-IL-1β serum had no effect on fever (ANOVA, $P<0.05$). LPS-induced suppression of voluntary running and food intake were attenuated in rats receiving anti-IL-6 serum, while the decrease in food intake was totally abolished in rats receiving anti-leptin serum (ANOVA, $P<0.05$). Injection of anti-TNF-α or anti-IL-1β serum had no effect on LPS-induced sickness behavior. Peripherally released IL-6 and leptin therefore appear to be important in regulating LPS-induced fever and sickness behavior.

Keywords: Pro-inflammatory cytokines; Antiserum; Endotoxin; Food intake; Voluntary wheel-running

1. Introduction

During an infection host defense mechanisms are activated in animals and humans, and include both thermal responses, such as fever, and non-thermal responses, such as sickness-type behaviors [13]. The behavioral responses accompanying fever such as sleep, lethargy and anorexia can be observed in experimental animals following the administration of the Gram-negative bacterial cell wall product lipopolysaccharide (LPS) [13]. Systemic administration of LPS activates immune cells in the periphery to release pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β and IL-6 [9,18,38]. A number of hypotheses have emerged on how peripherally released cytokines may regulate the central mechanisms of fever and sickness behavior, some of which include the activation of neuronal afferent pathways and transport systems from blood to the brain [12,13].

Interleukin-6 has emerged as an important circulating mediator of the LPS-induced fever response [46]. Neutralization of endogenous IL-6 in rats [9] and the absence of IL-6 in knock-out mice [10] attenuate fever induced by LPS. The synthesis and release of IL-6 into the circulation following LPS administration may be due to the action of IL-1β [9,34], synthesized soon after LPS administration, at local tissue sites and in the brain, where it has been shown to mediate fever in its own right [8,34,35,37,56]. Tumor necrosis factor-α also is synthesized early on in an LPS-induced febrile episode, but its involvement in fever is complex, with reports suggesting both pyrogenic and antipyretic properties [22,29–31,44].

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In contrast to the established importance of peripherally released IL-6 in mediating LPS-induced fever, the peripheral involvement of cytokines in regulating sickness behavior is less clear [7]. Studies showing that central administration of IL-1β and TNF-α cause decreased body weight and locomotor activity suggest that cytokines can act in the brain to induce sickness behavior [6,21]. Another cytokine-like hormone, leptin, also apparently plays a role in LPS-induced fever and anorexia in rats [19,36,49]. However, whether these or other peripherally released cytokines are involved in the behavioral changes observed following LPS administration remains uncertain.

Many techniques can and have been used to elucidate the roles of cytokines in the generation of the fever response. Arguably, the most physiological approach is to antagonize endogenously released cytokines, following a pyrogenic stimulus. We have administered species-specific antisera to examine whether the endogenously released cytokines, IL-1β, IL-6, TNF-α and leptin are physiologically active in the generation of the fever response, and two aspects of sickness behavior, induced by LPS injection into rats. We monitored body temperature responses and two disparate, quantifiable behaviors, voluntary exercise and food intake, after administration of cytokine-specific antiserum and a pyrogenic dose of LPS. We hypothesized that if a peripherally released cytokine is involved in mediating the generation of fever or sickness behavior, then endogenous neutralization of this cytokine using species-specific antisera would attenuate the body temperature rise, and/or the sickness behavior induced by LPS. Our results confirm roles for endogenous IL-6, TNF-α and leptin in the fever response, and uncover roles for endogenous IL-6 in suppression of voluntary running and food intake and confirm leptin's role in the control of feeding behavior.

2. Methods

2.1. Animals

Male Sprague–Dawley rats (initial body mass 100–120 g) were housed individually in cages to which exercise-training wheels had been attached, at an ambient temperature of 21 ± 2 °C on a 12-h:12-h light/dark cycle (lights on 07:00). Food (pelleted rat chow, Epol, Johannesburg, South Africa) and water were provided *ad libitum*. All procedures were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (ethics no. 2003/39/5).

2.2. Body temperature

Core body temperatures of rats were measured by remote biotelemetry, using temperature-sensitive radiotelemeters (TA10TA-F40, Data Sciences, St. Paul, MN, USA), which were implanted intraperitoneally in animals anaesthetized with 80 mg/kg ketamine hydrochloride (Anaket-V, Bayer, SA) and 20 mg/kg xylazine (Chanazine, Bayer, SA). Transmitter output frequency (Hz) was monitored at 5-min intervals, by a receiver plate (RTA 500, Mini-Mitter, Sunriver, OR, USA) situated beneath the cage of each animal. The frequency received by each plate was fed into a peripheral processor (Datacol-3 Automated Data Acquisition System, VitalView, Minimitter, Sunriver, OR, USA) connected to a personal computer and the output expressed in degrees centigrade. The telemeters were calibrated against a quartz thermometer (Quat 100, Heraeus, Germany) to an accuracy of 0.1 °C.

2.3. Voluntary wheel-running

The exercise-training wheels had a circumference of 1.06 m and each wheel was equipped with a counter (cyclocomputer, Cat eye velo 2, CC-VL200, CAT EYE Co., Ltd., Osaka, Japan) attached to a sensor (No. 169-9771, CAT EYE Co., Ltd., Osaka, Japan), which detected complete wheel revolutions when a magnet (No. 166-5120, CAT EYE Co., Ltd., Osaka, Japan) placed on the edge of the wheel, passed under the sensor. The wheels were designed to allow rotation in only one direction. Night-time running activity was recorded daily at 08:00 and day-time running activity was recorded daily at 17:00.

2.4. Food intake

Food containers were filled daily with a measured amount (60 g) of standardized pelleted rat chow. Twenty-four-hour food intake was measured at 17:00 and quantified by subtracting the food remaining in the food container and on the cage floor from the amount of food given the preceding day. Food powder in the cage was ignored as it has previously been reported to be similar amongst rats and generally weighs less than a gram [39].

2.5. Pyrogens and antisera

Lipopolysaccharide (LPS) derived from *Escherichia coli* endotoxin (serotype, 0111:B4, Sigma, St. Louis, MO, US) was reconstituted in saline (sterile, pyrogen-free 0.9% saline Sabax, Johannesburg, South Africa) and injected subcutaneously (s.c.) at a dose of 250 μg/kg.

We used four rat-specific cytokine antisera; anti-rat TNF-α serum, anti-rat IL-1β serum, anti-rat IL-6 serum, anti-rat leptin serum (NIBSC, South Mimms Potters Bar, Herts UK) and pre-immune sheep serum (PIS, NIBSC) for the control injections, all with an endotoxin content < 0.24 ng/ml (2.4 IU) and injected intraperitoneally in a volume of 1.5 ml. The anti-rat TNF-α, anti-rat IL-1β, anti-rat IL-6 and anti-rat leptin polyclonal antibodies were raised in sheep as previously described [41,42]. The neutralizing ability of the anti-rat TNF-α, IL-1β and IL-6 sera on rat cytokines was determined *in vitro* using a two-site sandwich ELISA as described previously [41]. One hundred microlitres of the antiserum was found to neutralize up to 10,000 pg of the specific recombinant rat cytokine against which it was raised ("LM Harden, unpublished observations"). Results from previous studies using the same anti-rat TNF-α, IL-6 and leptin sera raised in sheep have confirmed the neutralizing ability of these specific antisera *in vivo* [9,49,52]. Each of the cytokine-specific antisera has been shown to not cross-react with the following rat recombinant cytokines: (rr) TNF-α, IL-6, IL-1β, IL-1-α, IL-1ra and IL-10 [41,42].

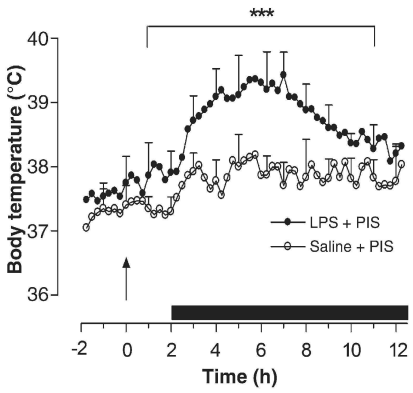

Fig. 1. Body temperature of rats injected s.c. with LPS (250 μg/kg) (n=6) or saline (n=7) and an i.p. injection of pre-immune sheep serum (PIS) (1.5 ml). ***Indicates a significant increase in body temperature of rats injected with LPS compared to rats injected with saline (P<0.001). The results are represented as mean (S.D.). The arrow indicates the time of the injection; the black bar indicates lights out (19:00 clock time).

2.6. Experimental procedure

Rats with a voluntary daily running distance of on average 2 km monitored over a 14-day period were selected for the study. After surgery for implantation of the radiotelemeters, all animals were returned to their cages and the running wheels were locked for a period of 3 days so that animals could not exercise. Thereafter, the wheels were unlocked and experimentation started a minimum of 10 days post-surgery once rats had reached their pre-surgery nightly running distance.

Groups of rats (n=6–8 per group) were injected intraperitoneally (i.p.) with 1.5 ml of one of: anti-rat TNF-α serum, anti-rat IL-1β serum, anti-rat IL-6 serum, anti-rat leptin serum or pre-immune sheep serum (PIS), followed by a s.c. injection of either LPS (250 μg/kg) or sterile saline (1 ml/kg). All injections were administered between 17:00 and 18:00 prior to the onset of the dark phase when rats are most active. Body temperature, spontaneous running and food intake were monitored for 72 h before and 24 h after injections.

2.7. Data analysis

All data are expressed as mean (S.D.). The body temperature responses were plotted as abdominal temperature–time curves in 15-min intervals. For statistical purposes, the original 5-min temperature recordings of each rat were averaged over 2 h for the 10-h period following injections and two-hourly means were analyzed using two-way analysis of variance with intervention and time as main effects. A Student–Newman–Keul's (SNK) *post hoc* test was used to detect for differences within and between groups when the ANOVA detected significant main effects or interactions. Because of negligible daytime running, only night-time running distance was analyzed. Running distance was determined from the number of wheel turns and was expressed as a percentage change from pre-injection (average of three nights) running distance. Food intake was expressed as grams of food consumed per 100 g of rat body weight. The effect of LPS on the percentage change in running and grams of food consumed for the night of injection was analyzed using a t-test with Bonferroni correction for multiple comparisons. The effect of each antiserum on the LPS-induced change in running and food consumption for the night of injection was analyzed using a one-way analysis of variance followed by a SNK *post hoc* test.

3. Results

3.1. Body temperature

Fig. 1 shows that s.c. injection of LPS induced a significant rise in body temperature after a latent period of approximately 2 h. The peak in body temperature (39.3 (0.5) °C) occurred between 5 and 7 h after the injection. The fever lasted for up to 10 h following injections.

Body temperatures of rats injected with LPS and PIS were significantly higher than the body temperatures of rats injected with saline and PIS for most of the observation period. The main effects of time ($F_{(4,44)}$=18.93, P<0.0001), group ($F_{(1,11)}$= 102,24, P<0.0001) and interaction ($F_{(4,44)}$=6.53, P=0003)

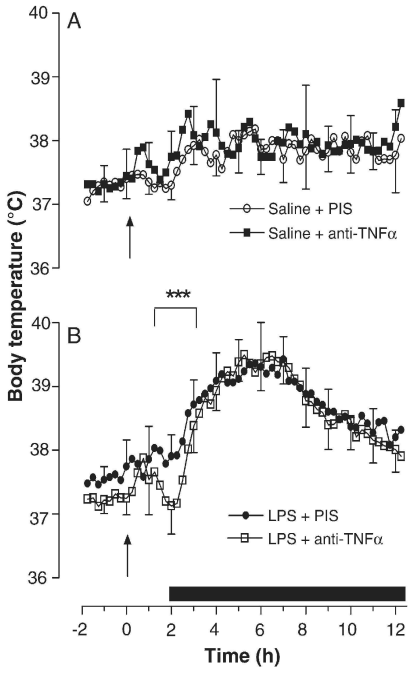

Fig. 2. Body temperature of rats injected with: (A) saline (s.c.) and anti-TNF-α (1.5 ml, i.p.) (n=7) or PIS (n=7); (B) LPS (250 μg/kg, s.c.) and anti-TNF-α serum (1.5 ml, i.p.) (n=8) or PIS (n=6). ***Indicates a significantly lower body temperature of rats injected with LPS+anti-TNF-α serum 1–3 h after the injections when compared to rats injected with LPS+PIS (P<0.001). The results are represented as means (S.D.). The arrow indicates the time of the injection; the black bar indicates lights out (19:00 clock time).

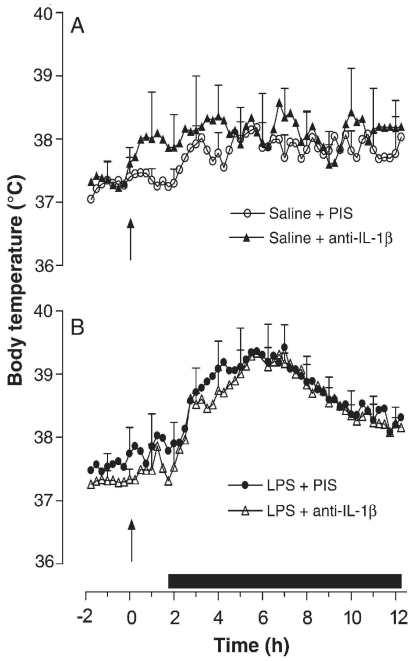

Fig. 3. Body temperature of rats injected with: (A) saline (sc) and anti-IL-1β serum (1.5 ml, i.p.) (n=6) or PIS (n=7); (B) LPS (250 μg/kg, s.c.) and anti-IL-1β serum (1.5 ml, i.p.) (n=6) or PIS (n=6). No significant differences were found in the body temperatures of rats injected with LPS+PIS and LPS+anti-IL-1β serum (P>0.05). The results are represented as means (S.D.). The arrow indicates the time of the injection; the black bar indicates lights out (19:00 clock time).

were significant such that rats injected with LPS (250 μg/kg) developed a fever, while rats injected with saline did not.

3.1.1. Effects of cytokine antisera on body temperature

Fig. 2 shows effects of injection of anti-TNF-α serum or PIS on body temperature of rats when the sera were administered i.p. prior to s.c. saline (Fig. 2A) or LPS (Fig. 2B). Anti-TNF-α serum had no effect on normal body temperature (Fig. 2A), as no significant differences were found between the body temperatures of rats injected with saline and PIS and saline and anti-TNF-α serum (P>0.05). Anti-TNF-α serum injected before LPS caused a significant fall in body temperature 1–3 h post-injection compared with rats receiving LPS+PIS ((Fig. 2B, main effects of time ($F_{(4,96)}$=70.57, P<0.00001), group ($F_{(3,24)}$=34.38, P<0.0001) and interaction ($F_{(12,96)}$=14.76, P<00001)). For the remainder of the course of the fever there was no significant difference in the average temperature of rats receiving LPS with anti-TNF-α serum and rats receiving LPS with PIS.

Fig. 3 shows effects of injection of anti-IL-1β serum or PIS on body temperature of rats when the sera were administered i.p. prior to s.c. saline (Fig. 3A) or LPS (Fig. 3B). Anti-IL-1β serum had no effect on normal body temperature when saline was administered (Fig. 3A). Moreover, anti-IL-1β serum had no significant effect on the fever induced by LPS injection (Fig. 3B, P>0.05).

Fig. 4 shows effects of injection of anti-IL-6 serum or PIS on body temperature of rats when the sera were administered i.p. prior to s.c. saline (Fig. 4A) or LPS (Fig. 4B). Anti-IL-6 serum had no significant effect on normal body temperature (Fig. 4A, P>0.05). Anti-IL-6 serum significantly lowered rat body temperatures, for a 6-h period during the expected peak of the fever, when compared to rats injected with LPS+PIS ((Fig. 4B, main effects of time ($F_{(4,84)}$=23.07, P<0.00001), group ($F_{(3,21)}$= 15.13, P<0.00001) and interaction ($F_{(12,84)}$=3.68, P=0001)). Injecting anti-IL-6 serum abolished the LPS-induced fever such that the average body temperature of the rats for the 10-h post-injection period was not significantly different from rats injected with saline+anti-IL-6 serum (P>0.05).

Fig. 5 shows effects of injection of anti-leptin serum or PIS on body temperature of rats when the sera were administered i.p. prior to s.c. saline (Fig. 5A) or LPS (Fig. 5B). Anti-leptin serum had no effect on normal body temperature (Fig. 5A, P>0.05). Anti-leptin serum injected before LPS in the rats significantly lowered body temperatures over the entire 10-h period of the fever, when compared to rats injected with LPS+PIS ((Fig. 5B, main effects of time ($F_{(4,84)}$=25.68, P<0.0001), group ($F_{(3,21)}$= 3.23, P<0.0001) and interaction ($F_{(12,84)}$=3.71, P=0001)). Injecting

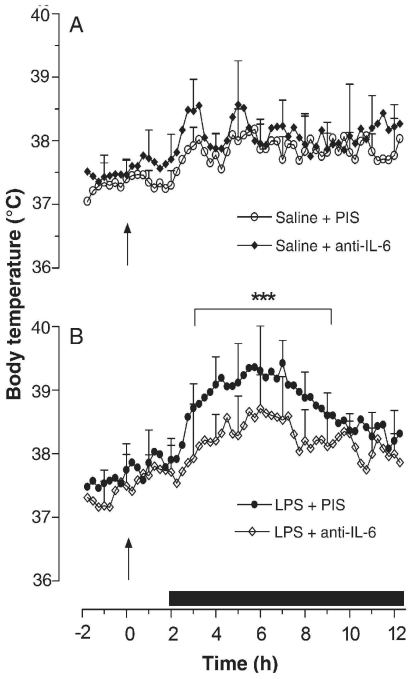

Fig. 4. Body temperature of rats injected with: (A) saline (s.c.) and anti-IL-6 (1.5 ml, i.p.) (n=6) or PIS (n=7); (B) LPS (250 μg/kg, s.c.) and anti-IL-6 serum (1.5 ml, i.p.) (n=7) or PIS (n=6). ***Indicates a significantly lower body temperature of rats injected with LPS+anti-IL-6 serum for a 6-h period during the peak of the fever when compared to rats injected with saline+PIS (P<0.001). The results are represented as means (S.D.). The arrow indicates the time of the injection; the black bar indicates lights out (19:00 clock time).

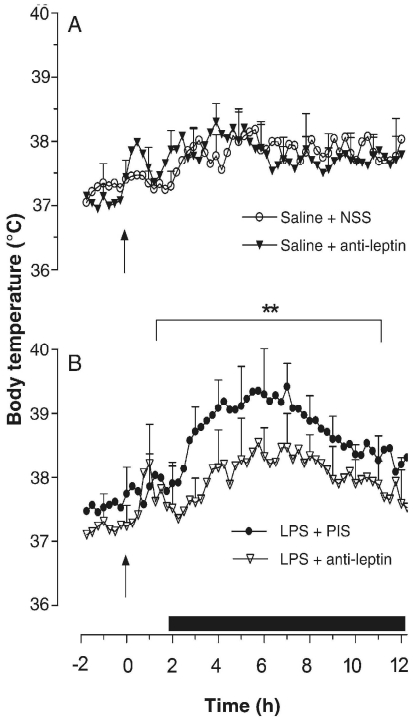

Fig. 5. Body temperature of rats injected with: (A) saline (s.c.) and anti-leptin serum (1.5 ml, i.p.) (n=6) or PIS (n=7); (B) LPS (250 μg/kg, s.c.) and anti-leptin serum (1.5 ml, i.p.) (n=6) or PIS (n=6). **Indicates a significantly lower body temperature of rats injected with LPS+anti-leptin serum for the duration of the fever (10 h) compared to the rats injected with LPS+PIS (P<0.01). The results are represented as means (S.D.). The arrow indicates the time of the injection; the black bar indicates lights out (19:00 clock time).

anti-leptin serum abolished the LPS-induced fever such that the average body temperature of the rats for the 10-h post-injection period was not significantly different from rats injected with saline+anti-leptin serum (P>0.05).

3.2. Voluntary-wheel running

On average, rats ran between 2 and 4.5 km per night for three nights before the experimental interventions. Fig. 6(A–D) shows the effects of s.c. administration of LPS or saline with anti-cytokine sera or PIS on nightly running distance in rats. Fig. 6(A–D) shows that injection of LPS+PIS in rats significantly reduced nightly running distance by 93 (8.6)%. Saline+PIS injected animals also demonstrated a fall in running the night after injection (Fig. 6A–D), but only to the extent of 23 (21.01)% which was significantly less than the fall in running induced by LPS+PIS (t=8.13, P<0.0001).

3.2.1. Effects of cytokine antisera on voluntary-wheel running

Fig. 6A shows that anti-TNF-α serum had no effect on the night-time spontaneous running irrespective of whether LPS or saline was co-injected (P>0.05).

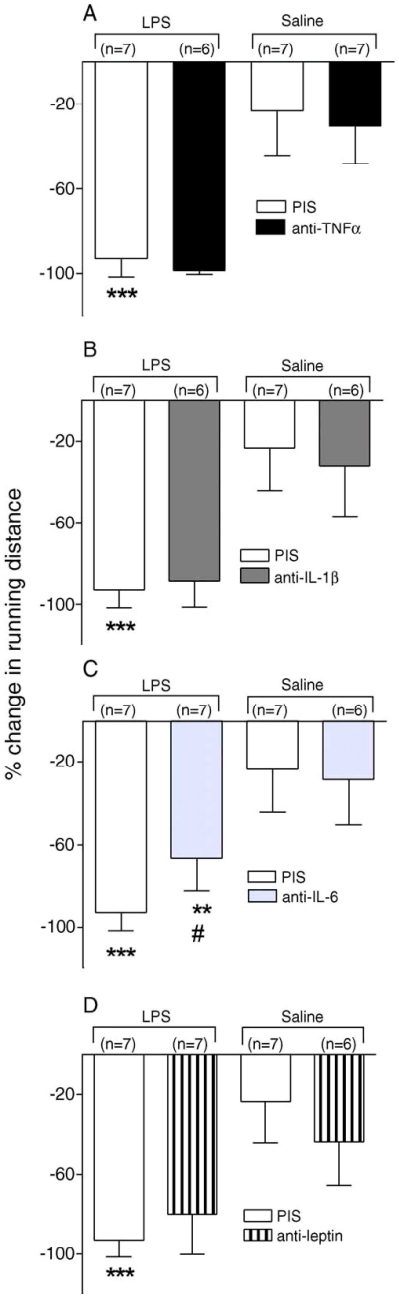

Fig. 6. Percentage change from pre-injection nights, in night-time running distance of rats after s.c. injection of LPS (250 μg/kg) or saline, and i.p. injection (1.5 ml) of PIS or: (A) anti-TNF-α serum, (B) anti-IL-1β serum, (C) anti-IL-6 serum, (D) anti-leptin serum. The results are represented as means (S.D.). ***Indicates P<0.001, LPS+PIS versus saline+PIS; **indicates P<0.01, LPS+PIS versus LPS+anti-IL-6 serum; #indicates P<0.001, LPS+anti-IL-6 serum versus saline+anti-IL-6 serum.

Fig. 6B shows similarly that anti-IL-1β serum had no effect on the night-time spontaneous running irrespective of whether LPS or saline was co-injected ($P>0.05$).

Fig. 6C shows that anti-IL-6 serum injected together with saline had no effect on night-time spontaneous running ($P>0.05$). Anti-IL-6 serum injected before LPS caused a significant attenuation of the LPS-induced decrease in spontaneous running (one-way ANOVA, $F_{(3,23)}=24.38$, $P<0.01$). However, the presence of anti-IL-6 antibodies in the circulation only partially reversed the LPS-induced suppression of voluntary running, as distance run still was significantly less in rats receiving LPS+anti-IL-6 serum, compared to rats injected with saline+anti-IL-6 serum ($P<0.001$).

Fig. 6D shows that anti-leptin serum had no effect on the night-time spontaneous running irrespective of whether LPS or saline was co-injected ($P>0.05$).

3.3. Food intake

Food consumed by the rats in the 24-h period before injections ranged between 10 and 12 g of food/100 g of body weight. Fig. 7(A–D) shows that LPS+PIS injections in rats significantly reduced 24-h food intake compared to rats injected with saline+PIS ($t=5.231$, $P=0.0002$).

3.3.1. Effects of cytokine antisera on food intake

Fig. 7A shows that anti-TNF-α serum had no effect on 24-h food intake irrespective of whether LPS or saline was co-injected ($P>0.05$).

Fig. 7B shows similarly that anti-IL-1β serum had no effect on 24-h food intake irrespective of whether LPS or saline was co-injected ($P>0.05$).

Fig. 7C shows that anti-IL-6 serum injected together with saline had no significant effect on 24-h food intake ($P>0.05$). However, anti-IL-6 serum injected before LPS significantly attenuated the LPS-induced decrease in food intake observed in the rats when LPS was injected with PIS (one-way ANOVA, $F_{(3,20)}=14.06$, $P<0.05$). However, the presence of anti-IL-6 antibodies in the circulation only partially reversed the LPS+PIS-induced decrease in food intake, as the food intake still was significantly less in rats receiving LPS+anti-IL-6 serum compared to rats injected with saline+anti-IL-6 serum ($P<0.05$).

