# LEPTIN, A MOLECULAR LINK BETWEEN NUTRITIONAL STATUS, BRAIN AND INFLAMMATION.

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March, 2009

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Doctor of Philosophy

## Acknowledgements

This thesis is dedicated to all my family, friends, supervisors and colleagues whose help and advice enabled me to complete my PhD and to experience all the excitement of research.

First of all, I would like to express my deepest gratitude to Dr. Giamal N. Luheshi, my PhD supervisor, without whom this thesis would simply have been impossible. He provided continuous support, encouragement and guidance throughout the time I spent in his laboratory and I am especially grateful for his dedication to training students without regard to the amount of personal time and energy involved. I would also like to acknowledge his efforts in helping me to adjust to life in Canada, such kindness was very important for me as I was living in a foreign country for the first time in my life. Thank you very much Giamal for giving me the opportunity to do a PhD in your laboratory.

I would also like to thank the members of my advisory committee, Dr. Sylvain Williams and Dr. Samuel David for their kind support, helpful discussions and constructive feedback throughout my PhD, and also my mentor, Dr. Joseph Rochford, for his encouragement.

I would never have found myself doing a PhD in science if I had not met my MSc. supervisor, Dr. Kiyoshi Matsumura, who introduced me to the joy of research and inspired me to go abroad for a new experience. I would also like to extend my gratitude to Prof. Shigeo Kobayashi for his guidance throughout my MSc. study.

A large part of the thesis research would not have been possible without support from Dr. Andrew Bristow and Dr. Stephen Poole, who generously provided recombinant leptin, anti-leptin antiserum and ELISA reagents for cytokine assay. A collaboration with Dr. Emmanuel Pinteaux significantly influenced the direction of the thesis project and I would also like to thank him for his expert advice concerning primary microglial culture.

The Nakajima Foundation, Japan, provided generous financial support for my entire study. Their regular and thoughtful encouragement is deeply appreciated and I admire their continued commitment to science.

I would also like to acknowledge former and present colleagues from the Luheshi laboratory. Christelle was my older sister in the lab and I owe her so much for everything. Christoph was an advisor, collaborator, friend and roommate, so perhaps he knows a little too much about my PhD life. Véronique, I admire your patience and the hard work you put into the microglial culture, and I also thank Bernard for his help in microglia experiments. I learned a lot from Helen and her expert advice was very helpful. Thank you very much Gokce for your excellent job with ELISA, PCR, animal handling, lab management, *i.e.*, virtually everything. I also thank Argel for coffee time discussions and his constructive comments, and Joanna for her "help" writing in French. It was very enjoyable to work with Kei, Brandy and Natalia, you are all wonderful.

Many people at the Douglas made my time here so precious and I cannot mention them all by name but special thanks are due to Dr. Marc Danik for his kindness, hospitality and mentorship during my time in Montreal. Also deserving of a special mention are my drinking/softball/hockey friends; Germaine, Mandy, Michelle, Melissa, Guillaume, Jessie, Carey, and Leora – it's been fun!

My heartfelt thanks go to Dr. Bríd Nic Niocaill for her professional work and kindness in correcting my English, and to her assistant Mr. Beag Mac Manseau for his continuous encouragement during the writing of this thesis.

Lastly I cannot thank enough my partner Rinko Kawano for her never ending support and patience, even whilst living with a stressed PhD student, and my family in Japan for their encouragement.

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## Abstract

Leptin is an adipocyte-derived cytokine originally identified as an anti-obesity hormone but is implicated in other functions including immunity. The work described in this thesis addressed leptin's role as a neuroimmune mediator during systemic inflammation and the physiological significance of this role. We first demonstrated that leptin stimulates the production of interleukin-1β, a critical pro-inflammatory cytokine, through action on brain-resident macrophages, microglia and endothelial cells known to play key roles in the brain's innate inflammation. Moreover, leptin displayed atypical inflammatory effects, acting as an enhancer/modulator rather than a *bona fide* stimulator of brain inflammation. In subsequent studies, we addressed the leptin regulation of inflammation in a physiological context and examined whether malnutrition (which reduces basal leptin levels) attenuates the brain's innate inflammatory response in a leptin-dependent manner. Food deprivation prior to induction of brain inflammation blunted the up-regulation of cytokines, chemokines and adhesion molecules, and the recruitment of neutrophils to the brain. Repletion of leptin during fasting reversed all the indices of neuroinflammation, demonstrating that leptin modulates the brain's innate inflammation in relation to the host's nutritional status. These studies were extended to examine the role of leptin in fever, an energy demanding response to inflammation. Food deprivation significantly attenuated fever, in part through a leptin-dependent mechanism, supporting a role for leptin in linking energy balance and fever. However, leptin regulation of fever was dissociated from the febrigenic inflammatory response, highlighting the complexity of leptin's role which depends to some degree on the severity of the pathogenic stimulus. Leptin reversed the fever attenuation most likely by activating thermoregulatory functions in the fasted animals.

In summary, this thesis demonstrated the interaction between energy balance and host defence responses to infection. Leptin, an adipose tissue-derived cytokine, plays a critical role in this relationship in part by linking energy balance with the brain's innate inflammatory response and fever.

## Résumé

La leptine est une cytokine provenant d'adipocytes identifié à l'origine comme hormone d'anti-obésité. En plus de cette fonction, la leptine est impliquée dans la réponse au stress, la reproduction, le métabolisme et la fonction immunitaire. Cette thèse a étudié le rôle de la leptine sur la réponse inflammatoire du cerveau et sur la fièvre. Les résultats des deux premiers manuscrits ont démontrés que la leptine stimule la production de la cytokine pro-inflammatoire interleukin-1β in vivo et in vitro, en agissant sur les macrophages résidants du cerveau, les microglies et les cellules endothéliales connues pour leurs rôles dans l'inflammation du cerveau. En plus, la leptine a eu des effets inflammatoires atypiques, agissant en tant que renforceur/modulateur plutôt qu'un véritable stimulateur de l'inflammation. La troisième expérience a étudié les effets inflammatoires atypiques de la leptine dans un contexte physiologique et a examiné si la malnutrition (qui réduit les niveaux basiques de leptine) atténue la réponse inflammatoire du cerveau d'une façon dépendante de la leptine. Le jeûne avant l'induction de l'inflammation au cerveau a arreté la production de cytokines, de chémokines et de molécules d'adhérence, ainsi que le recrutement de neutrophiles au cerveau. La réplétion de la leptine pendant le jeûne a renversé tous ces indexes de neuroinflammation, ainsi démontrant que la leptine module l'inflammation du cerveau. Les deux derniers manuscrits ont examiné le rôle de la leptine sur la fièvre. La privation de nourriture a atténué la fièvre de manière significative, en partie par un mécanisme dépendant de la leptine, soutenant un rôle pour celle-ci dans le lien entre le statut énergétique/nutritionnel et la fièvre. Cependant, l' éffet de la leptine sur la fièvre a été attribué à ses effets sur la thermorégulation/métabolisme et a été dissocié de la réponse inflammatoire du cerveau, démontrant une fois de plus les fonctions multiples de la leptine. En résumé, cette thèse a démontré l'interaction entre le bilan énergétique et la reponse inflammatoire. La leptine, une cytokine dérivée du tissu adipeux, joue un rôle critique dans ce rapport en liant le bilan énergétique avec la réponse inflammatoire du cerveau et la fièvre.

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# Introduction

Infection or injury triggers, in addition to local inflammation, subjective and systemic symptoms of illness, such as lethargy, cognitive dysfunction, fever and loss of appetite (Dantzer, 2001; Konsman et al., 2002). These pathogen non-specific symptoms form, along with the immunologic response, important mechanisms to adapt to and fight against infection (Kluger et al., 1975; Hart, 1988) and are collectively referred to as "sickness behaviours". The responses are controlled by the central nervous system (CNS) (Dantzer, 2001; Konsman et al., 2002) following activation of non-neuronal cells in the brain, such as brain-resident macrophages, microglia and endothelial cells, by afferent immune signals (Van Dam et al., 1995; Matsumura et al., 1998a; Quan et al., 1998; Laflamme and Rivest, 1999). This immune-to-brain signalling generally involves *de novo* synthesis of classical innate inflammatory mediators such as interleukin (IL)-1β and prostaglandin (PG)E2 by non-neuronal cells, whose actions on neurons in turn orchestrate sickness behaviour (Van Dam et al., 1993b; Van Dam et al., 1995; Matsumura et al., 1998c; Matsumura et al., 1998a; Quan et al., 1998). In general, the brain's inflammatory response is a transient and tightly regulated process, and is believed to cause no harm to the brain (Mouihate and Pittman, 1998; Cunningham et al., 2005; Teeling et al., 2007). However, it has become increasingly evident that the same mechanism could be neurotoxic when over-activated. Accumulating evidence suggests that when peripheral inflammation continues unabated or is combined with ongoing neuropathology (which primes the non-neuronal cells) the brain's innate inflammatory response could be exaggerated resulting in irreversible brain damage (Papadopoulos et al., 2000; Perry et al., 2007; Dantzer et al., 2008). Previously, we and others have proposed that leptin, an adipose tissue-derived hormone, acts as an important mediator of systemic inflammation and activates brain mechanisms responsible for sickness behaviour (Luheshi et al., 1999; Sachot et al., 2004; Wisse et al., 2004; Harden et al., 2006; Inoue et al., 2006).

Leptin is a cytokine-like peptide originally identified as an important satiety factor that prevents obesity by decreasing appetite and increasing energy expenditure via its actions on the hypothalamus (Friedman and Halaas, 1998). Although this remains the best known function of leptin, biological effects of this cytokine have been revealed to be far more pleiotropic; implicated in the stress response (Ahima et al., 1996; Heiman et al., 1997; Korbonits et al., 1997), reproduction (Barash et al., 1996; Chehab et al., 1996), metabolism (Stehling et al., 1996; Doring et al., 1998; Overton et al., 2001), immune function (Lord et al., 1998; Howard et al., 1999) and inflammation (Fantuzzi and Faggioni, 2000; Matarese et al., 2005). In addition, available data supports a role for leptin in sickness behaviour. The circulating levels of leptin rise acutely following an inflammatory challenge (Grunfeld et al., 1996b; Sarraf et al., 1997; Faggioni et al., 1998; Mastronardi et al., 2000). Administration of exogenous leptin induces physiological responses that resemble sickness behaviour, including fever and loss of appetite (Luheshi et al., 1999; Turek et al., 2004). Neutralization of endogenous leptin by a specific antiserum blunts fever and anorexia during inflammation (Sachot et al., 2004; Harden et al., 2006). Of interest to this thesis is leptin's ability to trigger the brain's innate inflammation and to stimulate brain IL-1β and PG synthesis (Brunetti et al., 1999; Hosoi et al., 2002b; Wisse et al., 2004; Inoue et al., 2006). These observations, coupled with its established role in energy balance, suggested that contributors to sickness behaviour and the brain's innate inflammatory response can originate from a source other than the immune system (*i.e.*, adipose tissue), adding yet another dimension to the host defense response against infection and inflammation.

Given the obvious negative impact of anorexia (decreased energy intake) and fever (increased energy expenditure) on energy balance, it is reasonable to think that an appropriate regulation of sickness behaviour according to the host's energy status is of fundamental importance to survival. Such an inter-relationship is supported by studies reporting that malnutrition prior to the induction of inflammation ameliorates, or even abolishes, anorexia (Kent et al., 1994; Lennie et al., 1995; Lennie, 1998; Gautron et al., 2005) and fever (Hoffman-Goetz and

Kluger, 1979a; Szekely, 1979; Kleitman and Satinoff, 1981; Shojoony, 1985; Shido et al., 1989). In addition to sickness behaviour, there is growing interest in the potential role of systemic inflammation in the pathogenesis and/or progression of a wide range of brain diseases including sepsis associated encephalitis, Alzheimer's diseases, epilepsy, major depression, prion's disease and ischemic brain injury (Papadopoulos et al., 2000; Bohatschek et al., 2001; Kitazawa et al., 2005; McColl et al., 2007; Perry et al., 2007; Dantzer et al., 2008; Riazi et al., 2008). The proposed mechanism involves active roles for non-neuronal cells (*e.g.*, brain-resident macrophages, microglia and endothelial cells) in regulating the brain's innate inflammation. Interestingly, some of these brain pathologies have been suggested to be under the influence of the nutritional status of the individuals (Bercault et al., 2004; Scott et al., 2004; Mattson, 2005; Pasinetti and Eberstein, 2008; Plunet et al., 2008). Given the proposed dual role of leptin in the energy balance regulation and immune-to-brain signalling, this cytokine may serve as a point of convergence where nutritional status affects sickness behaviour and brain diseases. This thesis investigated the role of leptin in linking energy balance, sickness behaviour and the brain's innate inflammation.

# Chapter I: Comprehensive review of the literature

# I.1. Sickness behaviour and neuroimmune interaction

Anyone who has experienced an episode of infection knows what it is to be sick. Systemic infections, either viral or bacterial, as well as some localized infections dramatically alter our behaviour, with symptoms including lethargy, disinterest in rewarding activities, decrease in social interaction and impaired concentration. These subjective feelings of sickness are often accompanied by physiological changes such as increased body temperature (fever), loss of appetite and increased sleep (Dantzer, 2004). In our daily life, these pathogen non-specific symptoms are sometimes ignored, seen as merely undesirable side-effects of infection or debilitation resulting from the disease process. However, much of the available evidence indicates that they form, along with the immunologic response, an important mechanism to adapt to and fight against infection. These alterations in our behavioural and metabolic states are collectively referred to as "sickness behaviour" and are controlled by the CNS.

## 1.1. Survival values of sickness behaviour

Classically, these pathogen non-specific symptoms are evolutionarily conserved, homeostatic mechanisms which, together with immunologic strategies, work to facilitate the elimination of invading pathogens [see (Hart, 1988) for review]. This concept initially evolved alongside the description of the adaptive value of fever in the host defence response to pathogens. Kluger and his colleagues (Kluger et al., 1975) first demonstrated that in ectothermic reptiles, which can only thermoregulate by environmental means, the survival rates following bacterial infection were substantially increased when they are kept in, or allowed to move to, a warmer ambient temperature. Later this observation was extended to endothermic rabbits, whereby preventing fever using an antipyretic agent after bacterial infection increased the mortality rate (Kluger and Vaughn, 1978). The survival value of fever in humans is supported by clinical data which showed that patients suffering from bacterial or fungal infection who developed low or no fever had a high mortality rate (Bryant et al., 1971; Weinstein et al., 1983; Weinstein et al., 1997). These data are consistent with the fact that higher

temperatures achieved during fever inhibit the growth of at least some of bacterial and viral pathogens (Mackowiak, 1981; Small et al., 1986) and stimulates the activities and proliferation of immune cells (Sebag et al., 1977; Smith et al., 1978; Duff and Durum, 1983). The fever response to infection or inflammation is an evolutionarily conserved mechanism, having been reported in some invertebrates (*e.g.* cockroaches and grasshoppers), as well as in a range of vertebrates including fish, amphibians, reptiles, birds and mammals [see review (Kluger, 1991)]. This widespread occurrence of fever provides strong support for its role as an adaptive survival mechanism. Another compelling argument is that fever is an energy demanding process and thus, must have some survival value to be preserved in the course of evolution.

Given the metabolic cost of fever, the occurrence of anorexia upon infection, another aspect of sickness behaviour, appears to be somewhat paradoxical. Nevertheless, it was shown that force-feeding in mice during bacterial infection shortens survival time and increases lethality as compared to *ad lib* feeding (Murray and Murray, 1979). One of the mechanistic explanations for the survival value of anorexia is that, by reducing food intake, infected organisms lower the chances of increasing plasma levels of free iron derived from ingested food. Iron obtained from blood is an essential nutrient for many bacteria to grow (Elin and Wolff, 1974; Mackowiak, 1981; Small et al., 1986). Administration of iron to mice suffering bacterial infection dramatically increased mortality rate as compared to control infected mice (Holbein, 1981). Thus, anorexia forms, along with increased body temperature, an additional inhibition mechanism for the replication of infectious pathogens. Additionally, behavioural aspects involved in food intake activities can also explain the advantage of anorexia. Remaining sedentary during sickness will save the energy required for muscular activity during foraging which represents a significant proportion of energy expenditure by healthy animals in their natural habitat. In relation to the fever response, retention of body heat is an important mechanism for raising and maintaining body temperature without consuming energy. Therefore, “curling-up” (minimizing the surface area) in the nest with less motivation for foraging activity

can be seen as an effective strategy to conserve heat as it reduces both convection and body surface exposure for heat radiation. More subjective experiences of sickness, such as depressive feelings, fatigue and reduced social interaction which together reduce the activities of infected organisms, can be interpreted in the same context; that infected organisms are in an energy saving mode. Moreover, it has also been suggested that this could work as a mechanism to prevent the spread of infection among the population by reducing close contact, at least in amphibians (Kiesecker et al., 1999). Recently it has been proposed that sickness behaviour is an expression of one type of "motivational state" that is different from the healthy situation but normal in the time of infection. Dantzer and his colleagues (Dantzer, 2001; Konsman et al., 2002) proposed that it is a reorganization of the priorities of the host in a way to potentiate the immunologic process for the elimination of pathogens and to make the body suboptimal for microbial replication. This occurs along with the reallocation of the energy resources of the body; energy is used for metabolic and immunologic responses rather than for foraging and social activities.

As mentioned earlier, sickness behaviour is regulated by the brain. So, how? Signals from the activated immune system must reach the CNS and stimulate particular areas of the brain to initiate the response. The following sections provide an overview of this mechanism.

## 1.2. Cytokine production in the periphery

Pathogenic microorganisms that invade the body encounter a first line of defence by innate immune cells, such as tissue macrophages and dendritic cells. The initiation of innate inflammation by these phagocytic cells depends on their recognition of specific components of bacteria or viruses, which are collectively termed pathogen-associated molecular patterns, via pattern-recognition receptors expressed on their surface. Among these are Toll-like receptors (TLRs), an evolutionarily conserved family of pattern-recognition receptors (Akira et al., 2001; Janeway and Medzhitov, 2002). To date, as many as ten mammalian TLRs have been identified and a number of ligand-receptor combinations reported

(Akira et al., 2001; Janeway and Medzhitov, 2002). Perhaps the best-characterized example is TLR-4, which recognizes an outer membrane component of Gram-negative bacteria, widely known as bacterial endotoxin lipopolysaccharide (LPS). The gene analysis of the LPS-hyporesponsive C3H/HeJ mice strain revealed that this strain carries a missense point mutation within the *tlr4* gene region encoding the cytoplasmic domain (Poltorak et al., 1998; Qureshi et al., 1999). Subsequently TLR-4−/− mice were generated and the essential role of TLR-4 in LPS signalling was confirmed (Hoshino et al., 1999). TLR-4, however, is not the sole receptor involved in LPS recognition but rather it forms a critical signalling subunit of the LPS receptor complex. Although how the TLR-4 complex binds to LPS is not fully determined, the participation of both CD14, a GPI-linked cell surface protein that binds to LPS, and a small protein called MD-2 that associates with the extracellular region of TLR-4 are critical for optimal LPS signalling (Akira et al., 2001; Janeway and Medzhitov, 2002). The recognition of LPS by the TLR-4/CD14/MD-2 complex initiates a rapid production of key innate inflammatory mediators, most importantly tumour necrosis factor (TNF) and IL-1β. These pro-inflammatory cytokines then induce their own synthesis as well as other pro-inflammatory (*e.g.*, IL-6) and anti-inflammatory [*e.g.*, IL-1 receptor antagonist (IL-1RA) and IL-10] cytokines. Thus, the ensuing cytokine cascade amplifies and propagates the inflammatory signal: cytokines not only regulate the immunologic responses at the site of infection but in addition enter the circulation and induce various systemic host defence responses, and ultimately reach the brain to activate the sickness behaviours.

### 1.3. Immune-to-brain signalling

The vast majority of the literature reviewing sickness behaviour state that the main endogenous mediators of sickness behaviour during infection or inflammation are cytokines TNF, IL-1β and IL-6 (Kluger, 1991; Luheshi and Rothwell, 1996; Rothwell, 1997; Dantzer, 2001; Konsman et al., 2002). Prior to its molecular identification in 1984 (Auron et al., 1984), IL-1β was termed "endogenous pyrogen" that could be isolated from blood monocytes stimulated by

bacterial pathogens (Cooper et al., 1967). Similarly, TNF was initially termed "cachectin", a critical endogenous mediator of cachexia/anorexia wasting syndrome (Beutler and Cerami, 1988; Tracey et al., 1988) which turned out to be a molecule identical to TNF cloned independently for its anti-tumour activity (Beutler et al., 1985). Consistent with their historical background, systemic or central administration of recombinant TNF or IL-1β potently induce a spectrum of sickness behaviour including fever, anorexia and behavioural depression (Dinarello et al., 1986; Dinarello, 1988; Kluger, 1991; Dantzer, 2001). Smaller quantities are needed when injected centrally as compared to systemically to induce a response of similar intensity (Cooper et al., 1967; Kluger, 1991; Dantzer, 2001), indicating a central action of these cytokines in regulating sickness behaviour. In contrast, administration of recombinant IL-6 has yielded mixed results concerning its capacity to induce fever (and possibly other sickness behaviour), resulting in moderate (Dinarello et al., 1991; Harre et al., 2002; Rummel et al., 2006) or no (LeMay et al., 1990b; Cartmell et al., 2000) fever when injected systemically. Nevertheless the evidence that supports the importance of IL-6 in fever response is compelling. The lack of endogenous IL-6 activity, either following neutralization with a specific antibody (Cartmell et al., 2000; Rummel et al., 2006) or in IL-6-deficient mice (Chai et al., 1996; Sundgren-Andersson et al., 1998), abolished the fever response induced by LPS, TNF or IL-1β. These findings are supported by earlier studies which reported that IL-6 is consistently increased in the circulation as well as in the cerebrospinal fluid (CSF) of sick animals in a manner that correlates significantly with the magnitude and duration of the fever response (LeMay et al., 1990b; Roth et al., 1993). Based on these results, it has been proposed that IL-6 acts as a downstream mediator of IL-1β (LeMay et al., 1990c; Chai et al., 1996) or as a co-factor that potentiates the actions of IL-1β (Lenczowski et al., 1999; Cartmell et al., 2000). However, the exact mechanism IL-6 uses to interact with other cytokines to regulate the fever response (and other sickness behaviour) still remains unknown (Nilsberth et al., 2008). It has been suggested that the contribution of IL-6 during infection is rather specific to certain facets of sickness behaviour, being more important for the fever

response than for behavioural depression (Lenczowski et al., 1999), although there exists contradictory data (Bluthe et al., 2000). The relative importance of individual cytokines for different components of sickness behaviour is not fully understood. This is mainly because of the complexity of cytokine actions, as cytokines possess cross-reactivity (one cytokine induces or inhibits other cytokines) and redundancy (the effects of one cytokine overlaps with others): it is difficult to attribute the role of a single cytokine to specific physiological consequences. Although TNF, IL-1β and IL-6 have been established as the "main" mediators, a number of other cytokines and chemokines have been reported to be capable of inducing at least one of the sickness behaviours when administered peripherally or centrally; these include interferon (IFN)-γ (Dinarello et al., 1984), IL-2 (Mier et al., 1988), ciliary neurotrophic factor (Shapiro et al., 1993), macrophage inflammatory protein-1β (Minano et al., 1991a), β-endorphin (Rezvani et al., 1982; Fraga et al., 2008), endothelin (Fabricio et al., 2005a; Fabricio et al., 2005b) and leptin (Luheshi et al., 1999). The physiological importance of these cytokines in sickness behaviour relative to the "main" mediators remains largely undefined.

## 1.4. The brain's innate inflammatory response to peripheral signals

It was originally thought that cytokines mediating systemic immune-to-brain signalling primarily originate at the locus of infection/inflammation and activate the brain after being transported via the circulation (Banks et al., 1995; Matsumura et al., 1998a; Rivest, 1999) and/or act peripherally on sensory nerve endings (Watkins et al., 1995b; Watkins et al., 1995a). Although this scenario is generally true, cytokines are also produced *in situ* in the brain in response to peripheral inflammation. In fact, the brain mounts a well-organized innate immune reaction, not only to the direct injury or infection within the brain but also by responding to peripheral stimuli. This response takes place in the immune cells of the brain, namely brain-resident macrophages and microglia, and involves the *de novo* synthesis of some of the same inflammatory mediators implicated in

the peripheral innate immune reaction (*i.e.*, TNF, IL-1β and IL-6). Brain cytokine production is a key component in the development of sickness behaviour.

### *1.4.1. Brain IL-1β*

Preceding the molecular identification of IL-1, Fontana et al. (Fontana et al., 1984) reported the appearance of IL-1-like bioactivity in brain homogenates of mice that received an intraperitoneal (*i.p.*) injection of LPS, indicating the ability of the brain to produce IL-1 in response to systemic inflammation. Van Dam et al. (Van Dam et al., 1992) provided the first neuroanatomical evidence that systemic administration of LPS in rats induced IL-1β immunoreactivity in the brain that was found localized in brain-resident macrophages in the meningis and parenchymal microglia. Similarly, it was shown that an intravenous (*i.v.*) injection of LPS resulting in the appearance of IL-1β mRNA and protein in macrophages in the CVOs, perivascular macrophages and parenchymal microglia in rabbit, a species widely used for fever studies in the early 90s (Nakamori et al., 1993). Subsequent studies confirmed the finding using peripheral LPS injection as a stimulus (Nakamori et al., 1994; Van Dam et al., 1995; Quan et al., 1998). The brain synthesis of IL-1β in phagocytic cells as a result of peripheral LPS challenge follows a specific spacio-temporal pattern with rapid synthesis (0.5–3 h) taking place in macrophages residing in perivascular spaces, the meningis, the CVO's and the choroids plexus. After 4–24 h, the focus of sustained production moves toward parenchymal microglia surrounding these structures (Quan et al., 1998). This migratory pattern of IL-1β synthesis from the boundary of the brain towards the parenchyma implies a paracrine action of IL-1β among brain macrophage/microglia in mediating inflammatory signals, given that IL-1β can induce its own up-regulation and release (Dinarello et al., 1987). A subsequent study reported that this was a dose-dependent phenomenon, in that a high dose of LPS (0.5–1 mg/kg) was required to induce widespread IL-1β production reaching deep into the parenchyma whereas a low dose (0.001–0.01 mg/kg) induced the production of IL-1β only in macrophages at the boundary of the brain (Quan et al., 1999). Although all of the aforementioned studies used LPS as a stimulus, it was also shown that other models of inflammation, such as intramuscular (*i.m.*)

turpentine injection (a model of sterile inflammation) (Horai et al., 1998) and *i.p.* injection of IL-1β (mimicking the endogenous mediator of peripheral inflammation) (Hansen et al., 1998) were able to induce brain IL-1β synthesis, indicating the pathogen non-specificity of the response. The anatomical characteristic of brain IL-1β synthesis indicates the major role played by humoral factors (*i.e.*, circulating cytokines and also exogenous pathogens themselves) in this process. However, other studies suggested, contribution of the neuronal pathway to brain IL-1β production supported by observations that; electrical stimulation of the vagus nerve induces brain IL-1β (Hosoi et al., 2000b), whereas vagotomy (surgical transection of the vagus nerve) attenuates the increase of brain IL-1β levels in response to *i.p.* injection of LPS or IL-1β (Laye et al., 1995; Hansen et al., 1998).

As central administration of IL-1β is a potent inducer of the full-range of sickness behaviour, it is reasonable to think that the IL-1β produced locally in the brain contributes to the development of sickness behaviour. A number of studies utilizing pharmacological tools inhibiting the actions of IL-1β including IL-1RA and neutralizing antiserum for IL-1RI (the only known functional receptor for IL-1) have demonstrated that brain IL-1β plays an essential role in fever, anorexia and depressive behaviour during systemic inflammation [see review (Konsman et al., 2002)]. For instance, intracerebroventricular (*i.c.v.*) infusion of IL-1RA suppressed anorexia in mice (Laye et al., 2000) and fever in rats (Luheshi et al., 1996) evoked by systemic injection of LPS. This was also the case for other models of fever induced by *i.m.* injection of turpentine (Luheshi et al., 1997) or LPS injection into a subcutaneous air pouch (a model of local inflammation) (Miller et al., 1997).

### *1.4.2. Brain TNF*

It was also reported that systemic injection of LPS induces *de novo* brain TNF synthesis in macrophages and microglia that follows a spacio-temporal expression pattern very similar to that of IL-1β; early synthesis occurs in brain macrophages in the CVOs, meningis and perivascular space, followed by a delayed expression in parenchymal microglia (Nadeau and Rivest, 1999a; Quan et

al., 1999). Infusion of anti-TNF antibody into the lateral ventricle prior to systemic LPS challenge almost completely abolished both the initial and delayed expression of TNF in the brain, suggesting an autocrine/paracrine induction mechanism (Nadeau and Rivest, 2000). Another study demonstrated that the inhibition of TNF bioactivity in the brain by using anti-TNF antibody attenuated the fever response evoked by turpentine injection (Luheshi et al., 1997), supporting the importance of brain TNF in sickness behaviour.

*1.4.3. Brain IL-6*

Along with IL-1β and TNF, a significant increase of IL-6 mRNA levels has been reported in mice treated systemically with LPS, with a temporal profile slightly delayed from the former two cytokines (Laye et al., 1994). Central infusion of IL-1RA abolished brain IL-6 induction during LPS-induced systemic inflammation (Laye et al., 2000), indicating the causal role of IL-1 in IL-6 expression. Unfortunately, much less is known about the neuroanatomy of IL-6 expression compared to IL-1β and TNF. One study has reported that systemic LPS injection induces IL-6 almost exclusively in the CVOs and the choroid plexus, but little expression was observed in the meningis (Vallieres and Rivest, 1997), a structure that expresses high levels of IL-1β and TNF (Nadeau and Rivest, 1999a; Quan et al., 1999). Moreover, the cells expressing IL-6 in the CVOs bore neuron-like morphology indicating that cell populations responsible for IL-6 are distinct from those responsible for IL-1β and TNF. At the functional level, IL-6 synthesis and actions appear to be critical for sickness behaviour, probably mediating signals downstream of IL-1β and TNF actions, because IL-6 KO mice are resistant to the IL-1β- and TNF-induced fever response, whereas central IL-6 injection in the KO mice restores the fever response (Chai et al., 1996; Sundgren-Andersson et al., 1998).

## 1.5. Key components of the brain innate immune system

### *1.5.1. Brain-resident macrophages*

As described above, brain-resident macrophages are an important site of brain cytokine synthesis in response to peripheral inflammation. Although it has been thought that endogenous afferent signals [*i.e.*, circulating cytokines and sensory nerve inputs, see (section 1.3)] trigger this response in the brain, an alternative but not mutually exclusive mechanism has emerged recently. There is growing appreciation that exogenous pathogens such as LPS, once in the general circulation, may directly act on the brain to trigger a local innate immune response. Although it is generally true that access of circulating LPS to the brain is limited by the BBB, there are structures with leaky blood vessels, namely the CVOs and the compartment of the choroid plexus outside of the epithelial lining forming the blood–CSF barrier. Lacroix et al. (Lacroix et al., 1998) first reported neuroanatomical evidence that macrophages residing in these structures constitutively express the LPS receptor subunit CD14. Interestingly, CD14 expression was further up-regulated in response to systemic injection of LPS. TLR4, the signalling subunit of the LPS receptor complex, was subsequently shown to be expressed constitutively in the group of cells expressing CD14, although TLR4 expression was not increased by systemic LPS challenge (Laflamme and Rivest, 2001). In addition to the CVOs and the choroid plexus, significant levels of both CD14 and TLR4 were also found to be expressed in macrophages in the meningis and perivascular space (Lacroix et al., 1998; Laflamme and Rivest, 2001). The positioning of brain macrophages capable of recognizing LPS at the boundary of the brain are closely associated with the phenomenon that systemic LPS challenge rapidly induces the expression of IL-1β and TNF in these brain macrophages, implying a functional role for these cells in sensing circulating pathogens. A more recent study demonstrated a critical role played by TLR4 on brain resident cells in *in situ* brain cytokine synthesis during systemic LPS-induced inflammation. Chakravarty et al. (Chakravarty and Herkenham, 2005) examined the relative contribution of TLR4 expressed on

haematopoietic cells versus brain resident cells (essentially brain macrophages and microglia) by creating reciprocal bone marrow chimeras between wild-type (C3H/HeOuJ) and TLR4 mutant (C3H/HeJ) mice. Although haematopoietic TLR4 was required for an increase in cytokine levels in the circulation after systemic LPS challenge, TLR4 expression on brain-resident cells alone was sufficient for the *in situ* brain synthesis of cytokines, including TNF and IL-1β, as well as for the activation of the stress response (*i.e.*, blood corticosterone elevation). Thus, this study provides compelling evidence that the direct recognition of exogenous pathogens by brain macrophages, independent of the actions of circulating cytokines, has significant functional consequences during systemic inflammation. However, there is another brain structure that is very important for the immune-to-brain signalling.

### *1.5.2. Endothelial cells of cerebrovasculature*

It is now clear that peripheral inflammation triggers brain synthesis of cytokines either directly via circulating exogenous pathogens or indirectly through endogenous cytokines. How the centrally-produced cytokines trigger sickness behaviour was a question that was addressed by many investigators in this area and is still not fully resolved. Neurons in various brain nuclei involved in autonomic, neuroendocrine and cognitive functions are reported to express receptors for TNF (Nadeau and Rivest, 1999a), IL-1 (Ericsson et al., 1995) and IL-6 (Vallieres and Rivest, 1997; Cao et al., 2001), thus it is conceivable that some components of sickness behaviour are mediated by the direct actions of these cytokines on neurons. However, the contributions of the neuronal cytokine signalling to sickness behaviour remain largely unknown. In contrast, it is now well documented that the actions of brain cytokines leading to sickness behaviour involve yet another downstream mediator, namely PGE2. Indeed, pyrogenic activity of almost all cytokines characterized to date including TNF, IL-1β and IL-6 is attributable to the downstream action of PGE2. For example, mice deficient in the EP3 subtype of the PGE receptor were shown to be completely resistant to fever induced by PGE2, IL-1β or LPS (Ushikubi et al., 1998). Similarly mice with a genetic deficiency of either cyclooxygenase (COX)-2 or microsomal PGE

synthase (mPGES)-1, enzymes involved in the PGE2-synthesis cascade, mount no fever response to various cytokines (IL-1β and IL-6), LPS or turpentine (Li et al., 1999; Li et al., 2001; Engblom et al., 2003; Li et al., 2003; Saha et al., 2005). Furthermore, it has been shown that PGE2 also plays an important role in mediating anorexia (Pecchi et al., 2006) and activation of the hypothalamus-pituitary-adrenal (HPA)-axis (Matsuoka et al., 2003), both of which occur in conjunction with the fever response. Therefore PGE2 synthesis is an important point of convergence for the actions of a variety of cytokines or exogenous pathogens in inducing sickness behaviour.