Fig. 7D shows that anti-leptin serum injected together with saline significantly increased 24-h food intake (one-way ANOVA, $F_{(3,25)}=25.11$, $P<0.01$). Anti-leptin serum injected before LPS significantly attenuated the LPS-induced decrease in food intake observed in the rats when LPS was injected with PIS ($P<0.001$). The presence of anti-leptin antibodies in

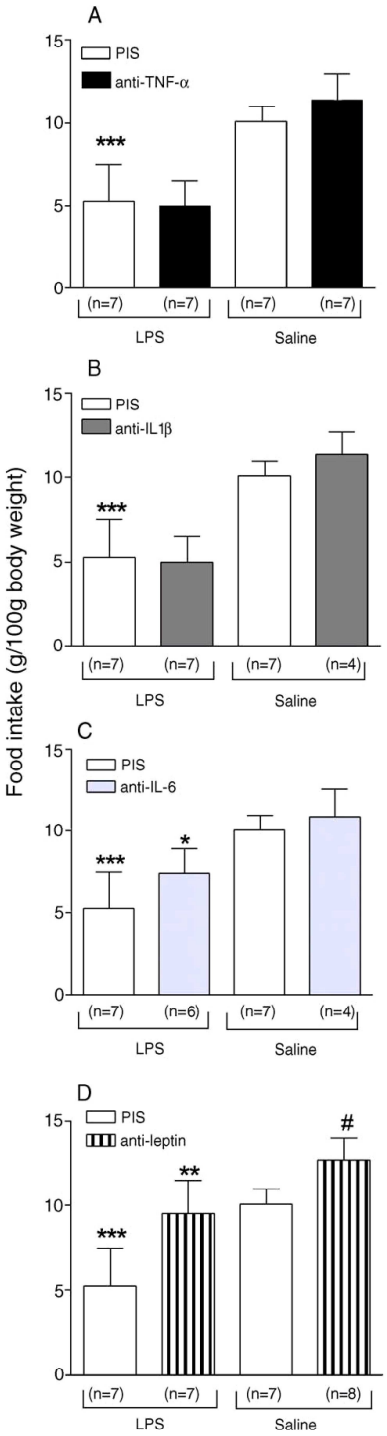

Fig. 7. Food intake of rats for 24 h after s.c. injection of LPS (250 μg/kg) or saline, and i.p. injection (1.5 ml) of PIS or: (A) anti-TNF-α serum, (B) anti-IL-1β serum, (C) anti-IL-6 serum, (D) anti-leptin serum. The results are represented as means (S.D.). ***Indicates $P<0.001$, LPS+PIS versus saline+PIS; *indicates $P<0.05$, LPS+anti-IL-6 serum versus LPS+PIS and saline+anti-IL-6 serum; **indicates $P<0.01$, LPS+anti-leptin serum versus LPS+PIS and saline+anti-leptin serum; #indicates $P<0.01$ saline+PIS versus saline+anti-leptin serum.

the circulation abolished the LPS+PIS-induced decrease in food intake to such an extent that the food intake of the rats for the 24-h post-injection period was not significantly different from rats injected with saline+PIS ($P>0.05$). However, the 24-h food intake in rats receiving anti-leptin serum+LPS was still significantly less than the increased food intake measured in rats receiving anti-leptin+saline ($P<0.01$).

4. Discussion

We have shown that subcutaneous administration of lipopolysaccaride (LPS) before the night-time active period in rats induces fever and sickness behavior. While this observation is not new, what is novel in our study is the finding that a particular form of voluntary activity, voluntary wheel-running, is suppressed after an LPS challenge. Indeed, voluntary exercise on running wheels was reduced by about 93% by a single injection of LPS. To our knowledge this is the first report that voluntary exercise, that is wheel-running, is suppressed as part of LPS-induced sickness behavior and our results indicate that this form of voluntary activity is almost completely abolished by LPS administration. The LPS-induced fever was abolished if either anti-IL-6 serum or anti-leptin serum were administered before LPS administration. Injecting rats with anti-TNF-α serum affected only the early phase of the fever response and had no effect on sickness behavior and anti-IL-1β serum had no effect on either fever or sickness behavior induced by LPS.

Our findings support the view that endogenously produced IL-6 is a primary mediator of LPS-induced fever in rats [9,46]. We can also support the more recently postulated role for peripherally released leptin in LPS-induced fever [49]. We have also uncovered, by using injection of cytokine-specific antisera, roles for peripherally released IL-6 and leptin, but not for TNF-α and IL-1β, in two specific behavioral changes induced by LPS, suppression of voluntary physical activity (wheel-running) and food intake. We have shown for the first time that peripheral IL-6 plays a role in the suppression of voluntary physical activity as well as in the suppression of appetite, following LPS injection. Leptin, on the other hand, and not surprisingly, affected food intake; not only was the LPS-induced suppression of food intake reversed by co-injection of anti-leptin serum with LPS, but injection of anti-leptin serum with saline significantly enhanced food intake compared to animals injected with saline+PIS (Fig. 7D).

Our results showing that LPS-induced fever is completely abolished following systemic administration of anti-IL-6 serum in rats adds to current evidence confirming the importance of circulating IL-6 in the development of fever. Interleukin-6 appears to be the only cytokine that is consistently measurable in significant quantities in the circulation during both systemic and localized inflammatory responses to LPS [7,9,18,38,44,47]. The importance of IL-6 in LPS-induced fever also is supported by findings of strong correlations between plasma concentrations of IL-6 and the magnitude of fever [26,45], systemic pre-treatment with neutralizing IL-6 antiserum abolishing the febrile response induced by s.c. LPS administration [9] and the failure of IL-6-knockout mice (lacking the gene for IL-6) to develop fevers in response to i.p. injection of LPS [10]. Regardless of the route of LPS administration, it has been proposed that IL-6, released into the circulation after an injection of LPS, gains entry to the brain via the sensory circumventricular organs, specifically the area postrema, and causes direct genomic activation of target gene transcription in brain cells [47]. Thus, circulating IL-6 indeed appears to be acting as the afferent signal for the development of fever [9].

Given the established importance of endogenous circulating IL-6 in the development of LPS-induced fever, failure to detect an increase in body temperature following exogenous administration of IL-6 has lead others to hypothesize that endogenous IL-6 is not working in isolation but requires additional co-factors to initiate the febrile response [9]. Co-factors shown to act with IL-6 to induce fever include IL-1β and the soluble IL-6 receptor (sIL-6R); both increased in animals treated with LPS [9,26,38,51].

Not only is IL-6, but so too is the cytokine-like adipocyte-product leptin significantly increased in the circulation following LPS administration [15,17,50]. Using leptin-specific antiserum administered i.p., we were able to completely abolish the fever induced by LPS in rats. The same antiserum, in other hands, attenuates fever induced by a lower dose of LPS (100 μg/kg, i.p.) in rats [49]. The different degrees of fever suppression in the two studies may be due to the higher dose of leptin antiserum used in our study. However, studies in obese (*f/f*) Koletsky rats which lack the leptin receptor and (*fa/fa*) Zucker rats in which the receptor-mediated transport and intracellular signaling of leptin are defective, do not support a role for leptin in LPS-induced fever [20,54]. It has been proposed that humoral mediators, other than leptin, may compensate for the absence of functional leptin receptors in obese (*fa/fa*) Zucker rats, thereby possibly explaining the reported discrepancies on leptin's role in LPS-induced fever [20,49,54].

Unlike IL-6 and leptin, IL-1β is not detected in measurable concentrations in the plasma following LPS administration, yet exogenous administration of recombinant IL-1β has been shown to induce fever in rats [1,14]. Surprisingly, therefore, in our study, we found that administering anti-IL-1β serum had no effect on fever induced by administration of a high dose (250 μg/kg) of LPS. In other hands, those who administered a lower dose (10 μg/kg) of LPS, a partial attenuation in the fever response was found with the administration of anti-1L-1β serum [32]. A number of differences in the experimental design between our study and that of Long et al. (1990) may explain these apparently contradictory findings. Firstly, the different anti-IL-1β sera used in the studies (ours being species-specific) may have had different neutralizing effects on the IL-1β release induced by LPS. Secondly, in our study, we used a greater dose of LPS (25-fold), which could have resulted in LPS entering the circulation from the site of administration and activating the same intracellular signaling pathway as IL-1β through an independent receptor, such as toll-like receptor 4 and hence bypassing the action of IL-1β [11]. Lastly, it has been shown that IL-1β is primarily produced at the local tissue site following s.c. administration of LPS where it can induce the release of IL-6 into the circulation to act as the humoral signal

for fever induction [9]. Therefore, by administrating the antisera i.p., we may not have influenced the synthesis of IL-1β induced by LPS at the tissue site of injection or in the brain, and therefore IL-1β's subsequent pyrogenic effects.

In addition to IL-6 and IL-1β, a third pro-inflammatory cytokine, TNF-α, also is reported to be involved in LPS-induced fever. However, its exact role in fever is not clear with studies reporting both pyrogenic and antipyretic actions for TNF-α [22,29,31,32,44]. Tumor necrosis factor-α is the first of the pro-inflammatory cytokines to be measurably increased in the circulation following systemic injection of LPS [18]. Its concentrations increase within 60 min of LPS injection and remain elevated for 3 h [18,44]. When we administered anti-TNF-α serum to rats, together with LPS, we found an attenuation in the rise in body temperature in the initial 1–3 h of the febrile episode, corresponding to the reported time period when plasma concentrations of TNF-α are increased [32,44]. The significantly enhanced peak and plateau phase of the fever response found by others using peripherally administered anti-TNF-α serum is in contrast to our findings which support a pyrogenic role for TNF-α [30,32]. With regards to these contradictory findings for TNF-α, it has been stated that the pyrogenic or antipyretic effects of endogenous TNF-α depend on the dose of the fever-inducing stimulus, the species of experimental animal and whether species heterologous or homologous protein is used, as heterologous proteins have different receptor affinities [53,55]. In our study, we used species-specific antisera and a greater (25-fold) dose of LPS than that used by others supporting an antipyretic role for TNF-α [22,30,32].

In contrast to the data supporting a role for circulating cytokines mediating LPS-induced fever, few data exist examining the involvement of such circulating mediators in LPS-induced sickness behavior. Moreover, the involvement of circulating cytokines in mediating the suppression of voluntary running behavior, a sensitive and highly quantifiable measure of sickness behavior, known to be different from general cage activity observed in rats [40], has not been established. Our results show, for the first time, that circulating IL-6 plays a role in mediating the reduction in voluntary activity induced by LPS. In addition, we also found that administering anti-IL-6 serum attenuated LPS-induced suppression of food intake, a role for IL-6 supported by findings using IL-6 knockout mice [5].

Interestingly, whereas the presence of IL-6 antibodies in the circulation abolished the fever response to LPS injection, suppression of voluntary activity was reversed by the presence of IL-6 antibodies only by 27%, and appetite also was not returned to normal levels in the presence of IL-6 antibodies. Thus, IL-6 may be an essential component of LPS-induced fever but an additional factor or factors, possibly working in parallel with IL-6, may be required to mediate sickness behaviors. Interleukin-1β could be this additional factor; as intracerebroventricular administration of IL-6 on its own had no effect on locomotor activity in rats, yet when administered in combination with a non-pyrogenic dose of IL-1β, locomotor activity was significantly reduced [27].

Interleukin-1β also is proposed to act with another proposed circulating mediator of fever, leptin, to induce sickness behavior [49]. In this case, circulating leptin could be acting as a peripheral signal to induce the release of IL-1β in the hypothalamus to mediate the anorexic effects induced by LPS [25,36,49]. Results from our study suggest a role for circulating leptin in mediating the suppression of food intake following LPS administration, supporting other studies [49] and those which have used leptin receptor-deficient (*db*/*db*) mice [16]. Not only did we completely inhibit the suppression of food intake induced by LPS, but also significantly increased food intake in the afebrile rats receiving saline+anti-leptin serum. The presence of circulating antibodies to leptin, therefore, has a significant effect on enhancing food intake, in the absence of illness. Other studies using rats in which the receptor-mediated transport and intracellular signaling of leptin is defective suggest that leptin in not the sole mediator of LPS-induced anorexia, but may rather have a modulatory role [16,33].

Our failure to attenuate LPS-induced suppression of voluntary running and food intake using antisera raised against rat IL-1β and rat TNF-α is in agreement with findings from a number of earlier studies [24,28,29,40]. Using the following knockout mice, IL-1β, IL-1r1 (in which neither IL-1α or IL-1β is able to induce a biological signal) and TNFR (which lack functional genes for both p55 and p75 TNF receptors), as an experimental model, it was found that the same degree of lethargy and anorexia induced by LPS was observed in both the knockout and wild-type mice [24,28,29]. Tollner et al. (2000) inhibited endogenously released TNF-α using a TNF-binding protein injected i.p. following LPS administration, but found no significant effect on the LPS-induced suppression of night-time activity in rats and only minimal effects on attenuating the LPS-induced decrease in food intake. Furthermore, administration of antibodies specific to TNF-α and IL-1β had no effect on *Corynebacterium parvum*-antigen induced suppression of wheel running in mice [40]. Together with our findings, these observations tend not to support a role for circulating TNF-α and IL-1β in regulating sickness behavior. However, central administration of TNF-α and IL-1β can induce sickness behavior [6,21] and mRNA expression for TNF-α and IL-1β in the brain is elevated at time points corresponding to the development of sickness behavior [40]. Thus, TNF-α and IL-1β may play a role in regulating sickness behavior, but if they do, then it is via central rather than peripheral mechanisms.

Irrespective of which cytokines appear to have pivotal roles in the generation of fever and/or sickness behavior, it is clear that they must activate brain mechanisms to do so, seeing the mechanisms regulating body temperature [23], activity [43] and appetite reside centrally [21]. We, and others, have shown IL-6 to have a key role to play in fever generation [9,10,46], and our current studies have demonstrated that IL-6 also plays a role in suppressing appetite and voluntary running, following LPS administration. The mechanisms by which peripherally circulating IL-6 mediates the centrally controlled responses remain unclear. It has been proposed that cytokines gain entry into the brain via an active transport system [2,3] or via sites of the brain

lacking a blood brain barrier, such as the circumventricular organs (CVOs) [4,12]. Also, endothelial cells in CVOs are activated by peripheral cytokines and may result in further cytokine release in the brain to induce fever and sickness behavior [4,12,48]. This question is one deserving of further investigation.

In conclusion, we have used cytokine-specific antisera, which neutralize cytokines released in response to LPS injection, to unveil pivotal roles for both IL-6 and leptin, as peripheral mediators of LPS-induced fever. Peripherally released IL-6 also has a role in the suppression of voluntary running and food intake accompanying infection but full suppression of these sickness behaviors may require synergistic action of other cytokines, such as IL-1β. Peripherally released leptin appears to also have a role, both in inducing fever and suppression of food intake accompanying infection, but not in suppression of voluntary running. Suppression of the predisposition to exercise is another behavior induced by LPS injection and is IL-6-mediated, at least in part. Our results have contributed significantly to the understanding of peripheral mechanisms mediating fever and accompanying sickness behaviors, and have implications for the management of sick individuals, particularly sick athletes.

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CHAPTER 3

Interleukin (IL)-6 and IL-1β act synergistically within the brain to induce sickness behavior and fever in rats.

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Interleukin (IL)-6 and IL-1β act synergistically within the brain to induce sickness behavior and fever in rats

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Abstract

Pro-inflammatory cytokines interleukin (IL)-6 and IL-1β can act in the brain (centrally) to cause fever. Sickness behaviors which accompany fever also appear to involve the central action of IL-1β. We injected species-homologous rat IL-6 and IL-1β directly into the brains of conscious rats to examine the effect of these cytokines on fever, and two behaviors affected by sickness, voluntary wheel-running and food intake. Male Sprague–Dawley rats selected for their predisposition to spontaneously run on running wheels were used in the experiment. Each rat was anaesthetized and had a temperature-sensitive radiotransmitter implanted intra-abdominally, and a 23-gauge stainless steel guide cannula inserted stereotaxically over the lateral cerebral ventricle. Rats were randomly assigned to receive intracerebroventricular injections of three doses of either IL-1β or IL-6 (100 ng, 1 ng or 0.1 ng IL-1β and 200 ng, 20 ng or 2 ng IL-6), or one of three different combinations of IL-1β and IL-6. Rats receiving either IL-1β or IL-6 showed a dose-dependent increase in body temperature and decrease in wheel-running (ANOVA, $p < 0.0001$). Only rats receiving the highest dose of IL-1β significantly decreased food intake and body mass compared to rats receiving vehicle (ANOVA, $p < 0.001$). Doses of IL-1β and IL-6 which, when injected on their own were non-pyrogenic and did not affect food intake and body mass, induced fever and anorexia when they were co-injected centrally. These results show that species-homologous rat IL-6 and IL-1β can act directly within the brain to decrease voluntary activity and suggest they also can act synergistically to induce anorexia and fever.

Keywords: Voluntary wheel-running; Anorexia; Cachexia; Pro-inflammatory cytokines; Synergy

1. Introduction

Various brain-mediated behavioral responses, more commonly referred to as sickness behaviors, are observed together with the fever response in both animals and humans during an acute infectious illness (Hart, 1988; Dantzer, 2001). Some of the behavioral changes typically observed in sick individuals and animals include a loss of appetite and interest in social activities, increased sleep and depressed activity (Dantzer, 2001). Of these behavioral changes one of the most commonly reported by patients in various disease states or observed in infected animals, is fatigue accompanied by a decrease in daily activity (Ottenweller et al., 1998; Johnson, 2002; Hewlett et al., 2005; Kramer et al., 2005; Schubert et al., 2007).

Activity is routinely assessed in experimental animals by measuring voluntary wheel-running and recently we and others have shown that voluntary wheel-running in mice and rats is markedly reduced during infection (Sheng et al., 1996; Ottenweller et al., 1998; Sherwin, 1998; Katafuchi et al., 2003; Harden et al., 2006). Results from a study conducted in our laboratory investigating the functional importance of endogenous interleukin (IL)-6 in lipopolysaccharide (LPS)-induced sickness behavior, have shown that peripherally released IL-6 only partially mediates the suppression of voluntary activity induced by LPS administration (Harden

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et al., 2006). However, administration of human recombinant IL-6 induces a sensation of fatigue in healthy humans at rest and decreases athletic performance in trained runners; treatment with IL-6 antibodies induces an immediate disappearance of previously debilitating fatigue reported by patients with multicentric Castleman disease, a disease characterized by a dysregulated overproduction of IL-6 (Spath-Schwalbe et al., 1998; Nishimoto et al., 2000; Robson-Ansley et al., 2004; Nishimoto et al., 2005). These data, obtained from animal and human studies therefore suggest some involvement of IL-6 in mediating sickness behavior responses such as fatigue and the associated reduction in physical activity thought to be triggered by the brain during infection. Nevertheless, to date it has been difficult to elicit sickness behaviors by central administration of IL-6 (Oitzl et al., 1993; Lenczowski et al., 1999).

In contrast to the lack of evidence for the direct involvement of IL-6 within the brain in mediating sickness behavior, there is substantial evidence that another pro-inflammatory cytokine, IL-1β, acts centrally to mediate sickness behavior. Central administration of IL-1β reproduces symptoms of sickness which include decreased food intake, body mass and locomotor activity (Anforth et al., 1998; Nadjar et al., 2005; Carmichael et al., 2006; Pecchi et al., 2006; Elander et al., 2007). These findings would tend to suggest that IL-1β is the more likely mediator of fatigue and associated reduction in physical activity observed during infection.

Cytokines probably do not function independently to induce host responses to infection, but rather they interact with other cytokines. In particular, cytokine interactions observed as physiological synergy in which the effect of a combination of substances exceeds the effect of the individual constituents, has been demonstrated *in vivo* between IL-1β and IL-6 (Lenczowski et al., 1999; Cartmell et al., 2000).

To discern direct effects in the brain of IL-6 and IL-1β in mediating sickness behaviors, we have injected species-homologous rat IL-6 and IL-1β directly into the brains of conscious rats and have examined the dose–response effects on voluntary activity, that is, voluntary wheel-running and food intake. We also measured body temperature to determine whether the effects of IL-6 and IL-1β on voluntary activity and feeding were secondary to fever. Moreover, we further assessed the possible physiological synergy between central IL-6 and IL-1β in mediating changes in behavior and body temperature by co-injection of the cytokines. Our results uncover a role for both central IL-6 and central IL-1β in mediating suppression of voluntary activity, and identify a synergistic effect of IL-6 and IL-1β in inducing anorexia and fever.

2. Methods

2.1. Animals

Male Sprague–Dawley rats (initial body mass 120–150 g) were housed individually in cages to which exercise-training wheels had been attached. The rats were kept at an ambient temperature of 21 ± 2 °C and on a 12 h:12 h light:dark cycle (lights on at 07:00). Rats with an average voluntary daily running distance of 1 km, monitored over a 21-day training period, were selected for the study. Food (pelleted rat chow, Epol, Johannesburg, South Africa) and water were provided *ad libitum*. All procedures were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (Ethics No. 2004/95/5).

2.2. Surgery

Rats selected for the study (body masses 300–350 g), were anesthetized with an intramuscular (i.m.) injection of 0.4 mg/kg domitor (Novartis, SA) and 40 mg/kg ketamine hydrochloride (Anaket-V, Bayer, SA) and had a temperature-sensitive radiotransmitter (TA10TA-F40, Data Sciences, St. Paul, MN, USA) implanted intra-abdominally. Thereafter the rats were placed in a stereotaxic frame (Stoelting, IL, USA), a heating pad was placed beneath the rat to maintain core body temperature, and they were given an injection (0.1 ml) of adrenaline (10 μg) (Merck, SA) and lignocaine hydrochloride (0.02 g) (Bayer, SA) subcutaneously over an area of skull. An incision was made in the midline of the cranium to expose the skull. A 23-gauge stainless steel guide cannula (Plastics One, Roanoke, VA, USA) was placed over the right lateral cerebral ventricle. Coordinates for the guide cannula were 0.8 mm posterior to bregma, 1.5 mm lateral to the midline and 3.5 mm below the skull surface at the point of entry (Paxinos and Watson, 1998). The cannula was secured to the skull with three screws and dental cement. After surgery each rat was given a subcutaneous injection of 0.3 mg buprenorphine hydrochloride (Temgesic, Schering-Plough, SA) and ringer lactate (1.5 ml) (SABAX, Adcock, Ingram, SA) and allowed a minimum of 21 days for recovery.

2.3. Body temperature

We measured core body temperatures of rats continuously, by remote biotelemetry using temperature-sensitive radiotransmitters which had been implanted intraperitoneally (see above). Transmitter output frequency (Hz) was monitored at 5 min intervals, by a receiver plate (RTA 500, Mini-Mitter, Sunriver, OR, USA) situated beneath the cage of each animal. The frequency received by each plate was fed into a peripheral processor (DP-24 DataPort, VitalView, Mini-Mitter, Sunriver, OR, USA) connected to a personal computer and the output expressed in degrees centigrade. The telemeters were calibrated by water immersion against a high-accuracy thermometer (Quat 100, Heraeus, Germany), to an accuracy of 0.1 °C.

2.4. Voluntary wheel-running

The exercise-training wheels had a circumference of 1.06 m and each wheel was equipped with a magnet and a magnetic switch (VitalView, Mini-Mitter, Sunriver, OR, USA). Each time the wheel rotated the magnet within range of the magnetic switch, the switch closed and a turn was counted. The mechanical switches were connected to an activity input module (QA-4, VitalView, Mini-Mitter, Sunriver, OR, USA) which in turn was fed into a peripheral processor (DP-24 DataPort, VitalView, Sunriver, OR, USA) connected to a personal computer which monitored the number of wheel turns at 5 min intervals using VitalView software version 4.1 (Mini-Mitter, Bend, OR, USA).

2.5. Food intake and body mass

Food intake and body mass were measured twice daily at 08:00 (1 h after lights on) and 18:00 (1 h before lights off). Food containers were filled daily at 08:00 with 100 g of the pelleted rat chow. Food intake was quantified by subtracting the food remaining in the food container and on the cage floor from the amount of food measured at the preceding time point.

Food powder in the cage was ignored, as it has been reported previously to be similar among rats, and generally to weigh less than a gram (Mueller et al., 1997).

2.6. Rat recombinant protein

Rat recombinant (rr)IL-6 (*Escherichia coli*-derived, specific activity 250,000 IU/μg, as measured in a B9 mouse hybridoma bioassay, National Institute for Biological Standards and Control, Potters Bar, UK) and rrIL-1β (*E. coli*-derived, specific activity 100,000 IU/μg, National Institute for Biological Standards and Control, Potters Bar, UK) were used in the study. All experiments were carried out using a single batch of IL-6 and IL-1β. The endotoxin content of the two recombinant cytokines was <4 ng (40 IU)/mg of protein, as measured in a *Limulus* Amoebocyte Lysate test. The cytokines were diluted in a vehicle of pyrogen-free saline containing 0.1% bovine serum albumin (BSA, fatty acid free, low endotoxin, Roche, Germany) on the day of the experiment and injected intracerebroventricularly (i.c.v.).

2.7. Experimental procedure

After surgery for implantation of the radiotransmitters and guide cannulae, all animals were returned to their cages and the running wheels were locked for a period of 7 days so that animals could not exercise. Thereafter, the wheels were unlocked and experimentation started 3 weeks post-surgery once rats had reached their pre-surgery nightly running distance. During this post-surgery period the rats were habituated to handling and injection procedures. To confirm correct placement of the guide cannula a week before i.c.v. injections of the recombinant proteins, rats were injected i.c.v. (see below) with angiotensin II (10 ng/5 μl; A-2900, Sigma, St. Louis, MO, USA) and then monitored for a drinking response for 30 min after the injection. Rats with a positive drinking response (>10 ml of water in 30 min) were used in the study.

For microinjections into the lateral cerebral ventricle, a 30-gauge injection stylette (Plastics One, Roanoke, VA, USA), connected by polyethylene tubing (0.58 mm i.d., 1.27 mm o.d.) to a 50 μl Hamilton gas-tight microliter syringe (Hamilton, Switzerland) was lowered into the guide cannula so that it protruded 0.5 mm beyond the tip of the guide cannula into the ventricle. Each microinjection was administered to freely moving rats over a period of 60 s in a volume of 5 or 6 μl of either vehicle or the specific rat recombinant protein. The injection stylette was left in the guide cannula for an additional 5 min to ensure dispersion of the injected substance within the ventricle.

To determine the dose–response relationship of centrally administered IL-1β and IL-6 on wheel-running, food intake, body mass and body temperature, rats were randomly assigned to receive either 100 ng, 1 ng or 0.1 ng of IL-1β or 200 ng, 20 ng or 2 ng of IL-6 i.c.v. in a volume of 5 μl. In addition to the dose–response experiments the effects of co-administration of IL-1β and IL-6 on wheel-running, food intake, body mass and body temperature, were also determined, using three different groups of rats which were randomly assigned to receive one of the following combinations: IL-1β (0.1 ng) + IL-6 (2 ng), IL-1β (1 ng) + IL-6 (2 ng) or IL-1β (0.1 ng) + IL-6 (20 ng). For the combination injections IL-1β and IL-6 were prepared separately at the given concentrations in a volume of 3 μl and then injected as a single bolus injection of 6 μl, with each rat therefore receiving only one injection. During both the cytokine dose–response and combination experiments rats were also randomly assigned to the control group and received an i.c.v. injection of the vehicle solution (5 μl). The IL-1β and IL-6 dose–response injections, the IL-6 and IL-1β co-injections and control (vehicle) injections were therefore administered in a randomized manner under the same experimental conditions.

All i.c.v. injections were administered between 17:00 and 18:00 before the onset of the dark phase when rats are most active. Wheel-running, food intake, body mass and body temperature were all monitored for 72 h before and 12 h after injections.

Rats were euthanized with 1 ml sodium pentobarbital injected intraperitoneally (Euthapent, 200 mg/ml; Kyron Laboratories (Pty) Ltd., South Africa) and correct placement of guide cannulae was verified post-mortem by infusion of 5 μl of blue dye (Kyro-quick stain, Kyron Laboratories, SA) through the guide cannula assembly. After 5 min the brain was removed and placed on ice and the distribution of the ink within the ventricles was visually inspected in approximately 1-mm sections of the brain.

2.8. Data analysis

All data are expressed as means ± SD. The body temperature responses to the injections were plotted as temperature–time curves, with the 5 min recordings averaged over 15 min intervals. For statistical analysis of the dose–response relationship, we calculated thermal response indexes (TRI, °C h) for each rat over 12 h (19:00–07:00), a time interval chosen in order to exclude the stress-induced hyperthermia that occurred after handling and injections. The TRIs were calculated as the area between the temperature-versus-time curve following an injection and the curve depicting the mean nychthemeral temperature rhythm for that rat, obtained for the 3 days before the injection. Because of negligible daytime running, only night-time running distance was analyzed. Night-time running distance (19:00–07:00) was determined from the number of wheel turns and was expressed as a percentage change from the mean running distance measured over three nights before the injection. Food intake was expressed as grams of food consumed per 100 g of rat body mass. Change in body mass was determined by subtracting the body mass measured 12 h after the injection from the body mass measured immediately prior to injection. The effects of central injections of IL-6 and IL-1β on the TRIs, percentage change in wheel-running, food consumption and body mass gain for the night of injection were analyzed using a one-way analysis of variance followed by a Student–Newman–Keul's (SNK) *post hoc* test.

3. Results

3.1. Voluntary wheel-running

On average, rats ran between 1 and 4 km per night (19:00–07:00) during the three nights before the experimental interventions. Fig. 1A shows that the inhibition of night-time running activity was dependent on the dose of IL-6 injected i.c.v. (one-way ANOVA, $F_{(3,20)} = 31.9$, $p < 0.0001$). The decrease in running activity of rats receiving 200 ng was significantly different to the decrease in running activity of rats receiving 20 ng ($p < 0.01$, SNK) and 2 ng ($p < 0.001$, SNK). Furthermore, the decrease in running activity of rats receiving 20 ng was also significantly different to the decrease in running activity of rats receiving 2 ng ($p < 0.05$, SNK). Rats injected i.c.v. with each of the three doses of IL-6 (200 ng, 20 ng and 2 ng) significantly decreased their nightly running distance compared to rats receiving vehicle ($p < 0.01$, SNK). Of the three doses of IL-6 injected, rats receiving the highest dose of IL-6 (200 ng) showed the greatest decrease in nightly running distance (85 ± 10.7%).

Fig. 1B shows that the inhibition of night-time running activity was dependent on the dose of IL-1β injected i.c.v. (one-way ANOVA, $F_{(3,20)} = 92.5$, $p < 0.0001$). The decrease in running activity of rats receiving 100 ng was significantly different from the decrease in running activity of rats receiving 1 ng ($p < 0.001$, SNK) and 0.1 ng

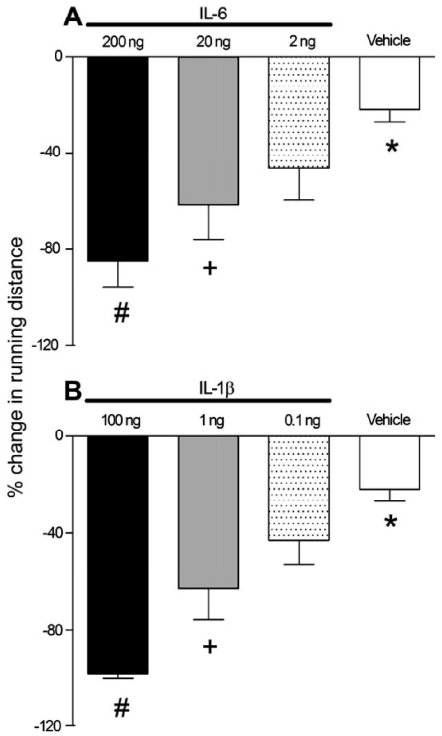

Fig. 1. Percentage change from that on pre-injection nights, in night-time running distance of rats after i.c.v. injection of vehicle ($n = 6$) or: (A) IL-6 at 3 doses 200 ng ($n = 6$), 20 ng ($n = 6$), 2 ng ($n = 6$); (B) IL-1β at three doses 100 ng ($n = 6$), 1 ng ($n = 5$), 0.1 ng ($n = 7$). The results are represented as means ± SD. *Significant decrease in running distance of rats receiving either IL-6 (200 ng, 20 ng and 2 ng) or IL-1β (100 ng, 1 ng and 0.1 ng) compared to rats receiving vehicle ($p < 0.01$, SNK). #Significant decrease in running distance of rats receiving either the highest doses of IL-6 (200 ng) or IL-1β (100 ng) compared to rats receiving the middle and lowest doses of IL-6 or IL-1β ($p < 0.01$, SNK). +Significant decrease in running distance of rats receiving the intermediate doses of IL-6 (20 ng) or IL-1β (10 ng) compared to rats receiving the lowest doses of IL-6 or IL-1β.

($p < 0.001$, SNK). Furthermore, the decrease in running activity of rats receiving 1 ng was also significantly different to the decrease in running activity of rats receiving 0.1 ng ($p < 0.001$, SNK). Rats injected i.c.v. with each of the three doses of IL-1β (100 ng, 1 ng and 0.1 ng) significantly decreased their nightly running distance compared to rats receiving vehicle ($p < 0.001$, SNK). Of the three doses of IL-1β injected, rats receiving the highest dose (100 ng) had the greatest decrease in nightly running distance (93 ± 8.6%). Injection of the vehicle (0.1% BSA) appeared to induce a small significant decrease (~22%) in running activity, for reasons which are not known, as the percentage change in running activity of rats receiving vehicle on the night of injection was significantly different to zero ($t = 10.5$, $p < 0.0001$). Injection of the vehicle did not have any effect on any of the other measurements taken (body temperature, food intake and body mass).

Fig. 2 shows the results of injecting three different combinations of IL-1β and IL-6 which when injected on their own all decreased running activity of the rats, but by different magnitudes. Fig. 2A shows the results of injecting a dose of IL-6 (2 ng) which induced the smallest decrease in running activity of the three doses of IL-6 administered, in combination with a dose of IL-1β (0.1 ng) which induced the smallest decrease in running activity of the three doses of IL-1β administered. Rats receiving combination i.c.v. injections of 0.1 ng IL-1β and 2 ng IL-6 decreased nightly running distance by a significantly greater percentage compared to when IL-1β (0.1 ng) and IL-6 (2 ng) were injected separately (one-way ANOVA, $F_{(3,20)} = 38.2$, $p < 0.001$). Fig. 2B shows that rats receiving combination i.c.v. injections of an intermediate dose of IL-1β (1 ng) and the lowest dose of IL-6 (2 ng) also decreased nightly running distance by a significantly greater percentage compared to when IL-1β (1 ng) and IL-6 (2 ng) were injected separately (one-way ANOVA, $F_{(3,19)} = 32.3$, $p < 0.01$). Fig. 2C shows that rats receiving combination i.c.v. injections of an intermediate dose of IL-6 (20 ng) and the lowest dose of IL-1β (0.1 ng) also decreased nightly running distance by a significantly greater percentage compared to when IL-6 (20 ng) and IL-1β (0.1 ng) were injected separately (one-way ANOVA, $F_{(3,20)} = 42.1$, $p < 0.001$).

3.2. Food intake and body mass

On average, rats consumed between 6 and 10 g of food/100 g of body mass per night (19:00–07:00) during the three nights before the experimental interventions. Figs. 3 and 4A show that the food intake and body mass of rats receiving i.c.v. injections of IL-6 at any of the three doses was not significantly different to the food intake (one-way ANOVA, $F_{(3,20)} = 1.4$, $p > 0.05$) and weight gain (one-way ANOVA, $F_{(3,20)} = 0.72$, $p > 0.05$) of rats receiving vehicle over the 12 h period after injection. Figs. 3 and 4B show that rats receiving 100 ng IL-1β consumed significantly less food (one-way ANOVA, $F_{(3,20)} = 34.5$, $p < 0.001$) and gained significantly less body mass (one-way ANOVA, $F_{(3,20)} = 26.76$, $p < 0.001$) compared to rats injected with vehicle. However, the food intake and weight gain of rats receiving 1 ng or 0.1 ng IL-1β was not significantly different compared to rats receiving vehicle over the 12 h period after injection ($p > 0.05$, SNK).

Figs. 5 and 6 show the results of injecting three different combinations of IL-1β and IL-6 which when injected on their own had no significant effect on the food intake and body mass of the rats. Figs. 5 and 6A show the results of injecting the lowest dose of IL-6 (2 ng) which, on its own had no effect on food intake and body mass, in combination with the lowest dose of IL-1β (0.1 ng) which, also had no effect on food intake and body mass. Rats receiving this combination of i.c.v. injections of IL-1β and IL-6 significantly decreased food intake (one-way ANOVA, $F_{(3,19)} - 5.5$, $p < 0.01$) and gained less body mass (one-way ANOVA, $F_{(3,19)} = 6.9$, $p < 0.01$) over the 12 h period

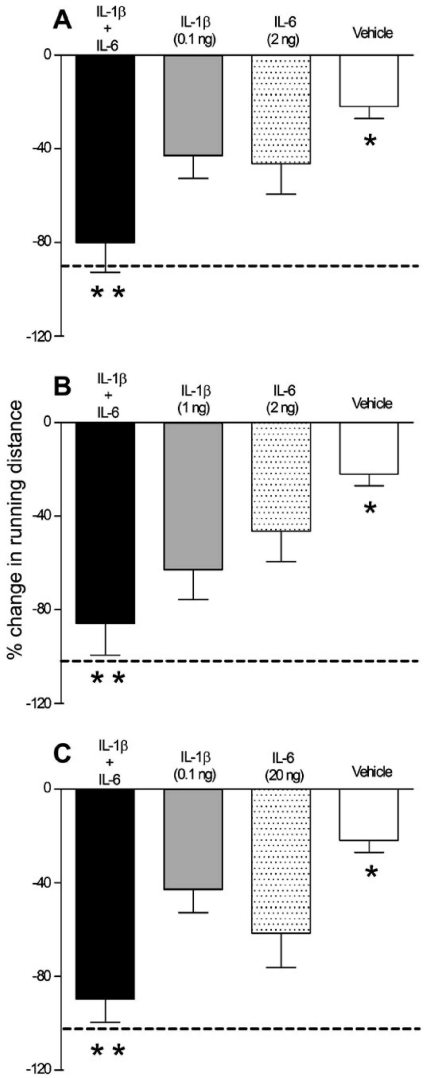

Fig. 2. Percentage change from that on pre-injection nights, in night-time running distance of rats after i.c.v. injection of vehicle ($n = 6$) or: (A) co-injection of 0.1 ng IL-1β + 2 ng IL-6 ($n = 6$); (B) co-injection of 1 ng IL-1β + 2 ng IL-6 ($n = 6$); (C) co-injection of 0.1 ng IL-1β + 20 ng IL-6 ($n = 5$). In (A), (B) and (C) the effects of injecting each cytokine dose separately, also is shown and is the same as the results in Fig. 1A and B. The results are represented as means ± SD. *Significant decrease in running distance of rats receiving combinations of IL-1β and IL-6 compared to rats receiving vehicle ($p < 0.01$, SNK). **Significant decrease in running distance compared to rats receiving the single doses of IL-6 and IL-1β ($p < 0.01$, SNK). The dashed line (---) indicates the sum of the individual responses of the cytokines.

after injection compared to rats receiving vehicle. Figs. 5B and C and 6B and C show the results of i.c.v. co-injections of other combinations of IL-1β and IL-6, at doses which even though higher than the doses depicted in Figs. 5

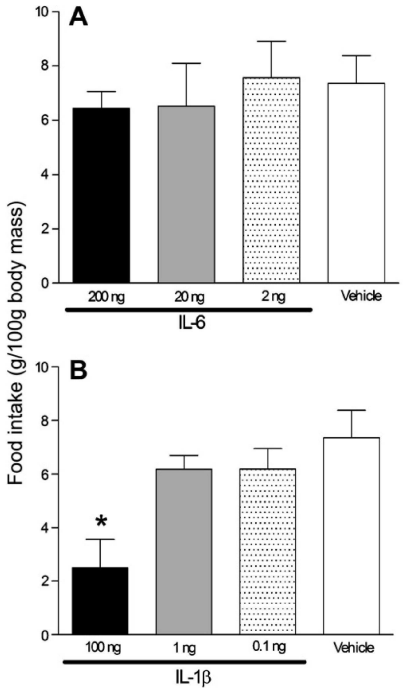

Fig. 3. Food intake of rats for 12 h after i.c.v. injection of vehicle ($n = 6$) or: (A) IL-6 at three doses 200 ng ($n = 6$), 20 ng ($n = 6$), 2 ng ($n = 6$); (B) IL-1β at three doses 100 ng ($n = 6$), 1 ng ($n = 5$), 0.1 ng ($n = 7$). The results are represented as means ± SD. *Significant decrease in food intake of rats receiving the highest dose of IL-1β (100 ng) compared to rats receiving vehicle ($p < 0.001$, SNK).

and 6A, also themselves had no effect on food intake and body mass, but when injected in combination, resulted in a significant decrease in food intake and weight gain compared to rats receiving vehicle ($p < 0.05$, SNK).

3.3. Body temperature

Fig. 7 shows the body temperature responses over ~14 h for rats that received i.c.v. injections of 200 ng, 20 ng or 2 ng IL-6 or vehicle. Body temperatures of rats injected i.c.v. with 200 ng or 20 ng of IL-6 started to increase significantly after a latent period of approximately 1–2 h (Fig. 7A and B). The increase in body temperature of the rats receiving either 200 ng or 20 ng IL-6 peaked at 39.8 ± 0.2 °C and 39.3 ± 0.3 °C, respectively, approximately 5 h after the injection. Body temperatures of rats injected with 2 ng IL-6 were not significantly different when compared to the body temperatures of rats receiving vehicle over the 12 h period after injection (Fig. 7C). Twelve-hour TRIs calculated for the period 19:00–07:00 showed that the increase in body temperature was dependent on the dose of IL-6 injected i.c.v. (one-way ANOVA, $F_{(3,19)} = 35.9$, $p < 0.0001$). The mean TRI for rats receiving

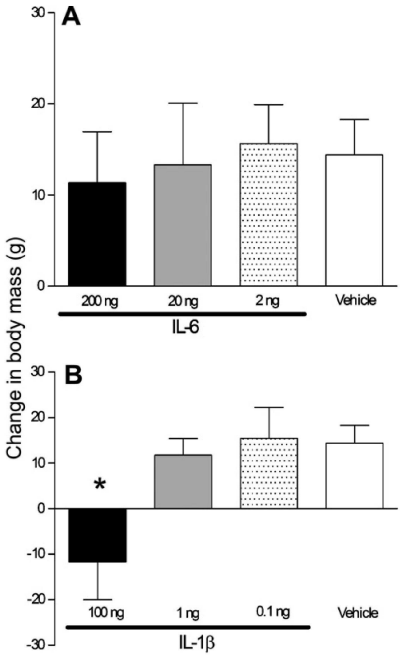

Fig. 4. Change in body mass of rats for 12 h after i.c.v. injection of vehicle ($n = 6$) or: (A) IL-6 at three doses 200 ng ($n = 6$), 20 ng ($n = 6$), 2 ng ($n = 6$); (B) IL-1β at three doses 100 ng ($n = 6$), 1 ng ($n = 5$), 0.1 ng ($n = 7$). The results are represented as means ± SD. *Significant decrease in body mass of rats receiving the highest dose of IL-1β (100 ng) compared to rats receiving vehicle ($p < 0.001$, SNK).

200 ng (12.4 ± 2.5 °C h) was significantly different from the mean TRI for rats receiving 20 ng (8.6 ± 2.1 °C h, $p < 0.05$, SNK) and 2 ng (2.6 ± 3.1 °C h, $p < 0.001$, SNK). Furthermore, the mean TRI of rats receiving 20 ng was significantly different to the TRI of rats receiving 2 ng ($p < 0.001$, SNK). The mean TRI (−0.2 ± 0.7 °C h) calculated for the body temperature of rats receiving vehicle was not significantly different from zero ($t = 0.6$, $p = 0.55$). Injection of the vehicle therefore did not have any effect on night-time body temperature of the rats.

Fig. 8 shows the body temperature responses over ~14 h for rats that received i.c.v. injections of 100 ng, 1 ng or 0.1 ng IL-1β or vehicle. Body temperatures of rats injected i.c.v. with a 100 ng or 1 ng of IL-1β started to increase significantly after a latent period of approximately 1–2 h (Fig. 8A and B). The increase in body temperature of the rats receiving either 100 ng or 1 ng IL-1β peaked at 39.8 ± 0.4 °C and 39.8 ± 0.3 °C, respectively, approximately 3 h after the injection. Body temperatures of rats injected with 0.1 ng IL-1β were not significantly different when compared to the body temperatures of rats receiving vehicle over the 12 h period after injection (Fig. 7C). Twelve-hour TRIs calculated for the period 19:00–07:00 showed that the increase in body temperature was dependent on the dose of IL-1β injected i.c.v. (one-way ANOVA,

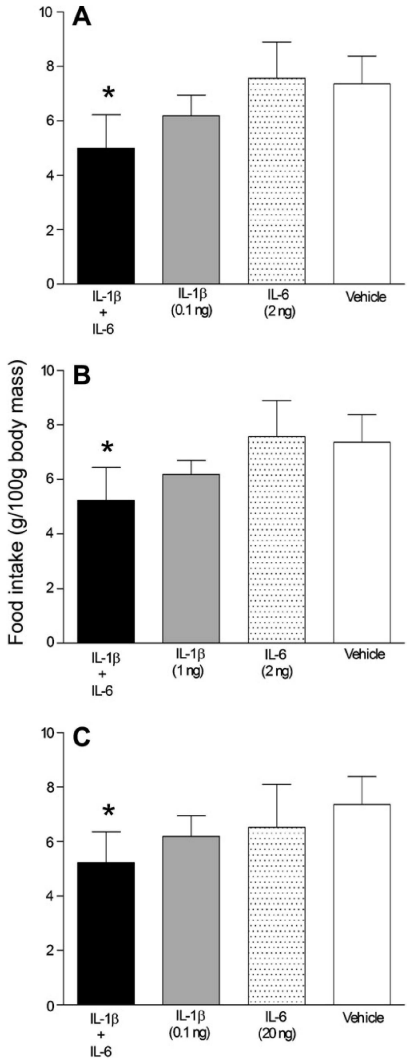

Fig. 5. Food intake of rats for 12 h after i.c.v. injection of vehicle ($n = 6$) or: (A) co-injection of 0.1 ng IL-1β + 2 ng IL-6 ($n = 6$); (B) co-injection of 1 ng IL-1β + 2 ng IL-6 ($n = 6$); (C) co-injection of 0.1 ng IL-1β + 20 ng IL-6 ($n = 5$). In (A), (B) and (C) the effects of injecting each cytokine dose separately, also is shown and is the same as the results in Fig. 3A and B. The results are represented as means ± SD. *Significant decrease in food intake of rats receiving combinations of IL-1β and IL-6 compared to rats injected with vehicle ($p < 0.05$, SNK).

$F_{(3,20)} = 35.3$, $p < 0.0001$). The mean TRI for rats receiving 100 ng (11.4 ± 3.8 °C h) was significantly different from the mean TRI for rats receiving 1 ng (8.2 ± 1.2 °C h, $p < 0.05$, SNK) and 0.1 ng (2.1 ± 1.8 °C h, $p < 0.001$, SNK). Furthermore, the mean TRI of rats receiving 1 ng was significantly different to the mean TRI of rats receiving 0.1 ng ($p < 0.001$, SNK).

Fig. 9 shows the body temperature responses over ~14 h for rats that received combination i.c.v. injections of doses

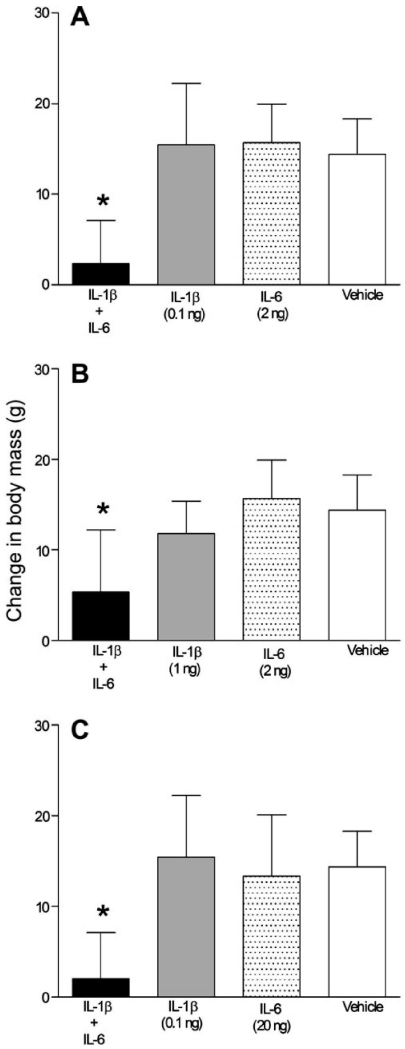

Fig. 6. Change in body mass of rats for 12 h after i.c.v. injection of vehicle ($n = 6$) or: (A) co-injection of 0.1 ng IL-1β + 2 ng IL-6 ($n = 6$); (B) co-injection of 1 ng IL-1β + 2 ng IL-6 ($n = 6$); (C) co-injection of 0.1 ng IL-1β + 20 ng IL-6 ($n = 5$). In (A), (B) and (C) the effects of injecting each cytokine dose separately, also is shown and is the same as the results in Fig. 4A and B. The results are represented as means ± SD. Asterisk indicates that rats receiving combinations of IL-1β and IL-6 gained significantly less body mass compared to rats receiving vehicle ($p < 0.05$, SNK).