A number of studies demonstrated that the brain structures responsible for PGE2 synthesis are endothelial cells forming the BBB, although a few studies also reported a role played by juxtaposed perivascular macrophages (Matsumura et al., 1998c; Matsumura et al., 1998a; Rivest, 1999; Schiltz and Sawchenko, 2002). The time course of fever after administration of cytokines or other exogenous pyrogens (*i.e.*, LPS and turpentine) is closely associated with the appearance of COX-2 and mPGES-1 in brain endothelial cells (Lacroix and Rivest, 1998; Ek et al., 2001; Yamagata et al., 2001; Inoue et al., 2002). In accordance with the causal relationship between cytokines and PGE2, brain endothelial cells express the receptors for TNF (Nadeau and Rivest, 1999b), IL-1 (Ericsson et al., 1995; Cao et al., 2001; Konsman et al., 2004) and IL-6 (Vallieres and Rivest, 1997; Cao et al., 2001). In fact, endothelial cells are the predominant group of cells in the brain that express IL-1RI (Konsman et al., 2004), although significant expression levels are also observed in brain macrophages and microglia (which are likely involved in the autocrine/paracrine activation of cytokine synthesis as described above). An immuno-electron microscopic study revealed that IL-1RI is expressed on both the luminal (blood) and abluminal (brain) sides of endothelial cells (Cao et al., 2001), providing an anatomical basis for the actions of IL-1β from both peripheral and central origins. There are two subtypes of TNF receptors, p55 and p75. It was shown that p55, but not p75, is constitutively expressed in endothelial cells as well as other barrier-related structures such as the CVOs and the choroid plexus (Nadeau and Rivest, 1999b).

In response to a systemic administration of LPS, both p55 and p75 expression are induced in endothelial cells. The functional IL-6 receptor is composed of two subunits, IL-6R and gp130. Under normal conditions, IL-6R is expressed mainly in neurons, whereas gp130 is heterogeneously expressed throughout the brain, in various brain nuclei, the CVOs and endothelial cells (Vallieres and Rivest, 1997; Cao et al., 2001). As in the case of TNF receptors, a systemic injection of LPS increases both IL-6R and gp130 expression in endothelial cells. The receptor activation of either IL-1β or TNF results in the nuclear translocation of nuclear factor (NF)κB, a transcription factor that regulates the transcription of a range of inflammation-related genes including COX-2 and mPGES-1. Peripheral or central injection of either cytokine produces a rapid (~30 min) activation of NFκB in endothelial cells after administration (Laflamme and Rivest, 1999; Konsman et al., 2000; Proescholdt et al., 2002). Similarly IL-6 receptor activation triggers the nuclear translocation of signal transducer and activator of transcription (STAT)3, whose activation rapidly occurs in endothelial cells following IL-6 administration (Lebel et al., 2000; Rummel et al., 2005; Rummel et al., 2006). Taken together, numerous data supports the importance of brain endothelial cells as a significant target of cytokines. Thus, endothelial cells are, along with brain macrophages, the key cells of the immune-to-brain signalling for sickness behaviour.

## 1.6. The brain's innate inflammatory response as a factor contributing to neuropathology

As emphasized in section 1.1, sickness is a normal physiological state in the face of infection, just as hunger is a normal response to starvation (Hart, 1988). The transient and fully reversible nature of sickness behaviour, together with its survival value, indicates that the innate inflammatory processes of the brain are self-limiting and cause little or no detrimental outcomes to the brain. This is generally true: it has been reported that apoptotic loss of neurons was not significantly increased by systemic LPS administration at a dose that is sufficient to trigger a robust brain synthesis of IL-1β and COX-2 and fever response (Mouihate and Pittman, 1998; Cunningham et al., 2005; Teeling et al., 2007).

However, it has become increasingly apparent that the same mechanism may be neurotoxic when over-activated. There is rapidly accumulating evidence that when peripheral inflammation continues unabated (*e.g.*, during sepsis) (Papadopoulos et al., 2000; Bohatschek et al., 2001; Semmler et al., 2005) or is combined with ongoing neuropathology (*e.g.*, ischemic injury, seizure, prion disease or Alzheimer's disease) (Sly et al., 2001; Cunningham et al., 2005; Kitazawa et al., 2005; McColl et al., 2007; Riazi et al., 2008), the brain's innate inflammatory responses triggered by afferent signals could be exaggerated resulting in irreversible brain damage.

### *1.6.1. Severe systemic inflammation could be neurotoxic*

It is widely understood that uncontrolled systemic inflammation, such as during sepsis, is a life-threatening situation involving a sequence of events including hypotension, inadequate organ perfusion, necrotic and apoptotic tissue damage, shock and ultimately death (Marshall, 2001). Because of such drastic systemic symptoms leading to multi-organ failure, most studies have been focusing on peripheral organs such as liver, lung and kidney which are frequently involved in sepsis-related complications (Marshall, 2001). However, brain dysfunction, so called septic encephalopathy, is also a common complication of sepsis reported to occur in up to 70% of patients, depending on the diagnostic criteria applied (Papadopoulos et al., 2000). Importantly, the incidence of septic encephalopathy is associated with a higher mortality rate (Sprung et al., 1990), and its onset often precedes multi-organ failure (Bolton and Perry, 1998), implying a contribution of brain dysfunction to the pathogenic course of sepsis. The mechanisms underlying septic encephalopathy remain poorly understood although cerebral hypoperfusion (Bowton et al., 1989; Maekawa et al., 1991) and functional effects of false neurotransmitters (Freund et al., 1986; Mizock et al., 1990) have been previously proposed. However, more recent studies indicated the direct involvement of the inflammatory process taking place within the brain. Semmler et al. (Semmler et al., 2005) reported that a single intraperitoneal injection of LPS at a septic dose in rats caused a robust astro- and microglial activation and inducible NO synthase (iNOS) induction, with parallel appearance

of apoptosis of brain cells including neurons. The apoptotic cell death was reversed by treatment with a NOS inhibitor, implicating the brain's inflammatory process in neuronal damage. A subsequent study by the same group demonstrated one of the potential functional outcomes of this phenomenon (Semmler et al., 2007). Three months after the LPS treatment, when the animals that survived were completely recovered from sepsis, they exhibited persistent memory deficits that were matched by a loss of neurons in the hippocampus and the prefrontal cortex. A different study also showed that peripheral LPS administration at a septic dose caused a dramatic invasion of granulocyte neutrophils into an otherwise uninjured brain, with parallel disruption of the blood-brain barrier (Bohatschek et al., 2001), indicating that exaggerated systemic inflammation can lead to a highly damaging inflammatory process (given that neutrophils can generate free radicals and release proteolytic enzymes) in an uninfected brain. Although the available data is still limited, it is conceivable that over-activation of immune-to-brain signalling can result in detrimental consequences to the brain. The role of cytokines and PGE2 in such neuroinflammatory conditions warrants further investigation. In addition to sepsis, recent research demonstrated a link between rather moderate to low-grade inflammation such as flu (which in itself causes no harm to the brain) and certain types of neuroinflammation.

### *1.6.2. Systemic inflammation exacerbates pre-existing neuroinflammation*

When the brain suffers a direct trauma, such as ischemic injury and stab wound, it is well established that local CNS inflammatory responses to the primary injury play a central role in the overall brain pathology (Allan et al., 2005). Similarly, there is much speculation on the role of brain inflammation in the progression of chronic neurodegenerative diseases (*e.g.*, Alzheimer's, Parkinson's and prion diseases) (Akiyama et al., 2000; Perry et al., 2002; McGeer and McGeer, 2008). Although it is obvious that peripheral infection itself has no reason to be the primary cause of these brain pathologies, there is accumulating evidence that systemic inflammatory events such as infection could enhance the neuroinflammation associated with acute brain injury as well as the progression of neurodegenerative disease (Emsley and Tyrrell, 2002; Perry et al., 2007). The

important point here is that low-grade systemic inflammation, which alone is insufficient to cause any damage to healthy brains, can trigger exacerbated neuroinflammation and neurotoxicity in pre-diseased brains. Recently, McColl et al. (McColl et al., 2007) have shown that a single *i.p.* injection of LPS or IL-1β at a moderate dose dramatically exacerbated ischemic brain injury and neurological deficit. The detrimental effects of systemic inflammation were nearly completely reversed by co-treatment with IL-1RA, pointing to the key role played by the IL-1 system. Similarly, in a mouse model of prion disease, systemic LPS administration was shown to induce strongly amplified brain synthesis of several inflammatory mediators including IL-1β in the pre-diseased brain, when compared to non-diseased brain receiving LPS alone (Cunningham et al., 2005). Importantly, the presence of prion disease itself did not cause significant brain cytokine production (*e.g.*, IL-1β) in the absence of LPS challenge, thus indicating that the observed difference was not a simple additive effect of two independent inflammatory processes. The exaggeration of LPS-induced brain synthesis of IL-1β and other cytokines in diseased mice was accompanied by a significantly increased apoptosis of neurons as compared to controls without LPS treatment, whereas LPS challenge alone in non-diseased mice did not cause any neuronal loss (Cunningham et al., 2005). A similar scenario is true for a mouse transgenic model of Alzheimer's disease: systemic LPS administration caused an amplified production of IL-1β in the diseased brain, which was associated with altered processing of amyloid-β (Sly et al., 2001) or increased TAU hyperphosphorylation (Kitazawa et al., 2005), both of which are key features of Alzheimer's disease pathology. Perry and his colleagues (Perry et al., 2007) proposed that the diseased brains are "primed" for systemic inflammatory stimuli, and the ensuing immune-to-brain signalling results in exaggerated neuroinflammation, which in turn contributes to the progression of the existing diseases. This hypothesis is supported by clinical observations that infectious episodes accelerate the progression of Alzheimer's disease (Holmes et al., 2003; Dunn et al., 2005) and that preceding or coinciding infectious episodes in stroke patients is associated with poorer outcomes (Emsley and Tyrrell, 2002).

## 1.7. Summary of immune-to-brain signalling

The first half of this literature review provided an overview of immune-to-brain signalling during systemic inflammation, and can be summarized by Figure I-1 below. This thesis primarily focuses on the brain's innate inflammatory response as a key mechanism which links the immune system and the brain. The set of experiments presented in this thesis study the potential role of leptin, an anti-obesity hormone produced primarily in adipose tissue, in activating/modulating the brain's innate inflammatory response, and thereby contributing to sickness behaviour and/or brain diseases.

**Figure I-1. An overview of the immune-to-brain signalling**

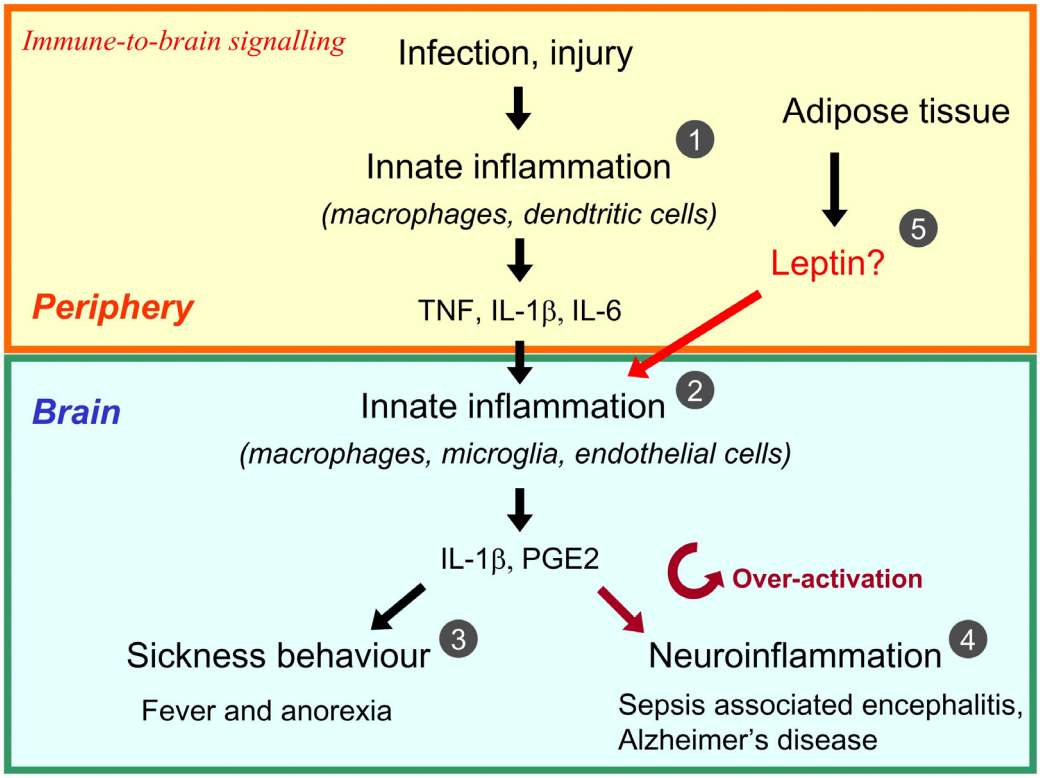


*Figure I-1. An overview of immune-to-brain signalling. (1) Infection or tissue injury triggers the innate inflammatory response in the periphery and the production of TNF, IL-1β and IL-6. (2) The afferent signal transmission, via immune mediators, activates brain-resident macrophages, microglia and endothelial cells leading to a secondary innate inflammatory response within the*

*brain. (3) Inflammatory mediators produced de novo in the brain in turn regulate sickness behaviour. (4) Over-activation of the brain's innate inflammatory response may contribute to various brain diseases. (5) Does leptin play a role in this process?*

# I.2. Leptin, an integrator of energy status, neuroendocrine function and immunity

## 2.1. Leptin as an anti-obesity hormone

### *2.1.1. Leptin—the long sought-after adipostatic factor*

The identification of the *ob* gene in 1994 led to dramatic progress in our understanding of the molecular mechanisms of body weight control (Zhang et al., 1994). Through positional cloning, Friedman and colleagues identified the *ob* gene whose mutation results in a marked obesity phenotype (*ob/ob) in* mice. This postulated anti-obesity hormone was named "leptin" derived from Greek root *leptos*, meaning thin. Shortly after, it was demonstrated that the administration of recombinant leptin in *ob/ob* mouse reverses the obesity by both dramatically reducing food intake and increasing energy expenditure (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995). In wild-type animals and humans, leptin is primarily produced by adipose tissue and secreted into the circulation (Frederich et al., 1995a; Frederich et al., 1995b; Maffei et al., 1995b), where levels correlate positively with body fat mass (energy storage); leptin levels are high in fed status and decline with food deprivation (Frederich et al., 1995a; Frederich et al., 1995b; Maffei et al., 1995b). These early studies provided an overview of leptin's function as a fat-derived systemic factor negatively regulating body weight. Approximately one year after the cloning of leptin, the leptin receptor (ObR) was identified (Tartaglia et al., 1995). This was soon confirmed to be identical to the *db* gene (Chua et al., 1996), whose mutation results in the obese phenotype of *db/db* mice. In fact, Coleman and his colleagues (Coleman and Hummel, 1969; Coleman, 1973) had predicted that the protein product of the *db* gene is the receptor for the *ob* gene product more than 20 years ago, based on parabiosis experiments between *ob/ob* and *db/db* mice or normal mice. Leptin-resistant obesity in *db/db* mice (C57Bl/Ks *db/db*) was attributed to a single nucleotide mutation in the *db* gene resulting in truncation of the intracellular domain of ObR (Chen et al., 1996b; Lee et al., 1996). In mice, five

splice variant forms of ObR were shown to exist (ObRa-e), among which the *db* mutation affects only the ObRb which has a long cytoplasmic region, indicating a critical role of this long form subtype in body weight homeostasis (Tartaglia et al., 1995; Chen et al., 1996b; Lee et al., 1996). These findings established the role of leptin as an important anti-obesity hormone. Indeed, it would be more accurate to say that the identification of leptin acted as a catalyst to prove many of the predicted properties of the long sought-after, putative adipostat signal regulating body weight homeostasis. In a very early review, Kennedy (Kennedy, 1953) proposed the "adipostat theory" to explain the relative stability of body weight (especially fat mass) in many animals despite short-term fluctuations in energy intake and expenditure. This theoretical model and numerous supporting experimental data (Hervey, 1959; Faust et al., 1977; Harris et al., 1986; Harris, 1990) predicted the existence of a circulating factor(s) reflecting the amount of body fat and its actions in the brain regulating appetite and energy expenditure.

*2.1.2. The hypothalamus—the target of the adipostat factor leptin*

The major, if not sole, target of leptin in regulating body weight homeostasis is the brain, more specifically the hypothalamus. This is supported by the fact that mice with hypothalamic lesions develop leptin-resistant obesity (Maffei et al., 1995a) and that central infusion of leptin in normal animals reduces body weight at doses that have no effect when delivered systemically (Campfield et al., 1995; Halaas et al., 1997). In accordance with these physiological data, the functional receptor ObRb was shown to be expressed in the hypothalamus, at high levels in nuclei of the ventrobasal hypothalamic area including the arcuate, dorsomedial, ventromedial and paraventricular hypothalamus, all of which had been implicated in feeding behaviour and thermogenesis (Mercer et al., 1996; Fei et al., 1997; Guan et al., 1997; Elmquist et al., 1998). Neurons in these hypothalamic nuclei express one or more neuropeptides that regulate food intake and/or energy expenditure (Inui, 1999). Important members of these neuropeptide families include cocaine amphetamine-related transcript (CART), α-melanocyte stimulating hormone [α-MSH, a derivative of pro-opiomelanocortin (POMC)] and corticotrophin releasing factor (CRF) that are anorexic/catabolic; and

neuropeptide Y (NPY) and agouti-related protein (AgRP) that are orexigenic/anabolic (Inui, 1999). A number of studies reported co-localization of these neuropeptides with ObRb-expressing neurons in the hypothalamus [see review (Elmquist et al., 1999; Elmquist, 2001; Meister and Hakansson, 2001)]. Moreover, the expression of hypothalamic CART, POMC and CRF are decreased in both leptin-deficient *ob/ob* mice and food deprived rodents (that have low leptin levels), whereas leptin administration in these animals reverses the expression levels (Schwartz et al., 1996; Schwartz et al., 1997; Kristensen et al., 1998). Conversely, *ob/ob* mice or fasted rodents show increased hypothalamic expression of NPY and AgRP, and leptin administration reduces the enhanced levels of these orexigenic peptides (Schwartz et al., 1996; Broberger et al., 1998; Hahn et al., 1998). Thus these neuroanatomical data are in line with leptin's function as a hormone that pushes the balance between anorectic and orexigenic brain signals towards the former.

**Figure I-2. Leptin as an adipostat signal**

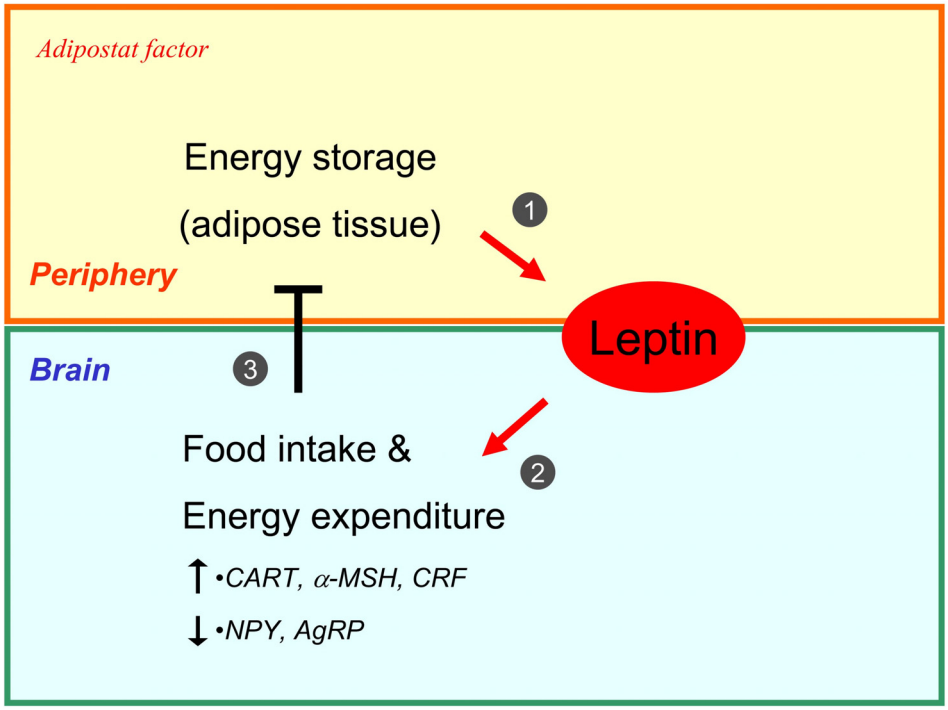


*Figure I-2. Leptin as an adipostat factor. (1) Adipose tissue produces and releases leptin in the circulation. The blood leptin levels (positively) reflect body*

*fat mass. (2) Leptin acts on the hypothalamus and suppress appetite and increases energy expenditure. Leptin shifts the balance between anorectic/catabolic and orexigenic/anabolic neuropeptides toward the former. (3) The actions of leptin negatively regulate body fat mass. The decreases in leptin signalling facilitate fat accumulation.*

In summary, leptin acts as an important negative feedback signal from adipose tissue to the brain (*i.e.*, is an adipostat factor) thereby playing a role in body weight homeostasis. An overview of this leptin function is depicted inFigure I-2.

Lastly, it has to be noted that the presence of leptin alone is not sufficient to prevent obesity, as diet-induced obesity or the numerous mouse strains with obese phenotype (without ObR mutation) show high circulating levels of leptin indicating that these animals are resistant to leptin (Frederich et al., 1995a; Halaas et al., 1997; Van Heek et al., 1997). Likewise, most obese human subjects are in a leptin-resistant state with high blood leptin levels (Considine et al., 1996), although very rare cases of human leptin and leptin receptor mutation have been reported (Montague et al., 1997; Clement et al., 1998; Strobel et al., 1998).

## 2.2. Leptin is a pleiotropic molecule regulating neuroendocrine function

Although it is indisputable that leptin is an essential adipostatic factor that prevents obesity by decreasing appetite and increasing energy expenditure, the biological function of leptin is not limited to this role but is far more pleiotropic. In fact, there exists long-standing evidence that both *ob/ob* and *db/db* mice exhibit, in addition to severe obesity, various neuroendocrine and autonomic abnormalities such as elevated glucocorticoid levels, suppressed thyroid and sex hormones, cold intolerance and functional infertility (Coleman, 1978). It was demonstrated that a reduction of body weight by diet restriction in *ob/ob* mice has little effect on, or in some cases even worsens, these symptoms, whereas leptin administration in these mutant mice reverses the abnormalities, even before their body weight approaches a normal range (Barash et al., 1996; Chehab et al., 1996;

Ahima et al., 1998; Huang et al., 1998). Therefore it was suggested that these abnormalities are unlikely to be the epiphenomena of obesity but rather are direct consequences of leptin deficiency. Available data now clearly show leptin's role in multiple physiological systems beyond the context of prevention of obesity. For example, it has been shown that, in both rodents and humans, blood leptin levels show a diurnal rhythm, reaching its peak at the end of the active period (dark for rodents and light for human) and nadir at the onset of the inactive period (Ahima et al., 1996; Sinha et al., 1996; Ahima et al., 1998; Dallman et al., 1999). Interestingly, this diurnal rhythm pattern is in an inverse temporal relationship with blood glucocorticoid levels (Ahima et al., 1996; Korbonits et al., 1997; Dallman et al., 1999), implicating a role for leptin in the feedback regulation of the HPA axis. In relation to the neuroendocrine role of leptin, a marked gender dimorphism has been reported: higher circulating leptin levels in females than males with similar body fat mass (Friedman, 1998). Acute leptin administration in mice from the time of weaning accelerates the onset of puberty (Barash et al., 1996; Chehab et al., 1996; Ahima et al., 1997), demonstrating its role in reproductive function. Similarly, leptin treatment in lean rodents increases sympathetic nerve activities, heart rate and blood pressure (Dunbar et al., 1997; Casto et al., 1998; Shek et al., 1998; Matsumura et al., 2000). In addition to these brain-mediated mechanisms, it has also been shown that some other biological actions of leptin may directly target peripheral tissues. Receptors for leptin, including the functional ObRb, are widely expressed in various peripheral organs/cells such as liver, lung, fat, muscle, vascular endothelial cells and immune cells (Friedman and Halaas, 1998). Studies have shown that leptin stimulates angiogenesis (Sierra-Honigmann et al., 1998; Park et al., 2001), glucose metabolism (Rossetti et al., 1997; Sivitz et al., 1997; Burcelin et al., 1999), lipogenesis (Chen et al., 1996a), haematopoiesis (Bennett et al., 1996) and immune cell activation (Lord et al., 1998) both *in vitro* and *in vivo*. The relative importance of central versus direct peripheral actions of leptin, however, warrants further investigation because many aspects of leptin-dependent abnormalities are corrected by central leptin administration (Campfield et al., 1995) or brain-

specific restoration of ObRb in receptor deficient *db/db* mice (Kowalski et al., 2001), presumably by modulating the hormonal milieu and/or the autonomic nerve tone. Whatever the underlying mechanisms are, these pleiotropic functions of leptin, not necessarily limited to those expected for body weight regulation, have extended the physiological significance of leptin beyond a simple anti-obesity factor. As circulating leptin levels generally reflect the amount of body energy storage (primarily in the form of fat) (Frederich et al., 1995a; Frederich et al., 1995b; Maffei et al., 1995b), this hormone is well-positioned to signal the nutritional status of the host to various biological functions. As a comprehensive view of leptin's biological status, it has been proposed that leptin is an important molecular link between the host's nutritional status and various biological functions (Friedman and Halaas, 1998). A hypothetical model is presented in Figure I-3.

**Figure I-3. Leptin as a pleiotropic factor**

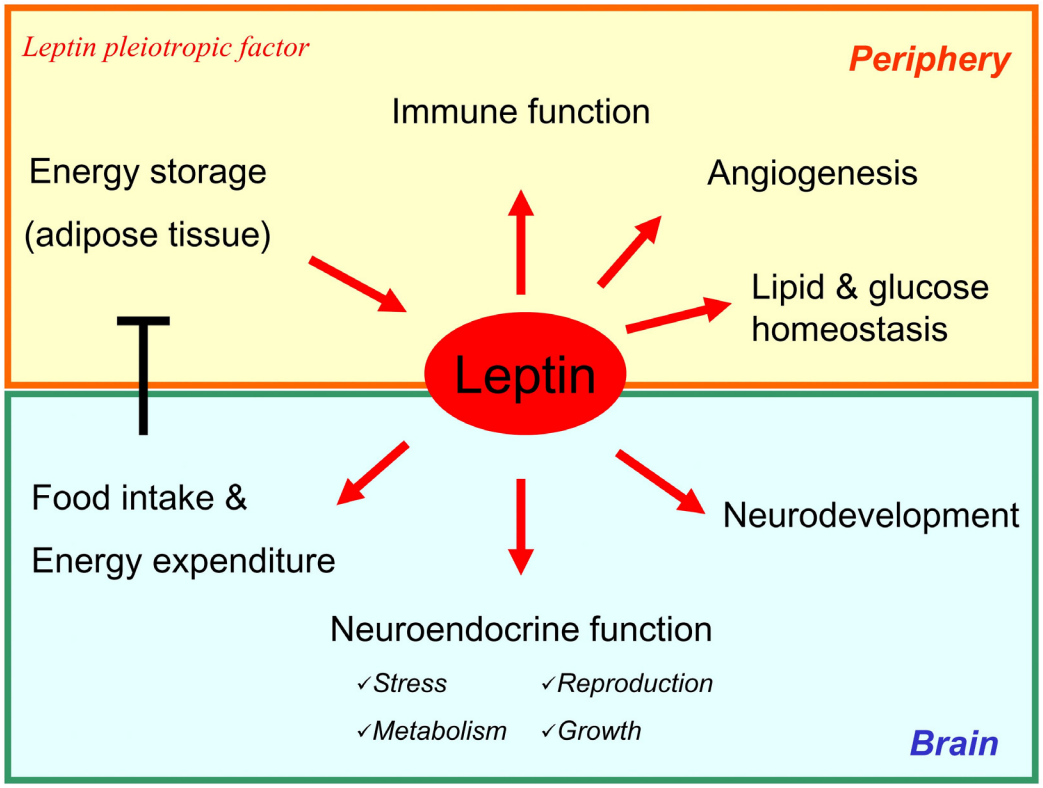


*Figure I-3. Leptin as a pleiotropic factor. In addition to regulating body weight homeostasis, leptin plays a role in a range of biological functions both in the*

*brain and the periphery. As leptin levels reflect energy balance (body fat mass), this hormone is well positioned to link host's nutritional status with various biological functions.*

### 2.3. Leptin as a mediator of the adaptive response to starvation

Although it is somewhat counterintuitive, the most dramatic effects of leptin as a mediator of nutritional status can be seen when leptin levels are decreasing rather than increasing (*i.e.*, at a time of insufficient energy balance). Ahima et al. (Ahima et al., 1996) first reported an important role for leptin in mediating neuroendocrine responses to starvation. In their study, 48 h of food deprivation in mice, which substantially decreased blood leptin levels, triggered a series of neuroendocrine responses including the stimulation of adrenal axis (increased ACTH and corticosterone levels) and the depression of thyroid (decreased thyroxine) and gonadal (decreased testosterone in male and luteinizing hormone in female) axis. Repletion of exogenous leptin during fasting abolished or blunted all of these neuroendocrine alterations as well as functional outcomes such as delay in ovulation in female mice (Ahima et al., 1996), demonstrating that the decrease of leptin is a key signal for the physiological responses to starvation. Importantly, these abnormalities are remarkably similar to those seen in leptin-deficient *ob/ob* mice or leptin-resistant *db/db* mice that are obese, despite the contrasting differences in the actual body fat amount. It is therefore possible to redefine the functional defects of these mutant mice as the perceived state of starvation, wherein the lack of leptin signalling triggers compensatory responses such as hormonal changes, unmitigated hunger and depression of energy expenditure, all of which lead to marked obesity with constant access to food. In accordance with this idea, it was further demonstrated that the fall in leptin levels at the onset of starvation is fairly rapid (within hours after food removal) and that the rate of decline is out of proportion to the actual loss of fat mass (Frederich et al., 1995b; Boden et al., 1996), indirectly supporting its role in signalling the switch from sufficiency to insufficiency of energy balance. Based on these

observations it was proposed that the physiological significance of leptin may be more dominant as a "signal of starvation" rather than its well-known role as an anti-obesity factor that signals the presence of excessive energy stores (Flier, 1998). This view of leptin can be strengthened by a theoretical consideration that such functions of this hormone would have more adaptive value in the course of evolution when periods of inadequate food supply have been dominant.

To date a number of papers have been published describing the role of leptin in this starvation paradigm where this hormone is implicated not only in neuroendocrine responses (Ahima et al., 1996; Bates et al., 2004) but also in metabolic (Stehling et al., 1996; Doring et al., 1998; Overton et al., 2001), thermoregulatory (Stehling et al., 1996; Geiser et al., 1998; Gavrilova et al., 1999; Ukropec et al., 2006) and behavioural (Hillebrand et al., 2005) alterations during starvation. Last, but not least, an important physiological effect of leptin is its regulation of the immune system.

## I.3. Leptin and the immune system

The role of this hormone in immunity now forms an important subfield of leptin research. In retrospect, it is not very surprising because leptin, from its molecular structure, is categorized as a cytokine: leptin shares structural similarity to the long chain helical cytokine family, which includes IL-2, IL-6, IL-12 and granulocyte-colony stimulating factor (Zhang et al., 1994); and its receptor belongs to the family of class I cytokine receptor gp130, a signalling subunit for IL-6, leukocyte inhibitory factor and granulocyte colony-stimulating factor (Tartaglia et al., 1995). On a functional level, leptin-deficient *ob/ob* or -resistant *db/db* mice exhibit severe immune dysfunction with marked atrophy of thymus and spleen (Mandel and Mahmoud, 1978; Chandra, 1980). As is the case for the endocrine and metabolic abnormalities, many aspects of the immune dysfunction observed in these mutant mice resemble those seen in starved mice. In fact, the first critical evidence of leptin's effects on immunity has been provided in the starvation paradigm where repletion of leptin during fasting restored immunosuppression (Lord et al., 1998; Howard et al., 1999). Later, additional

actions of leptin outside of the starvation paradigm have also been reported, where rising levels of leptin (in leptin-sufficient animals and humans) form a part of the cytokine cascade and play an active role in the course of inflammation. As a cytokine, leptin plays an essential role in immune homeostasis as well as in the development of inflammatory response. As a hormone, leptin is a critical molecular link between nutritional status and the immune system.

### 3.1. Leptin is essential for normal immune function.

There exists a substantial amount of data indicating that the absence of leptin results in severe immunosuppression, underscoring its essential role in immune homeostasis. Leptin deficiency, either genetically or due to nutritional deprivation, significantly compromises normal immune responses to pathogens and increases susceptibility and mortality to infection [see review (Ozata et al., 1999; Fantuzzi and Faggioni, 2000; Matarese et al., 2005)]. Replacement of leptin in *ob/ob* or starved mice reverses the immunodeficiency, at least in part through the proliferation of T-lymphocytes and improvement of thymic function (Lord et al., 1998; Howard et al., 1999), demonstrating a direct proliferative role of leptin in the immune system. *In vitro*, various immunocytes isolated from *ob/ob* mice, including T cells (Lord et al., 1998), macrophages (Loffreda et al., 1998; Lee et al., 1999), Kupffer cells (Ikejima et al., 2005) and neutrophils (Moore et al., 2003), exhibit abnormal cytokine production or impaired phagocytic functions which can be corrected by leptin treatment, thus demonstrating its direct effect on immune cells. Given the role of leptin in starvation-induced immunosuppression and the general notion that the immune system is energy costly, it is likely that leptin, at the physiological level, serves as a permissive signal for the immune system in the presence of sufficient energy storage. To date it has been shown that virtually every type of immune cell expresses ObR, and leptin directly stimulates or modulates their functions [see review, (La Cava and Matarese, 2004)].