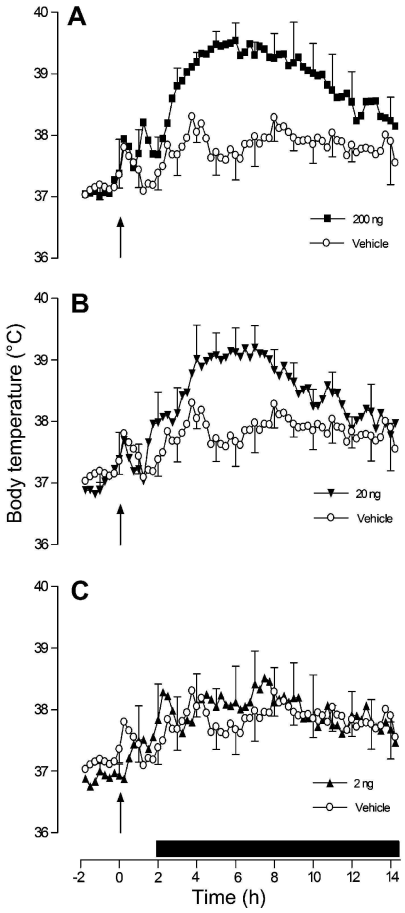

Fig. 7. Body temperature responses of rats injected i.c.v. with vehicle ($n = 6$) or: (A) 200 ng IL-6 ($n = 6$); (B) 20 ng IL-6 ($n = 6$); (C) 2 ng IL-6 ($n = 6$). The results are represented as means ± SD. The arrow indicates the time of the injection and the black bar indicates time of lights off (19:00 clock time).

of IL-1β and IL-6 both or one of which at least lacked pyrogenicity (see Figs. 7 and 8). Fig. 9A shows that rats receiving combination i.c.v. injections of doses of IL-1β (0.1 ng) and IL-6 (2 ng) neither of which themselves resulted in fever (see Figs. 7 and 8C), induced a significant increase in body temperature after a latent period of approximately 1–2 h. The increase in body temperature of the rats receiving this combination of cytokines peaked at 39.1 ± 0.2 °C approximately 5 h after the injection. The mean TRI for rats receiving this combination (9.6 ± 2.8 °C h) was significantly different to the mean TRI for rats receiving saline (−0.2 ± 0.7 °C h) ($t = 8.2$, $p < 0.0001$). The peak body temperature reached with injection of this combination of IL-1β and IL-6 is similar to that produced by either that of 10 times the dose of IL-6 alone (20 ng) (see Fig. 7B), or that of at least 10 times the dose of IL-1β alone (1 ng) (see Fig. 8B), but was of longer duration. Fig. 9B shows that rats receiving combination i.c.v. injections of a pyrogenic dose of IL-1β (1 ng) and a non-pyrogenic dose of IL-6 (2 ng) produced a fever over the 12 h period after injection almost identical to that of IL-1β alone. Thus, the addition of a non-pyrogenic dose of IL-6 did not enhance the pyrogenic response to IL-1β. Fig. 9C shows that rats receiving combination i.c.v. injec-

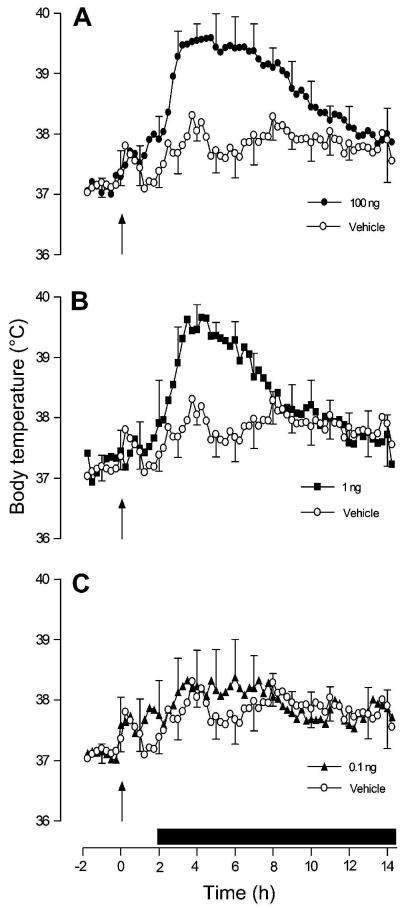

Fig. 8. Body temperature responses of rats injected i.c.v. with vehicle ($n = 6$) or: (A) 100 ng IL-1β ($n = 6$); (B) 1 ng IL-1β ($n = 5$); (C) 0.1 ng IL-1β ($n = 7$). The results are represented as means ± SD. The arrow indicates the time of the injection and the black bar indicates time of lights off (19:00 clock time).

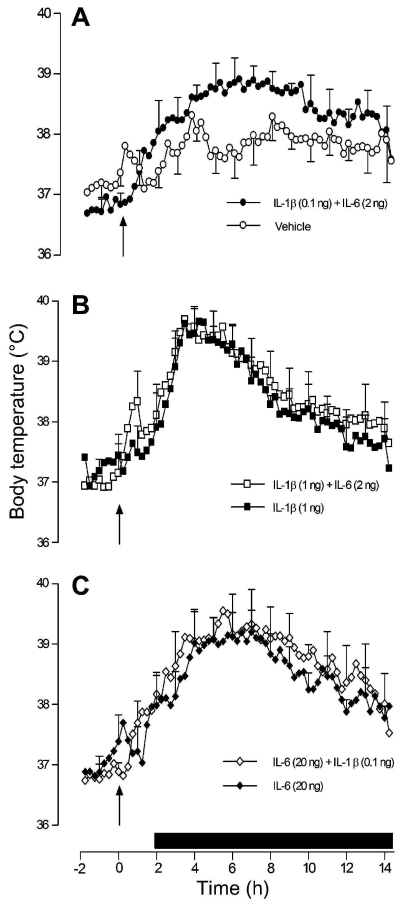

Fig. 9. Body temperature responses of rats injected i.c.v. with vehicle ($n = 6$) or: (A) a non-pyrogenic dose of IL-1β (0.1 ng) + a non-pyrogenic dose of IL-6 (2 ng) ($n = 6$); (B) a pyrogenic dose of IL-1β (1 ng) + a non-pyrogenic dose of IL-6 (2 ng) ($n = 6$); (C) a pyrogenic dose of IL-6 (20 ng) + a non-pyrogenic dose of IL-1β (0.1 ng) ($n = 5$). In (B) the effects of injecting IL-1β separately, also is shown and is the same as the results in Fig. 8. In (C) the effects of injecting IL-6 separately, also is shown and is the same as the results in Fig. 7. The results are represented as means ± SD. The arrow indicates the time of the injection and the black bar indicates time of lights off (19:00 clock time).

tions of a pyrogenic dose of IL-6 (20 ng) and a non-pyrogenic dose of IL-1β (0.1 ng) produced a fever of significantly greater magnitude and duration (12.4 ± 2.7 °C h) to that of IL-6 alone at that dose (8.6 ± 2.1 °C h) (one-way ANOVA, $F_{(2,14)} = 59.4$, $p < 0.0001$). The magnitude and duration of the febrile response with injection of this combination of IL-1β and IL-6 is similar to that produced by 10 times the dose of IL-6 alone (200 ng) (12.3 ± 2.5 °C h) (see Fig. 7A). Thus, the addition of a non-pyrogenic dose of IL-1β enhanced the pyrogenic response to IL-6 alone at that dose.

4. Discussion

We have shown that intracerebroventricular (i.c.v.) administration of species-homologous rat IL-6 and IL-1β before the night-time active period decreases voluntary activity in rats in a dose-dependent fashion. To our knowledge this is the first report that voluntary exercise, that is wheel-running, is suppressed by direct administration into the brain, of IL-6 and IL-1β. We also have shown that i.c.v. injection of IL-1β at the highest dose we used, but not IL-6 at any dose, induced a decrease in food intake in the rats. Furthermore, our results indicate that the effect of the two cytokines on voluntary activity is independent of their effects on body temperature and food intake, since suppression of voluntary activity induced by the lowest

doses of IL-6 and IL-1β occurred in the absence of fever and anorexia (Figs. 1, 3, 7 and 8).

While our observations that central administration of IL-1β and IL-6 can induce fever and that central administration of IL-1β but not similar administration of IL-6, can decrease food intake and body mass in rats are not new, they do confirm unequivocally the different effects, mediated centrally, of IL-1β and IL-6 on appetite despite their similar effects on inhibition of voluntary activity. Moreover, our study reveals new findings regarding the synergistic relationship between IL-1β and IL-6 in the brain, by showing that central co-administration of non-pyrogenic doses of IL-1β and IL-6 can induce fever (Fig. 9) and that central co-administration of doses of IL-6 and of IL-1β which alone do not induce anorexia, do so when co-injected (Figs. 5 and 6). Furthermore, it would appear that the synergistic action of IL-1β and IL-6 in the brain is not inevitable, as we did not observe a synergistic effect on the suppression of voluntary wheel-running when both cytokines were co-injected (Fig. 2).

Our results showing that central administration of IL-1β and IL-6 induces fever in rats confirm the observations made by others that both have pyrogenic effects within the brain (Dascombe et al., 1989; LeMay et al., 1990; Rothwell et al., 1991; Cao et al., 2001). There are two lines of evidence to show that direct central administration of IL-1β and IL-6 act via the proximal mediator of fever, prostaglandin-E_2 (PGE_2) to increase body temperature: (i) centrally injected IL-1β and IL-6 increase the production of PGE_2 in the brain (Dinarello et al., 1991); and (ii) inhibiting the production of the rate-limiting prostaglandin-synthesizing enzyme, cyclooxygenase-2 (COX-2) completely suppresses the fever induced by central administration of IL-1β and IL-6 (Dinarello et al., 1991; Cao et al., 2001). We and others (Dinarello et al., 1991; Rothwell et al., 1996; Cao et al., 2001) have shown that injecting lower doses of IL-1β into the brain induces fever of equal or greater magnitude and duration than higher doses of IL-6. Therefore while both are pyrogenic, IL-1β appears to be the more potent of the two cytokines (gram for gram). It has been suggested that the greater pyrogenic potency of IL-1β is due to its ability to induce a greater increase in the concentration of PGE_2 than IL-6 (Dinarello et al., 1991). The greater PGE_2 producing ability of IL-1β can be explained by the observation that i.c.v. administration of lower doses of IL-1β induces a greater intensity and number of COX-2 immunoreactive cells compared to i.c.v. administration of higher doses of IL-6 (Cao et al., 2001).

Not only has brain IL-1β been shown to be capable of inducing fever but also anorexia (Sonti et al., 1996; Pecchi et al., 2006). Moreover, as with IL-1β induced fever, the synthesis of PGE_2 has been confirmed as an important mediator of the IL-1β induced decrease in food intake and body mass (Bluthé et al., 1992; Swiergiel and Dunn, 2002; Pecchi et al., 2006; Elander et al., 2007). The involvement of PGE_2 in mediating anorexia has furthermore been supported by the finding that injection of PGE_2 decreases food intake in rats and mice (Levine and Morley, 1981; Pecchi et al., 2006). If cytokine-induced anorexia is indeed PGE_2 mediated then our finding that anorexia was not induced by central administration of IL-6 is surprising, if as mentioned above, central administration of IL-6 does increase PGE_2 in the brain. The finding that brain IL-6 *per se* is not a potent inducer of anorexia may therefore not be due to an inability to produce PGE_2. Differences in the sensitivity of brain sites controlling temperature and food intake to PGE_2 have been demonstrated with the finding that i.c.v. administration of PGE_2 increases body temperature at significantly lower doses (0.5 ng) than it decreases food intake (1 μg) (Levine and Morley, 1981; Oka et al., 1997). As IL-6 does not seem to be as potent as is IL-1β at inducing PGE_2 (Dinarello et al., 1991) one can therefore postulate that while IL-6 can induce PGE_2 it may not be able to increase PGE_2 to the threshold concentration necessary for the induction of anorexia. Possible differences in the threshold concentration of PGE_2 required to induce fever and anorexia may therefore also explain the different dose–response profiles we observed for body temperature and food intake following central administration of IL-1β. Body temperature was affected by i.c.v. injection of the high and intermediate doses of IL-1β while food intake was affected only by i.c.v. injection of the high dose of IL-1β.

In contrast to the different dose–response profiles we observed for the cytokine-induced fever and anorexia, central administration of IL-1β and IL-6 induced similar dose-dependent decreases in voluntary activity. Our finding that centrally administered IL-6 and IL-1β both can decrease voluntary activity and induce fever, could lead to the speculation that the cytokine-induced fever and decrease in voluntary activity are initiated via the same downstream mediators. However, our finding that all the administered doses of IL-6 and IL-1β decreased voluntary running, but not all doses induced fever, and that co-injection IL-6 and IL-1β within the brain has a synergistic action in inducing fever but not in decreasing voluntary activity, suggests that some dissociation exists between the physiological mechanisms by which central administration of IL-6 and IL-1β induce fever and inhibit voluntary activity within the brain. The explanation given above for the differences in the dose–response profiles observed for the cytokine-induced fever and anorexia, could also apply to the different dose–response profiles for voluntary activity and fever. It is possible that brain sites controlling activity are more sensitive to the presence of prostanoids and therefore respond to even lower doses of cytokines than do brain sites controlling temperature. Results obtained by others using a different experimental approach, that being administration of lipopolysaccharide and turpentine, have shown that reducing the synthesis of PGE_2 reduces fever but has no effect on the inhibition of cage activity, another measure of lethargy. These findings (Kozak et al., 1994; Saha et al., 2005) would tend to support a different view

regarding the relative role of PGE_2 in fever and suppression of voluntary activity.

The decrease in voluntary activity observed in our study following cytokine administration can be likened to the fatigue and decrease in daily activity experienced by sick individuals and animals during illness. In addition, fatigue accompanied by a decrease in activity is routinely experienced during exercise also, in which it is defined as an inability to continue exercising at a given work rate (Hawley and Reilly, 1997). The finding that IL-6 increases significantly in the central nervous system (Nybo et al., 2002) and plasma (Ostrowski et al., 1998) during prolonged exercise, together with the observations that administration of human recombinant IL-6 induces a sensation of fatigue in healthy humans at rest (Spath-Schwalbe et al., 1998) and decreases athletic performance in trained runners (Robson-Ansley et al., 2004) has lead to the hypothesis that IL-6 could be driving fatigue experienced during exercise. Results from studies showing that administration of IL-6 in rats increases brain tryptophan and serotonin metabolism (Zalcman et al., 1994; Wang and Dunn, 1999) together with evidence suggesting that serotonergic activity in the hippocampus is involved in locomotion (Takahashi et al., 2000), suggests that IL-6 may cause fatigue resulting in a loss of motivation to continue exercising by increasing serotonin activity during prolonged exercise (Robson-Ansley et al., 2004; Newsholme and Blomstrand, 2006). Not only can i.c.v. administration of IL-6 increase tryptophan and serotonin in the brain but so too can i.c.v. administration of IL-1β (Zalcman et al., 1994). The dose-dependent decrease in voluntary activity we observed following central administration of IL-6 and IL-1β may therefore be due to a dose-dependent increase in serotonin in the brain. While we can only speculate as to whether serotonin or PGE_2 is the downstream mediator of cytokine-induced inhibition of voluntary activity, our results demonstrate unequivocally that brain IL-6 and IL-1β have fatigue-inducing properties which result in lethargy and a lack of motivation to engage in physical activity.

The idea that the pro-inflammatory cytokines IL-1β and IL-6 exhibit mutual facilitation in inducing temperature and behavioral effects has been mooted before (Lenczowski et al., 1999; Cartmell et al., 2000), and we therefore deliberately co-administered doses of the two cytokines below the pyrogenic and behavioral thresholds into the brains of conscious rats to see if synergistic responses could be induced. Our results show that central co-administration of non-pyrogenic doses of IL-1β and IL-6 can induce fever, and confirm the observations (Cartmell et al., 2000), made after peripheral injection of non-pyrogenic doses of these two substances, of a synergistic relationship between IL-1β and IL-6 in mediating fever. Furthermore, we have shown that central co-injection of doses of IL-1β and IL-6 which, on their own have no effect on food intake and body mass, can induce anorexia, which extends the synergistic repertoire of these two cytokines and also demonstrates that the synergistic action of pro-inflammatory cytokines within the brain in inducing anorexia is not confined to the combined actions of IL-1β and the two cytokines, TNF-α and IL-8, as previously suggested (Bluthé et al., 1994; Sonti et al., 1996).

In addition to co-injecting the two lowest doses of IL-1β and IL-6 we also injected two additional combinations of intermediate and low doses of IL-1β and IL-6, to establish whether an existing pyrogenic effect could be enhanced by the presence of another cytokine in the brain, albeit in a non-pyrogenic concentration. Co-injection of a non-pyrogenic dose of IL-6 had no additional effect on the febrile response induced by IL-1β (Fig. 9B), while co-injection of a non-pyrogenic dose of IL-1β significantly enhanced the pyrogenic effect of IL-6 (Fig. 9C). Co-injection of both combinations of IL-1β and IL-6 did however; induce a significant decrease in food intake and body mass which was not evident when injecting either dose alone (Figs. 5 and 6). Thus we are lead to the conclusion that the synergistic action of IL-1β and IL-6 in inducing anorexia is a physiological phenomenon not primarily dependent on the metabolic or other effects of the rise in body temperature. The synergistic action of IL-1β and IL-6 within the brain in inducing fever and anorexia therefore appears to exist at varying concentrations of these two cytokines in the brain (Figs. 5, 6 and 9).

Synergy is deemed present when the effect of a combination of substances exceeds the effect of the individual constituents (Berenbaum, 1989). Two findings have suggested mechanisms for the synergy observed between IL-1β and IL-6. Firstly, IL-1β and IL-6 cause upregulation of not only their own receptors, but also each others' receptors in brain blood vessels (Cao et al., 2001). Secondly, IL-1β enhances the biosynthesis of IL-6 (Dinarello, 2005). One important possible consequence of these phenomena could be the enhanced transcription of the COX-2 gene and subsequent magnitude of PGE_2 synthesis in the brain (Cartmell et al., 2000; Cao et al., 2001). As PGE_2 has been established as an important mediator of cytokine-induced fever and anorexia (Cao et al., 2001; Pecchi et al., 2006), the most likely mechanism for the fever and anorexia we observed with co-injection of the two cytokines is a greater magnitude of PGE_2 synthesis.

It would appear however, that the synergistic action of IL-1β and IL-6 within the brain is not inevitable, as we did not observe a synergistic or even additive response in the suppression of voluntary wheel-running, when these two cytokines were co-injected (Fig. 2). If the mechanism for the synergistic effect of IL-1β and IL-6 in the brain is indeed dependent on the COX-2/PGE_2 pathway, then the lack of a synergistic effect of these two cytokines on voluntary activity is not unexpected, if, as we and others have suggested PGE_2 is not required to mediate lethargy (Kozak et al., 1994; Saha et al., 2005). The mechanism by which IL-1β and IL-6 individually mediate the suppression of voluntary activity in the brain therefore requires further investigation.

In conclusion, by injecting species-homologous rat IL-6 and IL-1β intracerebroventricularly we have identified that both cytokines can induce fever and decrease voluntary activity, two responses commonly observed during infection and inflammation. Our findings add support to the hypothesis that increased levels of pro-inflammatory cytokines in the brain mediate the debilitating fatigue reported by patients in various disease states. Moreover, we have confirmed that brain IL-1β, but not brain IL-6 plays a key role in mediating the anorexia of sickness. We also have shown that for some aspects of the acute phase response, such as fever and appetite suppression, IL-1β and IL-6 probably work synergistically in the brain. Our results have implications for the understanding of the relative roles of two important cytokines in the mediation of sickness behavior.

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CHAPTER 4

Endogenous antagonism of interleukin (IL)-6 or IL-1β significantly enhances the resolution of anorexia, lethargy and fever induced by lipopolysaccharide.

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Endogenous antagonism of interleukin (IL)-6 or IL-1β significantly enhances the resolution of anorexia, lethargy and fever induced by lipopolysaccharide

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ABSTRACT

Although fever, anorexia and lethargy may be beneficial sickness responses initially, they may become detrimental to the host if they continue for a prolonged period. We therefore investigated whether antagonizing the biological action of putative mediators of these sickness responses, interleukin (IL)-6 and IL-1β, could affect the duration of anorexia, lethargy and fever, measured as changes in food intake, voluntary activity and body temperature, induced by subcutaneous (s.c.) administration of lipopolysaccharide (LPS). Male Sprague-Dawley rats were randomly assigned to receive a caspase-1 inhibitor to reduce the synthesis of IL-1β or vehicle intracerebroventricularly and antiserum to IL-6 (IL-6AS) or pre-immune serum intraperitoneally, before receiving an injection of LPS (250 μg/kg) or saline. LPS administration induced a ~1.3 ± 0.2 ºC fever and reduced voluntary activity by 98.0 ± 2.4 %, food intake by 50.0 ± 9.8 % and body mass by 14.5 ± 2.7 g compared to rats injected with saline ($P < 0.05$, ANOVA). Increases in plasma IL-6 and IL-1β accompanied LPS administration on the day of injection. Within 2 days the fever resolved, while lethargy and anorexia continued for at least 3 days. Rats pre-treated with IL-6AS had reduced plasma levels of bioactive IL-6, no fever and attenuated sickness behaviors which resolved within 2 days. Rats pre-treated with the caspase-1 inhibitor exhibited attenuated fever and sickness behaviors which resolved within 2 days. Thus antagonizing the biological action of IL-6 in the circulation or IL-1β in the brain significantly reduces the duration of anorexia, lethargy and fever induced by LPS administration.

Keywords: Voluntary wheel-running; Anorexia; Pro-inflammatory cytokines

1. Introduction

Fever and sickness behaviors, such as anorexia and lethargy, experienced during infection appear to be advantageous short-term responses intended to support the immunological response of the host to eliminate the pathogen [18]. Although the feeling of fatigue, loss of appetite and increase in body temperature may be beneficial responses acutely, experiencing these sickness responses on a daily basis for prolonged periods may negatively impact the quality of life of patients and even worsen their condition, ultimately delaying recovery [19, 55]. From a clinical point of view identifying the physiological mechanisms underlying the onset and maintenance of these sickness responses during illness is particularly relevant because it may enable the design of appropriate therapeutic interventions to oppose the detrimental effects of the prolonged duration of sickness responses [23].

Most experimental investigations examining the physiological mechanisms mediating fever and sickness behavior responses have used purified lipopolysaccharide (LPS), the glycolipid pyrogenic moiety of the Gram-negative bacterial membrane, to trigger the innate immune system [9]. Part of the innate immune systems response to the presence of endotoxin in animals and humans includes the synthesis and release of pro-inflammatory cytokines from immune cells [13]. Results obtained from studies using specific antagonists to block the action of these cytokines released into the circulation following systemic administration of LPS, have uncovered a critical role for one particular cytokine, interleukin (IL)-6, in mediating the physiological response of fever [10, 16]. Although fever and sickness behavior both occur after systemic administration

of LPS, it has been established that these behavioral and febrile responses are not mediated via exactly the same cytokine-induced mechanisms [16, 24]. In particular, results from a study conducted within our laboratory showing that neutralizing the biological activity of IL-6 in the circulation completely abolishes fever, but only partially attenuates sickness behaviors, has identified one such distinct difference in the cytokine mechanisms mediating fever and sickness behavior [16]. It would appear therefore, that while IL-6 released in the periphery has a critical role to play in the events regulating fever, additional factors, possibly other cytokines, work in parallel with peripherally released IL-6 to regulate the sickness behavior responses induced by systemic administration of LPS. There is evidence to suggest that if other cytokines are working with peripherally released IL-6 to induce sickness behaviors, and two likely candidates are IL-1β and TNF-α [22], it is due primarily to their synthesis in the brain and not in the periphery, because antagonizing the actions of IL-1β and TNF-α in the periphery has been shown to have no effect on lethargy and no or minimal effect on anorexia induced in rats and mice during infection and inflammation [6, 16, 25, 27, 31, 32, 35, 48, 56].

Several studies have demonstrated that rats and mice treated intracerebroventricularly (i.c.v.) with IL-1β and TNF-α exhibit symptoms similar to those of LPS-treated animals, suggesting that both cytokines may be important central mediators of LPS-induced sickness behaviors [5, 17]. There are several lines of evidence to however suggest, that IL-1β mediates the synthesis and behavioral effects of TNF-α in the brain, because injecting IL-1 receptor antagonist (IL-1ra) i.c.v. has been shown to: (i) abrogate the mRNA expression of not only IL-1β but also TNF-α in the hypothalamus of mice injected peripherally with LPS [30] and (ii) to inhibit the behavioral effects of centrally

administered TNF-α [5]. It is reasonable to hypothesize therefore that endogenous brain IL-1β is the likely central cytokine working in parallel with IL-6 released in the periphery to induce sickness behavior following systemic administration of LPS. Although peripherally-released IL-6 has been implicated in mediating the development of LPS-induced sickness behaviors [4, 53], the involvement of IL-1β in the brain in mediating the development of LPS-induced sickness behaviors has not yet clearly been established [3, 25, 30]. Moreover the extent to which the absence of either of these cytokines affects the resolution of LPS-induced sickness behaviors has also not been established.

Thus we have chosen to antagonize the biological action of peripherally-released IL-6 or IL-1β in the brain following subcutaneous injection of LPS, and to monitor the behavioral responses until the symptoms of sickness induced by LPS have ceased, so as to determine the cytokine involvement not only in the induction of these responses, but also in their resolution. In particular we chose to decrease the biological activity of IL-6 in the circulation and IL-1β in the brain by administering species-specific antiserum to IL-6 (IL-6AS) intraperitoneally and a caspase-1 inhibitor, which prevents the cleavage of pro-IL-β to biologically active IL-1β, intracerebroventricularly. We monitored quantifiable behavioral responses known to be affected by systemic administration of LPS, such as food intake and voluntary wheel-running. Moreover, we measured body temperature to assess whether differences exist between the involvement of IL-6 in the periphery and IL-1β in the brain in mediating fever and sickness behavior. Our results reveal that antagonizing the biological action of IL-6 in the circulation or IL-1β in the brain significantly reduces the duration of anorexia, lethargy and fever induced by s.c. LPS administration.

2. Methods

2.1 Animals

Male Sprague-Dawley rats (initial body mass 80 - 120 g) were housed individually in cages to which exercise-training wheels had been attached. The rats were kept at an ambient temperature of 21 ± 2°C on a reversed 12 h:12 h light:dark cycle (lights on from 22:30 - 10:30). The light:dark cycle of the rats was reversed over a period of four weeks before the rats underwent surgery (described below). That the rats' circadian rhythm had been 12-h shifted, was confirmed before the start of the experimental interventions by evidence of a normal nychthemeral temperature rhythm for each rat. Food (pelleted rat chow, Epol, Johannesburg, South Africa) and water were provided *ad libitum*. All procedures were in accordance with the Animal Ethics and Control Committee of the University of the Witwatersrand animal care regulations and were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (ethics no 2005/86/5).

2.2 Surgery

Rats selected for the study (body masses 280 - 320 g) were anesthetized with an intramuscular (i.m.) injection of 80 mg/kg ketamine hydrochloride (Anaket-V, Bayer, SA) and 20 mg/kg xylazine (Chanazine, Bayer, SA) and had a temperature-sensitive radiotransmitter (TA10TA-F40, Data Sciences, St. Paul, MN, USA) implanted intraperitoneally. Thereafter the rats were placed in a stereotaxic frame (Stoelting, IL, USA), a heating pad was placed beneath the animal to maintain core body temperature

and they were given an injection (0.1 ml) of adrenaline (10 µg) (Merck, SA) and lignocaine hydrochloride (0.02 g) (Bayer, SA) subcutaneously over an area of skull. An incision was made in the midline of the cranium to expose the skull. A 23-gauge stainless steel guide cannula (Plastics One, Roanoke, VA, USA) was placed over the right lateral cerebral ventricle. Coordinates for the guide cannula were 0.8 mm posterior to bregma, 1.5 mm lateral to the midline and 3.5 mm below the skull surface at the point of entry of the guide cannula [39]. The cannula was secured to the skull with three screws and dental cement. After surgery each rat was given a subcutaneous injection of 0.3 mg buprenorphine hydrochloride (Temgesic, Schering-Plough, SA) and ringer lactate (1.5 ml) (SABAX, Adcock, Ingram, SA).

2.3 Body temperature

Core body temperatures of rats were measured by remote biotelemetry using temperature -sensitive radiotransmitters which had been implanted intraperitoneally (see above). Transmitter output frequency (Hz) was monitored at five minute intervals, by a receiver plate (RTA 500, Mini-Mitter, Sunriver, OR, USA) situated beneath the cage of each animal. The frequency received by each plate was fed into a peripheral processor (DP-24 DataPort, VitalView, Minimitter, Sunriver, OR, USA) connected to a personal computer and the output expressed in degrees centigrade. The telemeters were calibrated by water immersion against a high-accuracy thermometer (Quat 100, Heraeus, Germany) to an accuracy of 0.1°C.

2.4 Voluntary wheel-running

The exercise-training wheels had a circumference of 1.06 m and each wheel was equipped with a magnet and a magnetic switch (VitalView, Minimitter, Sunriver, OR, USA). Each time the wheel rotated the magnet within range of the magnetic switch, the switch closed and a turn was counted. The magnetic switches were connected to an activity input module (QA-4, VitalView, Minimitter, Sunriver, OR, USA) which in turn was fed into a peripheral processor (DP-24 DataPort, VitalView, Sunriver, OR, USA) connected to a personal computer which monitored the number of wheel turns at five minute intervals using VitalView software version 4.1 (Mini Mitter, Bend, OR, USA).

2.5 Food intake and body mass

Food intake and body mass were measured daily just before lights off. Food containers were filled daily with 100 g of standardized pelleted rat chow. Food intake was quantified by subtracting the food remaining in the food container and on the cage floor from the amount of food measured at the preceding time point.

2.6 Pyrogens and cytokine antagonists

Lipopolysaccharide injections

Lipopolysaccharide (LPS) derived from *Escherichia coli* endotoxin (serotype 0111:B4, Sigma, St. Louis, MO, US) was reconstituted in saline (sterile, pyrogen-free 0.9% saline Sabax, Johannesburg, South Africa) and injected subcutaneously (s.c.) at a dose of 250 μg/kg.

Cytokine antiserum injections

We used a species-specific antiserum to IL-6 (IL-6AS, NIBSC, South Mimms, Potters Bar, Herts UK) and pre-immune (normal) sheep serum (PIS, lot 056K8408, Sigma, St. Louis, MO, US) for the control injections, all with an endotoxin content < 0.24 ng/ml (2.4 IU). The IL-6AS and the PIS were injected intraperitoneally (i.p.) in a volume of 1.5 ml. The antibodies were raised in sheep as previously described [42, 43]. The IL-6AS used recognizes both natural and recombinant (*Escherichia coli*-derived) rat IL-6, but does not cross-react with the following rat recombinant cytokines: (rr) TNF-α, IL-1β, IL-1α, IL-1ra and IL-10 [42, 43].

Caspase-1 inhibitor injections

We used a specific caspase-1 inhibitor Ac.YVAD-cmk (Ac-Tyr-Val-Ala-Asp-chloromethylketone; lot B75699; Calbiochem, Darmstadt, Germany) to decrease the synthesis of IL-1β within the brain. The caspase-1 inhibitor (300 ng/rat) and the vehicle for its injection (0.6% DMSO in sterile saline) were injected intracerebroventricularly (i.c.v.) in a volume of 5 μl. This dose of caspase-1 inhibitor previously has been shown, when injected into the brain, to prevent the synthesis of brain IL-1β following i.p. administration of LPS [2].

2.7 Cytokine analysis

To assess the efficacy of IL-6AS in decreasing the biological activity of IL-6 in the periphery, we measured the levels of bioactive IL-6 in plasma using a bioassay. Determination of IL-6 was achieved by a bioassay based on the dose-dependent growth

stimulation of IL-6 on the B9 hybridoma cell line [1]. The assay was performed in sterile, 96-well microtiter plates. In each well, 5,000 B9 cells were incubated for 72 h with serial dilutions of biological samples or with different concentrations of a human IL-6 standard (code 89/548, National Institute for Biological Standards and Control, South Mimms, UK). The number of living cells after 72 h was measured by using the dimethylthiazoldiphenyl tetrazolium bromide (MTT) colorimetric assay [20]. Plasma samples were pre-diluted so that serial dilution of samples and standard dilution curves were parallel. The detection limit of the assay, after considering the dilution of samples into the assays, was 3 IU of IL-6/ml. As opposed to ELISA assays, use of the described bioassays does not exclude the possibility that some undiscovered substance might interfere in the assay by causing proliferation of the B9 cells. Therefore, we will refer to the measured IL-6 as IL-6-like-activity.

To exclude the possibility that trace amounts of the caspase-1 inhibitor injected i.c.v. entered the blood from the brain and attenuated the synthesis of IL-1β in the periphery following LPS administration, we measured the concentration IL-1β in plasma using a commercially available enzyme linked immunosorbent assay (ELISA) kit (R & D Systems, Minneapolis, MN). The microplate used had been pre-coated with an affinity purified polyclonal antibody specific for rat IL-1β. Fifty microliters of standard, control or sample was added to each well and incubated for 2 hours at room temperature. Each sample was measured in duplicate. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for rat IL-1β was added to each well (100 μl) and incubated for 2 hours at room temperature. Following a wash to remove any

unbound antibody-enzyme reagent, a substrate solution was added to each well (100 µl) and incubated for 30 minutes at room temperature. The enzyme reaction yielded a blue product that turned yellow when the reaction was terminated by addition of 100 µl of diluted HCl to each well. The optical density was measured at 450 nm and 540 nm. The readings obtained at 540 nm were subtracted from the readings obtained at 450 nm and the sample values were then read off the standard curve. The subtraction was used to correct for optical imperfections in the plate. The results were expressed as pg/ml. The detection limit of the assay was 5 pg/ml. Where the values obtained were below the detection limit, the detection limit of the assay was assigned.

2.8 Experimental procedure

We reversed the light/dark cycle of the rats so as to enable the collection of blood samples, describe below, during the day-time. To ensure that rats used in the experiments were exposed to the same experimental conditions, we reversed the light/dark cycle for all the rats used in the study. During the 4 week adaptation period required to reverse the light/dark cycle we monitored wheel-running of the rats. Rats with an average voluntary running distance per day (24 h) of 1 km, monitored over this 4-week period were selected for experiments. After surgery for implantation of the radiotransmitters and guide cannuale, all animals were returned to their cages and the running wheels were locked for a period of seven days so that animals could not exercise. Thereafter, the wheels were unlocked and experimentation started 4 weeks post-surgery once rats had reached their pre-surgery daily running distances. During this post-surgery period the rats were habituated to handling and injection procedures. To confirm correct placement of the

guide cannula, two weeks before injections rats were injected i.c.v. with angiotensin II (10 ng/5µl; A-2900, Sigma, St. Louis, MO, USA) and then monitored for a drinking response for 30 min after the injection. Rats with a positive drinking response (>10 ml of water in 30-min) were used in the study.

For microinjections, a 30-gauge injection stylette (Plastics One, Roanoke, VA, USA), connected by polyethylene tubing (0.58 mm i.d., 1.27 mm o.d.) to a 50 µl Hamilton gas-tight microlitre syringe (Hamilton, Switzerland) was lowered into the guide cannula so that it protruded 0.5 mm beyond the tip of the guide cannula into the ventricle. Each microinjection was administered to freely moving rats over a period of 60 s. The injection stylette was left in the guide cannula for an additional 5 min to ensure dispersion of the injected substance within the ventricle. Separate groups of rats were used in experiment 1 and experiment 2 described below. However, rats used in both experiments all underwent the same adaptations, surgical and experimental procedures described in the paragraphs above.

Experiment 1: Analysis of physiological responses

Rats received LPS or saline, together with IL-6AS or PIS, together with the caspase-1 inhibitor or its vehicle. They were randomly assigned to receive one of the following combinations of injections: LPS + PIS + vehicle (n = 8), LPS + IL-6AS + vehicle (n = 7), LPS + PIS + caspase-1 inhibitor (n = 8), saline + PIS + vehicle (n = 7), saline + IL-6AS + vehicle (n = 6) or saline + PIS + caspase-1 inhibitor (n = 6). To control for any different effects the two control injections (vehicle i.c.v. and PIS i.p.) may have, we chose to

include both control injections for all experimental groups. The caspase-1 inhibitor/vehicle (i.c.v.) injections and IL-6AS/PIS (i.p.) injections were administered at 08:00, 2 h before the injections of LPS or saline (s.c.) at 10:00, the latter being 30 min before the onset of the dark phase when rats are most active. Wheel-running, food intake, body mass and body temperature were monitored for 72 h before and after the injections.

Experiment 2: Circulating cytokine responses

Rats were randomly assigned to receive one of the following combinations of injections: LPS + PIS + vehicle (n = 9), LPS + IL-6AS + vehicle (n = 8), LPS + PIS + caspase-1 inhibitor (n = 7) or saline + PIS + vehicle (n = 10). Having determined in experiment 1 that neither injections of saline (s.c.) + IL-6AS (i.p.) or saline (s.c.) + caspase-1 inhibitor (i.c.v.) affected any of the physiological responses we measured, we chose to include only one control group, saline + PIS + vehicle, for experiment 2. The caspase-1 inhibitor/vehicle (i.c.v.) injections and IL-6AS/PIS (i.p.) injections were administered at 08:00, 2 h before the injections of LPS or saline at 10:00, the latter being 30 min before the onset of the dark phase. Based on the observations made by others [10, 33] that the increase in IL-6 concentration in plasma following LPS administration peaks at approximately the same time as does the increase in body temperature, and our observation on the course of fever from experiment 1, we chose to collect plasma samples 5 h after the s.c. injection of LPS or saline. Thus, blood was collected by cardiac puncture after the injections of LPS or saline from rats under terminal anaesthesia induced with an i.m. injection of 80 mg/kg ketamine hydrochloride (Anaket-V, Bayer, SA) and 20 mg/kg xylazine (Chanazine, Bayer, SA). Blood was collected into sterile

tubes containing EDTA and centrifuged (5300 g, 4ºC, 10 min). Following the cardiac puncture rats were euthanized by intracardiac injection of 1 ml sodium pentobarbital (Euthanase; Kyron, Johannesburg, South Africa). Plasma was stored at -70ºC until assayed.

Correct placement of guide cannulae was verified post mortem by infusion of 5 µl of blue dye (Kyro-quick stain, Kyron Laboratories, SA) through the guide cannula assembly. After 5 min the brain was removed and placed on ice and the distribution of the ink within the ventricles was visually inspected in approximately 1-mm sections of the brain.

2.9 Data analysis

All data are expressed as mean ± SD. The body temperature responses were plotted as abdominal temperature-time curves in 60 minute intervals. For statistical purposes, the original 5-minute temperature recordings of each rat were averaged over 2 h for the 12-h period of darkness (10:30 - 22:30) and 12-h period of light (22:30 - 10:30). The two-hourly means were analyzed using two-way analysis of variance with intervention and time as main effects. A Student-Newman-Keul's (SNK) *post hoc* test was used to detect differences within and between groups when the ANOVA detected significant main effects or interactions. Running distance was determined from the number of wheel turns per 24-h period (10:30 - 10:30) and expressed as a percentage change from the mean running distance measured over 3 days before the injections. Food intake was calculated as grams of food consumed in 24 h per 100 gram of rat body mass (measured daily) and expressed as a percentage change from the mean daily food intake measured over 3 days

before the injections. Change in body mass was determined by subtracting the body mass measured on each of the 3 days after the injection from the body mass measured immediately prior to injection. The change in wheel-running, food consumption and body mass between experimental groups for each of the days after injection was analyzed using a one-way analysis of variance followed by a SNK *post hoc* test. To determine whether the change in wheel-running, food intake and body mass on each of the 3 days post injection was significantly different from pre-injection values, a one sample *t* test was performed for each of the experimental groups. Because the values for cytokine concentrations are not normally distributed, a log transformation of the cytokine values was performed before the data were analyzed using a one-way ANOVA followed by a SNK *post hoc* test.

3. Results

3.1 Experiment 1: Three-day analysis of physiological responses

In this experiment body temperature, voluntary-wheel running, food intake and body mass were monitored for 3 days after rats were treated according to the triple-injection protocol (i.p. and i.c.v. injections followed 2 h later with an s.c. injection) described in the methods section. For the sake of brevity, the six different experimental conditions will be identified in the results section below as follows:

LPS (250 μg/kg s.c.) + PIS (1.5 ml i.p.) + veh (5 μl i.c.v.) = LPS alone;

LPS (250 μg/kg s.c.) + IL-6AS (1.5 ml i.p.) + veh (5 μl i.c.v.) = LPS + IL-6AS;

LPS (250 μg/kg s.c.) + PIS (1.5 ml i.p.) + casp-inh (300 ng/5 μl i.c.v.) = LPS + casp-inh;

Saline (s.c.) + PIS (1.5 ml i.p.) + veh (5 μl i.c.v.) = saline alone;

Saline (s.c.) + IL-6AS (1.5 ml i.p.) + veh (5 μl i.c.v.) = saline + IL-6AS;

Saline (s.c.) + PIS (1.5 ml i.p.) + casp-inh (300 ng/5 μl i.c.v.) = saline + casp-inh.

Body temperature

Fig. 1 shows that s.c. injection of LPS induced a significant rise in body temperature after a latent period of ~ 3 h. The body temperature of rats injected with LPS peaked at 39.5 ± 0.3°C between 5 and 7 h after the injection and remained significantly elevated for 8 h during the first lights off period after injection in comparison to rats injected with saline (the main effects of time ($F_{(5,130)} = 39.2$, $P < 0.0001$), group ($F_{(3,26)} = 19.2$, $P < 0.0001$) and interaction ($F_{(15,130)} = 5.1$, $P < 0.0001$)). On day 1 the body temperature of rats injected with LPS remained elevated for the entire 12 h light period also,

compared to the body temperature of rats injected with saline (the main effects of time ($F_{(5,130)}$ = 14.4 , P < 0.0001), group ($F_{(3,26)}$ = 20.7 , P < 0.0001) and interaction ($F_{(15,130)}$ = 0.9 , P > 0.05)). With lights off at the end of day 1 the body temperatures of rats injected with LPS remained at a similar level to that during the lights on period on day 1, while the body temperatures of the rats that received saline continued to follow a circadian rhythm and increased sharply with lights off. As such there was no significant difference between the body temperatures of rats injected with saline and LPS over the lights off period on day 2. With lights on, on day 2, the body temperatures of rats injected with saline and LPS both decreased. However, the body temperatures of rats injected with LPS remained slightly elevated for most of the lights on period (6 h) compared to the body temperatures of rats injected with saline (the main effects of time ($F_{(5,130)}$ = 22.5 , P < 0.0001), group ($F_{(3,26)}$ = 9.1 , P < 0.001) and interaction ($F_{(15,130)}$ = 1.9 , P < 0.05). During day 3 after the injections the circadian rhythm of body temperature for rats injected with LPS and saline was similar.

Fig. 2A shows that pre-treating rats i.p. with IL-6AS completely abolished the LPS-induced fever (P < 0.01, SNK), such that the circadian rhythm of body temperature of rats injected with LPS + IL-6AS were similar to those of rats injected with saline alone over the entire 3 days after injection, with the exception of a tendency for body temperature to be slightly elevated during the lights on period on day 1 (P < 0.05, SNK). Fig. 2B shows that the presence of the IL-6AS on its own in the circulation had no effect on the circadian rhythm of body temperature of rats.

Fig. 3A shows that rats injected with LPS and i.c.v. with the caspase-1 inhibitor had significantly lower body temperatures compared to rats injected with LPS alone during the expected peak of the fever after injections ($P < 0.01$, SNK). Pre-treating rats i.c.v. with a caspase-1 inhibitor before LPS did not completely abolish the LPS-induced fever, because the body temperatures of rats injected with LPS + caspase-1 inhibitor were still significantly elevated compared to the body temperatures of rats injected with saline alone during the entire first day after injection ($P < 0.05$, SNK). Fig. 3B shows that the presence of the caspase-1 inhibitor on its own within the brain had no effect on the circadian rhythm of body temperature of rats.

Voluntary wheel-running

On average, rats ran between 1 and 4 km per day during the 3 days before the experimental interventions. Fig. 4 shows that injection of LPS decreased running activity which was most reduced (by 98.1 ± 2.4 %) during day 1 after the LPS injection. The suppression of running activity continued for at least 3 days, because rats injected with LPS remained less active on day 3 after the injection when compared to before they received LPS ($t = 3.3$, $P < 0.05$). Rats injected with saline alone also decreased their running distance significantly on day 1 after injection, but only to the extent of 20.1 ± 13.2 %, which was significantly less than the decrease in running activity observed in rats injected with LPS alone (one-way ANOVA, $F_{(3,26)} = 76.5$, $P < 0.0001$). From day 2 onwards rats injected with saline ran similar distances to those before the injections.

Figs. 4A and 4B show that pre-treating rats i.p. with IL-6AS or i.c.v. with a caspase-1 inhibitor significantly attenuated the LPS-induced suppression of running activity on day

1 after the injections ($P < 0.05$, SNK). Furthermore, reducing the biological activity of IL-6 in the circulation appeared to resolve the LPS-induced suppression of running activity faster, because from day 2 onwards rats injected with LPS + IL-6AS were running similar distances to their pre-injection values ($t = 0.4$, $P > 0.05$) and to rats injected with saline alone ($P > 0.05$, SNK). The LPS-induced suppression of running activity also appeared to be resolved faster in rats injected i.c.v. with the caspase-1 inhibitor, but only from day 3 onwards ($t = 2.1$, $p > 0.05$). The presence of the IL-6AS on its own in the circulation or the caspase-1 inhibitor on its own within the brain had no effect on running activity of the rats.

Food intake and body mass

On average, the daily food intake of the rats was between 7 and 10 g of food per 100 g of body mass during the 3 days before the experimental interventions. Figs. 5 and 6 show that the injection of LPS decreased food intake and body mass which was most reduced during day 1 after the LPS injection. Following the initial decrease in food intake rats gradually began to eat more food and regain the body mass lost such that by the end of day 3 their body mass was similar to that before the injections ($t = 1.3$, $P > 0.05$).

Figs. 5 and 6 show that pre-treating rats i.p. with IL-6AS or i.c.v. with the caspase-1 inhibitor significantly attenuated the LPS-induced anorexia (one-way ANOVA, $F_{(3,26)} = 42.2$, $P < 0.0001$) and the LPS-induced decrease in body mass (one-way ANOVA, $F_{(3,26)} = 48.7$, $P < 0.001$) on day 1 after the injections. Furthermore, the presence of IL-6 antibodies in the circulation or the caspase-1 inhibitor within the brain appeared to

resolve the LPS-induced anorexia faster, because from day 2 onwards rats injected with LPS + IL-6AS ($t = 0.6$, $P > 0.05$) or LPS + caspase-1 inhibitor ($t = 0.9$, $P > 0.05$) were consuming similar amounts of food to their pre-injection values and to rats injected with saline alone ($P > 0.05$, SNK). In line with the increased food intake the LPS-induced stunting of growth also resolved faster, because rats injected with LPS + IL-6AS ($t = 2.2$, $P > 0.05$) or LPS + caspase-1 inhibitor ($t = 3.6$, $P < 0.01$) had regained the body mass lost on day 1 after injections by the end of day 2, and continued to gain weight similarly compared to rats injected with saline alone ($P > 0.05$, SNK). The presence of the IL-6AS on its own in the circulation or the caspase-1 inhibitor on its own within the brain had no effect on food intake and growth of the rats.

3.2 Experiment 2: Circulating cytokine responses

In this experiment circulating levels of bioactive IL-6 and concentrations of IL-1β were measured in the plasma 5 h after rats were treated according to the triple-injection protocol (i.c.v. and i.p. injections followed 2 hours later with an s.c. injection) described in the methods section. The different experimental conditions will be identified in the results section below as they were described for experiment 1 on page 18.

Levels of bioactive IL-6 in plasma

Fig. 7 shows that rats injected with LPS s.c. had a significant elevation in the biological activity of IL-6 in plasma compared to rats injected with saline alone (one-way ANOVA, $F_{(3,31)} = 119.0$, $P < 0.0001$). Pre-treating rats i.p. with IL-6AS significantly attenuated the LPS-induced increase in biological activity of IL-6 in the plasma ($P < 0.001$, SNK).

However, it did not completely abolish the LPS-induced increase in plasma IL-6, because rats injected with LPS + IL-6AS had greater levels of plasma IL-6 than rats injected with saline alone ($P < 0.001$, SNK). Pre-treating rats i.c.v. with the caspase-1 inhibitor had no significant effect on the biological activity of IL-6 in the plasma ($P > 0.05$, SNK).