### 3.2. Leptin is a pro-inflammatory cytokine

In addition to its role in maintaining immune homeostasis, rising levels of leptin have direct stimulatory effects on both innate and adaptive immune functions. In peritoneal and bone marrow-derived macrophages isolated from wild-type mice, leptin treatment dose dependently enhances their phagocytic activities against the parasites *Leishmania major* and *Candida parapsilopsis* (Gainsford et al., 1996; Loffreda et al., 1998), demonstrating that rising levels of leptin are capable of stimulating an aspect of innate immune reactions. Moreover, leptin treatment induces production of classical pro-inflammatory cytokines including TNF, IL-1β, IL-6, and IFN-γ *in vitro* in circulating monocytes from mice and humans (Zarkesh-Esfahani et al., 2001; Dixit et al., 2004; Maedler et al., 2004). In addition, co-treatment of immune cells with leptin significantly enhances the production of TNF, IL-6 and IL-12 in response to stereotypical innate immune stimuli, such as bacterial endotoxin LPS, in murine peritoneal macrophages (Loffreda et al., 1998) and human circulating monocytes (Santos-Alvarez et al., 1999). Likewise, leptin augments IFN-γ-induced production of nitric oxide and PGE2 in murine macrophage cell line (Raso et al., 2002).

In the adaptive immune system, the regulatory role of leptin in T helper (Th)1/Th2 balance has been well established. Based on the profile of cytokine production, T cell subpopulations are classically divided into two types; Th1 cells mainly produce pro-inflammatory cytokines (TNF, IFN-γ and IL-2) that activate macrophages and the cellular immune response, whereas Th2 cells produce anti-inflammatory cytokines (IL-4 and IL-10) that are important for the activation of B cells and basophiles, and humoral immune response. Leptin enhances the proliferation and activation of mature peripheral circulating T cells with bias towards the Th1 cytokine-production profile (Lord et al., 1998; Martin-Romero et al., 2000). Furthermore, leptin increases the expression of adhesion molecules including intercellular adhesion molecule (ICAM)-1 and very late antigen-2 that could then be responsible for the migration and clustering of immune cells to sites of inflammation (La Cava and Matarese, 2004). These *in vitro* data collectively provide strong evidence that leptin can act directly on various immune cells,

where this cytokine-like hormone plays an important role in both the maintenance and stimulation of the immune system.

However, it is also important to note that leptin affects various other systems including neuroendocrine function and autonomic nerve tones, which in turn indirectly affects immune function (Dantzer and Kelley, 1989; Pavlov and Tracey, 2005). In this regard, surprisingly strong (given a series of *in vitro* data discussed above) indirect influence of leptin on immune function *in vivo* has recently been reported. By using bone marrow transplantation, Palmer et al. (Palmer et al., 2006) demonstrated that the transplantation of *db/db* bone marrow cells into wild-type recipients resulted in normal size and cellularity of the thymus as well as humoral immune response. Conversely, the reverse WT bone marrow transplantation to *db/db* recipients decreased thymus size and cellularity, indicating that ObRb expression in the systemic environment, rather than immune cells, is important for normal T cell function. A different study, however, reported that systemic but not central leptin replacement in rats compensates starvation-induced immunosuppression (Zhang et al., 2002), thus favouring the importance of peripheral actions (most likely the direct action on immune cells) of leptin. Further studies are warranted to understand the relative importance of direct and indirect leptin action in the regulation of immune function in an *in vivo* setting.

### 3.3. Leptin in inflammation

Leptin is constitutively produced by adipose tissue and is present in nanogram concentrations in the general circulation (Friedman and Halaas, 1998). Although the levels of leptin under normal conditions are regulated primarily by factors related to body energy homeostasis (see section 2.2), inflammatory stimuli are additional strong signals that increase leptin synthesis above baseline levels. In experimental animals, administration of pro-inflammatory cytokines, such as TNF, IL-1β, and leukemia inhibitory factor have been reported to induce leptin production by adipose tissue and increase its circulating levels (Grunfeld et al., 1996b; Sarraf et al., 1997). Similarly, administration of exogenous pathogens, such as LPS, turpentine and carrageenin (all widely used acute inflammation

models) induce a transient elevation of circulating leptin levels (Faggioni et al., 1998; Gualillo et al., 2000; Mastronardi et al., 2001). The LPS- or turpentine-induced elevation of leptin was absent in IL-1β-deficient mice, demonstrating a critical role of IL-1β in this process (Faggioni et al., 1998). Similarly, pharmacological inhibition of endogenous TNF blunts leptin induction during bacterial peritonitis (Moshyedi et al., 1998). The temporal profile of leptin induction and its interactions with other cytokines (*e.g.*, IL-1β and TNF) strongly indicate that leptin is involved in the cytokine cascade during the course of inflammation.

Both *ob/ob* and *db/db* mice show abnormal inflammatory responses to various types of stimuli (Matarese et al., 2002). Depending on the nature of the inflammatory stimulus, lack of leptin signals results in either an exaggeration or attenuation of the response. In autoimmune disease, leptin generally acts as a pro-inflammatory cytokine that exaggerates the inflammatory process. For example, *ob/ob* mice are resistant to experimental autoimmune encephalomyelitis (EAE), an animal model of human multiple sclerosis (MS), at least in part, through an attenuated Th1-type cellular immune response (Matarese et al., 2001a). Repletion of leptin in these mutant mice reverses the susceptibility to the disease (Matarese et al., 2001a). These observations are in accordance with the aforementioned data that leptin-deficiency causes thymus atrophy (Mandel and Mahmoud, 1978; Chandra, 1980) and that leptin pushes the Th1/Th2 balance towards Th1 *in vitro* (Lord et al., 1998; Howard et al., 1999), as the Th1 immune response plays a key role in EAE pathogenesis (Owens et al., 2001). Interestingly, there exists a sexual dimorphism of blood leptin levels [higher in females than in males matched for the fat mass and age (Friedman and Halaas, 1998)], which corresponds with the gender bias of EAE/MS susceptibility [females have higher susceptibility to autoimmune diseases including MS and EAE in humans and mice, respectively (Matarese et al., 2001a; Whitacre, 2001)]. In line with this, treatment of an EAE-resistant mouse strain with recombinant leptin renders them susceptible to EAE (Matarese et al., 2001a), further supporting the contribution of leptin to disease development. Moreover, it has also been reported that a rapid increase in serum

leptin levels precedes the onset of EAE and the elevation of leptin is correlated with inflammatory anorexia and body weight loss (Sanna et al., 2003), implicating leptin in a more generalized disease process besides T cell immunity. *Ob/ob* or *db/db* mice show resistance to other autoimmune conditions including antigen-induced arthritis (Busso et al., 2002) and experimentally-induced hepatitis (Faggioni et al., 2000b).

Leptin also plays significant roles in acute inflammatory responses against infection or tissue injury, which are largely dependent on an activation of innate immunity. *Ob/ob* mice are more susceptible to bacterial infection, such as Gram-negative *Klebsiella pneumoniae* (Mancuso et al., 2002) and *Listelia. monocytogenes* (Ikejima et al., 2005), with reduced bacterial clearance and increased mortality. Leptin replacement in *ob/ob* mice improved resistance to infection *in vivo* and phagocytic activities of macrophages (Mancuso et al., 2002; Ikejima et al., 2005) and neutrophils (Moore et al., 2003) *in vitro*, indicating an important pro-inflammatory role of leptin in the clearance of exogenous pathogens by innate immune cells. On the other hand, *ob/ob* mice are more susceptible to LPS- and TNF-induced autotoxicity (Takahashi et al., 1999; Faggioni et al., 2000a), suggesting a certain anti-inflammatory role played by leptin in regulating the course of innate inflammation. Supporting this, it has been reported that *ob/ob* mice show reduced production of anti-inflammatory cytokines (IL-1RA and IL-10) during LPS-induced inflammation (Faggioni et al., 1999). Moreover, it has also been reported that *ob/ob* mice have a reduced number of $CD4^{+}$NK cells (Li et al., 2002), a predominant liver lymphocyte population that play a protective role in LPS- and TNF-induced hepatotoxicity by producing anti-inflammatory cytokines (Matsui et al., 1997; Guebre-Xabier et al., 2000). It appears that the effects of leptin, like any other cytokine, on inflammatory processes depend on the type of stimuli as well as the timing of its action. There is still much to learn about the specific roles of leptin in different inflammatory conditions. However, together these series of studies unequivocally demonstrate the important role of this cytokine like hormone in the immune system and inflammation.

### 3.4. Leptin a molecular link between nutritional status and immunity

The dual role of leptin in energy homeostasis and immunity raises the possibility that this hormone acts as a link between the nutritional status and the immune function of the host. For example, malnutrition is a well-known cause of immunosuppression with significantly increased incidence of infection and related mortality (Chandra, 1983, 1985). Although there are numerous nutritional factors, such as zinc, iron, vitamins A, C and E, and polyunsaturated fatty acid, which have been linked to immunological responses (Chandra, 1983, 1985), decreased leptin levels during nutritional deprivation can be an additional factor contributing to the compromised immunologic responses. Supporting this hypothesis, several studies reported the reversal of starvation-induced immunodeficiency by leptin supplementation in experimental animals. For example, decreases in thymus size and total thymocyte counts caused by 48 h of food deprivation were rescued by preventing the fall in leptin levels during fasting by repeatedly administering exogenous leptin in the mice (Howard et al., 1999). This leptin-mediated maintenance of immune cellularity was paralleled with functional recovery that was tested by the delayed-type hypersensitivity response (a measure of T cell priming with antigens) in starved mice with or without leptin treatment (Lord et al., 1998). Likewise, leptin replenishment during fasting in mice improves the host defence response against bacterial infection, in part, by recovering the impaired phagocytic activity and bacterial killing of macrophages, neutrophil mobilization and cytokine production of fasted mice (Mancuso et al., 2006). These leptin-dependent processes mirror those seen in genetic leptin (or its receptor) deficiency despite the contrasting differences in actual nutritional statuses (starvation *vs.* obesity). Therefore, as is the case for neuroendocrine functions, it is reasonable to conclude that a decrease in leptin levels plays a critical role in immunosuppression during starvation. This idea can be extended to some beneficial aspects of nutritional deprivation in autoimmune diseases, because, in the mouse model of EAE, the induction and progression of disease were shown to be successfully prevented by food restriction (Sanna et al., 2003).

It is also worth mentioning that food deprivation in humans has been effective in the treatment of rheumatoid arthritis (Fraser et al., 1999), further supporting the role of leptin linking dynamic changes in nutritional status with immunological responses. This would suggest that, in obesity, increased leptin levels might cause hyperactivation of immune function which will subsequently contribute to the development of autoimmune diseases and/or various health problems (cardiovascular diseases, some types of cancer etc.) which have in fact been linked to overweight. There is a myriad of data indicating that abnormal immune function is associated with obesity [see review (Calle et al., 2003; Lazar, 2005; Tilg and Moschen, 2006)]. However, at present, the specific involvement of leptin in such a process is less well defined than in the case of starvation or genetic leptin deficiency. This is partly because of the complexity of the situation, as most obese subjects, who are characterized by high blood leptin levels, are in a state of partial leptin resistance both at the level of the hypothalamus (Frederich et al., 1995a; Halaas et al., 1997; Van Heek et al., 1997) and individual immune cells (Papathanassoglou et al., 2006). In line with this, obesity has been associated with increased susceptibility to infectious disease (an indicator of immunosuppression) as is the case with starvation (Matarese et al., 2002; Martin et al., 2008). Specific contributions of leptin to obesity-related abnormal immune function await further study. Nevertheless, it is clear that the unique role of leptin in linking nutritional status and immune function will provide a valuable starting point to delineate the complex interaction between body energy status and immune function.

## I.4. The role of leptin in immune-to-brain signalling.

### 4.1. Leptin in anorexia and fever during inflammation

As outlined in Figure I-1, cytokines regulate not only local immunologic reactions but also mediate systemic components of inflammation including fever and anorexia by reaching the brain. Leptin suppresses appetite and increases thermogenesis through its central actions (Friedman and Halaas, 1998), and its circulating levels are acutely increased, as are those of other cytokines, during

inflammation or infection (Grunfeld et al., 1996b; Sarraf et al., 1997; Faggioni et al., 1998; Mastronardi et al., 2000; Mastronardi et al., 2001). Therefore, it is quite reasonable to hypothesize that leptin may act as an additional circulating mediator of anorexia, fever and other brain regulated sickness behaviour during inflammation. Faggioni et al. (Faggioni et al., 1997) first addressed this possibility by testing the anorexic response in *ob/ob* and *db/db* mice following systemic administration of LPS. This initial attempt, however, proved to be negative: it showed that LPS-induced anorexia still exists in *ob/ob* and *db/db* mice, although the latter strain was partially resistant, thus, indicating that leptin is not the major mediator of LPS-induced anorexia (and possibly other aspects of sickness behaviour). Indeed, in the same study, *ob/ob* mice unexpectedly showed more severe anorexia than wild-type counterparts. The exact mechanisms underlying the severe anorexia were undetermined (Faggioni et al., 1997), but different studies reported an enhanced pro-inflammatory reaction of *ob/ob* mice in response to systemic LPS and TNF administrations, rendering them more susceptible to septic shock (Faggioni et al., 1999; Takahashi et al., 1999). It is possible that permanent leptin deficiency alters the (peripheral) innate immune response to LPS [*e.g.*, increased sensitivity to and/or production of classical anorectic factors such as TNF, IL-1β and IL-6 (Faggioni et al., 1999; Takahashi et al., 1999; Madiehe et al., 2003)], which in turn may mask the specific contribution of leptin to anorexia in the mutant mice. Supporting this scenario, a correlation between anorexia and elevation of blood leptin levels has been reported following LPS, TNF and IL-1β injections in wild-type rodents (Grunfeld et al., 1996b; Sarraf et al., 1997; Mastronardi et al., 2000; Mastronardi et al., 2001). More recently, our own study demonstrated the contribution of leptin to LPS-induced anorexia in wild-type rats (Sachot et al., 2004) as opposed to the aforementioned report using *ob/ob* mice (Faggioni et al., 1997). To minimize the potential immune, neuroendocrine or other disturbances caused by chronic leptin-deficiency, we utilized an anti-leptin antiserum (LAS) to transiently neutralize endogenous leptin bioactivity during LPS-induced inflammation. The acute leptin deficiency partially but significantly prevented the reduction of food intake and nearly completely reversed body

weight loss in LPS-treated wild-type animals, supporting the role for leptin in mediating anorexia during inflammation. A subsequent study from a different group using the same antiserum reported similar results (Harden et al., 2006). It remains unclear however whether leptin induces the observed anorexia by activating the mechanisms involved in regulating body weight homeostasis or whether it targets different mechanisms specific for pathophysiological conditions. Available data supports the latter by showing that brain synthesis of cytokines, such as IL-1β, as a downstream mechanism of leptin-mediated anorexia during inflammation (Luheshi et al., 1999; Wisse et al., 2004), suggesting that leptin likely uses mechanisms specific for pathophysiological conditions in mediating the anorexia of disease.

With regard to the fever response, our previous study demonstrated, for the first time, that leptin administration, either centrally or systemically, triggers not only the suppression of food intake but also a transient elevation of core body temperature with a temporal profile similar to that of cytokine-induced fever (Luheshi et al., 1999). Importantly, the leptin-induced fever was associated with an increased synthesis of IL-1β in the hypothalamus and was blocked by central IL-1RA infusion in rats or was absent in IL-1RI-deficient mice. Similarly, co-administration of a COX inhibitor abolished leptin-induced fever, indicating that it is also PG-dependent. Together these results indicate that leptin triggers fever via a classical brain innate inflammatory mechanism in a manner similar to other pyrogenic cytokines (see section 1.4). Subsequent studies generally support this scenario by reporting that leptin administration induces fever (Turek et al., 2004), brain synthesis of IL-1β (Hosoi et al., 2002b; Wisse et al., 2004) and COX-2 (Inoue et al., 2006) in mice and rats, whereas one study reported negligible effects of leptin on body temperature and brain COX-2 mRNA up-regulation in rats (Kelly et al., 2004). The contribution of endogenous leptin to fever has been confirmed by the aforementioned LAS approach which significantly attenuated LPS-induced fever (Sachot et al., 2004; Harden et al., 2006), and brain up-regulation of IL-1β (Sachot et al., 2004) and COX-2 (Inoue et al., 2006) in rats. In accordance with these observations, a defective fever response was also reported

in *fatty* Zucker (*fa/fa*) rats, a mutant rat strain that express dysfunctional ObR (Phillips et al., 1996), as compared to lean wild-type (*Fa/Fa*) counterparts following various pyrogenic stimuli including LPS (Rosenthal et al., 1996; Ivanov and Romanovsky, 2002), IL-1β (Dascombe et al., 1989a; Busbridge et al., 1990), IL-2 (Plata-Salaman et al., 1998) and IL-6 (Plata-Salaman et al., 1998). Busbridge et al. (Busbridge et al., 1990) indicated that the inhibition of IL-1β-induced fever in obese (*fa/fa*) rats was largely attributable to inhibitory effects of corticosterone, which was known to be chronically elevated in the mutant rats (Cunningham et al., 1986). Given that glucocorticoids potently inhibits both transcription and translation of COX-2 (hence PGE2 synthesis) (Tanabe and Tohnai, 2002), these results suggest the suppression of the febrigenic inflammatory responses of the brain (*i.e.*, IL-1β → COX-2) in the absence of ObR signalling. On the other hand, obese *fa/fa* rats have functionally defective brown adipose tissue (BAT, an important thermogenic organ for rodents) and are cold intolerant (Seydoux et al., 1990; Bing et al., 1997), and a different study suggested that a compromised thermogenic capacity of obese Zucker (*fa/fa*) rats may be an alternative, or additional, underlying mechanism for the fever attenuation (Ivanov and Romanovsky, 2002). The relative importance of these two components warrants further investigation.

### 4.2. Leptin, a link between nutritional status, immune system and the brain

As with the case for immune and neuroendocrine functions, leptin appears to serve as a link between the influence of nutritional status and brain-regulated sickness behaviour (*i.e.*, anorexia and fever). Such regulations are of fundamental importance to the survival of organisms, given the immediate impacts of anorexia (reduced energy intake) and fever (increased energy expenditure) on the energy homeostasis. This inter-relationship was clearly shown by numerous studies reporting that reductions in body weight prior to the introduction of inflammation ameliorate, or even abolish, anorexia (Mrosovsky et al., 1989; Kent et al., 1994; Lennie et al., 1995; Lennie, 1998; Lennie et al., 2001; Gautron et al., 2005) and

fever (Hoffman-Goetz and Kluger, 1979b, a; Szekely, 1979; Kleitman and Satinoff, 1981; Molnar and Milner, 1983; Shojoony, 1985; Shido et al., 1989) in various species of experimental animals. In some of these studies, the diminution of anorexia was linked to changes in neuronal activities or expression levels of anorectic and orexigenic neuropeptides in the hypothalamus (Lennie et al., 2001; Gautron et al., 2005). Other studies attributed the attenuation of fever to a compromised metabolic thermogenesis (Kleitman and Satinoff, 1981; Molnar and Milner, 1983; Shojoony, 1985) or an attenuation of cytokine production in the peripheral monocytes and macrophages responding to pathogens (Hoffman-Goetz and Kluger, 1979b, a). At present, specific contributions of leptin to this complex physiological response remain largely unknown. As described above, there exists a functional link between leptin and each of the aforementioned systems (*e.g.*, peripheral immunologic reaction, centrally regulated appetite and thermogenesis). Thus, there are several potential scenarios whereby changes in leptin levels could affect the overall outcome of sickness behaviour. In addition to these, there is another plausible mechanism by which leptin could modulate sickness behaviour during inflammation.

Our own studies and those by others indicated that leptin may act as an afferent inflammatory signal to the brain as this cytokine-like hormone can induce classical pro-inflammatory mediators such as IL-1β and PG in the brain (Brunetti et al., 1999; Luheshi et al., 1999; Hosoi et al., 2002b; Sachot et al., 2004; Wisse et al., 2004). The leptin-induced IL-1β and COX-2 (an indicator of PG synthesis) are localized in brain-resident macrophages (meningeal and perivascular macrophages) and endothelial cells, respectively (Inoue et al., 2006), indicating that leptin targets these non-neuronal cells which comprise the barrier-related structures of the brain. Importantly, this central inflammatory action of leptin is distinct from its reported mode of action for body weight homeostasis, where leptin is believed to act directly on hypothalamic neurons (Friedman and Halaas, 1998; Elmquist et al., 1999; Saper et al., 2002). On the other hand, in relation to the immune-to-brain signalling and sickness behaviour (discussed in detail in section 1.4), these non-neuronal cells play key roles; they detect the peripheral

inflammatory signal, synthesize innate inflammatory mediators (*i.e.*, IL-1β and PG) and regulate the brain's innate inflammatory response. It is therefore plausible that leptin's involvement in this inflammatory mechanism may be an important point of convergence where the changes in nutritional status (via leptin) influence the magnitude and/or quality of sickness behaviour. In addition, elucidating such modulation of the brain's innate immune function would have broader implications beyond sickness behaviour. As discussed in section 1.6, aberrant activation of the brain's innate immune response may underlie the development and/or progression of various neuropathologies such as sepsis associated encephalitis, Alzheimer's diseases, epilepsy, major depression, prion's disease and ischemic brain injury (Papadopoulos et al., 2000; Bohatschek et al., 2001; Kitazawa et al., 2005; McColl et al., 2007; Perry et al., 2007; Dantzer et al., 2008; Riazi et al., 2008). Interestingly, some of these brain pathologies have been suggested to be influenced by the nutritional status of the individuals (Bercault et al., 2004; Scott et al., 2004; Mattson, 2005; Pasinetti and Eberstein, 2008; Plunet et al., 2008). At present, the potential impact of the nutritional status on the brain's innate immune function is largely unknown. This thesis investigates such a relationship by focusing on the role of leptin.

## I.5. Hypotheses and objectives of the thesis

Studies to date provided evidence that leptin is an important mediator of sickness behaviour during inflammation. Coupled with the fact that leptin is a hormone that links the energy status of the host with various biological functions, we proposed that leptin is an afferent inflammatory signal to the brain that modulates the brain's innate inflammatory response in relation to the nutritional status of the organism. A hypothetical model is presented in Figure I-4.

**Figure I-4. Hypothetical model**

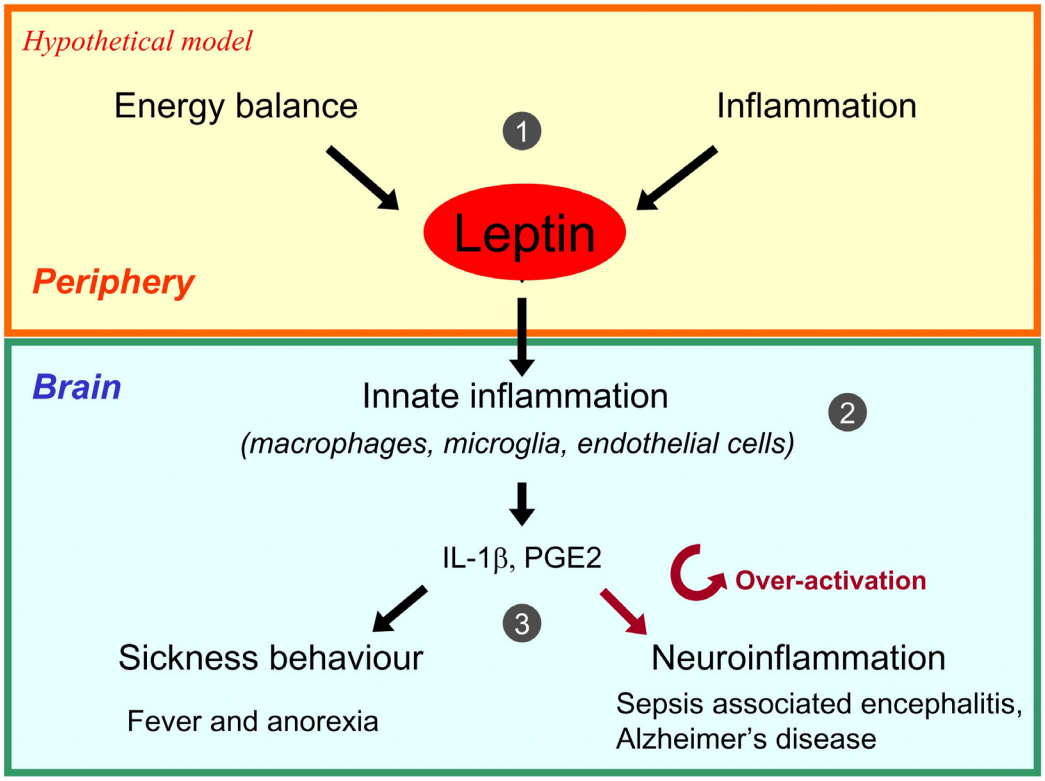


*Figure I-4. Hypothesis. (1) Leptin plays a dual role in energy balance regulation and inflammation. (2) Leptin acts as an afferent inflammatory signal to the brain, thereby linking brain's innate inflammatory response with energy balance. (3) Through this leptin-mediated mechanism, the host's nutritional status influences sickness behaviour and/or neuroinflammation.*

Based on these hypothesises the ***aims*** of the work described in this thesis are to:

***1. investigate if leptin activates the brain's innate inflammatory response***

by examining whether leptin stimulates brain cell production of innate inflammatory mediators, such as IL-1β and PGE2, and to identify the cellular targets of leptin *in vivo* and *in vitro*.

2. ***examine if leptin links energy balance with brain inflammation***

by investigating whether leptin contributes to the over-activation of the brain's innate inflammatory response during severe systemic inflammation and ensuing neuropathology, and by testing whether acute starvation, which decreases circulating leptin levels, ameliorates the observed neuropathology in a leptin-dependent manner.

***3. elucidate if leptin links energy balance with sickness behaviour***

by examining whether acute starvation compromises the fever response during systemic inflammation in a leptin-dependent manner, and by characterizing leptin's regulation of the febrigenic inflammatory response in the brain.

# Chapter II: Leptin induces cyclooxygenase-2 via interaction with interleukin-1β in the rat brain

## II.1. Preface

Leptin, an adipocyte-derived satiety factor, is a cytokine-like peptide that regulates body weight by reducing appetite and increasing energy expenditure via direct actions on hypothalamic neurons (Friedman and Halaas, 1998). Although this remains as the best known function of leptin, a substantial body of evidence indicates that leptin also regulates immune function and inflammation by directly stimulating various immunocytes in the periphery (Fantuzzi and Faggioni, 2000; La Cava and Matarese, 2004). We and others have previously reported that administration of leptin, either systemically or centrally, induces IL-1β, a prototypical pro-inflammatory cytokine in the brain (Luheshi et al., 1999; Hosoi et al., 2002b; Wisse et al., 2004), resulting in PG-dependent fever (Luheshi et al., 1999). These results suggested that leptin may also be an inflammatory signal within the brain. However, the precise mechanisms involved in leptin's stimulation of these pro-inflammatory mediators in the brain remained unknown. Therefore, the initial part of my PhD project aimed to further investigate such inflammatory actions and the cellular targets of leptin in the brain by examining leptin effects on the expression of IL-1β and COX-2 (a PG synthesizing enzyme), two recognized inflammatory signals in fever.

## II.2. Manuscript

**Title: Leptin induces cyclooxygenase-2 via an interaction with interleukin-1β in the rat brain.**

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European Journal of Neuroscience, Vol. 24 p2233-2245, 2006



Keywords: endothelium, fever, inflammation, prostaglandin

## Abstract

In addition to its central effects on appetite regulation, leptin has been implicated in immune function and inflammation. Previous data suggested that leptin acts as an inflammatory signal within the brain, since exogenously administered leptin induced fever, a typical brain-regulated inflammatory response. The present study aimed to delineate the inflammatory actions and cellular targets of leptin in the brain by examining its effects on the expression of interleukin (IL)-1β and cyclooxygenase (COX)-2, two important inflammatory components of the fever response. Intracerebroventricular injection of leptin (5 μg/rat) induced IL-1β and COX-2 mRNA and protein in the hypothalamus between 1 to 3 h after treatment as determined by RT-PCR and immunohistochemistry. Co-injection of IL-1 receptor antagonist (IL-1RA, 100 μg/rat, intracerebroventricular) attenuated leptin-induced COX-2, while IL-1RA had no effect on endogenous IL 1β levels, suggesting that leptin induces COX-2 via, at least partly, IL-1β action. IL-1β protein expression was induced in macrophages in the meningis and perivascular space after leptin treatment, while COX-2 induction was observed in endothelial cells, indicating the roles for these non neuronal cells in mediating inflammatory actions of leptin. In addition, neutralization of endogenous circulating leptin with anti-leptin antiserum attenuated intraperitoneal LPS (100 μg/kg)-induced brain IL-1β and COX-2 up-regulation, suggesting that leptin indeed acts as an inflammatory signal to the brain during systemic inflammation. These findings are in contrast to leptin's effects on appetite regulation where it is believed to act primarily on neurons, thus presenting a distinct anatomical basis for inflammatory and appetite regulatory actions of leptin in the brain.

## Introduction

Leptin, the product of ob gene, is an adipocyte-derived hormone that regulates body weight by reducing appetite and increasing energy expenditure via direct actions on hypothalamic neurons (Friedman and Halaas, 1998). Although this remains to be the major function of leptin, it is now evident that this cytokine-like peptide can directly regulate immune function and inflammation (Fantuzzi and Faggioni, 2000; La Cava and Matarese, 2004). Genetic deficiency of leptin, for example, causes immune suppression that impairs both innate and adaptive immunities (Lord et al., 1998; Howard et al., 1999; Li et al., 2002; Mancuso et al., 2002). In addition, studies have shown that leptin directly stimulates production of a variety of cytokines in immune cells (Fantuzzi and Faggioni, 2000; Matarese et al., 2005). We and others reported previously that systemic administration of leptin induced the pro-inflammatory cytokine interleukin (IL)-1β in the brain (Luheshi et al., 1999; Hosoi et al., 2002b; Wisse et al., 2004), resulting in a prostaglandin (PG)-dependent fever (Luheshi et al., 1999), a hallmark of brain-regulated inflammatory response. Our recent study further showed that neutralization of endogenous leptin with anti-leptin antiserum attenuated lipopolysaccharide (LPS)-induced brain IL-1β up-regulation with parallel amelioration in anorexia and fever (Sachot et al., 2004). Since circulating levels of leptin have been shown to rise acutely following various inflammatory stimuli including LPS (Grunfeld et al., 1996b; Sarraf et al., 1997; Faggioni et al., 1998), these findings suggest that leptin may be acting as an afferent inflammatory signal to the brain during systemic infection and inflammation, in a similar mode as pro-inflammatory cytokines.

Circulating cytokines, such as IL-1β, IL-6 and tumor necrosis factor (TNF)-α, play a major role in the immune-to-brain signalling triggering an array of neurobiological responses including fever and appetite suppression (Rothwell et al., 1996; Dantzer, 2004). The mechanisms by which these cytokines act in the brain involve *de novo* production of secondary mediators including PGE2. Indeed, PGE2 action or its synthesis by cyclooxygenase (COX)-2, an inducible form of

PG synthase, was shown to play a critical role in the fever response (Ushikubi et al., 1998; Li et al., 1999). Following inflammatory stimuli, COX-2 is induced in the brain, uniquely in endothelial cells and perivascular macrophages (Cao et al., 1995; Elmquist et al., 1997; Matsumura et al., 1998c; Schiltz and Sawchenko, 2002), indicating an important role of these non-neuronal cells in the development of sickness responses. Unlike these cytokines, however, very little is known about the inflammatory actions of leptin in the brain. Our earlier studies using a COX inhibitor demonstrated that the leptin-induced fever was PG-dependent (Luheshi et al., 1999). Interestingly, the same inhibitor had no effect on the appetite regulating effect of leptin in the same animals, suggesting that these two effects of leptin involve distinct mechanisms. In the present study, we sought to further delineate the inflammatory actions and cellular targets of leptin in the brain and show that leptin, unlike its effect on appetite regulation via direct actions on neurons, induces IL-1β and COX-2 distinctly in non-neuronal cells including macrophages and endothelial cells.

## Methods

*Preparation of recombinant murine leptin*

Recombinant murine 6-histidine (6-His)-leptin was purified from an E. coli expression system. DNA coding for the sequence of murine leptin with an N-terminal 6-His tag was cloned into a lac-inducible E. coli expression vector. The post-induction cell pastes, which contained induced leptin in the form of insoluble inclusion granules, were a kind gift from Dr. Brian Holloway, AstraZenecca Pharmaceuticals (Alderley Edge, UK). The leptin was recovered from inclusion granules by solubilization and refolding, followed by semi-purification using dialysis as described elsewhere (Rees et al., 1999b). Monomeric leptin was further purified by preparative size-exclusion chromatography on a column (350 ml) of Superdex200 (Amersham Pharmacia Biotech, Buckinghamshire, UK), equilibrated and eluted in 0.05 M ammonium bicarbonate. Fractions containing monomeric leptin, corresponding to Ve/Vt = 0.51 – 0.58 were pooled and lyophilized. The yield corresponded to approximately 2 mg purified leptin/g bacterial paste. The recovered leptin was characterised as follows: Purity was determined to be > 98% by SDS PAGE visualized with coomassie blue staining, with the only observable impurity corresponding to leptin dimers. Electrospray Mass spectroscopy confirmed a major peak corresponding to the predicted molecular weight of murine 6-His-leptin. Bioassay using HEK-293 co-transfected with ObRb/STAT-inducible promoter regulating firefly luciferase cDNA (Rosenblum et al., 1998) showed an equivalent bioactivity (w/w) with the WHO International Standard for murine leptin (Robinson et al., 2001).

Shortly before injection, the lyophilized leptin was dissolved in pyrogen-free saline (Abbott Laboratories Inc. Montreal, Quebec, Canada), and endotoxin levels were measured by a Limulus Amebocyte Lysate (LAL) assay kit (Cape Cod Inc., East Falmouth, MA, USA) and found to be 0.022 IU/μg of leptin. Therefore, the endotoxin potency in 1 μg of leptin was estimated to be equivalent to that in 3.8 pg of LPS (extracted from E. coli, 026:B6; Sigma, Oakville, Ontario, Canada) determined in the same assay (0.0058 IU/pg of LPS).

*Animals*

Adult male Sprague-Dawley rats (250-300 g; Charles River, Saint Constant, Quebec, Canada) were used in all experiments. They were housed individually in a controlled environment at an ambient temperature 21 ± 2 ˚C on 12 h light/dark cycle (light on at 08:00), with free access to water and standard laboratory chow. All experimental procedures were approved by the Animal Care Committee of McGill University pursuant to the Canadian Council of Animal Care guidelines.

*Surgery*

Rats were anesthetised with katamine/xylazine/acepromazine (50, 5, and 0.5 mg/kg, respectively) via intramuscular injection and stereotaxically implanted with 21G guide cannulae (Plastics One, Roanoke, VA, Canada). The coordinates using bregma as a reference point were the following: 0.8 mm posterior, 1.5 mm lateral and 3 mm ventral (from scull surface), resulting in the tip of guide cannula being situated 1 mm above the right lateral ventricle. The guide cannula was fixed to the skull with dental cement (Lang Dental Mfg. Co. Inc., Wheeling, IL, USA) holding two supporting screws with a diameter of 1.2 mm secured on the skull, and was sealed with dummy cannula that reaches the tip of the guide. Rats were allowed to recover for six to nine days post-surgery and were acclimatized to handling 3 days before injection. Injections were performed in conscious free moving animals, with a 26G injection cannula which extended 2 mm beyond the tip of the guide cannula and connected to a 10 µl Hamilton syringe via P50 tubing.

*Intracerebroventricular injections*

Each group of rats (n = 4) was injected intracerebroventriculary (*i.c.v.*) with 5 µg of leptin alone, 100 µg of recombinant human IL-1 receptor antagonist [IL-1RA; National Institute for Biological Standard and Control (NIBSC), Potters Bar, UK] alone, or leptin (5 µg) + IL-1RA (100 µg) at a final volume of 5 µl. Control animals received 5 µl of pyrogen-free saline. The dose of leptin (5 µg/rat) used here was based on our previous dose response study in which a dose of 4 µg/rat was found to produce an optimal fever (Luheshi et al., 1999). This is well

within the range of doses used previously by others which ranged between 0.5–30 μg, *i.c.v.* (Widdowson et al., 1997; Flynn et al., 1998; Hulsey et al., 1998). All injections were carried out between 09:00 to 10:30. The animals were sacrificed and their brains removed for analysis at 30 min, 1, or 3 h after treatment.

In a separate experiment, groups of rats (n = 5) were injected with either saline, 5 μg of leptin, or 20 pg of LPS in a volume of 5 μl to test potential influences of endotoxin contamination on the observed effects of leptin. This amount of LPS is slightly higher than that detected in 5 μg of leptin (3.8 pg/μg X 5μg = 19 pg) as assessed by the LAL assay. The brains from these animals were removed for analysis 3 h post treatment.

*Intraperitoneal injections*

The effects of neutralization of endogenous leptin on LPS-induced IL-1β and COX-2 expression in the brain were tested. Rats received intraperitoneal (*i.p.*) injections of 100 μg/kg of LPS in combination with 1 ml/kg of sheep anti-leptin antiserum (LAS; NIBSC). 1 ml/kg of pyrogen-free saline and normal sheep serum (NSS; Sigma) were used as control reagents for LPS and LAS, respectively. Namely, 4 groups of animals (n = 4) were injected with either saline + NSS, LPS + NSS, saline + LAS, or LPS + LAS by two consecutive *i.p.* injections performed at the same time. The dose of LPS (100 μg/kg) and LAS (1 ml/kg) used here were based on our previous study in which significant effects of LAS were observed on LPS-induced fever and appetite suppression (Sachot et al., 2004). Injections were carried out between 09:00 to 10:30. The animals were sacrificed 3 h following treatment.

*Tissue processing*

At appropriate time points, rats were deeply anesthetised with katamine/xylazine/acepromazine (100, 10, and 1 mg/kg, respectively) and perfused with ice-cold diethyl pyrocarbonate (Sigma)-treated saline via the ascending aorta in order to remove circulating blood cells from the brain. Brains were quickly removed, frozen in powdered dry ice and kept at 80 ˚C until analysis. Coronal cryostat sections (14 μm) were prepared by cutting through the

preoptic area and the anterior hypothalamus (bregma 0 to –1.8 mm) in a cryostat. In order to perform immunohistochemistry and RT-PCR on tissue taken from the same brain, seven consecutive sections were thaw mounted on glass slides for immunohistochemistry and the next one section was overlaid on a frozen glass slide for RT-PCR. This procedure was repeated 17 times until we reached bregma −1.8 mm. The hypothalamic region (2 mm right and left from the midline, and 4 mm from the bottom edge of the brain) was then punched out from the piled brain sections and stored in a cryotube at −80 ºC for total RNA extraction.

*Semiquantitative RT-PCR*

Total RNA was extracted using TRIzol (Invitrogen, Burlington, Ontario, Canada) according to the manufacturer's instructions. The first-strand cDNA was synthesized from 1 μg of total RNA using 200 units of Molony murine leukemia virus reverse transcriptase (Invitrogen), 5 μM of random hexamers (Applied Bioscience, Streetsville, Ontario, Canada), and 1 mM of dNTP mix (Sigma) in a total reaction volume of 20 μl. Following reverse transcription, the product was stored at −20 ºC. The cDNA product (0.9 μl) was added to 15 μl PCR reaction mix containing ReadyMix Taq PCR (Sigma) and 6 pmol of gene-specific primer sets for IL-1β, COX-2 and β-actin. Amplification was performed with a thermal cycler (GeneAmp PCR system 9700; Applied Bioscience) using the following cycling parameters:(1) denaturing; 95 ºC for 5 min, (2) amplification cycle; 95 ºC for 30 sec, annealing temperature for 30 sec and 72 ºC for 1 min (3) final extension; 72 ºC for 10 min. Primers were designed to span a sequence derived from different exons (separated by intron in genomic DNA sequence) in order to minimize amplification from non RNA derived templates. Inappropriate amplification from genomic DNA was negligible when amplification was performed with a template without reverse transcription. The gene accession numbers, primer sequences, annealing temperatures and cycle numbers used are listed as following: β-actin (NM_031144) sense 5'-GCCGTCTTCCCCTCCATCGTG-3', anti-sense 5'-TACGACCAGAGGCATACAGGGACAAC-3', 60 ˚C, 20 cycle; IL-1β (NM_031512) sense 5'-CCCAAGCACCTTCTTTTCCTTCATCTT-3' anti-sense

5'-CAGGGTGGGTGTGCCGTCTTTC-3', 60 ˚C, 36 cycle; COX-2 (NM_017232) sense 5'-TGATAGGAGAGACGATCAAGA-3', anti-sense 5'-ATGGTAGAGGGCTTTCAACT-3', 57 ˚C, 32 cycles. PCR products were separated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide staining. The band density was obtained for quantification using GeneTool image analysis software (Syngene, Frederick, MD, USA). To normalize the expression level of genes between different samples, the levels were estimated as the ratio of geneX/β-actin. In a pilot experiment, the amount of PCR product (on a log scale) was plotted against the number of cycles, and the linear range of template amplification was determined for 2 samples from each treatment group. The cycle numbers were determined within the exponential phase of amplification for all treatment groups.

*Immunohistochemistry*

On the day of the experiment, the frozen brain sections were air-dried at room temperature for 20 min and then fixed with 2% paraformaldehyde for 10 min. After briefly rinsing with PBS (pH 7.4), the sections were treated with blocking solution containing 10% normal donkey serum (Chemicon International Inc., Temecula, CA, USA) and 0.1% Triton-X100 in PBS, at room temperature for 1 h followed by the primary antibody of anti-IL-1β or anti-COX-2 at 4 ˚C for 48 h. The anti-IL-1β and anti-COX-2 antibodies were raised against a recombinant rat IL-1β protein (dilution; 0.5 μg/ml, raised in rabbit; cat: 500-P80; PeproTech Inc., Rocky Hill, NJ, USA) and a peptide corresponding to amino acids 584-598 of the murine COX-2 (dilution; 0.25 μg/ml, raised in rabbit; cat: 160126; Cayman Chemical, Ann Arbor, MI, USA), respectively. The primary antibodies were visualized with CY3-conjugated anti-rabbit IgG (1:500 dilution; cat: 711-065-152). All secondary antibodies were multiple labelling-grade raised in donkey (Jackson ImmunoResearch, West Grove, PA, USA). The specificity of the staining was confirmed by pre-absorption of the anti-IL-1β and COX-2 antibodies with rat recombinant IL-1β (0.1 μM; NIBSC) and murine COX-2 blocking peptide (0.1 μM; Cayman Chemical), respectively.

To identify the cell type(s), double immunhistochemistry was performed using specific cell marker antibodies. The antibody for IL-1β was mixed with a murine anti-ED2 monoclonal antibody (1 μg/ml; cat: MCA342R; Serotec, Raleigh, NC, USA), a cell marker for perivascular and meningeal macrophages in the brain. The COX-2 antibody was mixed with anti-ED2 or sheep anti-rat von Willebrand factor antibody (1.67 μg/ml; cat: SARTW-IG; Affinity Biologicals, Ancaster, Ontario, Canada), a cell marker for endothelial cells. Brain sections were incubated with the primary antibodies at 4˚C for 48 h, followed by secondary antibodies. IL-1β and COX-2 were visualized with CY3-conjugated anti-rabbit IgG (1:500 dilution), ED2 with FITC-conjugated anti-mouse IgG (1:500 dilution; cat: 715-095-151) and von Willebrand factor with FITC-conjugated anti-sheep IgG (1:500 dilution; cat: 713-095-147). In control experiments, the sections were incubated with the primary antibody mixture in which one of the primary antibodies was substituted with non-immunized animal IgG (rabbit IgG for IL-1β and COX-2, mouse IgG for ED2 and sheep IgG for von Willebrand factor), and inappropriate cross-reaction between antibodies was confirmed to be negligible. Non-immunized IgGs were obtained from Jackson ImmunoResearch.

Signal transducer and activator of transcription (STAT)3 proteins were detected by a rabbit polyclonal anti-STAT3 antibody (dilution 0.025 μg/ml, cat: sc-482, Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by CY3-conjugated anti-rabbit IgG. This antibody was clearly shown to immunohistochemically detect the activation of STAT3 by visualizing accumulation of its signal within cell nuclei in rat brains after *i.c.v.* leptin injection (Hubschle et al., 2001). In studies involving double immunostaining for STAT3 + IL-1β and STAT3 + COX-2, a sheep polyclonal anti-rat IL-1β raised against recombinant rat IL-1β (s328; NIBSC) and a goat polyclonal anti-COX-2 raised against C-terminus of murine COX-2 (cat: sc-1747; Santa Cruz Biotechnology) were used. Sheep anti-IL-1β (1 μg/ml) and goat anti-COX-2 (0.1 μg/ml) antibodies were mixed with rabbit anti-STAT3 (0.025 μg/ml). IL-1β and COX-2 signals were visualized with FITC-conjugated anti-sheep IgG (1:500

dilution) and FITC-conjugated anti-goat IgG (1:500 dilution; cat: 705-095-147), respectively. The specificity of the IL-1β and COX-2 staining was confirmed by pre-incubation of the antibodies with recombinant rat IL-1β (0.1 μM; NIBSC) and a specific blocking peptide for COX-2 (1 μM; cat: sc-1747P, Santa Cruz Biotechnology), respectively. Inappropriate cross-reaction between antibodies was negligible when primary antibodies were substituted with non-immunized animal IgGs.

*Data analysis*

All data are presented as mean values + standard error of the mean (SEM). IL-1β and COX-2 levels at different time points after *i.c.v.* injections were analyzed by parametric between-subject two-way analysis of variance (ANOVA) with treatment and time factors. Two-way ANOVA was also used to analyze the data from *i.c.v.* leptin injection in combination with IL-1RA. Bonferroni post-hoc pairwise comparison test was used to compare the effects of treatment. One-way ANOVA was used to analyze rest of the data. Statistical values (P value) less than 5% were deemed significant.

## Results

*Intracerebroventricular injection of leptin induces IL-1β and COX-2 expression in the hypothalamus*

Effects of leptin (5 μg/rat, *i.c.v.*) on IL-1β and COX-2 expressions in the hypothalamus were studied by semi-quantitative RT-PCR and immunohistochemistry at 30 min, 1, and 3 h post-injection. Figure II-1A shows that leptin administration significantly increased hypothalamic IL-1β mRNA levels by 1 h (ANOVA; $F_{1,18} = 39.25$, $P < 0.0001$, post-hoc; saline *vs.* leptin, $P < 0.001$) and even more pronounced at 3 h ($P < 0.001$) after injection as compared with saline treatment. COX-2 mRNA levels were significantly increased by leptin as early as 30 min after injection (ANOVA; $F_{1,18} = 28.72$, $P < 0.0001$, post-hoc; saline *vs.* leptin, $P < 0.05$) and remained elevated at 1 h ($P < 0.01$) and 3 h ($P < 0.05$) (Figure II-1A). Expression and distribution of IL-1β and COX-2 proteins were studied by immunohistochemisty. In saline-treated animals, IL-1β-like immunoreactivity (ir) was not detectable in the hypothalamus at any time point studied (30 min, 1 and 3 h; Figure II-1B). Sparse IL-1β-ir was evident in leptin-treated brains by 1 h after injection (data not shown) and further increased (number of positive cells and intensity of staining) by 3 h (Figure II-1B). This IL-1β-ir was mostly observed in the meningis, but weaker IL-1β-ir was also evident in the parenchyma along blood vessel-like structures (Figure II-1B). Pre-absorption of anti-IL-1β antibody with 0.1 μM of recombinant rat IL-1β protein completely abolished IL-1β-ir, confirming the specificity of the staining (Figure II-1B, inset). In agreement with earlier studies (Breder et al., 1995; Matsumura et al., 1998c), constitutive COX-2-ir was observed in neuron-like cells in several regions, such as the cerebral cortex, hippocampus and suprachiasmatic nuculei in both saline- and leptin-treated animals, but no change in the neuronal COX-2-ir was apparent at any time points after leptin as compared to saline treatment (data not shown). Besides the neuronal COX-2-ir, a weak, constitutive, COX-2-ir was also observed in blood vessel-like structures in saline-treated animals (Figure II-1B). The number and intensity of non-neuronal COX-2 staining were clearly

increased by leptin treatment by 1 h after injection as compared to saline treatment and further increased by 3 h (Figure II-1B). The non-neuronal COX-2-ir was observed throughout the parenchyma of the hypothalamus and in the meningis within blood vessel-like structures (Figure II-1B). The specificity of the staining was confirmed by repeating the immunohistochemical procedure using the COX-2 antibody pre-incubated with 0.1 μM COX-2 peptide. This treatment abolished all visible staining (Figure II-1B, inset).

The endotoxin level in the leptin preparation was found to be equivalent to 3.8 pg LPS/μg leptin, as determined by a LAL assay. Injection of LPS at slightly higher dose (20 pg/rat) than that found in 5 μg of leptin (the dose used for *i.c.v.* injections) failed to affect IL-1β and COX-2 mRNA levels at 3 h after injection (Figure II-1C, ANOVA; Ps < 0.0001, post-hoc; saline *vs.* LPS, Ps > 0.05, saline *vs.* leptin, Ps < 0.001), confirming that the observed effects of leptin on IL-1β and COX-2 induction were not due to endotoxin contamination.

*IL-1RA attenuates leptin-induced COX-2 expression*

We have previously reported that *i.c.v.* administration of IL-1RA attenuated leptin-induced fever (Luheshi et al., 1999). In the present study, we tested whether IL-1RA affects levels of leptin-induced COX-2. Recombinant IL-1RA (100 μg/rat, *i.c.v.*) was co-administered with either leptin (5 μg, *i.c.v.*) or saline, and the levels of IL-1β and COX-2 in the hypothalamus were studied by semi-quantitative RT-PCR and immunohistochemistry 3 h after injection. Treatment with IL-1RA alone had no effect on the levels of either IL-1β or COX-2 when compared to saline treated controls (Figure II-2A). While the level of leptin-induced IL-1β mRNA was not affected by IL-1RA (ANOVA; $F_{1,12} = 0.06$, $P > 0.05$, post-hoc; leptin + vehicle *vs.* leptin + IL-1RA, $P > 0.05$), the induction of COX-2 mRNA by leptin was almost completely abolished (ANOVA; $F_{1,12} = 12.41$, $P < 0.01$, post-hoc; leptin + vehicle *vs.* leptin + IL-1RA, $P < 0.01$). Similar results were obtained by immunohistochemistry, in which IL-1RA treatment clearly, albeit partially, attenuated leptin-induced COX-2-ir in non-neuronal cells (Figure II-2B), while it had no effect on IL-1β-ir (data not shown), suggesting that leptin induces COX-2 via, at least in part, an IL-1β action-dependant manner. The constitutive COX-2-ir

in neuron-like cells was not affected by IL-1RA treatment in the presence or absence of leptin (data not shown).

*Leptin induces IL-1β and COX-2 in non-neuronal cells*

The cell type(s) displaying IL-1β-ir were studied by dual immunohistochemistry using anti ED2 monoclonal antibody, a cell marker for perivascular and meningeal macrophages in the brain. At 3 h after leptin treatment, a subset of IL-1β-ir cells in the meningis was co-labelled with ED2-ir (Figure II-3). Furthermore, IL-1β-ir cells in the brain parenchyma that were associated with blood vessel-like structures were co-labelled with ED2-ir (Figure II-3), suggesting that they are either meningeal or perivascular macrophages. The non-neuronal COX-2-ir associated with blood vessel-like structures in the brain parenchyma and meningis was co-labelled with von Willebrand factor-ir, a cell marker for endothelial cells (Figure II-3). Since ED2-positive perivascular cells are also known to express COX-2 in response to inflammatory stimuli (Elmquist et al., 1997; Schiltz and Sawchenko, 2002), co-labelling of COX-2 and ED2 after leptin treatment was performed. Despite their immediate vicinity, COX-2-ir did not co-localize with ED-2-ir positive cells (Figure II-3).

*Stat3 activation in IL-1β- and COX-2-positive cells*

The Janus kinase signal transducer and activator of transcription (Jak-Stat) signalling pathway is the major intracellular signalling cascade triggered by leptin binding to its receptors in the brain (McCowen et al., 1998), thus activation of STAT3 is broadly used as a functional marker for leptin's action (Hubschle et al., 2001). To further clarify the link between leptin's action and the expression of IL-1β and COX-2 in the meningis and brain blood vessels, the distribution of STAT3 activation after leptin treatment was studied by immunohistochemistry. In saline-treated animals, moderate STAT3-ir was observed in neuron-like cells as well as in the meningis and blood vessel-like structures (Figure II-4A). This constitutive STAT3-ir was mostly observed in cytoplasma of these cells. Leptin injection strongly induced accumulation of STAT3-ir within the cell nuclei in neurons as well as in non-neuronal structures such as, ventrally located meningis and blood

vessel-like structures (Figure II-4A). The increase in the nuclear STAT3-ir by leptin was highest at 30 min, still prominent at 1 h, and thereafter decreased by 3 h. The double labelling of STAT3-ir with nuclear DAPI staining (Figure II-4A) depicts the nuclear accumulation of STAT3-ir both in neuron-like cells in the piriform cortex, as well as in cells along blood vessel-like structures and the meningis at 1 h after injection. Co-localization of STAT3-ir with IL-1β-ir and COX-2-ir was also studied. Double immunohistochemistry, however, showed no cellular co-localization of STAT3-ir with IL-1β-ir in the meningis at 1 h after treatment although these two signals were in close vicinity (Figure II-4B). On the other hand, COX-2-ir was clearly co-localized with STAT3-ir in blood vessel-like structures at the same time point (Figure II-4B). This time point was chosen as a compromise between the peak of STAT3-ir (30 min) and IL-1β-ir and COX-2-ir (3 h) by leptin.

*Endogenous leptin mediates brain IL-1β and COX-2 expression during LPS-induced systemic inflammation*

Production and circulating levels of leptin are known to acutely increase after various inflammatory stimuli including LPS (Grunfeld et al., 1996b; Sarraf et al., 1997; Mastronardi et al., 2001). Based on our recent observation that inhibition of endogenous leptin with a neutralizing antiserum attenuated LPS-induced fever and the accompanying elevation of IL-1β mRNA levels in the brain (Sachot et al., 2004), the present study evaluated the changes in brain COX-2 levels in the same context. Rats were injected with LPS (100 μg/kg, *i.p.*) or saline in combination with neutralizing sheep anti-leptin antiserum (LAS, 1 ml/kg, *i.p.*) or normal sheep serum (NSS, 1 ml/kg, *i.p.*). The levels of IL-1β and COX-2 expression in the hypothalamus were studied by semi-quantitative RT-PCR and immunohistochemistry 3 h after injection. This time point corresponds to a time when the highest COX-2 protein expression was observed in cerebral vasculatures after the same dose of *i.p.* LPS injection (Inoue et al., 2002). Treatment with LAS alone did not change the levels of either IL-1β or COX-2 mRNA in the hypothalamus when compared to NSS-treated animals (Figure II-5A, ANOVA; $Fs_{3,19} > 12.62$, $Ps < 0.001$, host-hoc; saline + NSS *vs.* saline + LAS, $Ps > 0.05$).

Injection of LPS in the presence of NSS significantly induced both IL-1β (saline + NSS *vs.* LPS + NSS, $P < 0.001$) and COX-2 ($P < 0.001$) mRNAs. In the presence of LAS, the LPS-induced IL-1β mRNA was significantly attenuated (LPS + NSS *vs.* LPS + LAS, $P < 0.01$). The attenuated level of IL-1β mRNA was not significantly different from that of saline + LAS treated group (saline + LAS *vs.* LPS + LAS, $P > 0.05$). In addition to the changes in IL-1β mRNA levels, LAS treatment partially but significantly attenuated LPS-induced COX-2 mRNA levels (LPS + NSS *vs.* LPS + LAS, $P < 0.05$). The attenuated level of COX-2 mRNA was still significantly higher than that of saline + LAS treated group ($P < 0.01$).

Immunohistochemical analysis revealed that LPS treatment induced relatively moderate expression of IL-1β-ir, as compared to the dramatic increase observed at the mRNA level. Treatment with LAS did not significantly affect the IL-1β-ir (Figure II-5B). On the other hand, COX-2-ir which was strongly induced by LPS in blood vessel-like structures, was clearly attenuated in the presence of LAS (Figure II-5B).

## Discussion

It has become increasingly apparent that leptin, besides its central effects on appetite regulation, plays a role in immune function and inflammation through direct influence on immune cells (Fantuzzi and Faggioni, 2000; La Cava and Matarese, 2004). Our previous data suggested that leptin could also be an inflammatory signal within the brain, since administration of recombinant leptin, either *i.c.v.* or *i.p.*, induced fever (Luheshi et al., 1999), a typical brain-regulated inflammatory response. The present study provides further support for this notion by demonstrating that *i.c.v.* injection of leptin induced IL-1β and COX-2 in the hypothalamus. Using immunohistochemistry, we found that leptin induced these pro-inflammatory mediators uniquely in non-neuronal cells including macrophages and endothelial cells while having no such effects on neurons. These findings are contrasting to its effects on energy homeostasis where leptin is believed to act primarily on neurons (Friedman and Halaas, 1998), thus presenting a distinct mechanisms for its inflammatory actions in the brain.

Previous studies have shown that leptin stimulates IL-1β production in various cells/tissues including macrophages/monocytes, T-lymphocytes, adipose tissue and brain (Luheshi et al., 1999; Hosoi et al., 2002b; Dixit et al., 2004; Lappas et al., 2005), indicating that IL-1β may act as a downstream regulator of leptin's action. Indeed, exposure of cultured human islets to leptin caused apoptosis and impaired function of βcells in an IL-1-dependent mechanism (Maedler et al., 2004). It has also been shown that leptin activates a subset of afferent vagal nerve fibres via interaction with IL-1β (Gaige et al., 2002; Gaige et al., 2004). In the brain, our recent study demonstrated that neutralization of endogenous leptin with anti-leptin antiserum attenuated LPS-induced IL-1β up-regulation, with parallel reversal of the anorexia and body weight loss and an attenuated fever response to LPS (Sachot et al., 2004). It has also been shown that leptin induced IL-1β mRNA in cultured mixed glial cells (Hosoi et al., 2000a). Given the potent anorectic and pyrogenic effects of IL-1β, these findings collectively indicate that IL-1β acts as a downstream signal of leptin under

inflammatory conditions. Interestingly, a recent study also suggested a role for brain IL-1β in leptin signalling under physiological conditions, by presenting similar reductions in hypothalamic IL-1β mRNA levels between obese Zucker (*fa/fa*) rats (impaired leptin signalling) and fasted wild-type animals (lowered leptin levels) (Wisse et al., 2004).

In the current study, we demonstrate that ED2-positive perivascular and meningeal macrophages were the major cell groups in the brain producing IL-1β in response to leptin. These brain macrophages appear to be important targets for leptin since various types of peripheral macrophages express ObR, and leptin directly enhances phagocytic function and cytokine production in these cells (Grunfeld et al., 1996b; Loffreda et al., 1998). We also observed that a subset of IL-1β-positive cells in the meningis were ED2-ir negative. Although previous histological studies have identified ObR expression in astrocyte-like cells in rodents (De Matteis and Cinti, 1998; Diano et al., 1998), the unidentified cells found in our study were uniquely localized in the meningis and therefore were unlikely to be astrocytes, but rather likely to be different types of meningeal macrophages labelled by other lines of cell markers.

The main finding of our current study is the demonstration that leptin induced COX-2 mRNA and protein expressions in the brain. Immunohistochemical approaches revealed that the induced COX-2-ir was localized in cells along blood vessels. The blockade of IL-1 action by IL-1RA clearly attenuated leptin-induced COX-2 expression, pointing to a potential mechanism that leptin induces vascular COX-2 expression via stimulation of IL-1β production. Several studies have shown that brain vasculature expresses the functional IL-1 receptor IL-1R1 (Ericsson et al., 1995; Konsman et al., 2004), and that IL-1β induces COX-2 in these cells (Cao et al., 1996; Schiltz and Sawchenko, 2002), collectively supporting the possibility of IL-1β-dependent effects of leptin on those cells. It is intriguing to note that, leptin, a cytokine originating from the periphery, shares common inflammatory properties with other circulating cytokines activating the interface between the periphery and brain parenchyma, namely the cerebral vasculature.

The kinetics of IL-1β and COX-2 mRNA induction after leptin injection (Figure II-1A), however, do not fully support the idea of an IL-1β-dependent mechanism, as the COX-2 mRNA levels peaked as early as 30 min after the injection when IL-1β levels were still low. This could be due to a number of possibilities; firstly, leptin triggers the release of IL-1β protein, which quickly increases COX-2 mRNA before *de novo* up-regulation of IL-1β mRNA is detectable. Secondly, leptin induces IL-1α, which like IL-1β signals through IL-1R1, and IL-1α is predominantly responsible for the early induction of COX-2. Thirdly, it may be possible that injection of leptin into the brain ventricles results in peripheral IL-1β induction which will in turn act on the endothelial cells lining the blood brain barrier to trigger COX-2 induction. Finally, leptin induces COX-2 in an IL-1-independent mechanism, in addition to an IL-1-dependent one.

To address the link between the actions of leptin and induction of IL-1β and COX-2, we further examined activation of STAT3 within the IL-1β- and COX-2-ir positive cells. Stat3 is a key, but not sole, signalling component for leptin-dependent gene expression mediated by the long form leptin receptor ObRb (Bjorbaek and Kahn, 2004), and is widely used as a functional marker for leptin's actions. As reported previously (Hubschle et al., 2001), leptin induced marked STAT3 activation in neuron-like cells in various brain structures by 30 min after the treatment. In addition, the current study showed that the leptin-induced STAT3 activation was readily detectable in non-neuronal structures, such as the meningis and cerebral vasculatures, an observation consistent with a recent study (Mutze et al., 2006). Although STAT3 activated cells and IL-1β-ir positive cells reside in close vicinity, STAT3-ir and IL-1β-ir did not co-localize (Figure II-4B). It should be noted that the absence of co-localization could be reflecting the time point (1 h) chosen here because of the compromise between the maximum expression of STAT3 (30 min) and IL-1β-ir (3 h). Another possible explanation is that leptin first induces other inflammatory mediator(s) via STAT3 activation (most likely in STAT3-ir positive cells proximal to the IL-1β-ir positive cells), and this factor in turn induces IL-1β. Lastly, leptin may induce IL-1β via a STAT3-independent mechanism. Hosoi et al. (Hosoi et al., 2002b) have addressed

this issue by using obese *db/db* mice, which lack ObRb and thus subsequent leptin-dependent STAT3 activation. In this study, they observed that systemic injection of leptin increased hypothalamic IL-1β mRNA in the *db/db* mice in comparable magnitude to that observed in wild-type mice, suggesting that leptin induces IL-1β in the brain via a STAT3-independent mechanism. Besides the Jak-Stat pathway, ObR is known to signal through other intracellular signalling molecules including MAPK (Bjorbaek et al., 1997) and PI3K (Wang et al., 1997). In fact, it was recently shown that leptin stimulated TNF-α production via activations of MAPKs (*i.e.* ERK1/2 and p38) in murine peritoneal macrophages (Zhao et al., 2005), and that leptin activates the IL-1RA promoter through ERK1/2 pathway (Dreyer et al., 2003), indicating an involvement of STAT3-independent pathways in the inflammatory actions of leptin.

Although IL-1β action appeared to play a major role in COX-2 induction by leptin, this observation does not totally exclude IL-1β-independent mechanisms. We observed, rather unexpectedly, that COX-2-ir co-localized with nuclear accumulation of STAT3-ir, indicating direct actions of leptin on COX-2-expressing cells (*i.e.,* endothelial cells). Recently, a study by Mutze et al. (2006) showed similar STAT3 activation in rat brain endothelial cells after systemic injection of leptin suggesting that it may play a direct role in COX-2 induction in brain endothelial cells. This is supported by our observation of an early induction of COX-2 mRNA preceding the increase of IL-1β mRNA (Figure II-1A). Although we cannot exclude the possibility that the STAT3 activation in COX-2-positive cells was induced by other factor(s) produced secondary to leptin's action (*e.g.* IL-6), studies have shown that leptin has direct effects on peripheral as well as retinal endothelial cells where it promoted angiogenesis and cell proliferation via STAT3 activation (Sierra-Honigmann et al., 1998; Suganami et al., 2004).

Our data showing leptin-induced brain COX-2 provides a molecular basis for our previous report that exogenous leptin is pyrogenic when administered *i.c.v.* or *i.p.* However, this is in contrast with another report (Kelly et al., 2004) showing negligible effects of intravenously injected leptin on brain COX-2 mRNA and body temperature. It is difficult to explain such discrepancies between

the two studies, but the source and species of leptin could be potential factors. In addition, it is worth noting that leptin induces IL-1RA in the brain (Hosoi et al., 2002a) as well as in circulating monocytes (Gabay et al., 2001), suggesting an intrinsic feedback mechanism for leptin's effects on COX-2 expression and fever as IL-1RA attenuated leptin's effects on COX-2 expression in the current study.

The dose of leptin (5 μg/rat, *i.c.v.*) used in the present study is supra-physiological, as leptin levels in rats were reported to be 1-2 ng/ml and 0.1-0.2 ng/ml in the plasma and CSF, respectively under normal condition (Ishihara et al., 2004). The plasma levels were shown to increase 2-3 fold after LPS injection (Mastronardi et al., 2001) although contradictory data exist showing no significant increase in circulating leptin levels after LPS (Gautron et al., 2005). However, doses similar to those used in our study (0.5-30 μg/rat, *i.c.v.*) were needed to induce a response by an *i.c.v.* injection, even for the well-established effects of leptin on food intake and body weight (Widdowson et al., 1997; Hulsey et al., 1998).

The question of possible endotoxin contamination is an important issue associated with the use of recombinant proteins to study inflammatory responses. In the present study, however, the lack of appreciable effects of LPS at a dose slightly higher than that found in our leptin preparation excluded any major influence of contaminating LPS on IL-1β and COX-2 expression. It is still worth mentioning, however, that leptin is known to augment LPS-induced cytokine productions in circulating monocytes (Zarkesh-Esfahani et al., 2001) and peritoneal macrophages (Loffreda et al., 1998), therefore we cannot totally exclude the possibility that our current observation could involve the effects of a sub-inflammatory dose of LPS, potentiated by the actions of leptin. Nonetheless the inflammatory effects of leptin in the brain itself are supported by several of our own studies and those of others. We have, for example, shown the lack of leptin-induced fever in obese Zucker rats (which possess defective leptin receptor but are responsive to LPS), confirming the specificity of pyrogenic effects of leptin (Luheshi et al., 1999). Others have also reported that *i.c.v.* injection of leptin or leptin anti-idiotype antibody that functionally mimics leptin action

induced similar increase in core body temperature (De Fanti et al., 2002; Turek et al., 2004), and that leptin enhanced PGE2 and PGF2α release from neonatal rat hypothalamic slices ex vivo (Brunetti et al., 1999). Finally, recent observations by us and those of others showing that the neutralization of endogenous leptin with anti-leptin antiserum, as opposed to using recombinant protein, resulted in the attenuation of LPS-induced fever (Sachot et al., 2004; Harden et al., 2006), strongly support the pyrogenic effects of leptin.

The same antiserum was used in the current study to assess the role of endogenous leptin in mediating the LPS induced fever signalling pathway in the brain, namely IL-1β and COX-2. In both cases, the anti-leptin antiserum significantly attenuated the expression of these signals at the mRNA level, but only COX-2 at the level of the protein. The IL-1β protein was only faintly induced by LPS, and no significant change was detected in the presence of anti-leptin antiserum. The reason for this is most likely due to the fact that only a very small amount of IL-1β protein was induced following LPS and any changes in the level of this expression would have been difficult to detect using immunohistochemical approaches. These data imply that, in contrast to direct administration of leptin into the brain, *in situ* production of brain IL-1β may not have a significant role in the induction of COX-2 expression in blood vessels during systemic inflammation. LPS however did induce significant amounts of COX-2 (both mRNA and protein) which was significantly attenuated in the presence of the anti-leptin antiserum. This response was not totally abolished by the antiserum treatment, with a substantial portion (71% at mRNA level) remaining intact, indicating either direct actions of LPS itself on COX-2 up-regulation or that of LPS-induced circulating pyrogens (*i.e.* IL-1β, IL-6 and TNF-α), possibly acting in conjunction with leptin (Loffreda et al., 1998).

Besides its pyrogenic role, COX-2 has been reported to mediate part of the LPS-induced anorexia (Johnson et al., 2002). Our finding that leptin partially mediates LPS-induced COX-2 expression could suggest that leptin suppresses appetite through COX-2 function, in addition to the COX-independent mechanisms (Luheshi et al., 1999), under inflammatory conditions. The specific

role of COX-2 in mediating the effects of leptin on food intake during systemic inflammation is an area that requires further studies, as does the precise role of leptin relative to other cytokines in mediating peripheral inflammatory signals to the brain. For example, systemic LPS injection was demonstrated to induce STAT3 activation in several non-neuronal cells including those reported in the present study after leptin injection (*i.e.,* endothelial cells and meningis) (Gautron et al., 2002; Rummel et al., 2005). However, these studies indicated that IL-6, which also signals through STAT3, was the primary mediator of this response (Gautron et al., 2002; Rummel et al., 2005). A different study (Gautron et al., 2005) also indicated that leptin is not involved in mediating LPS-induced brain STAT3 activation, by demonstrating that fasting (that strongly decrease plasma leptin levels) did not have marked effects on the amount or distribution of STAT3 activation after LPS injection. Based on these observations, it appears that under LPS-induced inflammation, IL-6, rather than leptin, acts as the primary mediator of STAT3 activation in the brain, and that the contribution of leptin, if any, would be masked in the presence of other readily inducible pro-inflammatory cytokines.