Concentration of IL-1β in plasma

Fig. 8 shows that rats injected with LPS s.c. had a significant increase in plasma IL-1β concentration compared to rats injected with saline alone (one-way ANOVA, $F_{(2,24)} = 5.3$, $P < 0.05$). Pre-treating rats i.c.v. with the caspase-1 inhibitor had no effect on the LPS-induced increase of IL-1β in plasma ($P > 0.05$, SNK).

4. Discussion

We have shown that subcutaneous administration of the pyrogenic moiety of the Gram-negative bacterial LPS (250 μg/kg), induces fever, lethargy and anorexia in rats. The fever however, which resolved within 2 days (Fig. 1), is outlasted by both the period of lethargy and anorexia (Fig. 4 and 5 respectively). In line with the LPS-induced anorexia continuing for at least 3 days, the growth of the rats also was significantly stunted (Fig. 6). Our study highlights several important findings regarding the role of cytokines released in the periphery and the brain in mediating the induction and duration of anorexia and lethargy induced in rats following subcutaneous administration of LPS. First, we have shown that endogenous antagonism of peripherally-released IL-6 or IL-1β in the brain significantly attenuated both the magnitude and the duration of anorexia induced by LPS administration (Figs. 5 and 6). We further showed that endogenous antagonism of peripherally-released IL-6 or IL-1β in the brain significantly reduced primarily the duration of lethargy induced by LPS administration (Figs. 4). Thus, we have identified that both peripherally-released IL-6 and IL-1β in the brain appear to be important mediators involved in the induction and maintenance of LPS-induced sickness behaviors. Moreover, we also identified that the roles of these two cytokines in the anorexia and lethargy induced by LPS is however, different from the roles they appear to have in inducing fever irrespective of the degree to which each was antagonized or inhibited; fever as a result of systemic administration of LPS is completely abolished in rats pre-treated i.p. with IL-6AS (Fig. 2), while it is only partially attenuated in rats pre-treated i.c.v. with a caspase-1 inhibitor (Fig. 3).

Our finding that reducing the biological activity of IL-6 in the circulation completely abolishes fever induced by LPS administration supports the consistent observations made within our own laboratory and in others, that while other cytokines may be involved, IL-6 appears to be the primary endogenous pyrogen mediating the fever response [10, 11, 16, 26]. We also have confirmed our previous finding that peripherally released IL-6 appears to contribute significantly to the mediation of LPS-induced anorexia [16]. The involvement of peripherally released IL-6 in mediating the anorexia induced by infectious agents has also been suggested previously following the finding that the decrease in food consumption observed in humans administered endotoxin is positively correlated with the secretion of IL-6 [44]. In the hands of others using a different experimental approach to investigate the involvement of IL-6 in sickness behavior, that being congenic IL-6 knockout mice which are deficient in IL-6 production, it was demonstrated, as in our study, that the absence of IL-6 also attenuates the loss of body mass induced by LPS administration [4]. The absence of IL-6 therefore appears to enable rats and mice to resist the anorexia and accompanying loss of body mass induced by systemic and local administration of LPS.

Our results showing that treating rats with serum containing IL-6 antibodies enables them to recover faster from the suppressive effects of LPS on voluntary activity, demonstrates that peripherally-released IL-6 is involved in mediating the duration of fatigue and lethargy induced in rats following LPS administration. In support of the hypothesis that IL-6 drives fatigue experienced by patients during illness are the findings that administration of human recombinant IL-6 induces a sensation of fatigue in healthy

humans at rest [52] and fatigue in patients with cancer is positively correlated with circulating levels of IL-6 [47]. Moreover, in an intervention in patients similar to ours experimentally in rats, in which a group of patients with multicentric Castleman disease, a disease characterized by a dysregulated overproduction of IL-6, were treated with IL-6 antibodies, the previously debilitating fatigue reported by these patients disappeared [36, 37]. Fatigue experienced by patients during illness results in a decrease in daily activity, probably not unlike the decrease in voluntary activity in the rats of our study.

Although we have identified that IL-6 released into the bloodstream from peripherally located immune cells appears to be involved in mediating lethargy, anorexia and fever during infection, our finding that reducing the biological activity of IL-6 in the circulation at most attenuates the behavioral responses to LPS but does not completely abolish them, highlights differences in the cytokine mechanisms mediating LPS-induced fever and LPS-induced anorexia and lethargy. There are two possible explanations for the differences we observed in the initial degree of attenuation, complete for the fever response versus partial for the anorexia and lethargy. Firstly, although we reduced the level of bioactive IL-6 in the circulation substantially (~ 80 %, see Fig 7) we did not completely neutralize it and there may have been sufficient IL-6 still to induce the anorexia and lethargy we observed on the first day after injection. Secondly, a more likely possibility is that mediators in addition to IL-6, possibly other cytokines, are also involved in regulating the LPS-induced anorexia and lethargy. Antagonizing the biological action of IL-1β in the brain by injecting a caspase-1 inhibitor, which prevents the cleavage of pro-IL-1β to biologically active IL-1β, we were able to identify that IL-1β

in the brain also is likely to be involved in mediating the anorexia and lethargy induced by LPS administration.

Our results showing that treating rats with a capsase-1 inhibitor enables them to recover faster from the suppressive effects of LPS on voluntary activity demonstrate a possible role for IL-1β within the brain in mediating the duration of fatigue and lethargy induced by LPS. In support of this finding is a previously reported observation from our laboratory that centrally administered IL-1β induces a dose-dependent decrease in voluntary activity [17]. Moreover, there is some preliminary evidence that supports the involvement of IL-1β in mediating the fatigue experienced by patients during illness, because administering IL-1ra to patients with rheumatoid arthritis induces rapid and persistent improvements in fatigue scores [38]. Not only did treating rats centrally with a caspase-1 inhibitor enable them to recover faster from the suppressive effects of LPS on voluntary activity, but so too on food intake and growth. Moreover, i.c.v. administration of the caspase-1 inhibitor attenuated the LPS-induced fever also.

Using a different agent, IL-1ra, to antagonize the biological action of IL-1β in the brain, others also have investigated the involvement of endogenous brain IL-1β in mediating fever and sickness behavior induced by i.p. administration of LPS [3, 8, 25, 30, 33, 34]. Although these studies have consistently shown that inhibition of endogenous brain IL-1β attenuates LPS-induced fever, unlike the findings in our study, they mostly have failed to show that it also can attenuate LPS-induced sickness behaviors. The failure of i.c.v administration of IL-1ra to attenuate LPS-induced sickness behaviors may be related to

IL-1ra being active for a significantly shorter period of time (2 -4 h) [3] in comparison to the prolonged duration (8 – 24 h) for which endogenous IL-1β is elevated in the brain [2]. The actions of brain-intrinsic cytokines, produced after the production of cytokines in the periphery, are generally believed to be more important in mediating sickness behaviors than fever during infection/inflammation [12]. Thus while the short duration of action of IL-1ra may be sufficient to inhibit the action of endogenous brain IL-1β in mediating fever, it may not be sufficient to significantly influence the prolonged sickness behaviors. The caspase-1 inhibitor used in our study is active for at least 24 h [41], therefore the longer period of brain IL-1β inhibition likely to be achieved with the caspase-1 inhibitor may have accounted for the reduction in the duration of the sickness behavior responses noted in our study.

While it has been established that administration of a caspase-1 inhibitor effectively reduces IL-1β activity *in vivo* following systemic administration of LPS [2], we did not measure the concentration of IL-1β in the brain and therefore cannot confirm the extent to which i.c.v. administration of the caspase-1 inhibitor inhibited the synthesis of IL-1β in the brain. It is therefore possible that if IL-1β was not completely inhibited by the dose of the caspase-1 inhibitor we injected, the contribution of endogenous brain IL-1β to mediating the anorexia and lethargy induced by s.c. administration of LPS we noted may be of a greater magnitude. It also is possible that the responses we noted, particularly for food intake and body mass, following i.c.v. administration of the caspase-1 inhibitor may not be entirely specific to the action of IL-1β, but may also be related to reducing the action of other pro-inflammatory cytokines implicated as putative mediators of appetite

such as IL-18 [57], because the enzyme caspase-1 is not only involved in the processing of IL-1β, but is also involved in the processing of IL-18 and IL-33 [14]. Irrespective of whether the synthesis of other cytokines were affected within the brain, the established effectiveness of the caspase-1 inhibitor at reducing endogenous IL-1β [2], indicates that a significant portion of the responses we noted are related to inhibiting the action of IL-1β.

How endogenous brain IL-1β influences the brain sites controlling appetite, activity and temperature during a systemic infection remains unclear however. Studies investigating the interaction of IL-1β with hypothalamic mechanisms involved in the regulation of feeding [40] and temperature [7, 49] provide evidence for the possibility of both direct (via neuronal mechanisms) and indirect (via modulation of brain chemistry) actions. In terms of the indirect actions, IL-1β can modulate hypothalamic chemistry by generating mediators such as prostaglandins, and by interacting with various neurotransmitters (e.g. serotonin) and peptides (e.g. neuropeptide Y) within the brain [9, 28]. The generation of proximal mediators such as prostaglandins and serotonin within the brain and the endothelial cells lining the vasculature throughout the brain, are also possible mechanisms by which IL-6 released into the bloodstream from peripherally located immune cells mediates fever and sickness behaviors during a systemic infection [15, 21, 46, 50]. In addition to inducing these proximal mediators it also is likely that peripherally-released IL-6 could be mediating lethargy, anorexia and fever via the induction of IL-1β in the brain, as others have reported that in the absence of IL-6 i.p. administration of LPS failed to induce IL-1β in the brain [51].

Although the results from our study clearly identify IL-6 as an important humoral mediator by which inflammatory challenges in the periphery can communicate with the brain to induce lethargy, anorexia and fever, they may not hold true under some experimental conditions. For example, injecting a lower does of LPS s.c. than the dose we used in our study (250 μg/kg) may induce a more localized response with significantly lower concentrations of IL-6 appearing in the blood. Under these conditions other signaling pathways, possibly afferent neural pathways from the skin to the central nervous system, are likely to be more important than humoral pathways in mediating fever and sickness behavior [45]. As activation of neural pathways during a peripheral immune challenge has been related to increased expression of IL-1β mRNA in the brain of mice [29] it is likely that brain IL-1β may remain an important endogenous mediator of fever and sickness behaviors induced during localized infection/inflammation.

In conclusion, we have shown that pre-treating rats with either antibodies to IL-6 i.p. or a caspase-1 inhibitor i.c.v. significantly enhanced the resolution of anorexia and lethargy by at least two days, but it did not completely prevent these sickness behaviors from occurring. Our inability to abolish the anorexia and lethargy with antagonism of individual cytokines appears to indicate the complexity of the cytokine involvement in mediating these two sickness behaviors during a localized Gram-negative bacterial infection. Using similar experimental tools, others [54] have also noted this trend of multiple cytokine involvement in mediating sickness behaviors during a systemic Gram-negative bacterial infection. Therefore while individual cytokines, such as IL-6 and IL-1β, are possible targets for therapies aimed at alleviating the debilitating consequences of

anorexia and lethargy in patients with prolonged bacterial infections, it appears that to abolish the presence of sickness behaviors multiple cytokines may need to be targeted. Although inhibiting the action of cytokines may provide some relief for patients experiencing sickness symptoms, this action could possibly compromise the ability of the host to fight infection in the situation of a live, replicating pathogen, as pro-inflammatory cytokines also perform important immune functions which facilitate pathogen elimination. Selectively inhibiting the downstream mediators by which cytokines induce sickness behaviors may prove to be a better tool therapeutically, as that could preserve the actions of pro-inflammatory mediators required for pathogen elimination yet improve the anorexia and fatigue in the patient.

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Legends

Figure 1. Circadian rhythms of body temperature over approximately 3 days (separated by vertical ----) after rats were injected i.p. with pre-immune serum (PIS 1.5 ml) and i.c.v. with the vehicle 2 h before receiving the s.c. injections of LPS (250 μg/kg) or saline 30 min before lights off on the first day. $\overline{}^{*}$ Indicates the period during which the body temperature of rats injected with LPS was significantly greater than the body temperature of rats injected with saline ($P < 0.01$, SNK). The results are represented as mean ± SD. The first arrow indicates the time of injection for the i.p. and i.c.v. injections and the second arrow indicates the time of injection for the s.c. injections. The black bars indicate lights off (10:30 - 22:30 clock time).

Figure 2. Circadian rhythms of body temperature over approximately 3 days after rats were injected with: (A) IL-6AS (1.5 ml) or PIS (1.5 ml) i.p. and the vehicle for the caspase-1 injection i.c.v. 2 h before receiving s.c. injections of LPS (250 μg/kg); (B) IL-6AS or PIS and the vehicle for the caspase-1 injection i.c.v. 2 h before receiving s.c. injections of saline. In A the effects of saline + PIS + veh also is shown and is the same as the results shown in B. $\overline{}^{*}$ Indicates the period during which the body temperature of rats injected with LPS + PIS + veh was significantly greater than the body temperature of rats injected with LPS + IL-6AS + veh ($P < 0.01$, SNK). $\overline{}^{\#}$ Indicates the period during which the body temperature of rats injected with LPS + IL-6AS + veh was significantly greater than the body temperature of rats injected with saline + PIS + veh ($P < 0.05$, SNK). The results are represented as mean ± SD. The first arrow indicates the time of

injection for the i.p. and i.c.v. injections and the second arrow indicates the time of injection for the s.c. injections. The black bars indicate lights off (10:30 - 22:30 clock time).

Figure 3. Circadian rhythms of body temperature over approximately 3 days after rats were injected with: (A) PIS (1.5 ml) i.p. and the caspase-1 inhibitor (300 ng/5μl) or its vehicle i.c.v. 2 h before receiving s.c. injections of LPS (250 μg/kg); (B) PIS i.p. and the caspase-1 inhibitor (300 ng/5μl) or its vehicle i.c.v. 2 h before receiving s.c. injections of saline. In A the effects of saline + PIS + veh also is shown and is the same as the results shown in B. $\overline{\ ^{*}\ }$ Indicates the period during which the body temperature of rats injected with LPS + PIS + veh was significantly greater than the body temperature of rats injected with LPS + PIS + casp-inh ($P < 0.01$, SNK). $\overline{\ ^{\#}\ }$ Indicates the period during which the body temperature of rats injected with LPS + PIS + casp-inh was significantly greater than the body temperature of rats injected with saline + PIS + veh ($P < 0.05$, SNK). The results are represented as mean ± SD. The first arrow indicates the time of injection for the i.p. and i.c.v. injections and the second arrow indicates the time of injection for the s.c. injections. The black bars indicate lights off (10:30 - 22:30 clock time).

Figure 4. Percentage change from pre-injection days, in daily wheel-running distance determined from the number of wheel turns measured during 3 days after rats were injected with: (A) PIS (1.5 ml) or IL-6AS (1.5 ml) i.p. and the vehicle for the caspase-1 injection i.c.v. 2 h before receiving a s.c. injection of LPS (250 μg/kg) or saline; (B) PIS i.p. and the caspase-1 inhibitor (300 ng/5 μl) or its vehicle i.c.v. 2 h before receiving a s.c.

injection of LPS or saline. * Indicates percentage change in running distances are significantly different to pre-injection days' running distances ($P < 0.05$). $^{\#}$ Indicates significant differences between rats injected with LPS + PIS + veh versus all other experimental groups ($P < 0.05$, SNK). $^{+}$ Indicates significant differences between rats injected with saline + PIS + veh versus rats injected with LPS + IL-6AS + veh and LPS + PIS + casp-inh on the first day ($P < 0.001$, SNK). There were no significant differences between rats injected with saline + PIS + veh, saline + IL-6AS + veh and saline + PIS + casp-inh. The results are represented as mean ± SD.

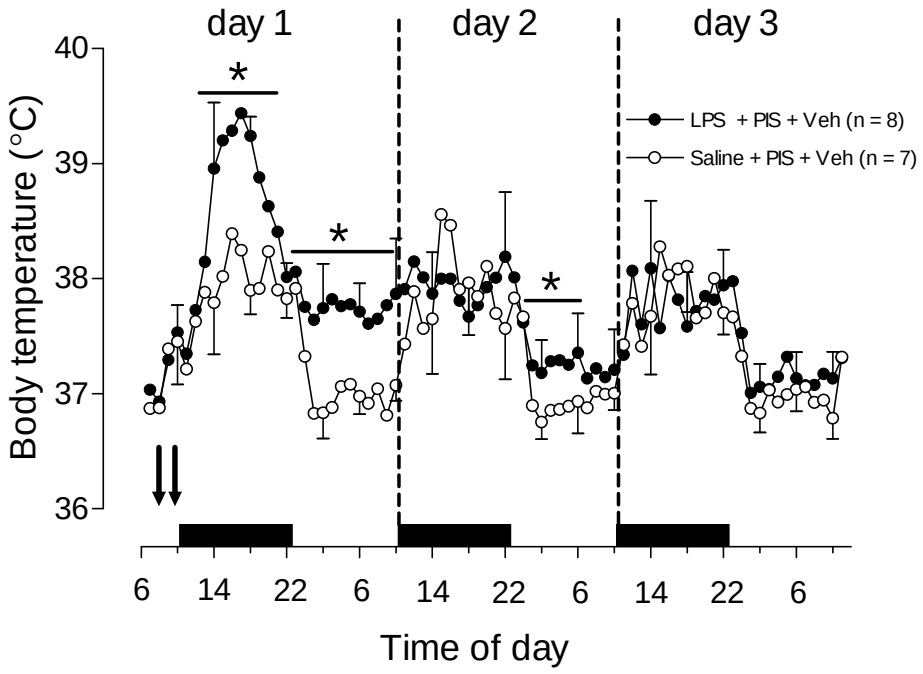
Figure 5. Percentage change from pre-injection days, in daily food intake calculated as grams of food consumed in 24 h per 100 gram of rat body mass, during 3 days after rats were injected with: (A) PIS (1.5 ml) or IL-6AS (1.5 ml) i.p. and the vehicle for the caspase-1 injection i.c.v. 2 h before receiving a s.c. injection of LPS (250 μg/kg) or saline; (B) PIS i.p. and the caspase-1 inhibitor (300 ng/5 μl) or its vehicle i.c.v. and 2 h before receiving a s.c. injection of LPS or saline. * Indicates percentage change in food intake is significantly different to pre-injection food intake ($P < 0.05$). $^{\#}$ Indicates significant differences between rats injected with LPS + PIS + veh versus all other experimental groups ($P < 0.05$, SNK). $^{+}$ Indicates significant differences between rats injected with saline + PIS + veh versus rats injected with LPS + IL-6AS + veh and LPS + PIS + casp-inh on the first day ($P < 0.05$, SNK). There were no significant differences between rats injected with saline + PIS + veh, saline + IL-6AS + veh and saline + PIS + casp-inh. The results are represented as mean ± SD.

Figure 6. Change in body mass from pre-injection values on each of the 3 days after rats were injected with: (A) PIS (1.5 ml) or IL-6AS (1.5 ml) i.p. and the vehicle for the caspase-1 injection i.c.v. 2 h before receiving a s.c. injection of LPS (250 μg/kg) or saline; (B) PIS i.p. and the caspase-1 inhibitor (300 ng/5 μl) or its vehicle i.c.v. 2 h before receiving a s.c. injection of LPS or saline. * Indicates a significant change in body mass compared to pre-injection values ($P < 0.05$). $^{\#}$ Indicates significant differences between rats injected with LPS + PIS + veh versus all other experimental groups ($P < 0.05$, SNK). $^{+}$ Indicates significant differences between rats injected with saline + PIS + veh versus rats injected with LPS + IL-6AS + veh and LPS + PIS + casp-inh on the first day ($P < 0.001$, SNK). There were no significant differences between rats injected with saline + PIS + veh, saline + IL-6AS + veh and saline + PIS + casp-inh. The results are represented as mean ± SD.

Figure 7. Plasma concentrations of bioactive IL-6 for rats injected i.p. with PIS (1.5 ml) or IL-6AS (1.5 ml) and i.c.v. with a caspase-1 inhibitor (300 ng/5 μl) or its vehicle 2 h before receiving s.c. injections of LPS (250 μg/kg) or saline. The plasma concentrations of bioactive IL-6 were measured 5 h after rats were injected with LPS or saline. * Indicates significant differences between rats injected with LPS + IL-6AS + veh versus all other experimental groups ($P < 0.001$, SNK). $^{\#}$ Indicates significant differences between rats injected with saline + PIS + veh versus all other experimental groups ($P < 0.001$, SNK). The detection limit of the assay was 3 IU of IL6 ml^{-1}. The results are represented as mean ± SD.

Figure 8. Plasma concentrations of IL-1β for rats injected with PIS (1.5 ml) i.p. and the caspase-1 inhibitor (300 ng/5 μl) or its vehicle 2 h before receiving s.c. injections of LPS (250 μg/kg) or saline. The plasma concentrations of IL-1β were measured 5 h after rats were injected with LPS or saline. * Indicates significant differences between rats injected with saline + PIS + veh versus all other experimental groups ($P < 0.05$, SNK). The results are represented as mean ± SD. Dashed line indicates level of detection of assay.

Fig. 1


day 1
day 2
day 3
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39
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36
Body temperature (°C)
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LPS + PIS + Veh (n = 8)
Saline + PIS + Veh (n = 7)
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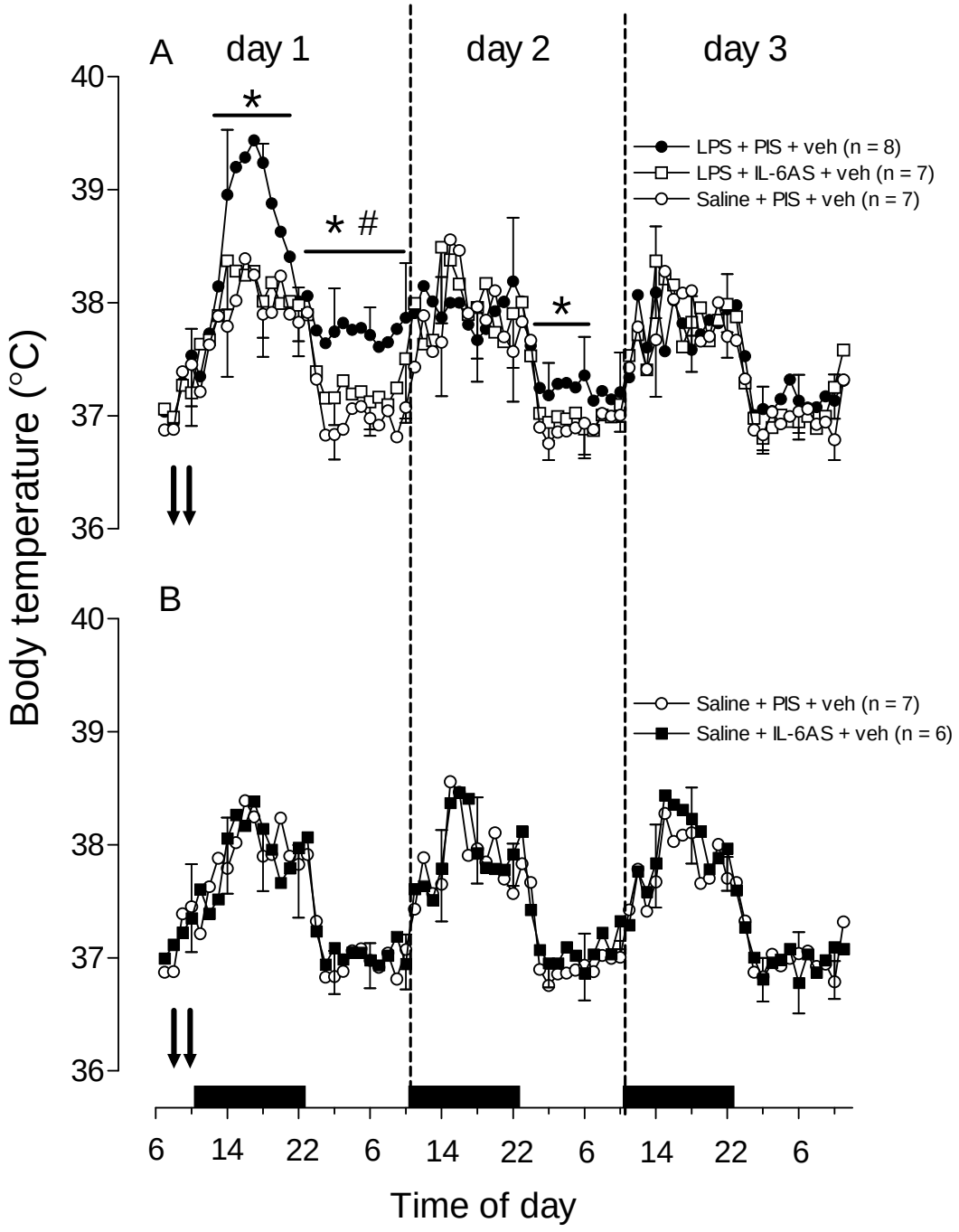
6
Time of day