In summary, we report here that leptin induces IL-1β and COX-2 in non-neuronal cells with negligible such effects on neurons, and that endogenous leptin contributes to LPS-induced up-regulation of these pro-inflammatory mediators. These findings collectively suggest that leptin plays a role in the brain-regulated sickness responses to infection and inflammation probably through distinct mechanisms from its appetite regulatory mechanisms.

**Acknowledgements**

This work was supported by the Canadian Institutes of Health Research and Natural Sciences and Engineering Research Council of Canada. W. Inoue is supported by a fellowship from The Nakajima Foundation, Japan.

## Figures and Tables

**Figure II-1. Leptin induced IL-1β and COX-2 expressions in the brain**

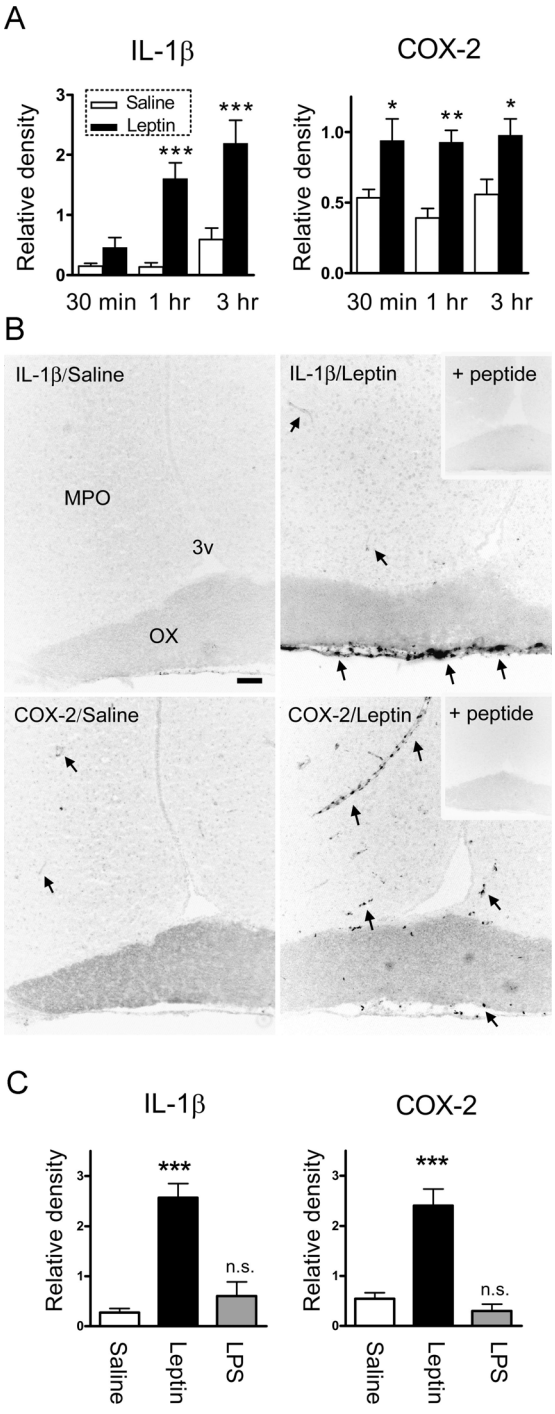

*Figure II-1. (A) IL-1β and COX-2 mRNA levels in the hypothalamus were determined by semi-quantitative RT-PCR. Rats were injected with either saline or leptin (5 μg/rat, i.c.v.). PCR products were separated by electrophoresis, and the band densities normalized to β-actin levels (referred to as relative density). Results represent the mean + SEM (n = 4 per group; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$; saline vs. leptin). (B) Fluorescent immunohistochemical analysis of IL-1β and COX-2 expression 3 h after injection. The fluorescent images are presented in brightfield for clarity of contrast at low magnification. Top row; IL-1β-ir in saline- (left) and leptin- (right) treated animals. Leptin induced strong IL-1β-ir (arrows) in the meningis and weaker IL-1β-ir along blood vessel-like structures in the parenchyma. Bottom row; faint COX-2-ir (arrows) was observed in saline-treated animals (left). Leptin induced COX-2-ir along blood vessel-like structures and in the meningis (arrows). Both IL 1β- and COX-2-ir were completely abolished when the primary antibodies were pre-incubated with respective antigen peptide (insets). Scale bar 100 μm; 3v, 3rd ventricle; i.c.v., intracerebroventricular; ir, immunoreactivity; MPO, median preoptic nucleus; OX, optic chiasm. (C) In a separate experiment, the effects of LPS (20 pg/rat, i.c.v.) on hypothalamic IL-1β and COX-2 mRNA levels were compared to that of leptin (5 μg/rat) and saline by semi-quantitative RT-PCR 3 h after treatment. This dose of LPS represents a slightly higher dose than that found in 5 μg of leptin (19 pg of LPS). Results are presented as mean + SEM (n = 5 per group; $^{***}P < 0.001$, n.s. not significant; vs. saline).*

**Figure II-2. IL-1RA attenuated leptin-induced COX-2**

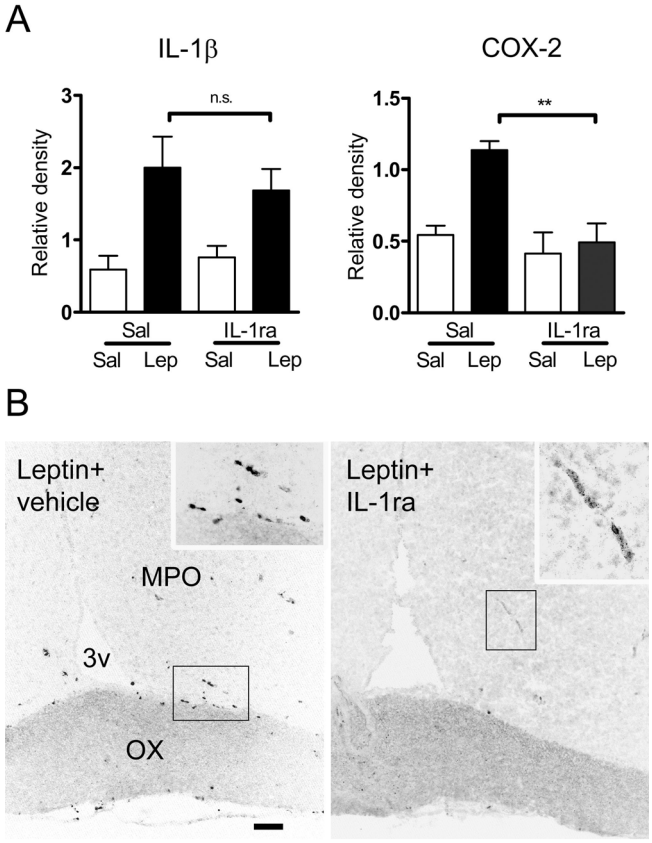


*Figure II-2. (A) IL-1β and COX-2 mRNA levels in the hypothalamus were determined by semi-quantitative RT-PCR. Rats were injected with saline or leptin (5 μg/rat, i.c.v.) in combination with or without IL-1RA (100 μg/rat, i.c.v.), and killed 3 h after treatment. Results are presented as mean + SEM (n = 4 per group; ** P < 0.01, n.s. not significant). Sal, saline; Lep, leptin. (B) COX-2-ir in the hypothalamus after leptin alone (left) or leptin + IL-1RA (right) injection. Fluorescent images are presented in brightfield for clarity of contrast at low magnification. The area enclosed by rectangle is enlarged in inset. Scale bar 100 μm; 3v, 3rd ventricle; i.c.v., intracerebroventricular; ir, immunoreactivity; MPO, median preoptic nucleus; OX, optic chiasm.*

**Figure II-3. Leptin induced IL-1β and COX-2 in brain macrophages and endothelial cells, respectively**

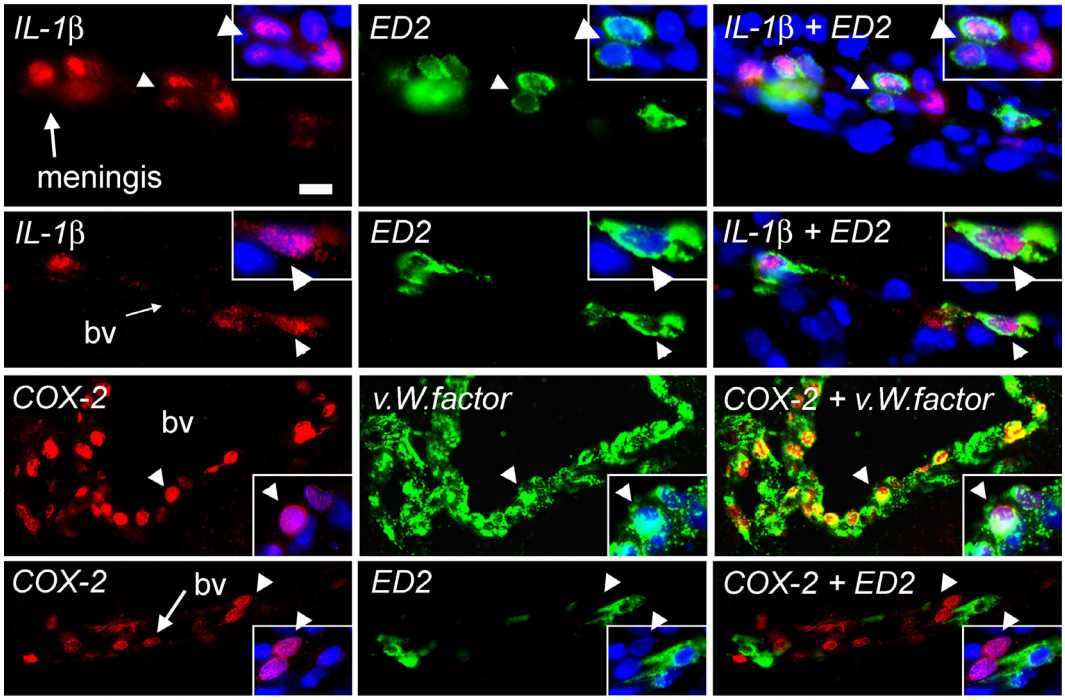


*Figure II-3. IL-1β- and COX-2-ir positive cells were double labelled with cell markers 3 h after leptin injection (5 μg/rat, i.c.v.). Top and 2nd rows; IL-1β-ir (red) and ED2-ir (green), a cell marker for perivascular and meningeal macrophages. IL-1β-ir positive cells were co-labelled with ED2-ir in the meningis (top row) and along blood vessel-like structures (2nd row). 3rd row; COX-2-ir (red) and von Willebrand factor-ir (green), a cell marker for endothelial cells. COX-2-ir positive cells were seen along an axially cut blood vessels and co-labelled with von Willebrand factor-ir. Bottom row; COX-2-ir (red) did not co-localize with ED2-ir (green) along blood vessel-like structures. Nuclear DAPI staining is shown in blue. Insets show enlarged images of cells indicated by arrowheads. Scale bar 20 μm; bv, blood vessel; i.c.v., intracerebroventricular; ir, immunoreactivity.*

**Figure II-4. Stat3 activation by leptin in non-neuronal cells, and its co-localization with IL-1β and COX-2**

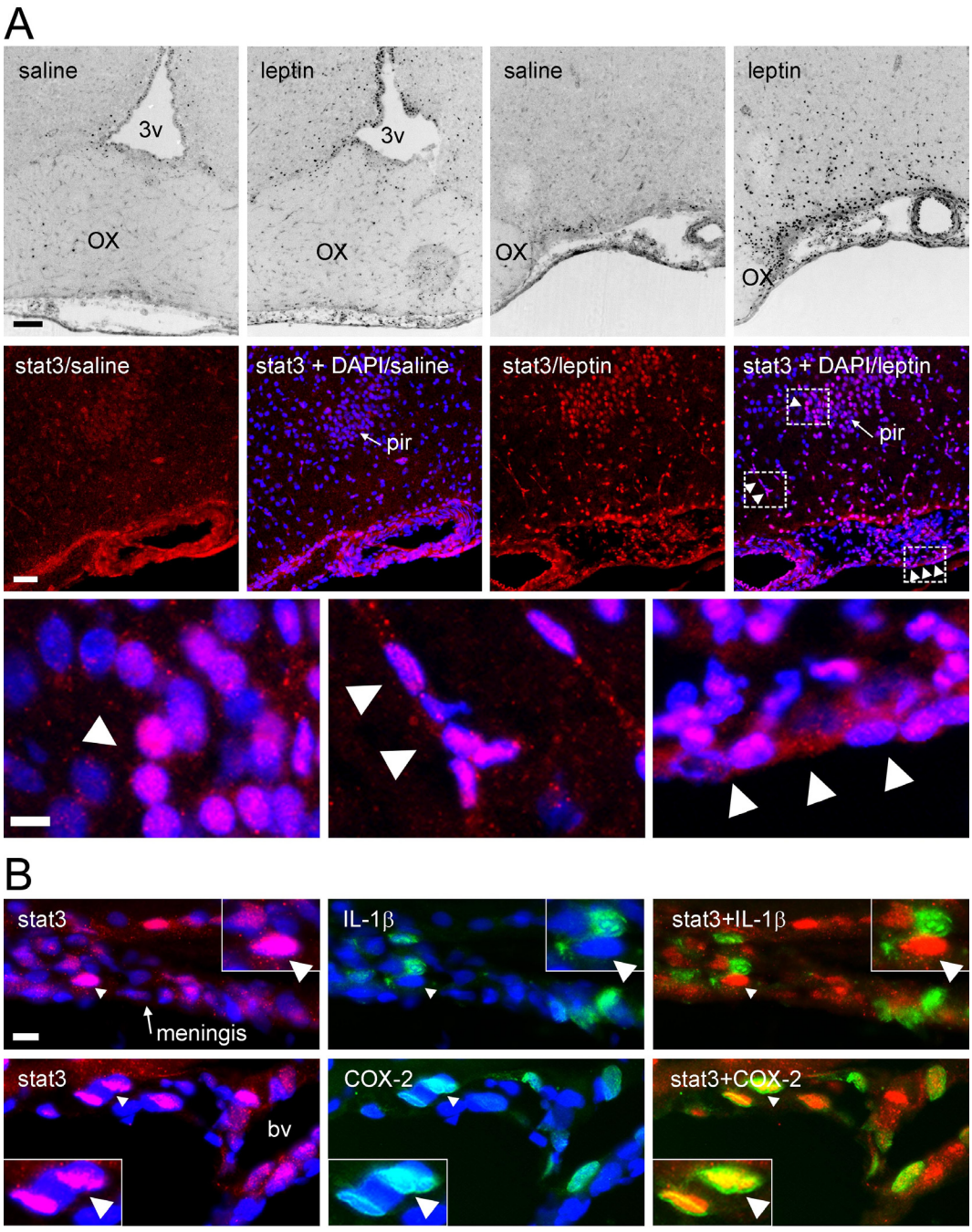


*Figure II-4. (A) Overview images of STAT3-ir at rostral hypothalamic levels (bregma -0.8 mm). Top row; brightfield images are shown for clarity of contrast. In leptin-treated animals, marked increase in STAT3-ir was observed in the meningis and the parenchyma along blood vessel-like structures as compared to*

*saline-treated controls 1 h after the treatment. 2nd and 3rd rows; double labelling of STAT3-ir (red) and nuclear DAPI staining (blue) are presented to depict accumulation of STAT3-ir within cell nuclei (activated form). In saline-treated animals, STAT3-ir was moderate and mostly cytoplasmatic (inactive form). Leptin treatment strongly increased nuclear accumulation of STAT3-ir in neuron-like cells in the piriform cortex as well as in the meningis and blood vessel-like structures (2nd row). The magnified images of these areas enclosed in squares are also shown (3rd row). Note that STAT3-ir (red) was clearly accumulated within cell nuclei in leptin-treated animals, as it co-localized with nuclear DAPI staining (blue), in neuron-like cells in the piriform cortex (left), and in non-neuronal cells in blood vessel-like structures (middle) and in the meningis (right). (B), COX-2-ir, but not IL-1β-ir, co-localized with nuclear STAT3-ir 1 h after leptin treatment. 1st row, STAT3-ir (red) and IL-1β-ir (green) were seen in close vicinity but did not co-localize in the meningis. 2nd row STAT3-ir (red) and COX-2-ir (green) co-localized in blood vessels. Insets show the enlarged images of cells indicated by arrowheads. Scale bar for A, top and 2nd row, 100 μm; 3rd row, 20 μm. Scale bar for B, 20 μm. 3v, 3rd ventricle; bv, blood vessel; OX, optic chiasm; pir, piriform cortex.*

**Figure II-5. Anti-leptin antiserum attenuated LPS-induced IL-1β and COX-2 induction**

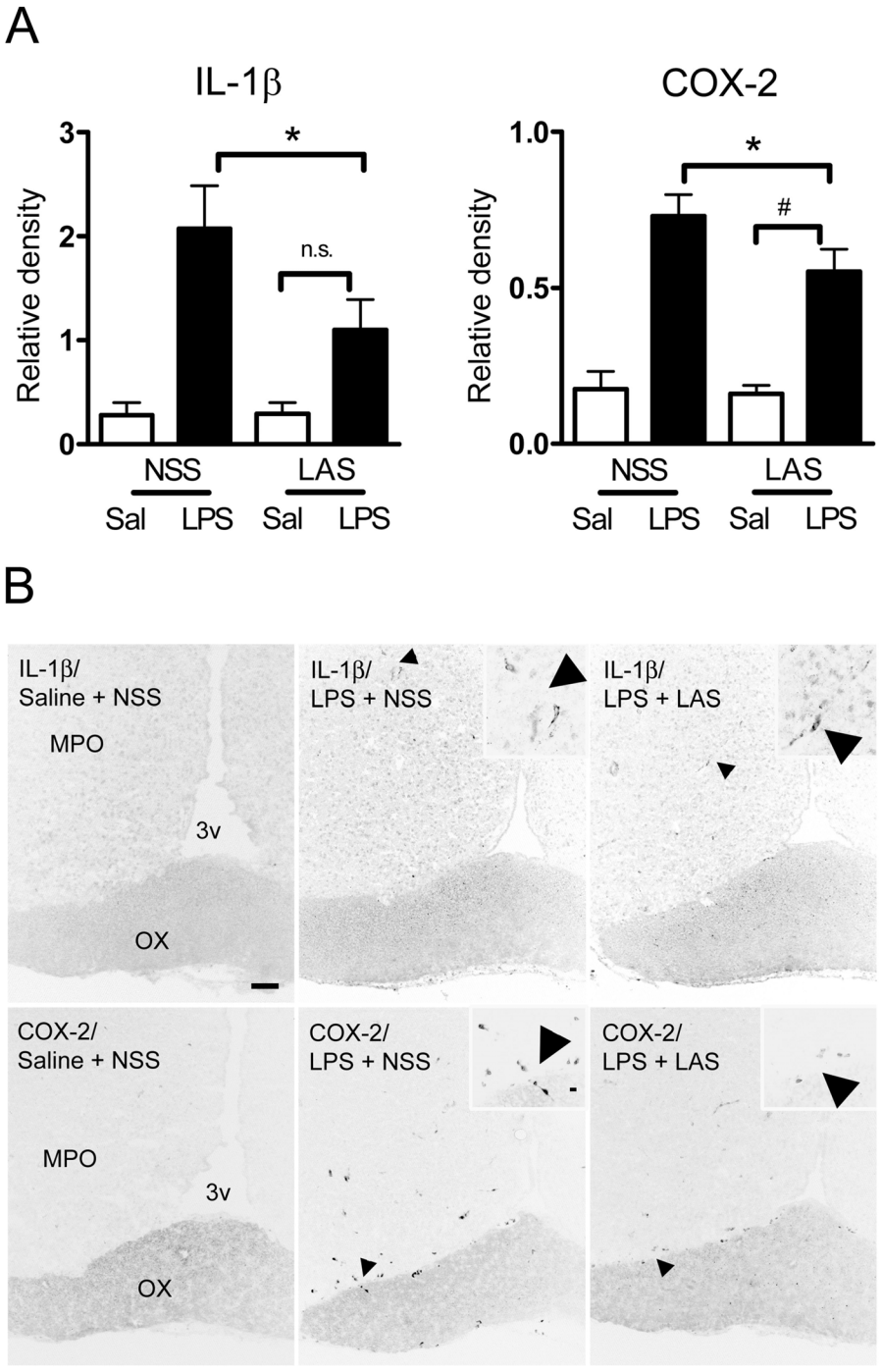


*Figure II-5. Rats were injected with LPS (100 μg/kg, i.p.) or saline in combination with either sheep anti-leptin antiserum (LAS; 1 ml/kg, i.p.) or normal sheep serum (NSS; 1 ml/kg, i.p.), and killed 3 h after injection. (A) IL-1β and*

*COX-2 mRNA levels in the hypothalamus were determined by semi-quantitative RT-PCR. Results are presented as mean + SEM (n = 4 per group; * P < 0.05 for LPS + NSS vs. LPS + LAS, # P < 0.001 and n.s. not significant for saline + LAS vs. LPS + LAS). (B) IL-1β- and COX-2-ir in the hypothalamus after saline + NSS (left), LPS + NSS (middle) and LPS + LAS (right). Fluorescent images are presented in brightfield for clarity of contrast at low magnification. The areas enclosed in squares are enlarged in insets. Scale bar 100 µm; 3v, 3rd ventricle; i.p., intraperitoneal; ir, immunoreactivity; LAS, anti-leptin antiserum; MPO, median preoptic nucleus; NSS, normal sheep serum; OX, optic chiasm.*

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## II.3. Supplementary results and discussion

*ObR expression in IL-1β and COX-2 positive cells.*

The main finding of this study is that leptin induces expression of IL-1β and COX-2 in non-neuronal cells of the brain, namely in brain-resident macrophages in the meningis and perivascular spaces (IL-1β), and endothelial cells (COX-2). Despite the reported expression of ObR on heterogenic cell populations in the brain (Mercer et al., 1996; Bjorbaek et al., 1998), the available *in vivo* data on the actions of this cytokine is, to the best of our knowledge, limited only to neurons with particular focus on the hypothalamus as this region is implicated in body weight regulation (Schwartz et al., 1997; Elmquist et al., 1999). Therefore, the data presented in this manuscript revealed novel functional targets of leptin in the brain. As leptin-mediated induction of IL-1β and COX-2 in non-neuronal cells was quite rapid (30 min and 1 h after leptin treatment for COX-2 and IL-1β mRNA, respectively), we hypothesized that leptin acts directly on these target cells. To further address the link between leptin and the expression of inflammatory mediators in non-neuronal cells, we studied the expression of ObR in these non-neuronal cells using immunohistochemistry. The anti-ObR antibody (dilution; 0.5 μg/ml, cat: GT15012; Neuromics, Northfield, MN) used in this study was raised against the extracellular domain of ObR (amino acids 1–839, a region shared by all isoforms) thus recognizing all its isoforms. Consistent with a previous report (Hakansson et al., 1998), a strong ObR-ir was observed in neuron-like cells in various brain regions including the medial preoptic nuclei (Figure II-6A, inset 1), a subregion of the hypothalamus important for fever/thermoregulation (Kluger, 1991). In addition to the neuronal staining, relatively strong intensity of ObR-ir was observed in non-neuronal structures including cerebral vasculatures and the meningis (Figure II-6A, inset 2 and 3). Pre-incubation of the antibody with recombinant ObR protein (0.02 μM; Neuromics) abolished all of these signals, confirming the specificity of the staining (data not shown). Dual immunohistochemical detection identified co-localization of ObR-ir with IL-1β-ir in the meningis and cerebral vasculatures

(Figure II-6B, top-middle), and with COX-2-ir in cerebral vasculatures (Figure II-6B, bottom), supporting direct effects of leptin on IL-1β and COX-2 expression. In accordance with the current observation, previous studies have reported substantial levels of ObR mRNA expression in the meningis and cerebral vasculatures in addition to the high level expression in hypothalamic neurons (Bjorbaek et al., 1998; Elmquist et al., 1998). Although an active transport of leptin across the BBB via vasculature ObR has been postulated (Banks et al., 1996), the physiological significance of the non-neuronal ObR expression has not been investigated in depth. Thus, the present data suggest a functional significance of the non-neuronal ObR expression in mediating inflammatory effects of leptin in cerebral vasculatures and the meningis.

*-see next page for Figure II-6-*

**Figure II-6. ObR expression in IL-1β- and COX-2-ir positive cells**

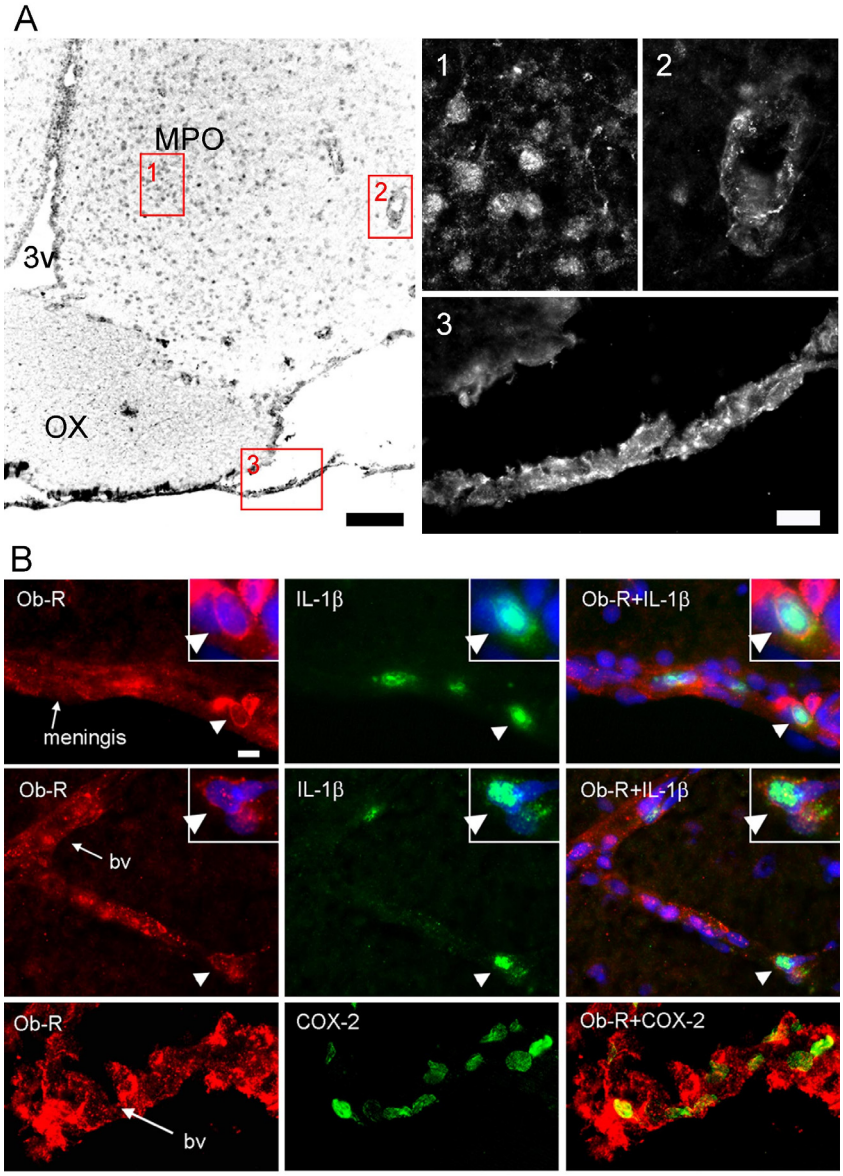


*Figure II-6. (A) ObR-ir distribution in the preoptic area. (Left) a brightfield image is shown for clarity with low magnification. ObR-ir was observed in neuron-like cells (1), blood vessel-like structures (2) and the meningis (3). Scale bar 100 µm. Higher magnification images for these areas are shown on the left (inset 1-3). Scale bar 20 µm. MPO, median preoptic nucleus; OX, optic chiasm; 3v, 3rd ventricle; bv, blood vessel. (B) IL-1β- and COX-2-ir co-localized with ObR-ir 3 h after leptin injection (5 µg/rat, i.c.v.). ObR-ir (red) was intensely*

*expressed throughout the meningis (top panels) and cerebral vasculatures (middle panels), and overlapped with IL-1β-ir (green). Nuclear DAPI staining is shown in blue. ObR-ir (red) also co-localized with COX-2-ir (green) in cerebral vasculatures (bottom panels).*

*Verification of the specificity of leptin*

It is important to establish that the observed inflammatory effects are specific to the actions of leptin and not due to non-specific effects of any potential contaminant in the leptin preparation. In the manuscript, we performed a standard control experiment (see Figure II-1C) and confirmed that the contribution of possible low levels of endotoxin contamination (it is impossible to completely remove endotoxin from recombinant protein prepared from *E. coli*) to the induction of IL-1β and COX-2 were negligible. Although unlikely, the effect of other potential impurities in the leptin preparation has remained undetermined. To further verify the specificity of our current observation, we carried out another complementary control experiment. As shown in Figure II-7A, 6-histidine (His)-tagged recombinant leptin was selectively removed from the leptin preparation by using a 6-His affinity column (TALRON® metal affinity resin; Clontech Laboratories, Mountain View, CA) that has an excess binding capacity over the amount of leptin loaded. Therefore, the flow-through of the column contains no (or very little) leptin but does contain other potential impurities from the preparation; this flow-through was named "column-treated leptin". The efficacy of the column treatment was confirmed by western blot (Figure II-7B). When injected centrally (*i.c.v.*), column-treated leptin was significantly less effective than leptin in inducing IL-1β and COX-2 mRNA measured 3 h after injection using semi-quantitative RT-PCR (Figure II-8A), thus excluding any major influence of contaminants on the observed effects of leptin. However, it has to be noted that IL-1β mRNA was significantly increased by column-treated leptin as compared to saline injection, leaving a possibility of minor effects of impurities. Therefore there still remains a slight possibility that this contaminant(s) could

have contributed to the observed STAT3, IL-1β and COX-2 induction *in vivo*. It is also possible that very low levels of leptin (although it was undetectable by western blot) remaining in column-treated leptin was responsible for this increase in IL-1β mRNA. At the protein level, column-treated leptin had little effect on IL-1β and COX-2 protein expression, whereas injection of leptin at the same dose/volume strongly induced these proteins (Figure II-8B).

**Figure II-7. An overview of 6His-affinity column treatment**

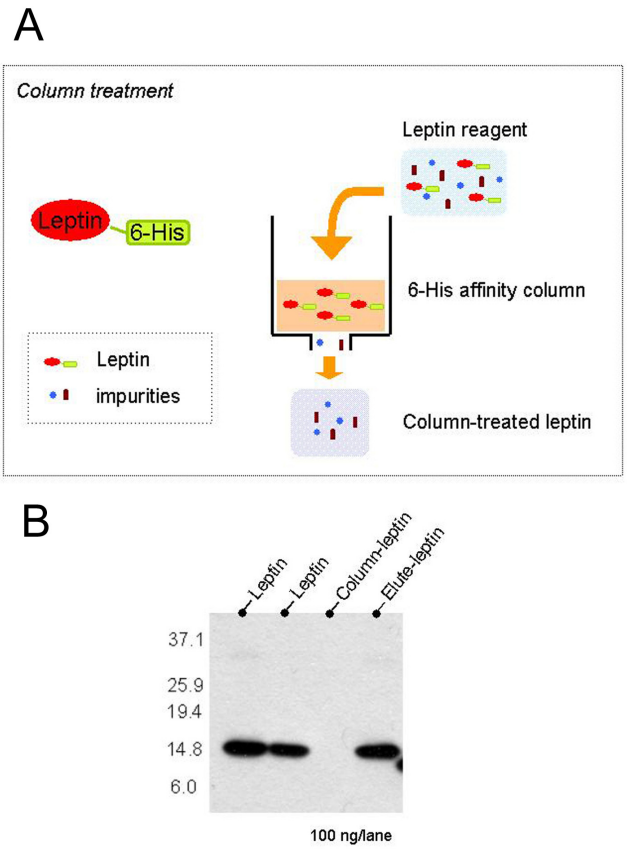


*Figure II-7. (A) The preparation of 6His-tagged recombinant murine leptin was incubated with a 6 His-affinity column that has an excess binding capacity over the amount of leptin loaded. The flow through of the leptin preparation (named column-treated leptin) contains no leptin but does contain other potential impurities from the original preparation. (B) The efficiency of column treatment was verified by western blotting using anti-murine/rat leptin antiserum. A clear single band at the predicted size (16 kDa) was detected in the lanes loaded with*

*6His-leptin (100 ng/lane). The equal volume of column-treated leptin (Column-leptin) contained an undetectable amount of leptin. Leptin was recovered from the column with EDTA elution buffer (Elute-leptin).*

**Figure II-8. The effects of column-treated leptin on IL-1β and COX-2 expression in the brain**

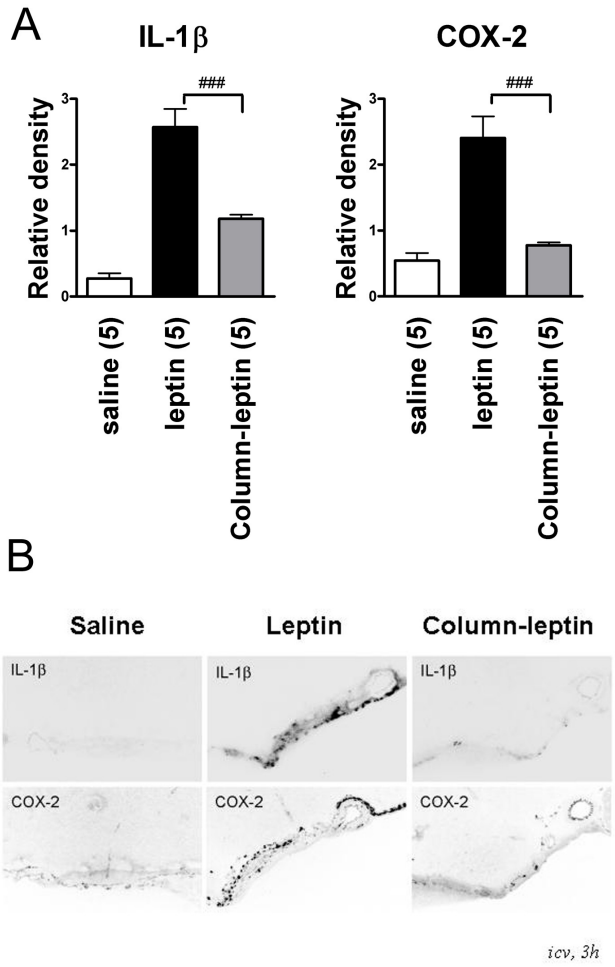


*Figure II-8. (A) Column-treated leptin (C-leptin) induced significantly lower levels of IL-1β and COX-2 mRNA. (n = 5, ANOVA $^{\#\#}$ P < 0.01 vs. saline, $^{\#\#\#}$ P < 0.001 vs. leptin) 3 h after i.c.v. injection. (B) C-leptin induced significantly lower amounts of IL-1β (upper panels) and COX-2 (lower panels) proteins 3 h after i.c.v. injection.*

# Chapter III: Leptin induces interleukin-1β release from rat microglial cells through a caspase-1 independent mechanism

## III.1. Preface

In parallel with the experiment described in the previous manuscript (Chapter II), we established an *in vitro* system to further characterize the inflammatory actions of leptin on brain cells. Earlier *in vivo* observations have suggested that the effects of leptin are, at least partly, mediated by IL-1β in the brain (Luheshi et al., 1999; Hosoi et al., 2002b; Wisse et al., 2004; Garcia et al., 2006). Given that leptin directly stimulates IL-1β production in macrophages and monocytes in the periphery (Zarkesh-Esfahani et al., 2001; Dixit et al., 2004) and that microglia, (the resident macrophages-type cells in the brain), constitute the major source of IL-1β in the brain (Allan and Pinteaux, 2003), we hypothesized that microglia play an important role in leptin-induced brain IL-1β production. Although data obtained from *in vitro* experiments may not always reflect what is actually happening *in vivo*, an *in vitro* setup will help to elucidate the underlying molecular mechanisms involved in leptin-induced IL-1β production in the brain. This could include the identification of specific receptor subtypes and intracellular signalling cascades involved. For this purpose, we studied the inflammatory effects of leptin in primary microglial culture.

## III.2. Manuscript

**Title: Leptin induces interleukin-1β release from rat microglial cells through a caspase-1 independent mechanism.**

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Journal of Neurochemistry, Vol, 102, p826-833, 2007



Keywords: Interleukin-1, leptin, microglia, neuroinflammation

## Abstract

Leptin regulates energy balance by suppressing appetite and increasing energy expenditure through actions in the hypothalamus. Recently we demonstrated that the effects of leptin are, at least in part, mediated by the release of interleukin (IL)-1β in the brain. Microglia constitute the major source of IL-1β in the brain but it is not known whether these cells express leptin receptors, or respond to leptin to induce the production and release of IL-1β. Using RT-PCR and immunocytochemistry, we demonstrate that primary rat microglial cells express the short (non-signalling) and long (signalling) isoforms of the leptin receptors (ObR)s. Immunoassays performed on cell medium collected 24 h after leptin treatment (0.01–10 μg/ml) demonstrated a dose-dependent production and release of IL-1β and its endogenously occurring receptor antagonist IL-1RA. In addition leptin-induced IL-1β release occurs via a STAT3-dependent mechanism. Western blot analysis demonstrated that leptin induced the synthesis of pro-IL-1β in microglial cells and the release of mature 17 kDa isoform into the culture medium. Leptin-induced IL-1β release was neither inhibited by the pan-caspase inhibitor BOC-D-FMK, nor by the caspase-1 inhibitor Ac-YVAD-CHO indicating that IL-1 cleavage is independent of caspase activity. These results confirm our earlier observations in vivo and demonstrate that microglia are an important source of IL-1β in the brain in response to leptin.

## Introduction

Leptin is a 16 kDa product of the ob gene produced mainly by adipose tissue and released into the circulation (Zhang et al., 1994). This hormone is best known for its action as an afferent adiposity signal to the brain that suppresses appetite and increases energy expenditure (Friedman and Halaas, 1998). Leptin also acts on peripheral targets to regulate immune function (Matarese et al., 2005). Leptin induces proliferation, differentiation, and functional activation of all types of haematopoietic cells (Gainsford et al., 1996) via binding to specific leptin receptors (ObRs) and the activation of STAT3, Ras and phosphatidylinositol-3 kinase signalling pathways (Shanley et al., 2002; Bates et al., 2003; Durakoglugil et al., 2005). Macrophages from leptin-deficient (*ob/ob*) mice, show impaired phagocytic function (Loffreda et al., 1998; Lee et al., 1999). Leptin also stimulates production of typical pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α in macrophages and circulating monocytes from normal animals and humans *in vitro* (Zarkesh-Esfahani et al., 2001; Dixit et al., 2004), and contributes to the development of a variety of inflammatory responses *in vivo* (Busso et al., 2002; Mancuso et al., 2002; Ikejima et al., 2005).

IL-1 is a major mediator of inflammation, exerting a wide range of effects on the immune, endocrine and central nervous systems (CNS). IL-1β, the main released form of IL-1, exists as an inactive precursor molecule which requires cleavage by the enzyme caspase-1 into its biologically active 'mature' form. All actions of IL-1 are inhibited by a naturally occurring receptor antagonist (IL-1RA), which blocks IL-1 binding to its signalling receptor (Dinarello, 1997). The activation of IL-1 signalling in the brain is an important regulator of systemic host defence responses to infection and inflammation including suppression of food intake and fever (*i.e.,* increased thermogenesis) (Horai et al., 1998; Josephs et al., 2000).

Under normal, physiological conditions, brain IL-1 levels are extremely low and in most cases undetectable (Vitkovic et al., 2000), suggesting that this

cytokine contributes little to physiological functions regulated by the brain. Recent evidence, however, suggests that, despite its low expression, IL-1 could play a role in the homeostatic regulation of body weight and/or fat metabolism. IL-1RA deficient mice exhibit a lean phenotype and are resistant to diet-induced obesity when compared to their wild-type controls, presumably due to enhanced/unchecked activity of IL-1 in the absence of the antagonist (Irikura et al., 2002; Matsuki et al., 2003; Somm et al., 2005). Conversely, mice lacking the IL-1R1 gene develop mature onset obesity (Garcia et al., 2006). Our own studies (Luheshi et al., 1999) and those of others (Garcia et al., 2006) demonstrated that the same mice (IL-1R1 knockouts) are resistant to the appetite suppressing effects of leptin. We also showed that administration of IL-1RA into the brain of normal rats abolishes the anorexic effect of leptin (Luheshi et al., 1999). We further found that neutralization of endogenous leptin by anti-leptin antiserum attenuated the increase of IL-1β mRNA expression in the hypothalamus, which was accompanied by reversal of the anorexia resulting from systemic inflammation induced by bacterial lipopolysaccharide (LPS) (Sachot et al., 2004). Collectively, these data indicate that the interaction between leptin and the IL-1 system in the brain plays an important role in body weight homeostasis both under physiological and inflammatory conditions. However, specific brain targets and cellular mechanisms of this interaction are largely unknown. Since leptin exerts profound effects on peripheral immune cells, and microglia are the key immune cells in the CNS, we hypothesized that microglial cells are important target of leptin in the brain. Here we show that leptin induces the production and release of mature IL-1β and IL-1RA proteins in primary culture of rat microglia, and that the maturation of IL-1β by leptin is caspase-1 independent via an as yet unidentified mechanisms.

## Methods

*Cell cultures*

Primary mixed glial cultures, and secondary cultures of astrocytes and microglia were prepared from brains of postnatal day 0–2 Sprague-Dawley rat (Charles River, Sandwich, UK) using a protocol previously described (Pinteaux et al., 2002). Primary cortical neuronal cell cultures were prepared from day 18 Sprague-Dawley rat embryos as previously described (Moore et al., 2002).

*Reverse transcriptase-polymerase chain reaction (RT-PCR)*

Total RNA was extracted from cell cultures using TRIzol reagent (Invitrogen, Paisley, UK). 1 μg of RNA was reverse transcripted using Moloney Murine Leukemia Virus (M-MLV) (Invitrogen, Paisley, UK) according to manufactures instructions. PCR was carried out with Biotaq DNA polymerase, contained in a ready-made 2 times reaction mix (BioMixRed) (Bioline, London, UK). Specific oligonucleotide primers, annealing temperatures and cycle numbers are as follows: ObRa, forward, 5'-ACACTGTTAATTTCACACCAGAG-3', reverse, 5'-AGTCATTCAAACCATAGTTTAGG-3', 59 °C and 40 cycle; ObRb, forward, 5'-ACACTGTTAATTTCACACCAGAG-3', reverse, 5'-TTCCAAAAGCTCATCCAACCC-3', 59 °C and 40 cycle; and β-actin, forward, 5'-GCCGTCTTCCCCTCCATCGTG-3', reverse, 5'-TACGACCAGAGGCATACAGGGACAAC-3', 60 ° C and 22 cycle. For all experiments, control reactions using total RNA were performed to ensure that amplification was not a result of contamination with genomic DNA.

*Immunocytochemistry*

Microglial cells seeded on glass coverslips were stained by incubation of living cells with a Fluorescein-isothiocyanate (FITC)-conjugated Lectin Griffonia Simplicifolia (GSL)-IB4 (Vector, Burlingame, UK) (1:400 dilution) for 30 min at room temperature. Cells were washed and ObR immunostaining was then carried out as previously described (Pinteaux et al., 2002), using an IgG anti-leptin

receptor (ObR) primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:500 dilution) in absence or presence of an excess (10 μg/ml) of recombinant ObR blocking peptide (Santa Cruz Biotechnology), and subsequent incubation with a Texas-Red-conjugated affinity-purified donkey anti-goat IgG (Chemicon, Temecula, CA, USA) (1:50 dilution).

*Treatments*

Cells were exposed to normal culture medium (Control) or treated for 24 h with increasing concentrations (0.01, 0.1, 0.5, 1, 5 or 10 μg/ml) of recombinant mouse leptin provided by Dr. Stephen Poole from the National Institute for Biological Standards and Control (NIBSC, Potters Bar, UK) or rat leptin (Peprotech, London, UK) diluted in phosphate buffered saline (PBS). To ensure that the IL-1β release was not due to endotoxin contamination, cells were incubated with heat-inactivated (95°C, 30 min) leptin (10 μg/ml) for 24 h. STAT3 activation was carried out by incubating cells with leptin (10 μg/ml) for 5, 15, 30 or 60 min, or with IFN-γ (100 IU/ml) for 30 min. Some cells were pre-treated with a specific STAT3 peptide inhibitor (H-Pro-Tyr-(PO3H2)-Leu-Lys-Thr-Lys-Ala-Ala-Val-Leu-Leu-Pro-Val-Leu-Leu-Ala-Ala-Pro-OH) (50 or 250 μM) (Calbiochem, Darmstadt, Germany) for 30 min prior to treatment with leptin (10 μg/ml) for 24 h. To investigate whether leptin-induced IL-1β release is dependent on caspase-1, cells were exposed with BOC-D-FMK (100 μM) (Merck Biosciences, Nottingham, UK) or with Ac-YVAD-CHO (10 μM) (Merck Biosciences) for 30 min prior to treatment with leptin. Cultures were also treated with LPS alone (0.1 μg/ml) (Sigma) for 24 h, or with ATP (5 mm) (Sigma, Poole, UK) in the absence or presence of Ac-YVAD-CHO (10 μM) for 2 h.

*ELISA*

IL-1β and IL-1RA release was assayed by specific rat sandwich ELISAs, generously provided by Dr. Stephen Poole (NIBSC, UK), using specific sheep anti-rat IL-1β or IL-1RA coating antibodies, and specific sheep anti-rat IL-1β or IL-1RA biotinylated antibodies. Recombinant rat IL-1β or IL-1RA was used as standards. The assays were specific for rat IL-1β and IL-1RA with no cross-

reactivity with other cytokines. Data were presented as absolute pg/ml values over basal expression. The detection limit of the assay was 20 pg/ml for IL-1β and 19 pg/ml for IL-1RA. The coefficients of variation were 0.5% and 2.1% (intra-assays), and 21.9% and 8.0% (inter-assays), for IL-1β and IL-RA respectively.

*Western blot*

Western blot analysis for IL-1β, IL-1RA and STAT3 activation was carried out as previously described (Brough et al., 2002), using primary antibodies diluted in PBS containing 0.1% Tween and 0.1% BSA: sheep anti-rat IL-1β (S328, NIBSC, UK) (1:1000 dilution), sheep anti-rat IL-1RA (S377, NIBSC, UK) (1:1000 dilution), rabbit anti-STAT3 (New England Biolabs, Hitchin, UK) (1:10000 dilution), rabbit anti-phospho-STAT3 (New England Biolabs) (1:500 dilution), and subsequent incubation with horseradish peroxidase-conjugated anti-rabbit or anti-sheep IgG secondary antibodies (Dako, Ely, UK) (1:2000 dilution).

*Data analysis*

Results are the mean values ± SEM from 3–4 experiments carried out on separate cultures and were analysed using a one-way analysis of variance (ANOVA), followed by a Tukey-Kramer post-hoc test. For all statistical analyses, a value of $P < 0.05$ was considered significant.

## Results

*Primary rat microglia express leptin receptors mRNA and protein*

RT-PCR experiments showed that microglia express mRNAs for the short isoform (ObRa) and the long isoform (ObRb) of leptin receptor (Figure III-1A). ObRa and ObRb mRNAs were also detected in mixed glia, astrocytes and neurons. Interestingly, ObRb mRNA expression was much higher in microglia than astrocytes and neurons, whereas ObRa mRNA expression was high in mixed glia, astrocytes and microglia compared to neurons. ObR immunostaining was then carried out on microglial cultures using an antibody raised against the C-terminus region of the mouse ObR known to recognize both the short and long form of the leptin receptor in rat cells (Diano et al., 1998; Hakansson et al., 1998). Strong immunostaining was detected on GSL-IB4 positive microglial cells, with the majority of staining found to be largely localised on the cell surface Figure III-1B, a–b). The specificity of the immunostaining was confirmed by pre-absorbing the primary antibody with a corresponding blocking peptide Figure III-1B, c).

*Leptin induces the release of IL-1β and IL-1RA from microglia*

Basal levels of IL-1β and IL-1RA in the medium of untreated microglial cultures were 0.2 ± 0.4 pg/ml and 1020 ± 44 pg/ml respectively. Treatment with recombinant mouse leptin induced a dose-dependent and statistically significant release of IL-1β (Figure III-2A) and IL-1RA (Figure III-2B) over basal levels. Responses to the maximal concentration of leptin (10 μg/ml) were completely abrogated by heat-treatment (95°C for 30 min), suggesting that the effects observed were not due to the presence of endotoxin contamination. Treatment of cells with LPS used as a positive control induced strong release of both IL-1β and IL-1RA.

*Leptin-induced release of IL-1β is mediated by activation of the STAT3 signalling pathway*

The concentration of leptin (10 μg/ml) that maximally stimulated IL-1βand IL-1RA release from microglia was used to investigate the activation of STAT3, a

major signalling component of the leptin signal transduction pathway. STAT3 activation was low in untreated microglia (Figure III-3A). Leptin induced a time-dependent activation of STAT3, reaching statistical significance after 15 and 30 min of treatment. After 60 min of treatment, the level of STAT3 activation returned to basal levels. IFN-γ (100 IU/ml) used as positive control, induced maximal activation of STAT3 in microglia after 30 min of treatment. Addition of a specific STAT3 inhibitor peptide (50 or 250 μM) 30 min prior to treatment with leptin significantly reduced leptin-induced IL-1β release, while the inhibitor alone had no effect (Figure III-3B).

*Leptin induces the release of mature IL-1β by a caspase-1-independent mechanism*

In order to determine whether IL-1β release from leptin-treated microglia is dependent on caspases activation (including caspase-1), cells were pre-treated with BOC-D-FMK, a broad spectrum irreversible pan-caspase inhibitor, for 30 min followed by treatment with leptin for 24 h. Leptin-induced IL-1β release was not blocked by BOC-D-FMK, while BOC-D-FMK alone had no effect (Figure III-4A). Similar results were obtained using another caspase inhibitor, Ac-YVAD-CHO, which failed to inhibit leptin-induced IL-1β release but significantly reduced release of IL-1β triggered by ATP from LPS-primed microglia (Figure III-4C). Western blot analysis of the cell lysates showed that leptin induced the synthesis of pro-IL-1β in the cells although at very low level compared to that induced by LPS (Figure III-4B). Leptin induced the release of pro-IL-1β and mature IL-1β from microglia, which were released at high level in LPS-treated cells. An additional unidentified IL-1β isoform of 14 kDa was also detected in the culture medium of leptin-treated cells. In addition to IL-1β, leptin induced the synthesis of the 21 kDa precursor isoform of soluble IL-1RA (pro-sIL-1RA) and the release of the 17 kDa soluble mature IL-1RA (Figure III-4B). A similar effect was observed in response to treatment with LPS which induced stronger expression of pro-sIL-1RA and the release of mature sIL-1RA from microglia. BOC-D-FMK had no effect on the cytosolic expression of pro-IL-1β or pro-sIL-

1RA, and had no effect on the level of mature IL-1β and sIL-1RA in the medium of leptin-treated cells (Figure III-4B).

*ATP induces the release of 17 kDa mature IL-1β in LPS-primed but not leptin-primed microglia.*

ATP is a well known activator of caspase-1 via binding to the P2X7 receptor, resulting in the processing and release of mature IL-1β from LPS-treated microglia (Brough et al., 2002). In order to determine whether ATP can induce the cleavage and release of IL-1β from leptin-treated or LPS-primed cells, microglia were pre-treated with leptin (10 μg/ml) or LPS (0.1 μg/ml) for 24 h, and then treated with ATP (5 mM) for 2 h. Western blot analysis showed that LPS induced strong synthesis of pro-IL-1β in the cells, and the release of some pro- and mature IL-1β in the culture medium (Figure III-5), as already shown in figure 4. ATP strongly induced the release of mature IL-1β from LPS-treated microglia, confirming the effect of ATP on IL-1β maturation and release (Brough et al., 2002). ATP also induced the release of pro-IL-1β in the culture medium of LPS-treated cells. In contrast ATP had no effect on the levels of pro- or mature IL-1β detected in the supernatant or cell lysate of leptin-treated microglia.

## Discussion

Earlier observations have demonstrated an important role of brain IL-1β in the anorexic, febrile and thermogenic effects of leptin (Luheshi et al., 1999; Sachot et al., 2004; Wisse et al., 2004; Garcia et al., 2006; Harden et al., 2006). The present study shows that microglial cells highly express both isoforms of leptin receptors, ObRa and ObRb, compared to astrocytes or neurons Figure III-1), and demonstrate that leptin can activate microglia to produce mature IL-1β (Figure III-2A and Figure III-4). These results add considerably to our understanding of the cellular actions of leptin in the CNS and suggest that the control of food intake and body weight by circulating leptin occurs at least partly via the production of IL-1β from microglial cells of the hypothalamus.

In contrast to undetectable amounts of IL-1β in the medium of untreated microglia, we detected considerable concentrations of its receptor antagonist IL-1RA (Figure III-2B). This observation is in agreement with published studies showing that IL-1RA is readily detectable in both the circulation and brain of normal human and rodents (Palin et al., 2004; Somm et al., 2005), as well as culture medium of untreated primary human liver cells (Gabay et al., 1997). However one cannot exclude the possibility that microglia in primary cultures are partially activated resulting in high levels of IL-1RA production. The treatment of microglia with leptin further induced the synthesis and release of IL-1RA over basal levels (Figure III-2B), demonstrating that leptin regulates the production of IL-1RA in parallel with that of IL-1β in microglia. The present *in vitro* data are in agreement with previous *in vivo* reports by Hosoi et al. (Hosoi et al., 2002a, b) demonstrating that systemic leptin injection increased mRNA expression of both IL-1β and IL-1RA. Because the balance between IL-1 and IL-1RA will determine the net IL-1 signalling, further investigation is required to characterize the precise role of leptin over the IL-1/IL-1RA ratio and its physiological significance. A recent study showed that serum IL-1RA concentrations are several fold higher in obese individuals, and they decreased after weight loss (Meier et al., 2002), suggesting a possible implication of IL-1RA in the mechanism of leptin resistance.

A separate study revealed that white adipose tissue constitutes a major source of IL-1RA under physiological conditions, and that IL-1RA production increases in obesity as well as in response to inflammatory stimuli (Juge-Aubry et al., 2003). These data combined with the fact that IL-1RA deficient mice show decreased fat mass and are resistant to high fat diet-induced obesity (Somm et al., 2005), implicate IL-1RA in energy balance regulation.

Although leptin induces the expression and release of IL-1β from microglia, we found that the levels of IL-1β produced after leptin treatment were relatively low when compared to those triggered by LPS, which is known to induce robust microglial activation. It is possible that higher dose of leptin could induce larger amount of IL-1β similar to that by LPS, however it is also plausible that the low amount of IL-1β produced by this dose of leptin reflects an alternative physiological role for microglia that is different to that associated with inflammatory responses to exogenous pathogens or injury. CNS injuries, for example, trigger microglial activation leading to the production of large (pathophysiological) amounts of IL-1 (Allan and Pinteaux, 2003). In contrast, leptin may trigger (or maintain) mild microglial activation, which could result in the production of smaller amounts of IL-1β via a mechanism different from that employed by inflammatory stimuli (caspase-independent), as discussed later. This low concentration of IL-1β could contribute to the maintenance of normal body weight as demonstrated by a recent *in vivo* study by Garcia et al. (Garcia et al., 2006) using IL-1R1 knockout mice. Furthermore, hypothalamic expression of IL-1β is significantly reduced in response to decreased levels of leptin induced by acute starvation, or in Zucker rats, characterized by dysfunctional leptin receptors (Wisse et al., 2004). These data indicate a role for IL-1β as one of the downstream signals of leptin under physiological conditions.

The hypothesis that microglia and IL-1β may play a physiological role in body weight homeostasis under the influence of leptin, however, does not rule out inflammatory actions of leptin in the brain under pathophysiological conditions. We have shown previously *in vivo* that administration of leptin in rats induces fever (Luheshi et al., 1999), and that neutralization of endogenous leptin with

anti-leptin antiserum attenuates LPS-induced fever (Sachot et al., 2004). More recently, we also reported that leptin induces cyclooxygenase-2 in the brain partly via IL-1 action (Inoue et al., 2006). Interestingly, Sanna et al. (Sanna et al., 2003) have reported that, in mice autoimmune encephalomyelitis, infiltrating T cells and macrophages produce leptin within the brain, suggesting a role of leptin for the development of certain neuroinflammatory diseases. Although some of observations made in the current study would support this notion further investigations are required to clarify the exact mechanisms that regulate the physiological and pathophysiological actions of this hormone.

Leptin regulates body weight through the activation of STAT3 (Bates et al., 2003), an intracellular signalling molecule activated by ObRb. In the present study, we show that leptin activates STAT3, and demonstrate that inhibition of STAT3 significantly suppressed the leptin-induced IL-1β release in microglial culture (Figure III-3). This finding is somewhat contradictory to previous observations *in vivo* showing that leptin increased IL-1β mRNA expression in the hypothalamus of obese *db/db* mice which lack ObRb/STAT3 signalling (Hosoi et al., 2002b). The reason for this discrepancy is unclear, but the different experimental conditions (mRNA levels in mouse brain tissue *vs.* protein levels in microglial culture from rats) can be one possible explanation.

Biological activation of the IL-1β protein depends on proteolytic cleavage of the inactive 32 kDa precursor into a 17 kDa mature form, a mechanism mediated by caspase-1 (Kuida et al., 1995; Li et al., 1995). In the present study, we found that leptin-induced IL-1β release was not inhibited by a broad spectrum pan-caspase inhibitor (BOC-D-FMK) (Figure III-4A and B). Similar results were obtained using a different caspase inhibitor (Ac-YVAD-CHO), which effectively inhibited ATP-induced release of IL-1β from LPS-primed cells (Figure III-4C), a prototypical IL-1β release mechanism mediated by caspase-1. In contrast to its effect on the release of mature IL-1β from LPS-primed cells, ATP failed to induce the release of mature IL-1β from leptin-treated microglia (Figure III-5). These results collectively indicate that leptin-induced IL-1β release occurs independently of caspase-1 activity, and thus involves different mechanisms from

LPS-induced IL-1β release. Caspase-1-independent processing of IL-1β has already been reported (Miwa et al., 1998) and other extracellular proteases have been proposed for an alternative mechanism of IL-1β cleavage (Schonbeck et al., 1998; Herzog et al., 2005). However whether or not these mechanisms are involved in the case of leptin was not addressed in the current study. Processing of 32 kDa pro-IL-1β by caspase-1 is thought to lead to the production of a 27 kDa intermediate form, which allows exposure of the cleavage site at Asp116 rendering it accessible to caspase-1 for full processing to the 17 kDa isoform. Western blot analysis showed the presence of an additional band of 14 kDa in the medium of leptin treated cells. An additional 14 kDa isoform of IL-1β has already been reported (Knudsen et al., 1986) and could be the product from direct cleavage of the 32 to 17 kDa isoform.

Although our data clearly demonstrate that leptin interacts directly with microglial cells *in vitro*, how this interaction might occur *in vivo* is still an open question. Given the wide distribution of systemically injected radiolabelled leptin in the brain (Banks et al., 1996) and the abundant distribution of microglial cells throughout the CNS, it is feasible that once in the brain this hormone will activate microglia, regardless of location, resulting in IL-1β production (Hosoi et al., 2002b). However, it is also likely that the action of leptin on microglia occurs more neuroanatomically restricted to areas of the brain (for example the hypothalamus) as a result of a more restricted entry to specific regions. A previous study has proposed a delivery mechanism for leptin mediated by astrocytes residing around the arcuate nucleus of the hypothalamus (Cheunsuang and Morris, 2005). In summary, these observations suggest that microglia are a target for leptin action which leads to the production of the pro-inflammatory cytokine IL-1β. In combination with our previous findings *in vivo* (Luheshi et al., 1999), these results add further support to the hypothesis that leptin acts as a neuroimmune modulator, and suggest that microglial cells play an important part in this process.

## Acknowledgements

This work was supported by the Medical Research Council UK, the Canadian Institutes of Health Research and Natural Sciences and Engineering Research Council of Canada. The authors would like to thank Drs. Adrian Bristow and Stephen Poole (NIBSC, UK) for providing recombinant mouse leptin and rat specific ELISAs.

## Figures and Tables

**Figure III-1. Expression of leptin receptors.**

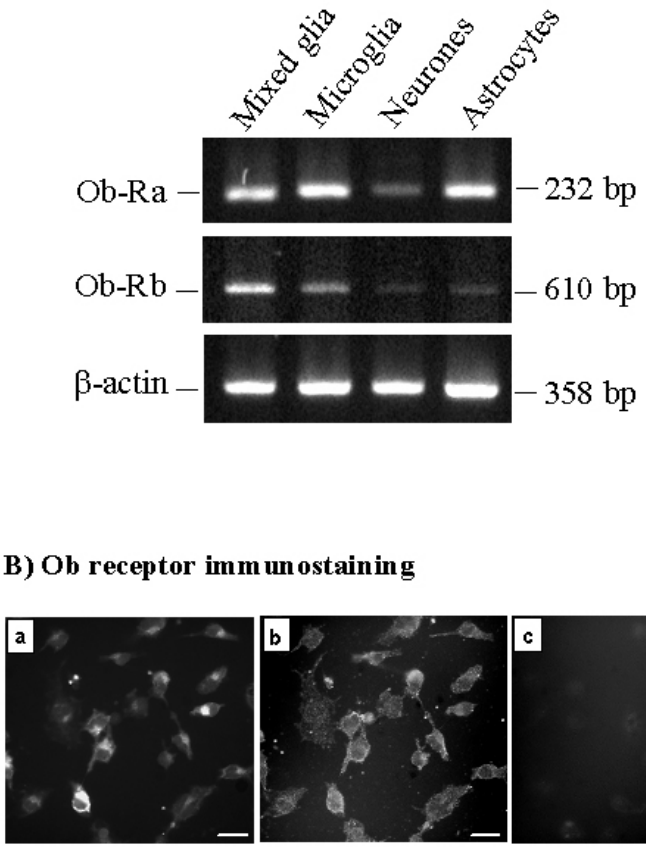


*Figure III-1. Expression of leptin receptors. A) mRNA expression of the short (ObRa) and long (ObRb) isoforms of leptin receptors in mixed glia, microglia, neurones or astrocytes, compared to expression of β-actin mRNA. Single experiment representative of three separate experiments. B) ObR immunostaining in rat microglial cultures using a specific antibody that recognises both short and long isoforms of ObR. a) Lectin GSL IB4 staining. b) ObR immunostaining. c) Neutralization of ObR immunostaining in presence of 10x excess specific blocking peptide. Bar scale = 20 µm*

**Figure III-2. Effect of treatment with recombinant rat leptin**

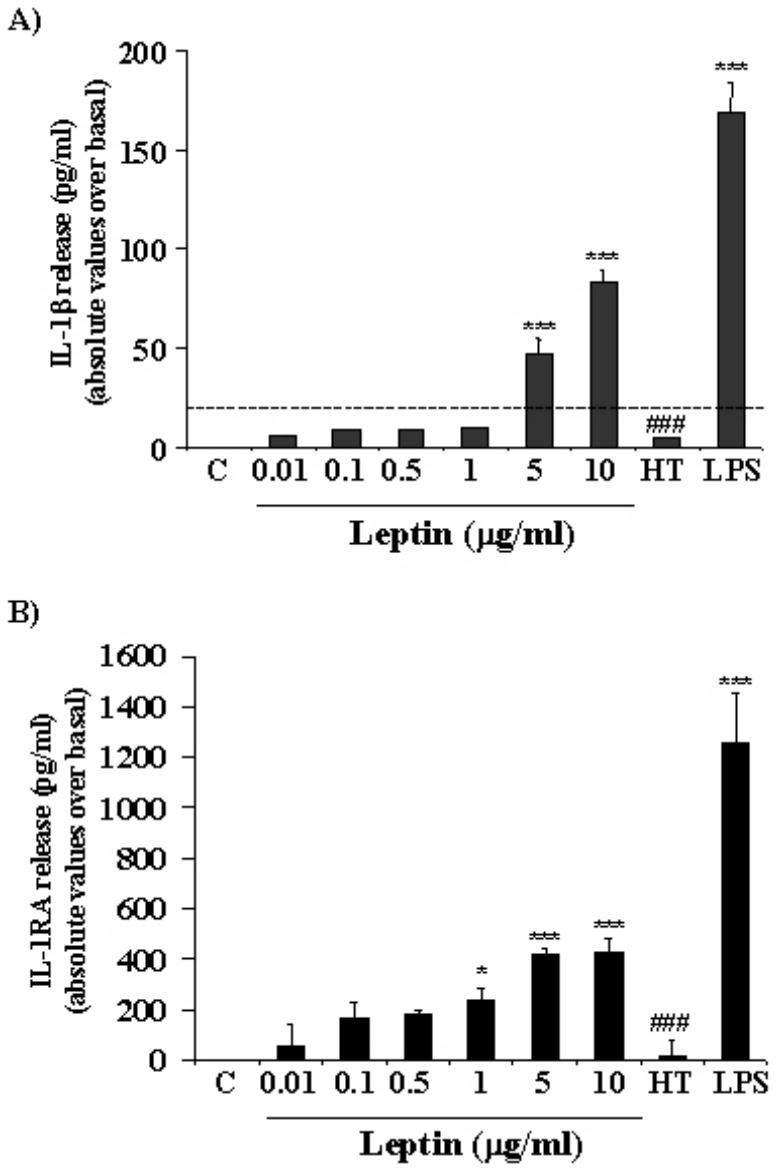


*Figure III-2. Effect of treatment with recombinant rat leptin (0.01, 0.1, 0.5, 1, 5, 10 μg/ml), LPS (0.1 μg/ml) or heat treated leptin (HT) (95°C, 30 min) for 24 h on the production of IL-1β or IL-1RA from rat microglial cultures. Data presented are the mean ± SEM of released levels over basal values from four independent experiments carried out on separate cultures.* $^{*}P < 0.05$ *and* $^{***}P < 0.001$ *vs. control (C),* $^{\#\#\#}P < 0.001$ *vs. leptin (5 and 10 μg/ml). Dotted line represents the detection limit of the assay for IL-β ELISA.*

**Figure III-3. Leptin-induced IL-1β release from microglia is dependent on STAT3 activation**

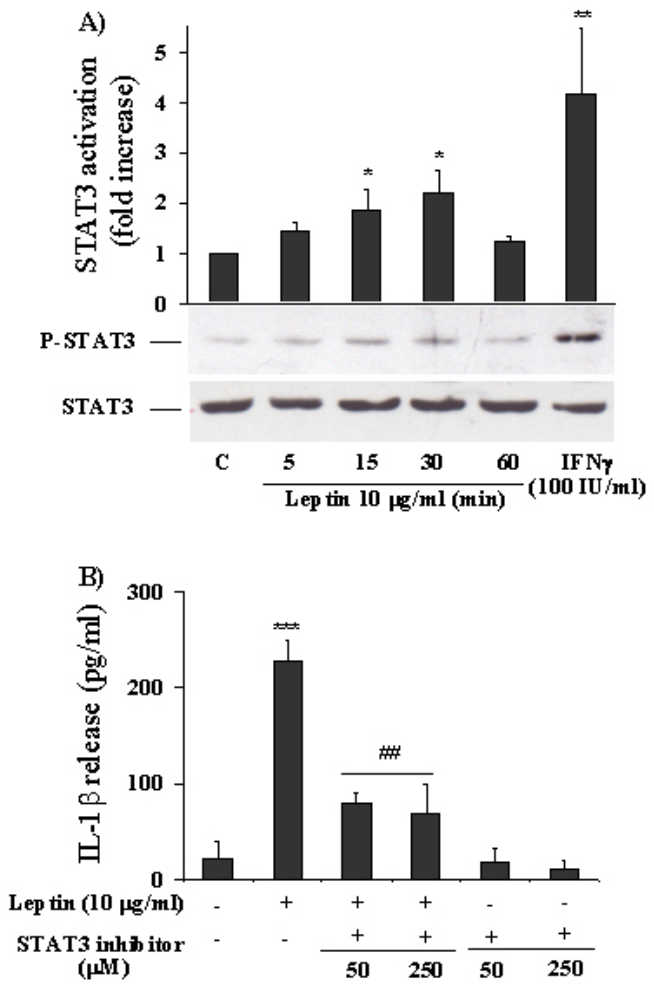


*Figure III-3. Leptin-induced IL-1β release from microglia is dependent on STAT3 activation. A) Microglial cultures were treated with leptin (10 μg/ml) for 5, 15, 30 or 60 min, or with IFNγ (100 IU/ml) for 30 min, and cell lysates were assayed for STAT3 activation by semi-quantitative Western blot analysis. Data presented are the mean ± SEM of three independent experiments carried out on separate cultures.* $^{*}P < 0.05$, $^{**}P < 0.01$ *vs. control (C). B) Microglial cells were treated with specific STAT3 inhibitor peptides (H-Pro-Tyr-(PO3H2)-Leu-Lys-Thr-Lys-Ala-Ala-Val-Leu-Leu-Pro-Val-Leu-Leu-Ala-Ala-Pro -OH) (50 or 250 μM) for 30 min prior to treatment with leptin (10 μg/ml) for 24 h, and IL-1β release was assessed by specific ELISA. Data presented are the mean ± SEM of three*