Fig. 2


A
day 1
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LPS + PIS + veh (n = 8)
LPS + IL-6AS + veh (n = 7)
Saline + PIS + veh (n = 7)
B
Saline + PIS + veh (n = 7)
Saline + IL-6AS + veh (n = 6)
Body temperature (°C)
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22
Time of day

Fig. 3

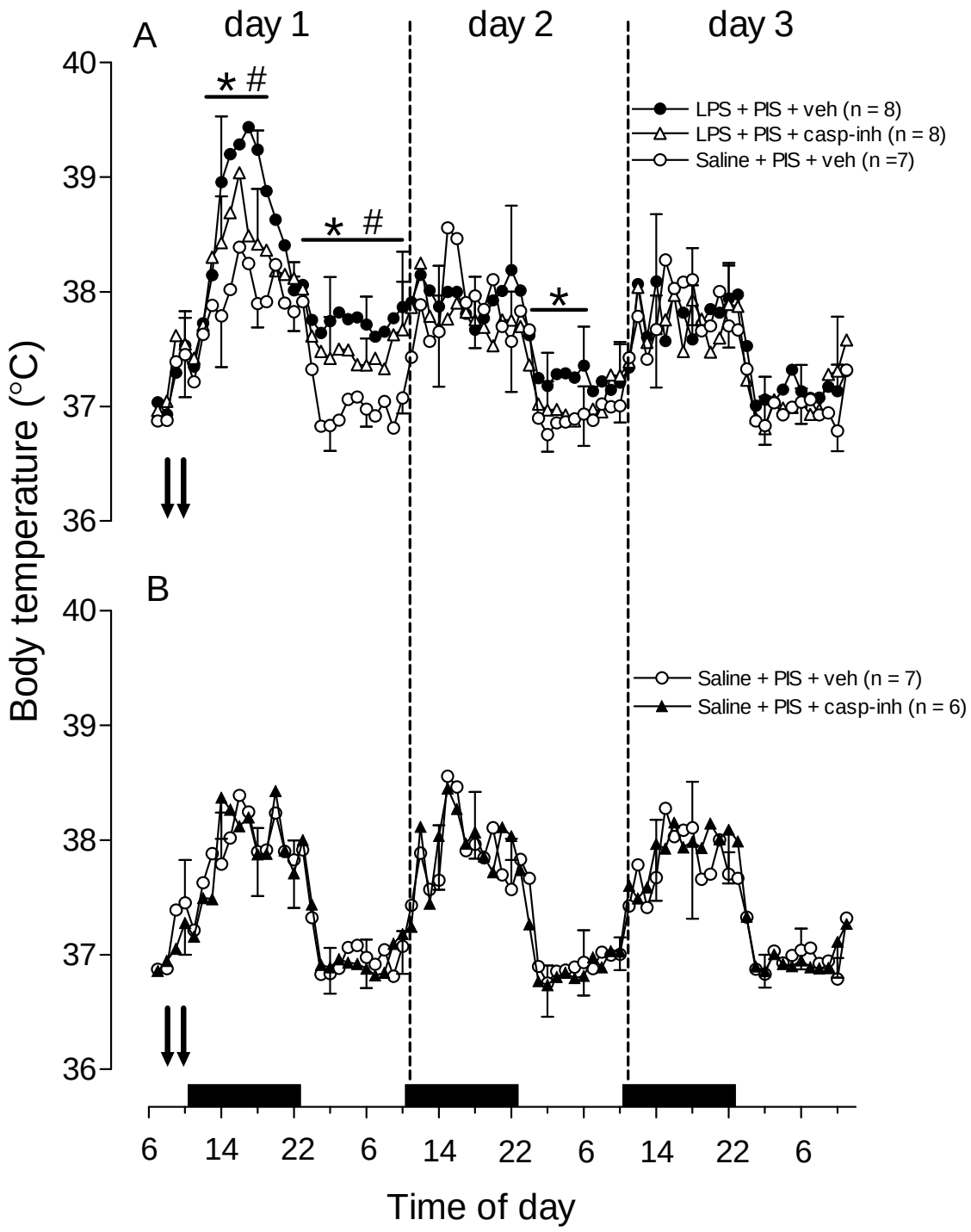

A
day 1
day 2
day 3
40
39
38
37
36
* #
* #
*
LPS + PIS + veh (n = 8)
LPS + PIS + casp-inh (n = 8)
Saline + PIS + veh (n =7)
B
40
39
38
37
36
Saline + PIS + veh (n = 7)
Saline + PIS + casp-inh (n = 6)
Body temperature (°C)
6
14
22
6
14
22
6
14
22
6
Time of day

Fig. 4

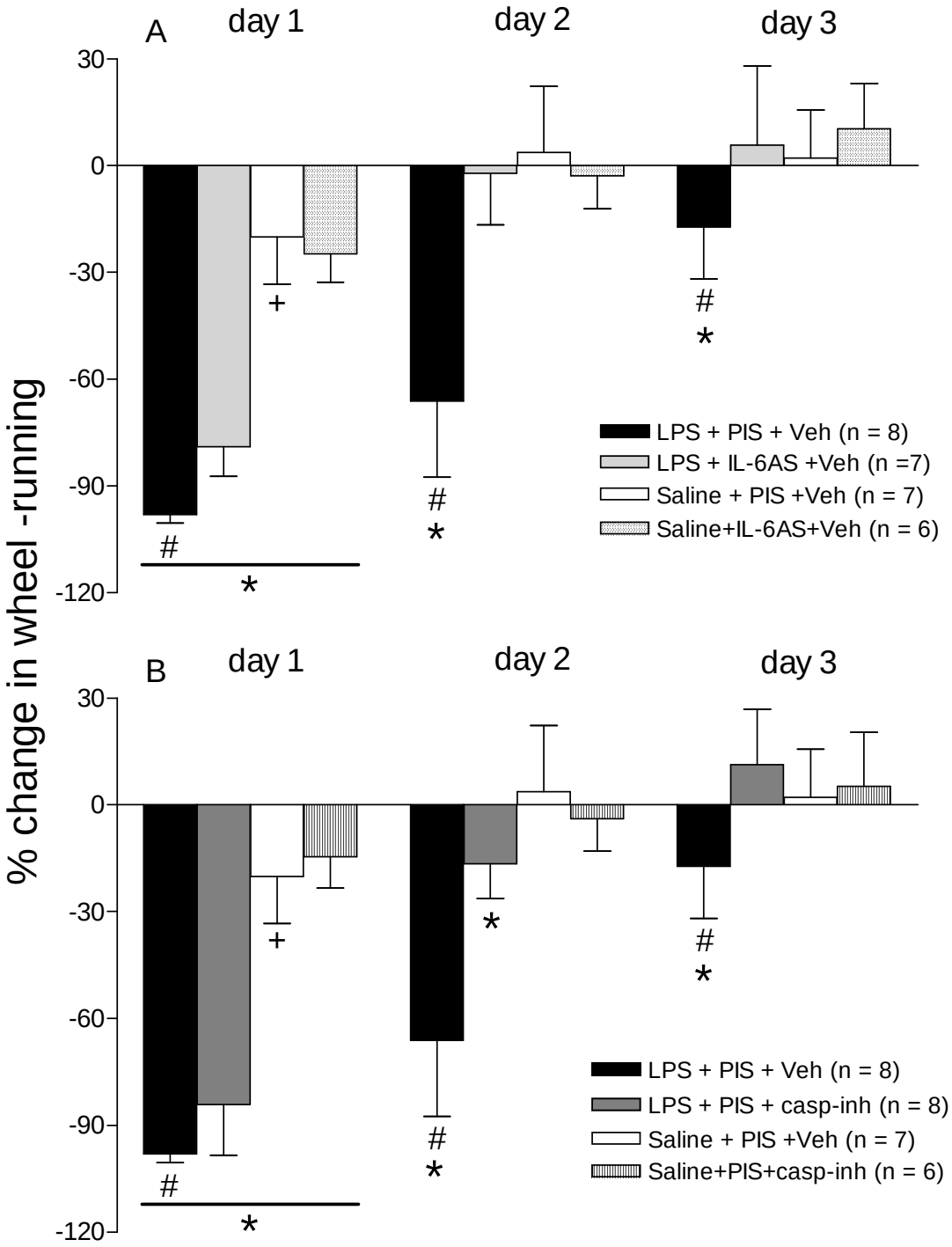

A
day 1
day 2
day 3
30
0
-30
-60
-90
-120
% change in wheel -running
+
#
*
LPS + PIS + Veh (n = 8)
LPS + IL-6AS +Veh (n =7)
Saline + PIS +Veh (n = 7)
Saline+IL-6AS+Veh (n = 6)
B
day 1
day 2
day 3
LPS + PIS + Veh (n = 8)
LPS + PIS + casp-inh (n = 8)
Saline + PIS +Veh (n = 7)
Saline+PIS+casp-inh (n = 6)

Fig. 5

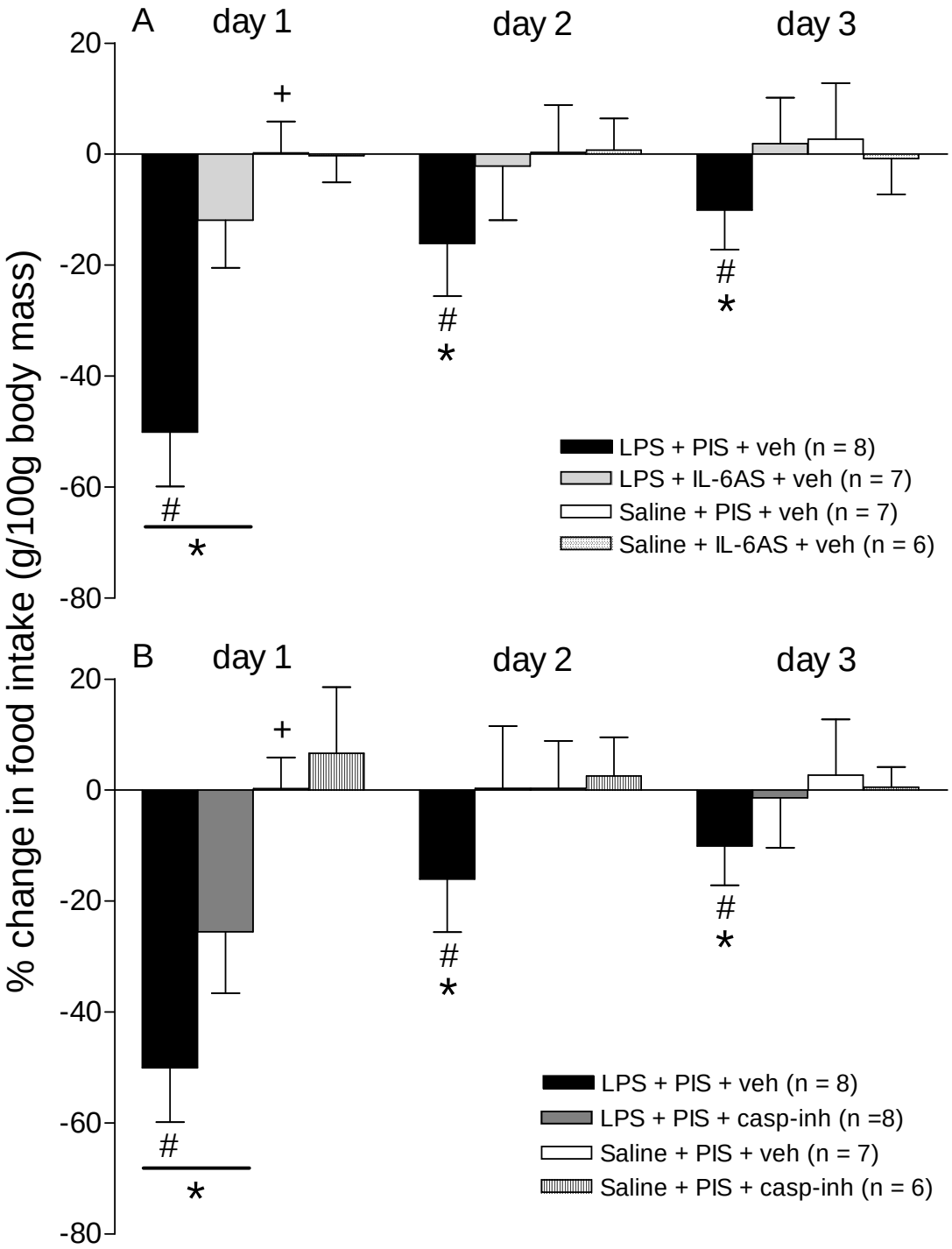

A
day 1
day 2
day 3
20
0
-20
-40
-60
-80
% change in food intake (g/100g body mass)
+
#
*
LPS + PIS + veh (n = 8)
LPS + IL-6AS + veh (n = 7)
Saline + PIS + veh (n = 7)
Saline + IL-6AS + veh (n = 6)
B
day 1
day 2
day 3
LPS + PIS + veh (n = 8)
LPS + PIS + casp-inh (n =8)
Saline + PIS + veh (n = 7)
Saline + PIS + casp-inh (n = 6)

Fig. 6

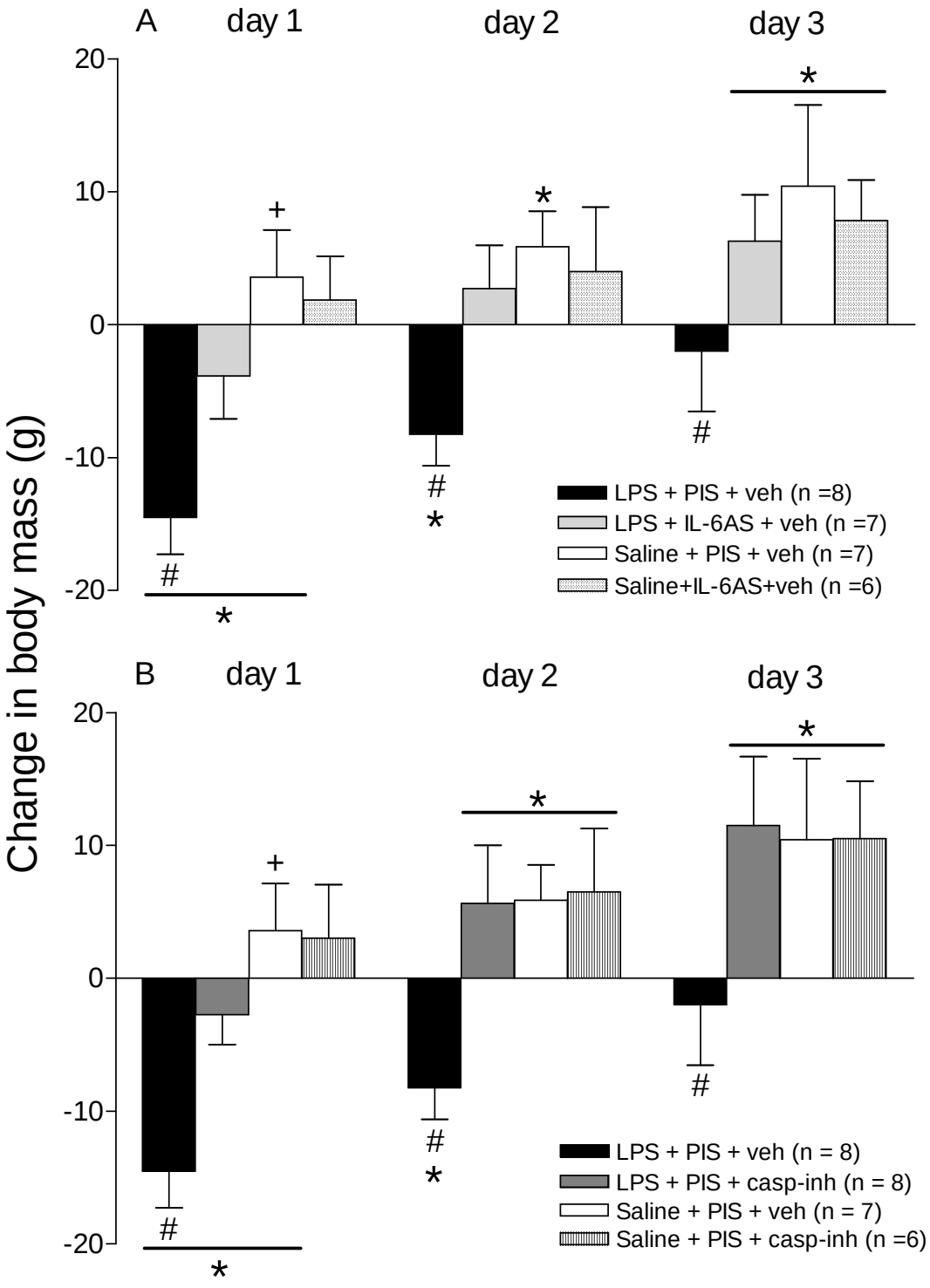

A
day 1
day 2
day 3
20
10
0
-10
-20
Change in body mass (g)
+
*
#
*
#
*
#
*
LPS + PIS + veh (n =8)
LPS + IL-6AS + veh (n =7)
Saline + PIS + veh (n =7)
Saline+IL-6AS+veh (n =6)
B
day 1
day 2
day 3
20
10
0
-10
-20
+
*
#
*
#
*
#
*
LPS + PIS + veh (n = 8)
LPS + PIS + casp-inh (n = 8)
Saline + PIS + veh (n = 7)
Saline + PIS + casp-inh (n =6)

Fig. 7

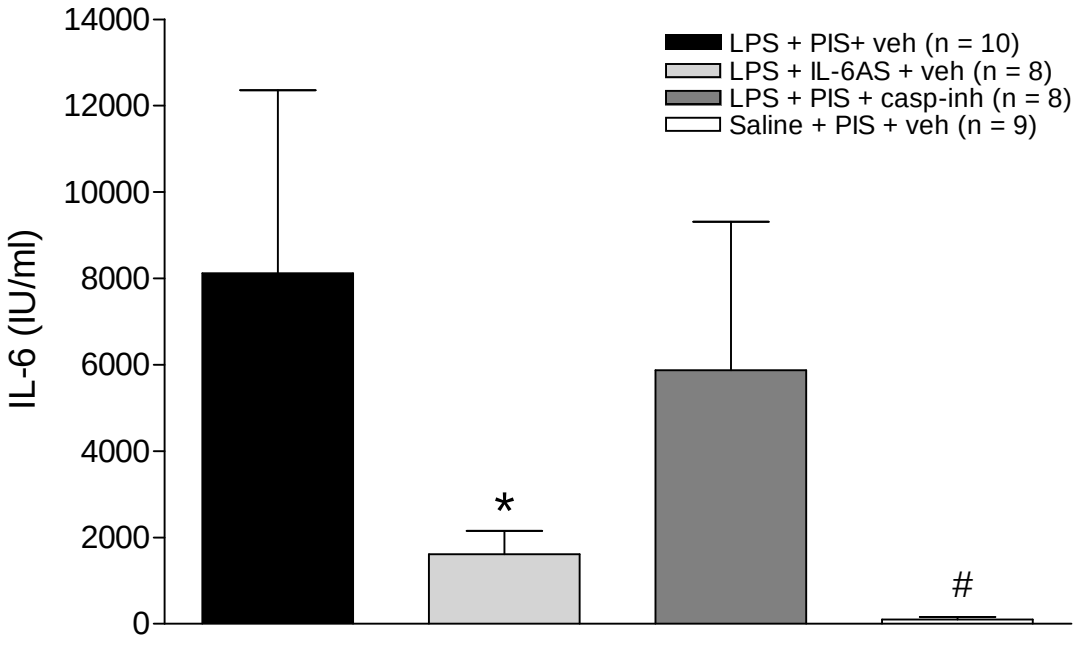
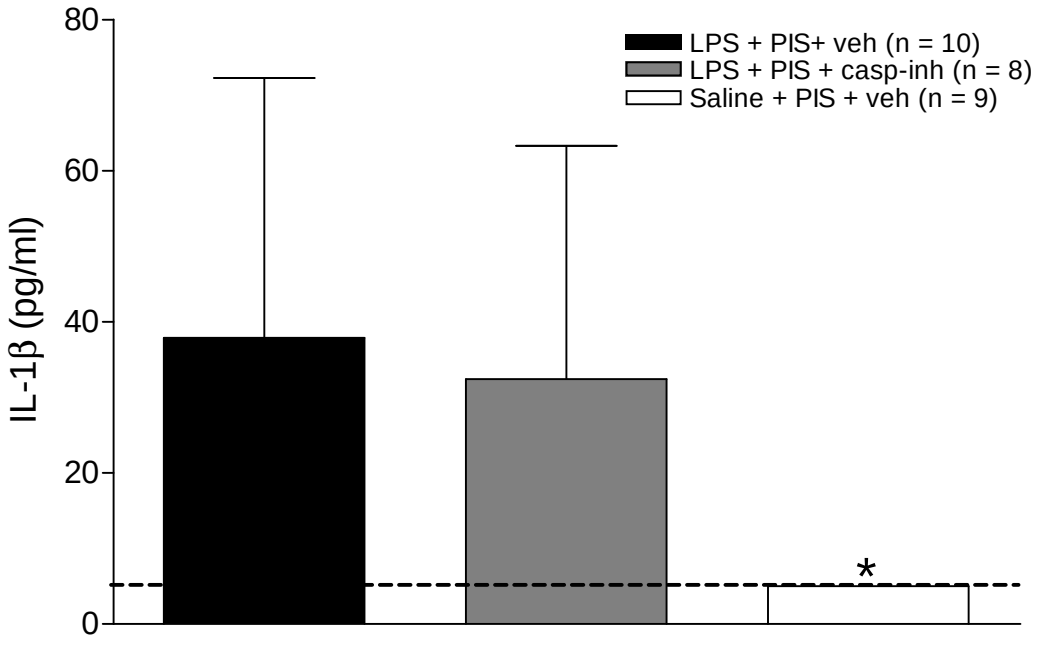

14000
12000
10000
8000
6000
4000
2000
0
IL-6 (IU/ml)
LPS + PIS+ veh (n = 10)
LPS + IL-6AS + veh (n = 8)
LPS + PIS + casp-inh (n = 8)
Saline + PIS + veh (n = 9)
*
#

Fig. 8


80
60
40
20
0
IL-1β (pg/ml)
LPS + PIS+ veh (n = 10)
LPS + PIS + casp-inh (n = 8)
Saline + PIS + veh (n = 9)
*

CHAPTER 5

CONCLUSIONS

Peripherally-released cytokines have been strongly implicated as important endogenous mediators of the brain-controlled fever mechanism activated following systemic and local administration of LPS. In contrast to the important role established for peripherally-released cytokines in mediating LPS-induced fever, no clear role has emerged as yet for these cytokines as endogenous mediators of anorexia and lethargy, two brain-controlled sickness behaviours, also known to be induced following systemic and local administration of LPS. Using endogenous antagonism of the biological action of cytokines as far as possible, and highly quantifiable measures of anorexia and lethargy, I therefore systematically investigated the contribution of endogenous pyrogenic cytokines in mediating anorexia and lethargy induced by local subcutaneous administration of LPS. I specifically chose to simulate infection using LPS (extracted from *Escherichia coli*) as it a powerful activator of the innate immune system and pro-inflammatory cytokine release, and understanding the physiological mechanism of its biological action has significant clinical application, as Gram-negative sepsis in humans is caused by Enterobacteriaceae such as *Escherichia coli* and *Klebsiella* species (Bochud & Calandra, 2003). The results obtained from the studies I undertook are summarized and discussed below in the context of the current literature.

5.1 The contribution of cytokines released in the periphery and the brain in mediating anorexia and lethargy induced by subcutaneous administration of LPS

To establish a role for molecules, such as cytokines, as endogenous mediators of a given biological response it is imperative that inhibition of the biological action or synthesis of the molecule at a site that relates to its observed synthesis and action, is shown to inhibit or abolish the biological response in question (Kluger, 1991). Injecting species-specific neutralizing antibodies to therefore effect endogenous antagonism, I investigated in chapter 2 whether peripherally-released IL-6, IL-1β, TNF-α and leptin, cytokines shown to be endogenous mediators of LPS-induced fever, also contribute in mediating two specific sickness behaviours, anorexia and lethargy in rats. I used highly quantifiable and sensitive measures, quantity of food consumed and voluntary wheel-running, to assess anorexia and lethargy, in the rats.

I found that injecting rats s.c. with LPS (250 μg kg^{-1}) before the night-time active period, dramatically affected their activity levels, as most of the rats did not run at all on the night after injection. Food intake was also affected following the LPS injection, but to a lesser extent than voluntary activity, with the rats reducing the quantity of food they normally consumed by about half (Chapter 2). Treating rats peripherally with species-specific IL-6 antiserum significantly attenuated the LPS-induced decrease in food intake and voluntary activity. Thus I have identified that peripherally-released IL-6 plays a role in mediating the lethargy and anorexia induced by local administration of LPS. The idea

that IL-6 is involved in mediating LPS-induced sickness behaviours has been mooted before by others (Bluthé *et al.*, 2000b) studying the effect of systemic administration of LPS on body mass and social behaviour, another behavioural response affected during infection. Bluthé *et al*., (2000) observed that IL-6 deficient mice (IL-6 -/-) were less sensitive than wild-type (IL-6 +/+) mice to the depressing effects of i.p. administration of LPS on social behaviour and body mass.