*independent experiments carried out on separate cultures.* $^{***}P < 0.001$ *vs. control (C),* $^{\#\#}P < 0.01$ *vs. leptin.*

**Figure III-4. Effect of BOC-D-FMK and Ac-YVAD-CHO on leptin-induced IL-1β or IL-1RA synthesis and release in microglial cultures**

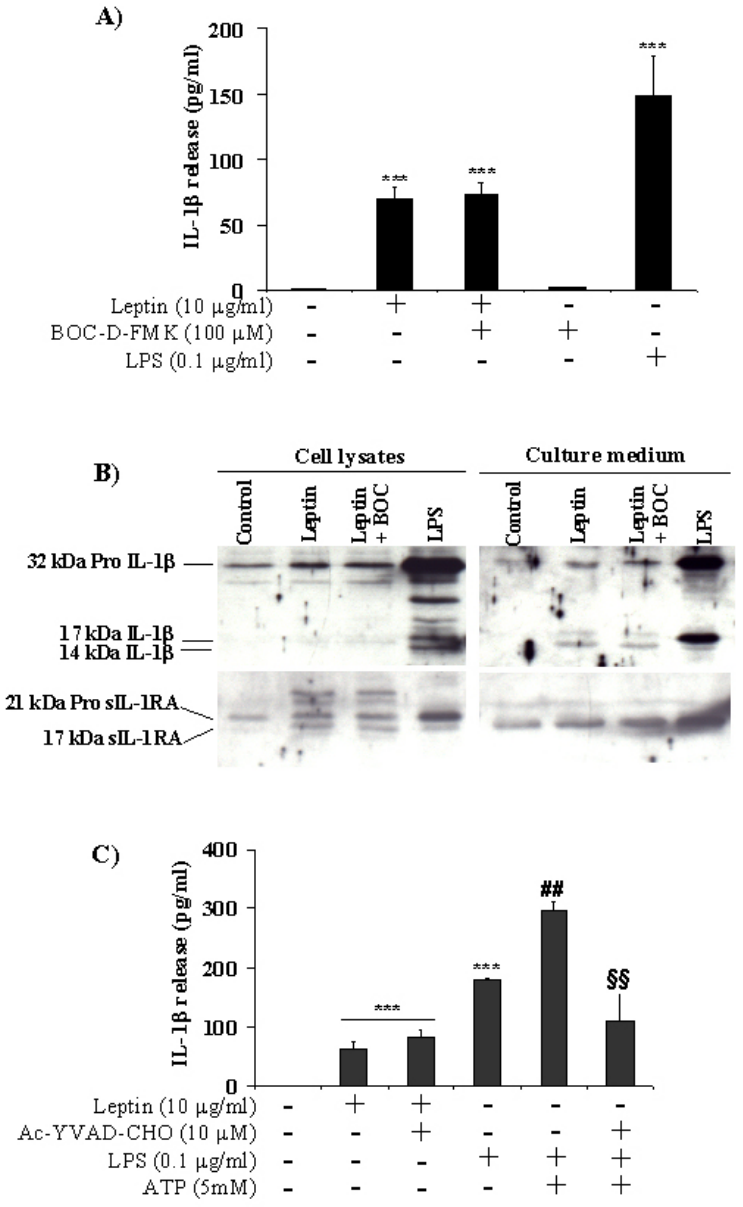


*Figure III-4. Effect of BOC-D-FMK and Ac-YVAD-CHO on leptin-induced IL-1β or IL-1RA synthesis and release in microglial cultures. Cells were treated with BOC-D-FMK (100 µM) or Ac-YVAD-CHO (10 µM) 30 min prior to treatment with*

*leptin (10 µg/ml), LPS alone (0.1 µg/ml) for 24 h, or with ATP (5 mM) (Sigma, Poole, UK) in the absence or presence of Ac-YVAD-CHO (10 µM) for 2 h. After 24 h, IL-1β released in the medium was assayed for IL-1β by ELISA (A) (C), and the amount of pro- and mature- IL-1β or IL-1RA was assessed in the cell lysates and the medium by Western blot analysis (B). For (A) and (C) data presented are the mean ± SEM of three independent experiments carried out on separate cultures.* $^{***}P < 0.001$ *vs. control,* $^{\#\#}P < 0.01$ *vs. LPS,* $^{\S\S}P < 0.01$ *vs. LPS + ATP. For (B) the results presented are representative of three independent experiments.*

**Figure III-5. Effect of ATP on leptin-induced or LPS-induced synthesis and release of IL-1β from microglial cultures**

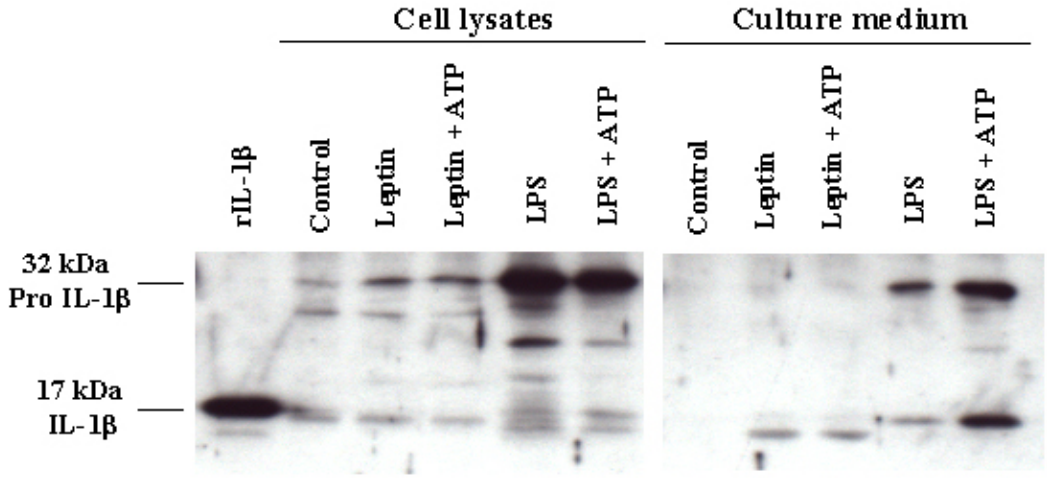


*Figure III-5. Effect of ATP on leptin-induced or LPS-induced synthesis and release of IL-1β from microglial cultures. Cells were treated with leptin (10 µg/ml) or LPS (0.1 µg/ml) for 24 h and then treated with ATP (5 mM) for 2 h. Culture medium and cell lysates were collected and analysed for pro- and mature- IL-1β expression by Western blot analysis. The results are representative of 3 independent experiments carried out on separate cultures.*

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## III.3. Supplementary results and discussion

The experiments in the present manuscript addressed the actions of leptin on microglia which we hypothesized to represent an important target of this cytokine in the CNS. The results revealed that leptin induces IL-1β in a STAT3-dependent manner. Among several subtypes of ObR, only ObRb is capable of activating STAT3 (Bjorbaek et al., 1997), therefore the results indicate that this long-form subtype is critical for leptin involvement in IL-1β induction. Perhaps the most interesting observation in the present manuscript is that the leptin-induced IL-1β maturation/release was independent of caspase-1 (Figure III-4). It is well established that prototypical inflammatory stimuli, such as LPS, trigger IL-1β maturation/release almost exclusively via a caspase-1-dependent mechanism (Kuida et al., 1995; Li et al., 1995), a phenomenon also confirmed in the present manuscript (Figure III-4). Thus, evidence is provided that leptin stimulates microglia in a way qualitatively different from LPS. We investigated this further using the same approach and obtained some very interesting and potentially significant results, as discussed below.

In these studies, a primary culture of rat microglia was incubated with either a serum-free medium alone (untreated), with leptin (1 μg/ml) or LPS (0.1 μg/ml) for 24 h, and cytokine release in the medium assayed by ELISA (NIBSC). As in the manuscript, leptin treatment significantly induced IL-1β release (Figure III-6). Along with IL-1β, LPS treatment triggered the release of other classical inflammatory cytokines TNF and IL-6, an observation in agreement with previous studies (Nakamura et al., 1999; Kim et al., 2004). In contrast, leptin treatment had no effect on TNF or IL-6 release (Figure III-6), thus further demonstrating the difference between leptin and LPS in their effects on microglia. However, despite the fact that leptin did not directly induce the release of TNF or IL-6 from microglia, it facilitated their release in combination with LPS. These results were obtained by pre-incubating the cells with leptin (1 μg/ml) for 24 h followed by LPS (0.1 μg/ml) for 3 h. As can be seen in Figure III-7 below, the pre-treatment with leptin significantly potentiated the LPS-induced release of TNF and IL-6 as

well as enhancing IL-1β release. Importantly, the enhancement of LPS-induced TNF and IL-6 release by leptin was not an additive effect of the two because leptin treatment alone had no effect on the release of these cytokines. The synergistic effects of leptin (pre-treatment) on LPS-induced TNF and IL-6 release suggests a role for leptin as a co-stimulator of inflammation, rather than as a classic inflammatory stimulus.

**Figure III-6. Leptin induces the release of IL-1β but not TNF or IL-6**

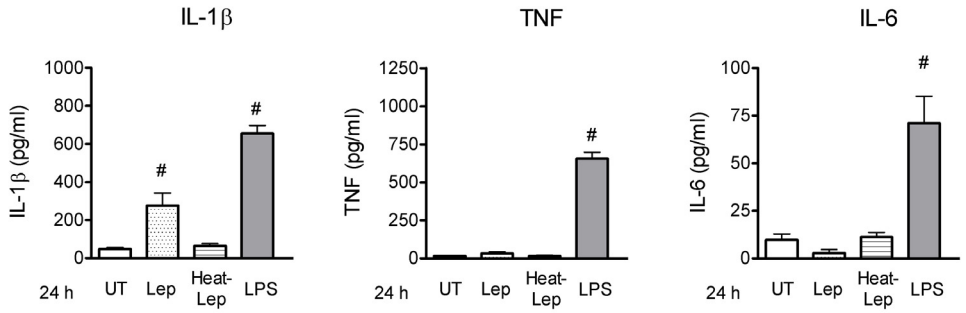


*Figure III-6. Leptin induces the release of IL-1β but not TNF or IL-6. Primary cultures of microglia were treated in a serum-free medium (UT, untreated), with leptin (Lep, 1 μg/ml), heat-inactivated leptin (Heat-lep, 1 μg/ml) or LPS (0.1 μg/ml) for 24 h. Cytokine release was measured in the medium by ELISA. LPS-treated cells released significantly higher amounts of IL-1β, TNF and IL-6 as compared to untreated cells. Leptin significantly increased IL-1β but not TNF or IL-6. Heat inactivation abolished the effects of leptin on cytokine release, confirming the specificity of leptin treatment.* $^{\#}$ *$P < 0.05$ vs. UT*

**Figure III-7. Leptin potentiates LPS-induced release of IL-1β, TNF and IL-6**

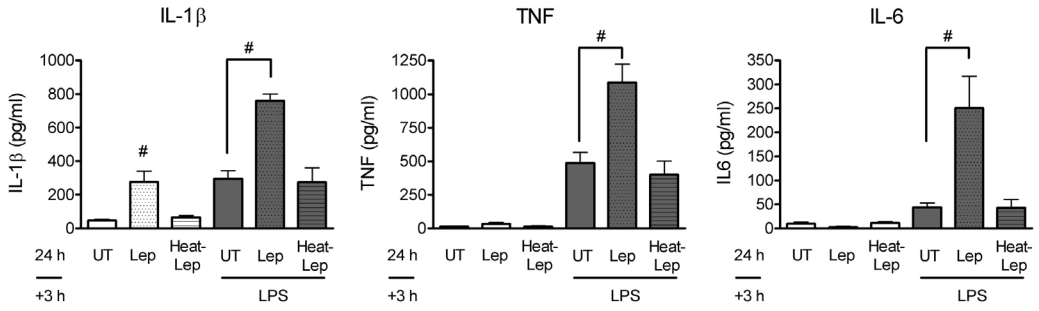


*Figure III-7. Leptin potentiates LPS-induced release of IL-1β, TNF and IL-6. Primary cultures of microglia were treated in a serum-free medium (UT, untreated), with leptin (Lep, 1 μg/ml) or heat-inactivated leptin (Heat-lep, 1 μg/ml) for 24 h, followed by 3 h incubation with or without LPS (0.1 μg/ml). Cytokine release was measured in the medium by ELISA. Leptin treatment alone increased IL-1β release but not TNF or IL-6. Leptin pre-treatment significantly enhanced LPS-induced IL-1β, TNF and IL-6 release. Heat inactivation abolished the effects of leptin on cytokine release, confirming the specificity of leptin treatment.* $^{\#}$ $P < 0.05$

In the course of performing this study, we in addition noted that leptin treatment causes a morphological change in microglia that is distinct from the morphological change induced by LPS. Although one can conclude little about the actual function of microglia from their morphology alone, there exists a general consensus that fine ramifications are indicative of the resting state of microglia whereas round amoeboid-shapes are associated with their activation (Perry and Gordon, 1988; Streit et al., 1988). Microglial cells seeded on glass cover slips were incubated in a serum-free medium alone (untreated), with leptin (1 μg/ml) or LPS (0.1 μg/ml) for 24 h, and then stained with anti-CD11b antibody (a marker for microglia; 1:200 dilution, cat: ARS1122, Biosouce International, Camalliro, CA) followed by visualization with CY3-conjugated anti-mouse IgG (1:500 dilution, cat: 715-165-151, Jackson ImmunoResearch, West Grove, PA). As shown in Figure III-8, untreated cells showed a round shape with no

ramification, indicating that the cells are in an activated state: this is most likely due to the mechanical stress associated with the isolation/seeding procedure. In support of this assumption, untreated cells formed a clear ramification when cells were kept for a longer period to allow for recovery (48 h, data not shown). In contrast, the majority of leptin-treated cells displayed a bipolar rod shape with fine ramifications within 24 h, suggesting that leptin treatment unexpectedly promoted the recovery of the newly seeded cells from the isolation/seeding stress. LPS-treated cells, on the other hand, presented round shape cell bodies smaller than the untreated cells, indicating their highly activated state.

**Figure III-8. Leptin induces morphological changes in microglia**

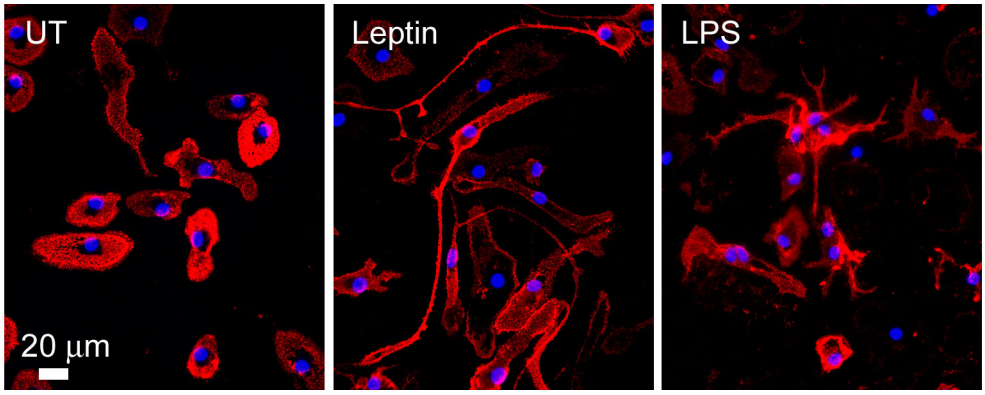


*Figure III-8. Leptin induces morphological changes in microglia. Microglia were seeded on glass cover slips and incubated in a serum-free medium (UT, untreated), with leptin (1 µg/ml) or LPS (0.1 µg/ml) for 24 h. Cells were stained with anti-CD11b antibody.*

Collectively, these results indicate that leptin's effects on microglia are distinct from those of a prototypical inflammatory stimulus, namely LPS. It will be important to elucidate the mechanisms underlying how leptin potentiates LPS responsiveness of microglia. For example, leptin may increase the expression of TLR4 (or other components of the LPS receptor complex) thereby enhancing LPS-induced cytokine release. Alternatively, leptin may stimulate transcription and/or translation of TNF and IL-6 without affecting their release, whereas the

subsequent LPS triggers the release. Of interest is if, and how the leptin-induced morphological change is related to the changes in microglial function. The downregulated morphology induced by leptin treatment, at first glance, appears to be contradictory to the enhancement of LPS-induced cytokine release. However, it was shown that preceding (inflammatory) stimuli alter the microglial response to LPS resulting in attenuation of cytokine production from these cells (Hausler et al., 2002). Therefore that the possibility that leptin could enhance LPS-induced cytokine release by promoting microglial recovery from stress incurred during the isolation procedure is still plausible. Given that leptin is a constitutive hormone with levels in the circulation and CSF of 1–2 ng/ml and 0.1–0.2 ng/ml, respectively, under normal conditions (Ishihara et al., 2004), it is possible that leptin may be an important homeostatic factor for optimal microglial function. A reduction in leptin levels, either by genetic mutation or by nutritional deprivation (which reduces leptin levels), may suppress microglial function and their response to inflammatory stimuli.

Lastly, it is important to mention that the data obtained from the present *in vitro* study may not directly reflect the *in vivo* mechanisms of leptin-microglia interactions. As described in the previous chapter, immunohistochemistry on brain sections did not detect IL-1β expression in microglia following leptin administration (5 μg/rat, *i.c.v.*) (Figure II-3). In the same brains, leptin did induce IL-1β which was found exclusively in meningeal and perivascular macrophages, brain-resident myeloid cells phenotypically distinct from parenchymal microglia (Guillemin and Brew, 2004; Davoust et al., 2008). It is possible that microglia produce less IL-1β than brain-resident macrophages in response to leptin and that the approach used (immunohistochemistry) was not sensitive enough to detect microglial IL-1β production. However, it is also possible that the responsiveness of microglia to leptin is altered (enhanced) in cell culture, which generally activates the cells (Kuipers et al., 2006). In the normal (healthy) brain, parenchymal microglia are characterized by a highly down-regulated phenotype with low expression levels of cell surface receptors such as MHC class II (Perry and Gordon, 1988; Mott et al., 2004; Deckert et al., 2006). Disturbances in their

microenvironment rapidly activate microglia, triggering morphological changes and up-regulation of a range of effecter molecules (Perry and Gordon, 1988; Kreutzberg, 1996). Therefore, it will be very interesting to examine whether leptin administration *in vivo* stimulates microglia in the diseased brain (*e.g.*, pre-treatment with LPS) where microglia are in an activated state. It was recently demonstrated, in rodent models of ischemic stroke (Valerio et al., 2009) and neuropathic pain (Lim et al., 2009), that leptin (and also its receptor) expression is up-regulated in the CNS at the inflammatory foci. Interestingly, one of these studies revealed that leptin expression was found in microglia/macrophage cells in ischemic penumbra (Valerio et al., 2009), indicating an as yet unknown leptin-microglia relationship in brain inflammation.

# Chapter IV: Leptin mediates neutrophil recruitment to the brain following systemic LPS inflammation

## IV.1. Preface

The results obtained in the previous two chapters demonstrated the ability of exogenous leptin to stimulate IL-1β production in the brain, as 1) an *i.c.v.* injection of leptin acutely induced IL-1β mRNA and protein expression in the brain (Chapter II), and 2) leptin treatment induced or, in combination with LPS, potentiated IL-1β release by primary cultures of microglia (Chapter III). These results, coupled with our previous observations that neutralization of endogenous leptin with LAS abrogated the up-regulation of IL-1β mRNA in the brain during LPS-induced systemic inflammation (Sachot et al., 2004; Inoue et al., 2006), strongly support a significant contribution of leptin to brain IL-1β production during systemic inflammation. Given the established roles of IL-1β actions in the brain in regulating fever and anorexia (Rothwell and Luheshi, 2000), these results reinforce the possible link between leptin and sickness behaviour. However, beyond the paradigm of sickness behaviour, brain IL-1β action has been implicated in various neuropathologies, such as inflammatory responses following ischemic brain damage or mechanical injury (Allan et al., 2005). In the following manuscript, we examined the potential roles of leptin in contributing to brain damage associated with severe systemic inflammation. To this end, we use a mouse model of sepsis induced by systemic injection of a high dose of LPS, and compared brain pathologies in wild-type, leptin-deficient (*ob/ob*) and leptin-resistant (*db/db*) mice. The effects of acute leptin deficiency were assessed by using the LAS approach in wild-type mice. Furthermore, to address the physiological relevance of leptin's involvement in this process we studied the impact of food deprivation, which dramatically reduces leptin levels, on LPS-induced brain inflammation.

## IV.2. Manuscript

**Title: Leptin mediates neutrophil recruitment to the brain following systemic LPS inflammation**

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Submitted

Keywords: Cytokines, innate inflammation, starvation

## Abstract

Leptin, an adipose-derived hormone known to control body weight, has emerged as a critical link between energy balance and host defence responses to pathogens. Severe and sustained systemic inflammation can lead to brain damage that is partly mediated by neutrophil recruitment into the brain. Herein we examined the contribution of leptin to this process in mice injected intraperitoneally with a septic dose of lipopolysaccharide (LPS). LPS-induced mRNA levels of interleukin-1β, intracellular adhesion molecule (ICAM)-1 and neutrophil chemokines were significantly attenuated in the brains of leptin-deficient (*ob/ob*) and -resistant (*db/db*) mice. In addition, the mutant mice were protected from the accumulation of neutrophils that were, in wild-type brains, closely associated with the barrier-related structures exhibiting significantly increased levels of ICAM-1. Importantly, leptin replenishment in *ob/ob* mice reversed both inflammatory gene expression and neutrophil recruitment. Moreover, 48 h food deprivation in wild-type mice, which decreased circulating leptin levels, attenuated the LPS-induced neutrophil recruitment in a leptin-dependent manner. Likewise, a single injection of an anti-leptin antiserum 4 h prior to LPS treatment in wild-type mice was sufficient to attenuate neutrophil recruitment. These results provide the first demonstration that leptin plays a critical role in neutrophil recruitment to the brain following severe systemic inflammation.

## Introduction

The energy status of an individual is coupled to its ability to mount an effective immune response to pathogenic challenges. Malnutrition, for example, has long been known to cause immunosuppression (Wing et al., 1988; Chandra, 1992), but the underlying mechanisms have remained elusive. Leptin, whose circulating levels are proportional to body fat mass (Friedman and Halaas, 1998), has recently emerged as one of the key molecules involved in this relationship. This is based on the multiplicity of its roles, which along with regulating energy balance as initially described, now includes significant involvement with other physiological systems, including immunity (Faggioni et al., 2001; La Cava and Matarese, 2004). The importance of this cytokine-like hormone in immune function is clearly demonstrated in mice with genetic leptin deficiency (*ob/ob*) or resistance (*db/db*) which exhibit various signs of immunodeficiency (Fernandes et al., 1978; Chandra, 1980), are susceptible to bacterial infection (Mancuso et al., 2002; Ikejima et al., 2005) or are resistant to autoimmune diseases (Matarese et al., 2001b; Busso et al., 2002). Importantly, immune abnormalities observed in leptin deficient mice are analogous to starvation-induced immunosuppression, which is accompanied by a dramatic reduction in circulating leptin levels and is reversible by leptin replenishment (Lord et al., 1998; Howard et al., 1999). These observations led to the proposal that leptin represents a critical signal which links the nutritional status of the host to its ability to effectively respond to a pathogenic challenge.

Peripheral infection or injury triggers, in addition to local inflammation, brain-regulated behavioural and metabolic responses, such as fever and anorexia, via the afferent actions of circulating cytokines (Dantzer, 2004). This generally involves *de novo* synthesis of classical innate inflammatory mediators such as interleukin (IL)-1β and prostaglandin (PG)s in the brain (Van Dam et al., 1993a; Quan et al., 1998) whose local actions regulate the sickness behaviours (Dantzer, 2004). Whereas these responses are thought to be adaptive and promote survival (Hart, 1988; Dantzer, 2004), it has recently been proposed that the same

mechanism when over-activated, may be neurotoxic (Perry et al., 2007; Dantzer et al., 2008). There is rapidly accumulating evidence that when peripheral inflammation continues unabated (*e.g.*, during sepsis) (Papadopoulos et al., 2000; Bohatschek et al., 2001; Semmler et al., 2005) or is combined with ongoing neuropathology (*e.g.*, injury, stroke, prion disease or Alzheimer's disease) (Sly et al., 2001; Cunningham et al., 2005; Kitazawa et al., 2005; McColl et al., 2007; Riazi et al., 2008), the brain's innate inflammatory responses triggered by the afferent signals could be exaggerated resulting in irreversible brain damage. Of particular interest to the present study is the previous report demonstrating that a peripheral challenge with bacterial endotoxin lipopolysaccharide (LPS), when applied at a 'septic' dose, leads to dramatic invasion of granulocyte neutrophils into an otherwise uninjured brain with parallel disruption of the blood-brain barrier (Bohatschek et al., 2001). Thus, this report provides an ideal experimental paradigm to elucidate the underlying mechanisms responsible for brain damage following severe systemic inflammation.

Previously we and others have proposed that leptin acts as an important circulating mediator of systemic infection or inflammation by activating brain mechanisms responsible for sickness behaviours (Luheshi et al., 1999; Hosoi et al., 2002b; Sachot et al., 2004; Harden et al., 2006). This hypothesis is based on the acute rise in circulating leptin levels following systemic LPS or other inflammatory agents (Grunfeld et al., 1996a; Sarraf et al., 1997; Faggioni et al., 1998), its ability to induce fever and appetite loss along with brain IL-1β and PG synthesis (Luheshi et al., 1999; Hosoi et al., 2002b; Wisse et al., 2004; Inoue et al., 2006), and the attenuation of LPS-induced sickness behaviours by a neutralizing antiserum against leptin (Sachot et al., 2004; Harden et al., 2006). In a recent study (Rummel et al., 2008), we characterized the sites of leptin's action on the brain during systemic LPS inflammation and found that leptin predominantly acts on the leptomeningis, cerebral fissures and vasculatures, that is, the barrier-related structures of the brain. These structures were shown to express leptin receptors (Mercer et al., 1996; Bjorbaek et al., 1998; Mutze et al., 2006), and both leptin and LPS were reported to induce IL-1β expression in brain macrophages residing

along these barrier-related structures (Van Dam et al., 1992; Quan et al., 1998; Inoue et al., 2006). While these results reinforce the proposed link between leptin and brain IL-1β and the ensuing sickness behaviours, the location of this interaction is also indicative of potential pathological consequences in the case of unabated production of IL-1β during severe systemic inflammation. In fact, these barrier-related structures form the site of primary contact for invading leukocytes, and it has been shown that sustained IL-1β action in the brain is a potent driver for the recruitment of these cells, in particular neutrophils (Ferrari et al., 2004; Shaftel et al., 2007). Recently, we have provided preliminary evidence implicating leptin in this process by showing that it is involved in the increase of chemokine mRNA expression in the brain following peripheral LPS challenge (Rummel et al., 2008). Therefore, in the present study we tested the hypothesis that leptin contributes to the induction of innate inflammatory mediators such as IL-1β and chemokines in the brain and is involved in subsequent pathological conditions associated with severe systemic inflammation. To this end, we used a mouse model of sepsis induced by systemic injection of a high dose of LPS, which triggers recruitment of neutrophils into the brain (Bohatschek et al., 2001), and then examined brain expression of innate inflammatory mediators and neutrophil recruitment in wild-type, leptin-deficient (*ob/ob*), and leptin receptor-deficient (*db/db*) mice. To address the physiological relevance of leptin's involvement in this process, we also studied the impact of food deprivation, which dramatically decreases circulating leptin levels (Lord et al., 1998; Howard et al., 1999), on the LPS-induced brain inflammatory responses. Finally, we examined the effects of transient leptin deficiency in otherwise normal (non-obese or -starved) animals by pharmacologically neutralizing endogenous leptin and assessing the LPS-induced brain inflammatory responses.

## Methods

*Animals*

Adult male *ob/ob* (B6.V-Lepob/J) and *db/db* (B6.Cg-m +/+ Leprdb/J) mice maintained on a C57BL/6J background, and C57BL/6J mice were purchased from the Jackson Laboratories (Bar Harbor, ME). In all studies 7 wk old animals, on the day of injection, were used, with a body weight of approximately 20 g for WT and 40 g for *ob/ob* and *db/db* mice. An age-dependent effect on the leukocyte recruitment to the brain has been reported in experimental models of brain inflammation (Lawson and Perry, 1995; Anthony et al., 1997), therefore animals were age-matched and mutant mice were heavier than control WT animals. All animals were housed in a controlled environment at an ambient temperature of 21 ± 2˚C on 12:12 h light-dark cycle (light on at 08:30) with free access to water and standard laboratory chow unless otherwise specified. The institutional Animal Care Committee pursuant to the Canadian Council of Animal Care guidelines approved all experimental procedures.

*Treatment and experimental protocols*

WT, *ob/ob* and *db/db* mice were injected with either LPS (E. coli 0111:B4; cat: L-2630; Sigma, Ontario, Canada) at a dose of 2.5 mg/kg (*i.p.*) or with pyrogen-free saline. A separate group of *ob/ob* mice were treated with either murine leptin (6 μg/day; PeproTec Inc, Rocky Hill, NJ) or saline delivered by Alzet miniosmotic pumps (0.25 μl/h; model 1002, Durect Corp., CA) implanted subcutaneously in the dorsal midline caudal to the scapulae of the animals under isoflurane anesthesia 10 days before the LPS treatment. To address the effect of the decline in leptin levels associated with acute starvation on the brain responses, additional groups of WT animals were fasted for 48 h with or without leptin replenishment (6 μg/day) using Alzet pumps prior to the LPS treatment. To test the effect of acute leptin deficiency in otherwise normal animals, additional groups of WT mice were treated with a neutralizing sheep anti-mouse leptin antiserum (LAS; S4159, NIBSC, Potters Bar, UK) or preimmune sheep serum

(PIS; NIBSC) at 0.5 ml/kg *i.p.* 4 h before LPS or saline treatment. The volume and timing of LAS injections were determined in accordance with a previous study using the same antiserum (Rummel et al., 2008).

In all experiments, animals were euthanized 24 h after LPS or vehicle treatment by terminal anesthesia (pentobarbital; 70 mg/kg, *i.p.*) and blood samples collected via cardiac puncture. Mice were then transcardially perfused with ice-cold saline with 5 IU/ml of heparin to flush-out any remaining blood from the brain. The brains were quickly removed, frozen in powdered dry ice and kept at -80˚C until analysis.

*Tissue processing*

In a preliminary study, appropriate time points and potential brain areas of neutrophil recruitment following systemic LPS challenge were examined in WT mouse collected 6, 24 or 48 h after LPS treatment (n = 2 per group). Every third serial coronal section (20 μm) from bregma 0.5 to 2.3 and 7.2 to 7.92 mm was processed for immunohistochemical detection of neutrophils. Very little neutrophils were detected 6 h after LPS treatment throughout the brain. By 24 h, a robust neutrophil recruitment was observed, which was prominent around the lateral ventricles, the ventrobasal hypothalamus and the brain stem mostly associating with ventricle walls, the leptomeningis, cerebral fissures and vasculatures. The recruited neutrophils were dispersed more evenly throughout the parenchyma by 48 h. Based on these results, the 24 h time point was chosen for subsequent analysis of brain sections collected from three levels of the brain (encompassing the anterior hypothalamus, bregma −0.1 to −0.82 mm; the posterior hypothalamus, bregma −1.22 to −2.3 mm; and the brain stem, bregma −7.2 to −7.92 mm) using coordinates from the mouse brain atlas (Paxinos and Franklin, 2001).

In order to perform immunohistochemistry and RT-PCR on the same tissue taken from the same brain, 9–18 consecutive frozen sections were thaw-mounted on glass slides for immunohistochemistry, and the remainder of the serial brain sections from the forebrain (bregma −0.10 to −2.3 mm) were overlaid on a frozen

glass slide, the hypothalamus dissected, and collected in a cryotube at −80°C for RNA extraction.

*Quantitative RT PCR*

Total RNA was extracted using TRIzol (Invitrogen, Ontario, Canada) according to the manufacturer's instructions. Following reverse transcription, qRT-PCR was carried out in duplicate using pre-optimized primer/probe mixture (TaqMan® Gene Expression Assays; Applied Biosystems, Ontario, Canada) and TaqMan® universal PCR master mix (Applied Biosystems) on 7500 Real-Time PCR System (Applied Biosystems). The cDNA quantities between different reactions were normalized by using a housekeeping gene β-actin (Cat: 4352341E, Applied Biosystems) as a reference. The sample values represent X-fold differences from a control sample (given a designated value of 1) within the same experiment. The assay ID for each gene are as follows; IL-1β (Mm00434228_m1), CD14 (Mm00438094_g1), ICAM-1 (Mm00516024_g1), TIMP-1 (Mm00441818_m1), KC (Mm01354329_g1), MIP-2 (Mm00436450_m1).

*Immunohistochemistry*

Fresh-frozen sections were post-fixed in 2 % paraformaldehyde, incubated with rat monoclonal anti-mouse neutrophil antibody (1:500 dilution; 7/4, cat: MCA771G, AbD Serotec, Oxford, UK) followed by CY3-conjugated donkey anti-rat IgG antibody (1:500 dilution; cat: 712-165-150, Jackson Immuno Research, West Grove, PA). Cell nuclei were stained with DAPI (1:4000 dilution in PBS; Molecular Probes, Eugene, OR). All antibodies were diluted in the blocking solution [10 % normal donkey serum (NDS; Chemicon International Inc., Temecula, CA), 0.2 % Triton-X100 in PBS]. ICAM-1 staining followed the same procedure with a few modifications; frozen sections were dipped in 100 % ethanol, kept at -20 °C for 15 min, air dried followed by paraformaldehyde fixation; the blocking solution contained 0.01 % Triton-X100. Rat monoclonal anti-mouse ICAM-1 (1: 200 dilution; cat: sc-52553, Santa Cruz Biotechnology, Santa Cruz, CA) was used as a primary antibody.