While peripherally-released IL-6 appears unequivocally to be contributing to mediating LPS-induced sickness behaviours, it is important to note that the sickness behaviours are only attenuated and not abolished when the biological activity of IL-6 is inhibited. There are two likely explanations for why these sickness behaviour responses were only attenuated. Firstly, it is possible that not all peripherally-released IL-6 was neutralized by the dose of IL-6 antiserum I administered. Secondly, additional endogenous mediators, possibly other cytokines, could be working in parallel with peripherally-released IL-6 to regulate anorexia and lethargy induced by s.c. administration of LPS. As others (Cartmell *et al.*, 2000) have demonstrated complete neutralization of the LPS-induced increase in circulating IL-6 in rats using an equivalent dose of IL-6 antiserum to the dose I used, the attenuated responses I noted appear to rather reflect the involvement of additional mediators. To determine if these mediators were possibly other cytokines released in the periphery following LPS administration I injected rats i.p. with species-specific antiserum to either TNF-α or IL-1β before administering LPS.

Treating rats peripherally with either TNF-α antiserum or IL-1β antiserum had no effect on the LPS-induced suppression of voluntary activity and food intake (chapter 2). Thus I identified that peripherally-released IL-1β and TNF-α do not appear to be involved in mediating lethargy and anorexia induced by local administration of LPS. Although I do not provide *in vivo* evidence of neutralization of TNF-α and IL-1β in the circulation, it is unlikely that my failure to attenuate the LPS-induced anorexia and LPS-induced lethargy was due to the neutralization being incomplete, as I determined using an *in vitro* two-site sandwich ELISA that one hundred microlitres of the antiserum could neutralize up to 10, 000 pg of rat recombinant TNF-α and IL-1β (unpublished observations). From the results presented in later experiments (chapter 4), where I measured the concentration of IL-1β in the plasma, it would appear that the dose of IL-1β antiserum I administered (1.5 ml) should have been more than sufficient to neutralize the increase in plasma concentrations of IL-1β (~ 38 pg ml^{-1}) induced by the dose of LPS I administered (250 μg kg^{-1}).

The increase in plasma concentrations of IL-1β I measured in my study following local LPS administration is substantially lower than the increase (~ 350 pg ml^{-1}) measured by others, at the same time point, injecting a ten-fold lower dose of LPS (25 μg kg^{-1}) systemically (Bilbo *et al.,* 2005). A similar difference also has been noted in the concentration of TNF-α measured in plasma when injecting LPS systemically versus locally into an air pouch. Injecting LPS into an air pouch did not significantly increase TNF-α in the plasma, however injecting the same dose of LPS (100 μg kg^{-1}) systemically did (peak concentration ~ 1636 pg ml^{-1}) (Miller *et al.*, 1997b). Injecting LPS s.c. as I did may therefore be a more representative model of localized infection, similar to injecting

LPS into a pre-formed subcutaneous airpouch. In this experimental model of localized infection only IL-6 is significantly elevated in the circulation, with the increase in TNF-α and IL-1β mostly being confined to the local infection site (Miller *et al.*, 1997b). From my findings that injecting rats peripherally with either TNF-α or IL-1β antiserum has no effect on the anorexia and lethargy induced by local administration of LPS, I hypothesized that if other cytokines are working with peripherally-released IL-6 to induce anorexia and lethargy, it is due primarily to their synthesis in the brain and not in the periphery.

In chapter 3 I therefore investigated whether specific cytokines likely to fulfil this role, such as IL-1β and IL-6, could act within the brain to induce anorexia and lethargy. I found that i.c.v. administration of either species-homologous rat IL-6 or IL-1β before the night-time active period decreased voluntary activity in rats in a dose-dependent fashion. These findings were the first to show that voluntary exercise, that is wheel-running, is suppressed by direct administration into the brain, of IL-6 and IL-1β. While voluntary exercise was significantly reduced by i.c.v. administration of either IL-6 or IL-1β, food intake was only decreased by i.c.v. injection of IL-1β at the highest dose I used, but not IL-6 at any dose. Having identified that IL-6 can act endogenously to induce anorexia and lethargy my finding that central administration of IL-6 affected voluntary exercise, but did not affect food intake was surprising. Although it is yet to be established how IL-6 released into the bloodstream from peripherally located immune cells influences the brain-mediated response of anorexia and lethargy during a peripheral immune challenge, my findings presented in chapter 3 identify that the direct action of IL-6 on the

hypothalamic neurones involved in the control of food intake may not be a prerequisite. Rather, the induction of anorexia may be more dependent on peripherally-released IL-6 inducing other signalling molecules within cells of the BBB, which then penetrate into the brain to affect feeding. Regardless of how peripherally-released IL-6 mediates anorexia and lethargy induced by s.c. administration of LPS it is evident that IL-6 is not the sole mediator of these responses. Based on my finding that central administration of IL-1β decreased both voluntary exercise and food intake I hypothesized that endogenous brain IL-1β could possibly be involved in mediating lethargy and anorexia induced by local administration of LPS.

To determine whether this indeed is the case, I specifically chose to antagonize the biological action of IL-1β in the brain by administering a caspase-1 inhibitor, which prevents the cleavage of pro-IL-β to biologically active IL-1β, i.c.v. (Chapter 4). Others have reported that central administration of a caspase-1 inhibitor is an effective strategy to use for reducing IL-1β activity in the brain following systemic administration of LPS (Bilbo *et al.*, 2005). In my study I monitored the behavioural responses until the symptoms of sickness induced by LPS had ceased, so as to determine the cytokine involvement not only in the induction, but also in the resolution of these sickness responses. I found that pre-treating rats with a caspase-1 inhibitor i.c.v. did not abolish the anorexia and lethargy induced by LPS, but it did significantly attenuate both the magnitude and the duration of the anorexia and lethargy induced by LPS. Thus I have identified that endogenous brain IL-1β contributes to mediating lethargy and anorexia induced by local administration of LPS in rats.

While it has been established that administration of a caspase-1 inhibitor effectively reduces IL-1β activity *in vivo* following systemic administration of LPS (Bilbo *et al.*, 2005), I did not measure the concentration of IL-1β in the brain and therefore cannot confirm the extent to which i.c.v. administration of the caspase-1 inhibitor inhibited the synthesis of IL-1β in the brain. It is therefore possible that if IL-1β was not completely inhibited by the dose of the caspase-1 inhibitor I injected, the contribution of endogenous brain IL-1β to mediating the anorexia and lethargy induced by s.c. administration of LPS I noted may be of a greater magnitude.

The results I obtained with peripheral and central antagonism of endogenous IL-1β presented in chapter 2 and 4 appear to identify that brain, but not peripherally-released IL-1β plays an important role in mediating sickness behaviours induced by local administration of LPS. My finding that IL-1β in the brain, but not IL-1β released in the periphery, contributes to mediating sickness behaviours appears to be at odds with the findings of others (Bluthé *et al.*, 1992b). Bluthé *et al*., (1992b) found that pre-treating rats with IL-1ra peripherally, but not centrally, attenuated the decrease in body mass and social exploration induced by systemic administration of LPS.

Differences in the experimental design between my study and that of Bluthé *et al*., (1992b) may explain these contradictory findings. Firstly, the difference noted with antagonism of peripherally-released IL-1β may have been related to the different route by which LPS was administered. While the same dose of LPS (250 μg kg^{-1}) was administered in both studies, I administered the LPS s.c. in my studies while they

administered LPS i.p in their study. Injecting LPS s.c. appears to be a more representative experimental model of localized infection, than a model of systemic infection. The lack of involvement of peripherally-released IL-1β I noted in mediating LPS-sickness behaviours may be related to s.c. administration of LPS not inducing sufficient IL-1β in the circulation, unlike the significant increase noted following i.p. administration of LPS (Bilbo *et al.*, 2005), to influence the areas of the brain regulating these behaviours. Secondly, the differences noted with antagonism of IL-1β in the brain may be related to the use of different substances to antagonize the biological action of endogenous brain IL-1β. The time course of action of these two substances differs quite substantially, with IL-1ra being active for about 2 to 4 h (Bluthé *et al.*, 1992b), while the caspase-1 inhibitor is active for at least 24 h (Rabuffetti *et al.*, 2000). Brain IL-1β has been shown to remain significantly elevated for prolonged periods of between 8 - 24 h following i.p. administration of LPS (Bilbo *et al.*, 2005). Thus in terms of investigating the involvement of endogenous brain IL-1β in mediating LPS-induced sickness behaviours, the longer period of IL-1β neutralization likely to be achieved with the caspase-1 inhibitor may have prolonged the inhibition of IL-1β in the brain and therefore produced the attenuated sickness behaviour responses noted in my study.

It is also possible that the effects I noted following i.c.v administration of the caspase-1 inhibitor may not be entirely specific to the action of IL-1β, but may also be related to the action of other pro-inflammatory cytokines, as the enzyme caspase-1 is not only involved in the processing of IL-1β, but is also involved in the processing of IL-18 and IL-33 (Dinarello, 2005a). Administration of the capase-1 inhibitor may therefore have reduced

the synthesis of these two cytokines in addition to the synthesis of IL-1β in the brain. Even though as of yet the involvement of IL-18 and IL-33 in LPS-induced lethargy has not been identified, IL-18 has been implicated as a putative mediator of energy homeostasis by suppressing appetite (Zorrilla *et al*., 2007). Thus the participation of these two cytokines in the biological processes described in my study cannot be excluded.

Due to the strong likelihood that the contradictory findings regarding the peripheral involvement of IL-1β in mediating LPS-induced sickness behaviours may be related to the experimental model of infection used, systemic versus local, investigating the effect of endogenous antagonism of peripherally-released IL-1β following LPS administration via different routes (s.c., i.p., i.v. and i.m.) may provide clarity on the role of peripherally-released IL-1β in mediating LPS-induced sickness behaviours. Moreover, to identify if the failure to inhibit sickness behaviours induced by local and systemic administration of LPS following i.c.v. injection of IL-1ra (Bluthé *et al.*, 1992b; Kent *et al.*, 1992b) is indeed related to the short half-life of IL-1ra, it would be useful to investigate the effect of antagonism of endogenous brain IL-1β using IL-1ra and the caspase-1 inhibitor in the same study.

In chapter 4 I also investigated the effect of antagonizing the biological action of peripherally-released IL-6 on the duration of the anorexia and lethargy induced by s.c. administration of LPS, as although peripherally-released IL-6 had been implicated in mediating LPS-induced sickness behaviours (Chapter 2 and Bluthé *et al*., 2000b), the extent to which its absence could facilitate recovery from LPS-induced sickness

behaviours had not been established. In addition to my initial findings in chapter 2 which showed that treating rats with serum containing IL-6 antibodies attenuated the magnitude of anorexia and lethargy induced by s.c. administration of LPS, I identified in chapter 4 that treating rats with serum containing IL-6 antibodies also facilitated a significantly faster recovery of the rats (by at least 2 days) from the suppressive effects of LPS on voluntary activity, food intake and growth. Moreover, in chapter 4 I was able to demonstrate that the species-specific antiserum I administered did indeed significantly neutralize the LPS-induced bioactive IL-6 (by ~ 80%) in the plasma of the rats injected with LPS and the IL-6 antiserum. Although I reduced the level of bioactive IL-6 in the circulation by a substantial margin it is likely that had I achieved complete neutralization the degree of attenuation I noted may have been of a greater magnitude.

Although peripherally-released IL-6 appears to be an important endogenous mediator of anorexia and lethargy, how IL-6 released into the bloodstream from peripherally located immune cells influences the brain-mediated responses of anorexia and lethargy during a peripheral immune challenge however, remains unclear. Others have reported that injecting LPS i.p. induces an increase in IL-1β mRNA in the brains of wild-type mice, however in IL-6 knockout mice the LPS-induced increase in IL-1β is greatly attenuated or entirely absent (Sparkman *et al.*, 2006). Thus, in the absence of IL-6, systemic administration of LPS failed to induce IL-1β in the brain. Having identified in chapter 4 that brain IL-1β is involved in mediating anorexia and lethargy induced by local administration of LPS, one mechanism by which peripherally-released IL-6 could be mediating anorexia and lethargy is via the induction of IL-1β in the brain. The possibility

that IL-6 can induce the synthesis of IL-1β is intriguing as the vast majority of the literature points towards IL-1β inducing IL-6 and not *vice versa* (Shalaby *et al.*, 1989; Sironi *et al.*, 1989; Schindler *et al.*, 1990).

Not only did I identify an important role for peripherally-released IL-6 in mediating anorexia induced by local administration of LPS in my thesis, but I confirmed the importance of another endogenous mediator, leptin (chapter 2). Treating rats peripherally with leptin antiserum abolishes the anorexia induced by s.c. administration of LPS. Using the same species-specific leptin antiserum, but administered at a lower dose, others have demonstrated an attenuation of anorexia induced by i.p. administration of LPS in rats (Sachot *et al.*, 2004). In contrast, studies investigating the involvement of leptin in mediating LPS-induced anorexia using rats with defective receptor-mediated transport and intracellular signalling of leptin, have shown that leptin signalling does not appear to influence LPS-induced anorexia (Faggioni *et al.*, 1997; Lugarini *et al.*, 2005). There are two likely explanations for the discrepancies noted between studies using rats with genetic defects in the leptin system and leptin antiserum. Firstly, due to the importance of food intake for survival, the control of food intake is not solely dependent on a single pathway, but rather is regulated by different pathways involving mediators other than leptin, such as cholecystokinin, insulin and glucagon. Thus it is possible that the absence of the leptin-mediated pathway occurring early on in the development of mutant animals may result in other pathways involved in food intake homeostasis compensating. These alternative pathways could be activated during pathological conditions like LPS stimulation, leading to the observed anorexia in animals with genetic defects in the leptin

system. Secondly, systemic administration of LPS has been shown to induce comparable concentrations of IL-6 in the circulation of leptin-deficient (*ob/ob*) mice and their respective lean littermates (+/?*ob* mice) (Faggioni *et al.*, 1999). Thus humoral mediators other than leptin, such as IL-6 identified in this thesis, may compensate for the absence of leptin in animals with genetic defects in the leptin system (Sachot *et al.*, 2004). My finding that peripherally-released leptin and peripherally-released IL-6 appear to share the same biological effect of suppressing food intake following s.c. administration of LPS, may be related to them both: (i) belonging to the same long-chain helical cytokine family and sharing a common signal transducer among their receptors (Zhang *et al.*, 1994; Vaisse *et al.*, 1996) or (ii) inducing the same important downstream mediator of food intake, IL-1β, in the brain (Sachot *et al.*, 2004).

Not only do leptin and IL-6 released in the periphery both appear to be involved in mediating the suppression of food intake following s.c. administration of LPS, but they also both appear to be involved in mediating the fever response, as treating rats peripherally with either leptin antiserum or IL-6 antiserum abolishes fever induced by s.c. administration of LPS (Chapter 2). The involvement of both peripherally-released leptin and peripherally-released IL-6 in mediating LPS-induced fever may be related to similarities in the actions of the two cytokines, as they both have been shown to induce COX-2, an important downstream mediator of fever, in the brain (Rummel *et al*., 2006; Inoue *et al*., 2006). The involvement of leptin in mediating LPS-induced fever may however not only be related to the induction of COX-2 in the brain, but it also may be related to the activation of thermoeffectors required for fever production, in particular the

activation of metabolic heat production. Leptin raises metabolic heat production by increasing sympathetic outflow to brown adipose tissue (nonshivering thermogenesis) in small rodents (Collins *et al*., 1996). It is unlikely however that the attenuated effects I noted on fever production following administration of leptin antiserum were related to blocking the thermogenic action of leptin, as leptin appears to have a critical role in chronic, but not acute activation of thermogenesis (Girardier *et al*., 1995; Steiner & Romanovsky, 2007) and fever is brought about by acute activation of thermoeffectors (Romanovsky *et al*., 2005). Moreover, in a thermally neutral environment activation of thermogenesis may not be a prerequisite to increase core body temperature during fever, as the increase in core body temperature may be achieved by constriction of skin vessels alone (Romanovsky *et al*., 2002). It therefore appears that the participation of leptin in LPS-induced fever is mostly related to its inflammatory action in the brain.

5.2 Differences in the cytokine-mechanisms mediating fever and sickness behaviour

Not only did I investigate the contribution of endogenous cytokines in mediating LPS-induced sickness behaviours in the studies presented in this thesis, but I also investigated their contribution in mediating fever induced by s.c. administration of LPS. My rationale for concurrently investigating the contribution of endogenous cytokines in mediating sickness behaviours and fever was to identify possible differences in the cytokine-mechanisms mediating these responses. The observation I made in chapter 4 and which others have also made previously (Hübschle *et al*., 2006), that sickness behaviours continue for a longer period of time than does fever during infection/inflammation, has

lead to the speculation that the cytokine-mechanisms regulating fever and sickness behaviours may not be the same (Kent *et al.*, 1992a; Dantzer, 2001).

In the studies presented in this thesis I uncovered some distinct differences in the cytokine-mechanisms regulating fever and sickness behaviours. For instance, LPS-induced fever could be abolished by inhibiting the action of peripherally-released IL-6, while it appeared that LPS-induced lethargy and LPS-induced anorexia could be abolished only if both endogenous brain IL-1β and peripherally-released IL-6 were inhibited (chapter 4). Moreover, central administration of the lowest dose of IL-1β or IL-6 decreased voluntary activity in rats in the absence of fever (chapter 4). These differences highlight that: (i) brain-intrinsic cytokines may be more important in mediating sickness behaviours and they are in mediating fever and (ii) the brain sites controlling sickness behaviours, in particular lethargy, may be more sensitive to the presence of cytokines than the brain sites controlling temperature. Both of these differences are likely to account for the prolonged duration of the sickness behaviours during illness. From a clinical point of view identifying the cause for the longer duration of sickness behaviours is particularly relevant, as it is the prolonged duration of the sickness behaviour responses that are most disabling and uncomfortable for patients to endure during illness (Bower *et al.*, 2002; Hewlett *et al.*, 2005). Moreover, having a better understanding of the physiological mechanisms inducing both fever and sickness behaviours such as anorexia and lethargy, may provide clinicians with more insight into managing not only the thermal but also the non-thermal responses to infection.

5.3 Clinical implications and recommendations

My finding in chapter 4 that reducing either the amount of biologically active IL-6 in the circulation or IL-1β in the brain significantly enhances the resolution of anorexia and lethargy by at least two days in rats, but does not completely prevent these sickness behaviours from occurring, appears to indicate the complexity of the cytokine involvement in mediating these two sickness behaviours during a localized Gram-negative bacterial infection. Using similar experimental tools, others (Swiergiel & Dunn, 1999) have also noted this trend of multiple cytokine involvement in mediating sickness behaviours during a systemic Gram-negative bacterial infection. Therefore while individual cytokines, such as IL-6 and IL-1β, are possible targets for therapies aimed at alleviating anorexia and lethargy in patients with bacterial infections, it appears that to abolish the presence of sickness behaviours multiple cytokines may need to be targeted.

Pro-inflammatory cytokines also perform important immune functions, however which facilitate pathogen elimination. They increase the microbial activity of phagocytic cells, activate adjacent epithelium tissues which result in the attraction of neutrophils and monocytes and induce the production of acute phase proteins by the liver (Kapetanovic & Cavaillon, 2007). Therefore, while inhibiting the action of multiple cytokines may resolve the sickness responses quicker, this action could possibly compromise the ability of the host to fight infection in the situation of a live, replicating pathogen. In instances of chronic infection where patients are placed on prolonged treatment regimens, inhibiting the action of cytokines may increase the prevalence of opportunistic infections.

Selectively inhibiting the downstream mediators by which cytokines induce sickness behaviours may prove to be a better tool therapeutically, as that could preserve the actions of pro-inflammatory mediators required for pathogen elimination yet improve the malaise and sense of sickness in the patient. To therefore identify the most beneficial treatment strategy for sickness behaviours in patients, future pathophysiological mechanism-based assessment studies of sickness behaviours in animal models of infection/inflammation are needed which not only look at specific cytokines, but also the activation status of their intermediate signalling pathways and their downstream products (Dantzer *et al.*, 2008).

Although the results presented in this thesis have important implications for the treatment of patients with bacterial infections, they may not be directly transferable to medially ill patients in general, as the cytokine profiles induced by different pathogens is not necessarily the same. The high prevalence of non-specific behavioural symptoms in medically ill patients not only with a "common cold" or flu, but also in patients with some of the most harmful, costly and debilitating diseases currently experienced in the Western World: coronary heart disease, cancer, obesity, type II diabetes and neurodegenerative disorders associated with aging (Dantzer *et al.*, 2008) necessitates the continuation of preclinical studies, such as those presented in this thesis to be carried out using established animal models of disease, to identify possible therapies for these symptoms in patients.

Not only does the work I have presented in this thesis have value in terms of identifying possible targets for the treatment of symptoms of medically ill patients, but it also may be of value in improving our approach to identifying and monitoring infection/inflammation in patients. From early on in the history of medicine, fever has been viewed by physicians and patients alike as the principal symptom of illness and thus decisions concerning the onset of and recovery from illness were primarily based on a patient's body temperature. The findings presented in this thesis showing that fever is outlasted by both the period of lethargy and anorexia in the presence of a simulated Gram-negative bacterial infection in rats, indicates that the behavioural symptoms associated with infection, such as lethargy and loss of appetite, should become part of the close monitoring of the condition of patients, as the presence of these sickness behaviours, may in fact be a more valuable clinical marker of illness and recovery in patients, than is body temperature alone.

CHAPTER 6

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APPENDIX

STRICTLY CONFIDENTIAL

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

ANIMAL ETHICS SCREENING COMMITTEE

CLEARANCE CERTIFICATE NO:

2003	39	5

APPLICANT: Professor H Laburn

DEPARTMENT: School of Physiology

PROJECT TITLE: Identification of Cytokines Involved in the Pressure Spontaneous Activity During Infection

Species	Number	Expiry Date
Sprague dawley	35	2005

i) Approval is hereby given for the experiment described in the above application.

The use of these animals is subject to AESC Guidelines for the use and care of animals, is limited to the procedures specified in the application form, and to:

APPROVED Subject to:
-a pilot study being done on a small number of rats in order to monitor potential problems
-reporting the results of the pilot study to the Chairman of the AESC
-confirmation that normal husbandry procedures can be followed by the CAS staff
-in addition the Committee suggests that the wheels should be left locked for 3-5 days after the operation

SIGNED D. A. Gray DATE: 29 April 2003
(Chairman: ~~Animal Ethics Screening~~ Committee)

ii) 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: 29 April 2003
(~~Registered Veterinarian~~)

NOTE:

First-time users of the CAS should contact the Director of the CAS in order to familiarise themselves with the facilities available, and the procedures required by the CAS for the carrying out of experiments.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
ANIMAL ETHICS SCREENING COMMITTEE

CLEARANCE CERTIFICATE NO:

2004	95	5

APPLICANT: L Harden

DEPARTMENT: School of Physiology

PROJECT TITLE: The effect of centrally injected cytokines and leptin on spontaneous running activity, body temperature and food intake.

Species	Number	Expiry Date
Rat	152	November 2006

i) Approval is hereby given for the experiment described in the above application.

The use of these animals is subject to AESC Guidelines for the use and care of animals, is limited to the procedures specified in the application form, and to:

Approved

SIGNED D. A. Gray (Chairman: Animal Ethics Screening Committee) DATE: 22 November 2004

ii) 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: 22 November 2004

NOTE:

First-time users of the CAS should contact the Director of the CAS in order to familiarise themselves with the facilities available, and the procedures required by the CAS for the carrying out of experiments.

AESC 3

STRICTLY CONFIDENTIAL

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

ANIMAL ETHICS SCREENING COMMITTEE

CLEARANCE CERTIFICATE NO:

2005	86	5

APPLICANT: Ms Lois Harden

DEPARTMENT: School of Physiology

PROJECT TITLE: The Effect of Centrally Injected Antibodies to IL-1Beta and IL-6 on LPS-induced Fever and Sickness Behaviour

Species	Number	Expiry Date
Rat	300	November 2007

i) Approval is hereby given for the experiment described in the above application.

The use of these animals is subject to AESC Guidelines for the use and care of animals, is limited to the procedures specified in the application form, and to:

APPROVED subject to;

- discussing the method of euthanasia, as well as the use of post-operative analgesics with CAS staff.

SIGNED D. A. Grey DATE: 1st December 2005

Chairman: Animal Ethics Screening Committee)

ii) 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: 1st December 2005

(Registered Veterinarian)