For the double labelling of neutrophil/ICAM-1 or neutrophil/vW factor (an endothelial cell marker), the anti-neutrophil antibody was mixed with a goat anti-mouse ICAM-1 (1:200 dilution; cat: sc-1511, Santa Cruz Biotechnology) or a sheep anti-rat vW factor (1:3000; cat: SARTW-IG; Affinity Biologicals, Ontario, Canada) antibody. All secondary antibodies were multiple labelling-grade, raised in donkey, and used at 1:500 dilution (Jackson Immuno Research): CY3-conjugated anti-rat IgG for neutrophil, FITC-conjugated anti-sheep (cat: 713-095-147) and anti-goat IgG (cat: 705-095-147) for vW factor and ICAM-1, respectively. In control experiments, the sections were incubated with the primary antibody mixture in which the primary antibody was substituted with an IgG from a non-immunized animal (rat, goat, and sheep IgG for 7/4, ICAM-1 and vW factor, respectively; Jackson Immuno Research). Inappropriate cross-reaction between antibodies was confirmed to be negligible (data not shown).

*Assessment of neutrophil recruitment*

Three different levels of the brain (as described above) were analyzed. First, for each animal, three to four sections were analyzed at each brain level and the mean value calculated. Second, the mean values from the three brain levels were summed to represent the total number of neutrophil. For each brain section, one image was taken with a 2.5X magnification objective lens using a fixed exposure time, gain and offset for all sections. This low magnification image covers more than 60 % of the brain area at the levels of the hypothalamus and more than 80 % of the area at the level of the brain stem. Representative images are shown in Figure IV-4. The number of neutrophil-positive labelling as defined by size and light intensity was determined using an automatic counting procedure (Scion Image 4.0.2, Scion Corporation, Frederick, MD). Obvious artefacts due to dust or air bubbles were manually excluded from the area of interest.

*Leptin ELISA*

Plasma leptin levels were measured in duplicate using a mouse leptin ELISA (NIBSC) as described previously (Rummel et al., 2008). The detection limit of the assay was 15.6 pg/ml.

*Data analysis*

All data were presented as mean values ± S.E.M. Data were analyzed using a one-way ANOVA followed by a Newman-Keuls multiple comparisons post hoc test. A parametric unpaired two-tailed Student's t-test was used to compare leptin replaced groups of *ob/ob* animals. Values (P value) less than 5 % were deemed significant.

## Results

*Leptin contributes to the up-regulation of innate inflammatory genes in the brain during systemic LPS inflammation.*

Systemic inflammation triggers *de novo* synthesis of various inflammatory mediators in the brain that are typical of innate inflammation in the periphery. To determine if leptin plays a role in this process, mRNA levels of innate inflammatory mediators were measured using quantitative (q)RT-PCR in the hypothalamus of WT, *ob/ob* and *db/db* mice during LPS-induced systemic inflammation. LPS injection (2.5 mg/kg *i.p.*) in WT mice resulted in a 120-fold increase in IL-1β mRNA levels ($P < 0.001$) when compared to saline controls 24 h after injection (Figure IV-1). This increase was dramatically attenuated in both *ob/ob* and *db/db* mice ($P < 0.01$). Importantly, replenishment of leptin in *ob/ob* mice partially but significantly reversed the attenuated IL-1β mRNA levels ($P < 0.01$). In contrast, the increase of CD14 mRNA, another LPS-inducible innate inflammatory gene (Lacroix et al., 1998), was comparable between WT, *ob/ob* and *db/db* mice, indicating that the impaired IL-1β mRNA induction was not due to a general reduction of the inflammatory response in *ob/ob* or *db/db* mice but rather to a specific contribution of leptin to IL-1β mRNA up-regulation in the brain. IL-1β actions in the CNS, either directly or indirectly via downstream inflammatory mediators, contributes to a wide range of brain inflammatory mechanisms including recruitment of granulocyte neutrophils (Andersson et al., 1992; Anthony et al., 1997). In this study we detected significant effects of leptin on the LPS-induced increases of the key neutrophil chemokines keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (MIP)-2, as well as the adhesion molecule intracellular adhesion molecule (ICAM)-1 (Figure IV-1). In contrast, no such effect was observed on tissue inhibitor of metalloproteinase (TIMP)-1, an endogenous metalloproteinase inhibitor also implicated in leukocyte infiltration into the brain (Crocker et al., 2006), which was strongly induced by LPS in the same animals (Figure IV-1). Plasma leptin levels when measured at the end of the experiment (24 h after injection) were not altered by LPS treatment in

WT mice (WT-saline *vs.* WT-LPS, 0.85 ± 0.12 *vs.* 1.53 ± 0.81 ng/ml) but were significantly increased in *db/db* animals ($P < 0.001$, *db/db*-saline *vs. db/db*-LPS, 27.40 ± 2.07 *vs.* 80.49 ± 9.25 ng/ml). As expected, leptin was not detectable in *ob/ob* mice in any of the treatment groups (< 0.16 ng/ml). Leptin infusion in *ob/ob* mice increased leptin levels to 12.52 ± 1.64 ng/ml.

*Leptin mediates LPS-induced ICAM-1 up-regulation and granulocyte neutrophils accumulation in the brain.*

A previous study demonstrated that ICAM-1 plays a crucial role in neutrophil recruitment into the brain following systemic administration of a high dose of LPS (Bohatschek et al., 2001). Therefore, we further examined protein expression and distribution of ICAM-1 in the brain by using immunohistochemistry. In WT mice, LPS treatment strongly increased ICAM-1-immunoreactivity (IR) along blood vessels throughout the brain, and in the leptomeningis, ependymal linings of the ventricles and the choroid plexus when compared with saline controls (Figure IV-2). Consistent with the mRNA expression profile, the increase in ICAM-1-IR was clearly attenuated in both *ob/ob* (Figure IV-2) and *db/db* mice (data not shown), and this effect was reversed by leptin replenishment in *ob/ob* mice (Figure IV-2). In saline-treated groups, a weak but clear ICAM-1-IR was observed along blood vessels and in the leptomeningis. This constitutive ICAM-1-IR was comparable between WT, *ob/ob* (Figure IV-2) and *db/db* (not shown) mice, indicating that leptin is not critical for basal ICAM-1 expression in the brain under non-inflammatory conditions.

We next examined the recruitment of neutrophils to the brain and their spatial relationship to ICAM-1-IR structures. LPS treatment, when examined 24 h after injection, caused the appearance of cells immunoreactive for the neutrophil specific antigen 7/4, which showed a segmented nucleus characteristic of granulocytes (Figure IV-3A-D). In saline treated controls, 7/4-IR cells were sparse (see below for quantitative data). The 7/4-IR cells were predominantly found localized and aggregated in structures with ICAM-1-IR, namely along cerebral vasculatures, fissures, in the leptomeningis and the choroid plexus. Double immunohistochemistry for 7/4 and ICAM-1 demonstrated that most, if not

all, of accumulating 7/4-IR neutrophils were closely associated with ICAM-1-IR structures (Figure IV-3A, D-F). Interestingly some 7/4-IR cells were found adhering to the abluminal (brain) side of the ICAM-1-IR vasculature-like structures (Figure IV-3B-C).

*LPS-induced neutrophil accumulation is abolished in ob/ob or db/db mice*

We next compared neutrophil accumulation in the brain between WT, *ob/ob* and *db/db* mice. Twenty four hours after the LPS challenge, a robust neutrophil accumulation in the brain of WT mice was observed, which was particularly intense at the level of the ventromedial hypothalamus and the brain stem as well as along the lateral ventricles, the leptomeningis and the cerebral fissure as determined in our preliminary studies (see Material and Methods). Based on these observations neutrophil numbers were counted in coronal sections collected at three levels of the brain [at the level of the subfornical organ (SFO), the arcuate nucleus (ARC) and the area postrema (AP), Figure IV-4A]. The average total cell number from the three brain levels (3–4 sections per level x 3 levels, n = 5–6 per group) are shown in Fig 4B. The neutrophil accumulation in LPS-treated *ob/ob* or *db/db* mice was markedly lower than in WT counterparts ($P < 0.001$) and was comparable to their respective saline-treated controls ($P > 0.05$, LPS *vs.* saline). Leptin replenishment in *ob/ob* mice partially, but significantly, reversed the attenuation of LPS-induced neutrophil accumulation ($P < 0.01$).

To examine the similarity of the distribution patterns of neutrophil accumulation and the sites of leptin's action previously observed on the barrier-related structures of the brain (Rummel et al., 2008), the anatomical distribution of leptin-mediated neutrophil accumulation was more precisely examined in brain sections from LPS-treated *ob/ob* mice with or without leptin replenishment (Figure IV-5). Briefly, leptin replenishment in *ob/ob* mice increased neutrophil numbers, almost all of them adhering to von Willebrand (vW) factor-IR endothelial cells, when compared with vehicle infusion. Representative images show vasculatures in the cortex, the leptomeningis at the level of the ventral hypothalamus, the paraventricular nucleus (PVN), the ventromedial preoptic nucleus (VMPO), the supraoptic nucleus (SON) and the hippocampal fissures

(Figure IV-5). The neutrophil distribution pattern in these brain structures was similar to the previously reported leptin-dependent activation of the barrier-related structures (Rummel et al., 2008).

*Acute starvation suppresses the brain innate inflammatory response via a decrease in circulating leptin levels.*

Given leptin's role linking nutritional status, immunity and the brain, we tested if the reduction in leptin levels during acute starvation will influence the inflammatory mechanisms linked to neutrophil trafficking into the brain. To this end, WT mice were fasted for 48 h with or without leptin replenishment prior to LPS administration (2.5 mg/kg, *i.p.*), and the inflammatory responses in the brain assessed 24 h after the injection. Plasma leptin levels, when measured at the end of experiment, were 2.06 ± 0.64 ng/ml and below the detection limit of the assay (0.16 ng/ml) for *ad lib* fed mice and fasted mice with vehicle infusion, respectively. Leptin infusion in fasted mice increased leptin levels to 12.9 ± 2.1 ng/ml. Fasting impaired the LPS-induced innate inflammatory gene expression in the brain in a manner almost identical to leptin or leptin receptor genetic deficiency (Figure IV-6). Briefly, fasted mice with vehicle infusion showed impaired LPS-induced increase in IL-1β, KC, MIP-2 and ICAM-1 mRNA levels ($P < 0.05$) while having comparable CD14 and TIMP-1 mRNA increases when compared with *ad lib* fed counterparts. Leptin replenishment during fasting reversed the induction of IL-1β and ICAM-1 mRNA levels ($P < 0.05$). Although there was a tendency for KC mRNA levels to recover, it did not reach statistical significance. MIP-2 mRNA levels were not affected by leptin replacement (Figure IV-6). The LPS-induced neutrophil accumulation was significantly attenuated by fasting without leptin infusion as compared to *ad lib* feeding ($P < 0.01$), and leptin replenishment significantly reversed neutrophil accumulation ($P < 0.05$, Figure IV-6).

*A transient neutralization of leptin alleviates neutrophil accumulation in the brain during systemic LPS inflammation.*

Given the significant role of leptin in LPS-induced neutrophil recruitment to the brain, which is potentially neurotoxic, it is important to elucidate whether a transient neutralization of leptin with anti-leptin antiserum (LAS) could be used as a potential tool for therapeutic intervention. To this end, WT mice received a single injection of LAS (0.5 ml/kg, *i.p.*) or a control preimmune serum (PIS) 4 h prior to the administration of LPS (2.5 mg/kg, *i.p.*) or saline, and the brain innate inflammatory response was examined 24 h after LPS treatment. Plasma leptin levels, when measured at the end of the experiment, were 1.12 ± 0.28 and 1.15 ± 0.44 ng/ml for the PIS-saline and PIS-LPS groups, respectively. The values for both the LAS-saline and LAS-LPS groups were below the detection limit of the assay (0.16 ng/ml), confirming the effectiveness of LAS treatment. Among the genes that were found to be regulated by leptin (in *ob/ob*, *db/db* or fasted mice) during LPS-induced inflammation, LAS-treatment attenuated only KC and MIP-2 mRNA induction ($P < 0.01$), whereas it did not affect the induction of IL-1β and ICAM-1 mRNA (Figure IV-7). In addition, LPS-induced TIMP-1 mRNA expression was attenuated by LAS ($P < 0.01$). LAS treatment effectively suppressed LPS-induced neutrophil accumulation to the brain ($P < 0.001$, Figure IV-7) to a degree similar to that seen in fasted animals.

## Discussion

The main finding of the present study is that leptin mediates the recruitment of neutrophils to the brain during severe systemic LPS inflammation. This conclusion is based on the following observations. First, the LPS-induced neutrophil recruitment was almost completely abolished in mice deficient in leptin (*ob/ob*) or the long form leptin receptor ObRb (*db/db*), and replenishment of leptin in *ob/ob* mice significantly restored the neutrophil recruitment process. Second, 48 h food deprivation in WT mice (which reduced endogenous leptin levels) prior to LPS injection similarly inhibited neutrophil recruitment in a leptin-dependent manner. Third, a single injection of LAS in WT mice 4 h before LPS treatment was sufficient to attenuate neutrophil recruitment. Collectively, the present data provide compelling evidence that leptin has a specific role in this process and argues against the possibility that the reduction of neutrophil accumulation in the brain was due to other hormonal and metabolic abnormalities resulting from obesity or starvation. Most importantly, our observations support the role of leptin as a physiologically relevant link between the energy status of the host and its ability to mount an innate immune response in the brain following systemic inflammation.

The attenuation of LPS-induced neutrophil recruitment as well as the accompanying gene expression were comparable between *ob/ob* and *db/db* mice, the latter have intact short forms of ObR but lack the long form ObRb, indicating that these leptin-mediated processes were dependent primarily on ObRb. This observation in turn indicates that the neutrophil recruitment and brain production of inflammatory mediators are independent of the reported direct actions of leptin on neutrophils (which only express the short form ObRs) where this hormone has been shown to stimulate chemotaxis (Montecucco et al., 2006) or inhibit spontaneous apoptosis (Bruno et al., 2005), suggesting that its action on alternative targets including the brain is the major contributing factor to the leptin-mediated processes observed in this study.

We have recently reported (Rummel et al., 2008) that, during systemic LPS inflammation, leptin-dependent activation of the signal transducer and activator of transcription-3, an established indicator of ObRb signalling (Hubschle et al., 2001), was observed predominantly in the cerebrovasculature, the leptomeningis, and the longitudinal and hippocampal fissures, all of which reportedly express leptin receptors including ObRb (Mercer et al., 1996; Bjorbaek et al., 1998; Mutze et al., 2006), indicating that leptin directly acts on these barrier-related structures of the brain. Although the functional significance of leptin's action on these structures was not clear, the pattern of leptin activity demonstrated remarkable similarity to the distribution of leptin-mediated neutrophil accumulation (Figure IV-4 and Figure IV-5). Although speculative, one of the functional outcomes of leptin's action at these sites may be to facilitate the recruitment of neutrophils into the brain during severe systemic inflammation. The present results, together with previous reports on leptin's actions in the brain, provide some insight into the potential underlying mechanisms downstream of leptin's action (*e.g.*, mediators and cell types involved) as discussed below.

Parallel to the suppression of the neutrophil recruitment, LPS-induced IL-1β mRNA up-regulation was strongly blunted in the brains of *ob/ob*, *db/db* or fasted WT mice when compared to (*ad lib* fed) WT counterparts. IL-1β is rapidly induced in the brain by various inflammatory stimuli of both central and peripheral origin, and is a potent contributor to subsequent brain innate inflammatory responses including leukocyte recruitment (Rothwell and Luheshi, 2000; Allan et al., 2005). IL-1β alone, when acting centrally, is sufficient to trigger recruitment of neutrophils to the brain (Anthony et al., 1997; Proescholdt et al., 2002; Ferrari et al., 2004; Ching et al., 2005), whereas blockade of IL-1β actions by using IL-1RA or genetic deletion of its functional receptor IL-1R1 prevents the neutrophil recruitment associated with neuroinflammation (Garcia et al., 1995; Yang et al., 1998; Schiffenbauer et al., 2000; Ching et al., 2005). In the context of the present study, we and others have previously demonstrated that leptin administration, either centrally or systemically, induces brain IL-1β both at the mRNA and protein levels (Luheshi et al., 1999; Hosoi et al., 2002b; Wisse et

al., 2004; Inoue et al., 2006). In primary microglia-enriched culture, leptin stimulates the synthesis and release of IL-1β protein (Pinteaux et al., 2007), supporting direct actions of leptin on these cells. *In vivo*, shortly after *i.c.v.* leptin administration (1–3 h), the majority of IL-1β protein expression was found localized in macrophages in the meningis and perivascular spaces (Inoue et al., 2006). Interestingly, the cellular and regional expression of leptin-induced IL-1β is similar to that triggered by a systemic injection of LPS where brain IL-1β production is known to be largely dependent on brain macrophages that reside in perivascular spaces, the meningis, the choroid plexus and the cerebroventricular organs, as well as parenchymal microglia (Van Dam et al., 1992; Quan et al., 1998; Eriksson et al., 2000; Garabedian et al., 2000). More specifically, the appearance of brain IL-1β after systemic LPS challenge follows a specific spacio-temporal pattern; early (0.5–2 h) IL-1β expression appears in the brain-resident macrophages associated with the barrier-related structures, and thereafter, the focus of sustained (12–24 h) IL-1β expression moves from the macrophages to neighbouring parenchymal microglia (Quan et al., 1998). This time line correlates well with the spacio-temporal process of neutrophil recruitment, which peaked 24 h after LPS administration and later (48 h) spread into parenchyma (see Materials and Methods), a finding that is in agreement with a previous report (Bohatschek et al., 2001). These data suggest that defective brain IL-1β production resulting from leptin deficiency is one of the underlying mechanisms responsible for reduced neutrophil recruitment during systemic inflammation.

Neutrophil attachment to brain endothelium is the prerequisite step for their eventual recruitment into the CNS. In the present study, the replenishment of leptin in leptin-deficient *ob/ob* or fasted WT mice almost completely restored the attenuated ICAM-1 mRNA up-regulation by LPS, indicating a significant influence of leptin on the LPS-induced brain ICAM-1 expression. Moreover, immunohistochemical approaches demonstrated that the leptin-mediated ICAM-1 expression was predominantly localized in the cerebrovasculature, the leptomeningis, cerebral fissures and the choroid plexus, all of which were primary sites of neutrophil accumulation (Figure IV-2 and Figure IV-3). In fact, double

labelling studies clearly showed that the majority of neutrophils were closely associated with ICAM-1-IR cells, on both the luminal and abluminal sides of the vasculature-like structures (Figure IV-3). As the brains examined in this study were well perfused with heparinized saline (see Materials and Methods), we presume that this close contact may represent an integrin (ligand for ICAM-1)-mediated neutrophil arrest to the endothelium, the essential step for eventual neutrophil infiltration into the parenchyma. In fact, Bohatschek et al. (Bohatschek et al., 2001) reported that neutrophil infiltration into the brain following peripheral LPS challenge was strongly reduced in ICAM-1-deficient mice to levels below 50% of WT controls. The mechanism underlying leptin's regulation of ICAM-1 is not yet clear but IL-1β is likely to be an important factor. It is well established that, in the brain, IL-1R1 is constitutively, and indeed predominantly, expressed in the barrier-related structures (*e.g.*, vasculatures, the leptomeningis and the choroid plexus) (Ericsson et al., 1995; Konsman et al., 2004), and IL-1β readily induces ICAM-1 in these structures (Proescholdt et al., 2002). It was also reported that IL-1R1 is expressed in both luminal and abluminal sides of the brain vascular endothelium (Cao et al., 2001). Interestingly, in brain endothelial monolayer culture, IL-1β stimulation of the abluminal surface was reported to preferentially increase ICAM-1 expression at the luminal surface (Staykova et al., 2000). Given that leptin induces IL-1β in perivascular and meningeal macrophages *in vivo* (Inoue et al., 2006) which reside in the abluminal side of the vasculature, it is conceivable that leptin-induced IL-1β acts on the barrier-related structures in a paracrine manner and regulates leukocyte recruitment via ICAM-1.

A transient neutralization of leptin with LAS effectively attenuated neutrophil recruitment during LPS inflammation. This is an important observation as it might provide a potential therapeutic strategy for combating some neuropathologies such as cerebral ischemia in which neutrophil recruitment is linked with detrimental outcomes (Emerich et al., 2002). However, this approach also revealed some issues regarding the actions of leptin worth consideration. In contrast to other models (genetic deficiency or fasting), LAS treatment did not alter LPS-induced IL-1β and ICAM-1 mRNA expression. The efficiency of

neutralization is certainly a factor to be considered, however the plasma leptin levels in LAS-treated animals were verified at the end of the experiment and were all below the detection limit of the assay. Alternatively, this observation implies that the depletion of leptin signalling (for a certain time period) preceding inflammation, rather than in the course of, is critical for the attenuation of IL-1β and ICAM-1 levels. In contrast, LAS treatment was able to attenuate, similar to other models of leptin deficiency, the LPS-induced mRNA increase of KC and MIP-2, both of which are potent and well-characterized neutrophil chemoattractants in mice (Kielian et al., 2001). This indicates that although IL-1β has been demonstrated to be a powerful inducer of KC and MIP-2 (Anthony et al., 1998; Shaftel et al., 2007), leptin could be an additional factor influencing their brain expression independent of IL-1β. In line with this rationale, it was previously demonstrated that, in the periphery, leptin directly stimulates the production of KC and MIP-2 (Fenton et al., 2007; Wong et al., 2007). Since KC and MIP-2 are essential for neutrophil recruitment to the brain even in the presence of IL-1β and ICAM-1 (Kielian et al., 2001; Shaftel et al., 2007), the LAS-mediated attenuation of these chemokines explains the reduced neutrophil recruitment by LAS treatment without affecting the expression of either IL-1β or ICAM-1.

Perhaps one of the most important findings in this study is the impact of food deprivation on the brain's innate inflammatory response during systemic inflammation. Leptin has emerged as a critical mediator of starvation-induced immunosuppression (Faggioni et al., 2001; La Cava and Matarese, 2004). A number of studies have shown that replenishment of leptin in starved animals restores their abnormal host defence responses in various experimental models of inflammation (Lord et al., 1998; Howard et al., 1999; Faggioni et al., 2000a; Mancuso et al., 2006), highlighting the physiological importance of this hormone. Despite this, surprisingly little is known about the potential impacts of malnutrition and/or declined leptin signalling on the brain's innate inflammatory response. This is important to know as the inherent brain orchestrated responses which form part of the host's defence mechanisms actively contribute to the

course of inflammation (*e.g.*, through sickness behaviours and/or activation of the stress axis) (Hart, 1988; Dantzer, 2004). The present study demonstrated that acute starvation attenuates brain production of IL-1β, a prototypical innate inflammatory mediator, in a leptin-dependent manner after a systemic LPS challenge. This was paralleled by the changes in the levels of some other inflammatory mediators (ICAM-1 and KC) as well as the reduction in neutrophil recruitment. Importantly, these changes are specific to a certain facet of the brain inflammatory response and are not a result of a generalized suppression of the immune response because the expression of CD14 and TIMP-1 mRNA in the brain, which were also induced by LPS, were unaffected by fasting or leptin levels. However, the present study does not exclude the possibility that some of the leptin-mediated changes observed here were indirect, resulting from the established role of leptin in the peripheral innate inflammatory response (*e.g.*, cytokine production in the periphery), which subsequently affects the brain response. Among several possibilities, previous studies by us and others support the scenario that at least part of the brain's responses are attributable to direct actions of leptin as 1) leptin stimulates IL-1β synthesis in microglia and mixed glia *in vitro* (Hosoi et al., 2000a; Pinteaux et al., 2007), 2) central administration of leptin induces IL-1β in the brain (Luheshi et al., 1999; Inoue et al., 2006), and 3) during systemic LPS inflammation, leptin acts directly on the barrier-related structures (Rummel et al., 2008), the known sites of IL-1β production after systemic LPS challenge (Van Dam et al., 1992; Quan et al., 1998; Eriksson et al., 2000; Garabedian et al., 2000). In fact these observations, in combination with our earlier studies (Luheshi et al., 1999; Sachot et al., 2004) further point to a role for leptin in immune-to-brain signalling and resultant sickness behaviours during systemic inflammation. Furthermore, the present study, by demonstrating significant contributions of leptin to neutrophil recruitment caused by severe systemic inflammation, suggests a potential role for this hormone in the development and/or progression of neuropathological conditions linked to neutrophil-mediated pathogenesis (Papadopoulos et al., 2000; Bohatschek et al., 2001; McColl et al., 2007; Perry et al., 2007). Our finding that leptin is

significantly involved in the brain's innate inflammatory response may have broad implications on how nutritional status can impact the etiology of brain diseases.

**Acknowledgements**

We would like to thank Ms. G. Somay for excellent technical assistance. This study was supported by the Canadian Institute of Health Research and the Natural Sciences and Engineering Research Council of Canada (G.N.). C.R. is a fellow of the Deutsche Forschungsgemeinschaft (DFG project RU 1397/1-1). W.I. is a recipient of a PhD scholarship from the Nakajima Foundation, Japan. Authors declare no conflicting financial interests.

## Figures

**Figure IV-1. mRNA expression of innate inflammatory mediators in the brain following systemic LPS challenge in WT, ob/ob and db/db mice**

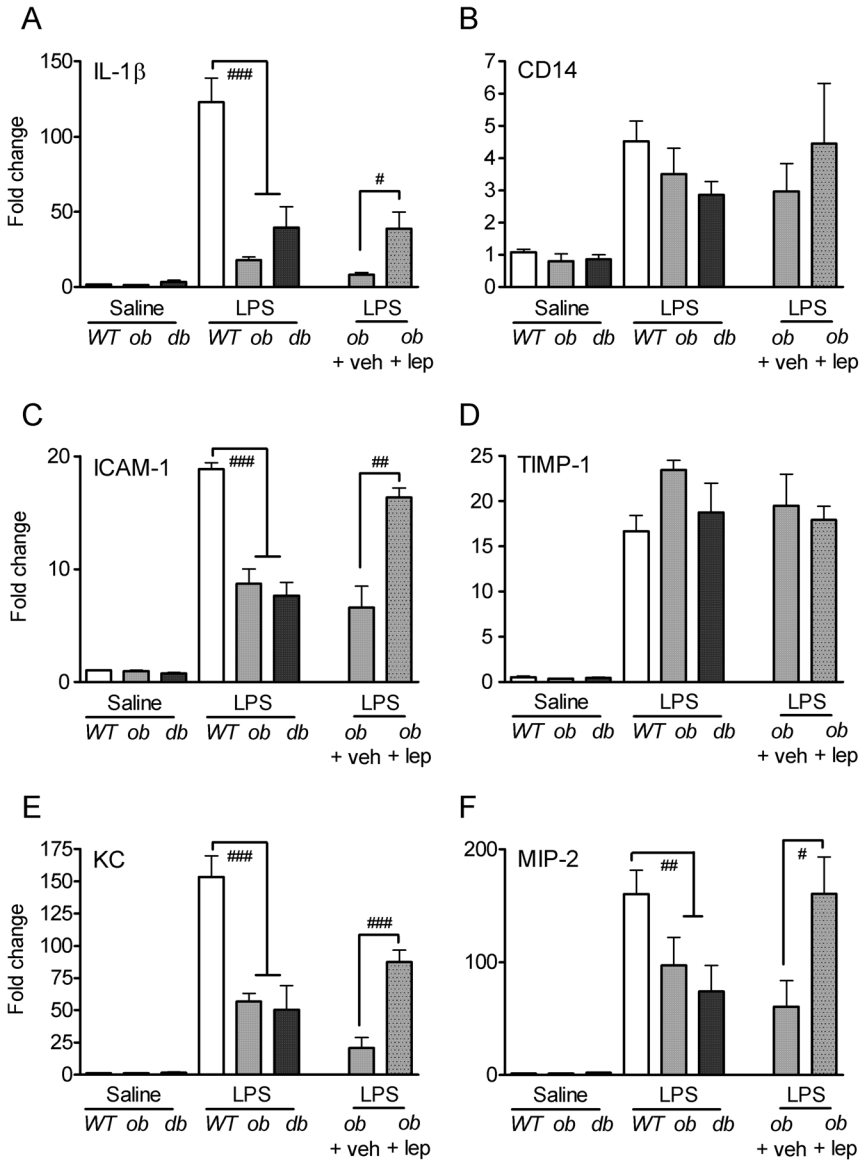



*Figure IV-1. mRNA expression of innate inflammatory mediators in the brain following systemic LPS challenge in WT, ob/ob and db/db mice. mRNA levels in the brain were analyzed 24 h after LPS challenge (2.5 mg/kg i.p.) using qRT-PCR. LPS-induced mRNA levels of IL-1β, ICAM-1, KC and MIP-2 were dramatically reduced in ob/ob and db/db mice when compared with WT controls. The LPS-*

*induced CD14 and TIMP-1 mRNA levels were similar between WT, ob/ob and db/db mice. Replenishment of leptin in ob/ob mice significantly increased the LPS-induced mRNA levels of IL-1β, ICAM-1, KC and MIP-2, whereas it had no effect on CD14 and TIMP-1 mRNA increase, as compared with vehicle infusion. Values are presented as means + S.E.M. P values:* $^{\#} < 0.05$, $^{\#\#} < 0.01$, $^{\#\#\#} < 0.001$.

**Figure IV-2. ICAM-1 protein expression in the brain following systemic LPS challenge in WT, ob/ob, and leptin-treated ob/ob mice**

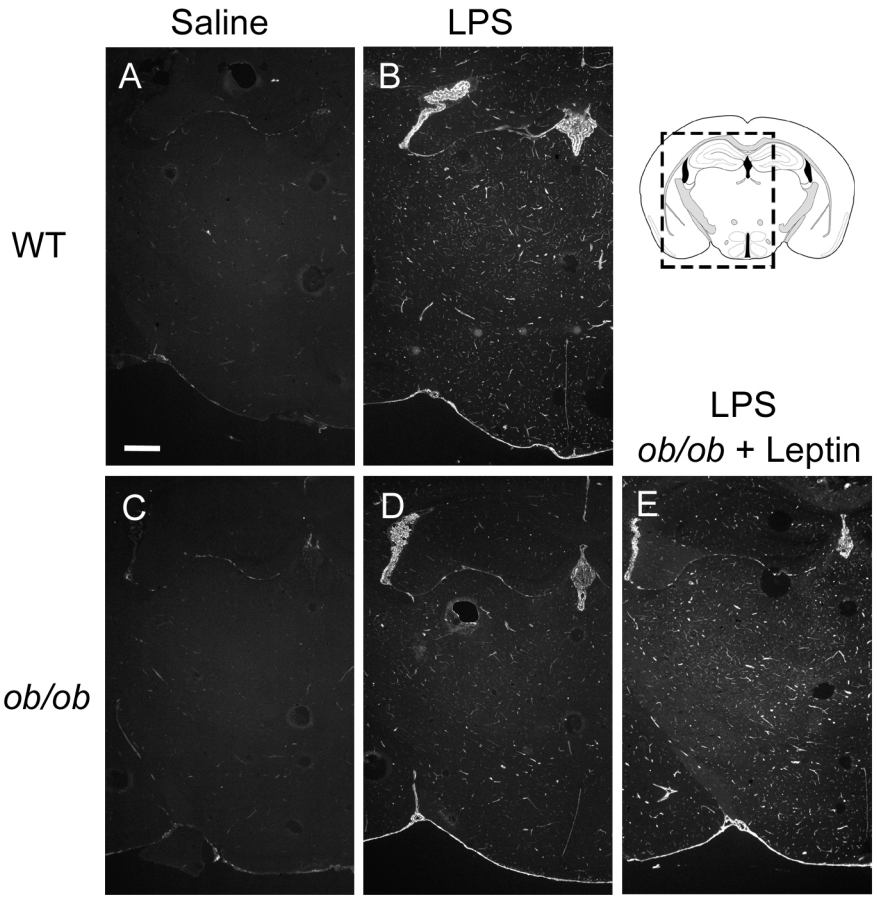



*Figure IV-2. ICAM-1 protein expression in the brain following systemic LPS challenge in WT, ob/ob, and leptin-treated ob/ob mice. ICAM-1 protein expression in the brain was examined by immunohistochemistry 24 h after systemic LPS challenge (2.5 mg/kg i.p.). A constitutive ICAM-1-IR was observed in saline-treated mice at similar levels between WT and ob/ob mice (A and C). LPS treatment dramatically increased ICAM-1-IR in the leptomeningis, cerebral*

*vasculatures and fissures, and the choroid plexus in WT mice (B). The increase of ICAM-1-IR was clearly attenuated in ob/ob mice and was reversed by leptin treatment in ob/ob mice (D and E). Scale bar in A represents 400 μm.*

**Figure IV-3. Double immunohistochemistry for ICAM-1 and granulocyte neutrophils in the brain of WT mice following systemic LPS challenge**

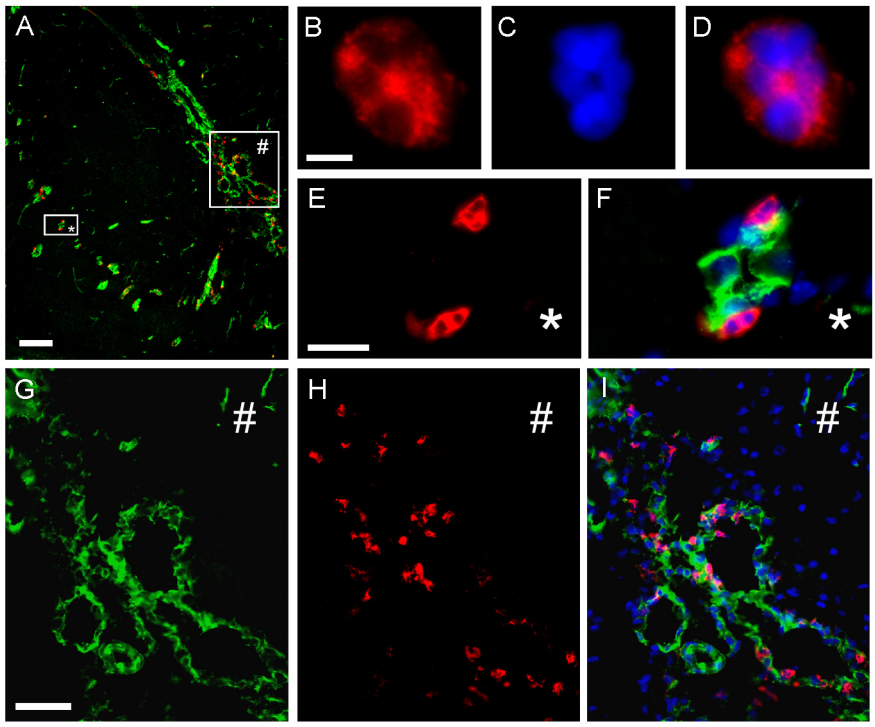



*Figure IV-3. Double immunohistochemistry for ICAM-1 and granulocyte neutrophils in the brain of WT mice following systemic LPS challenge. (A) Neutrophil antigen 7/4-IR (red) was detected in the brain 24 h after systemic LPS challenge (2.5 mg/kg i.p.) and was closely associated with ICAM-1-IR (green). (B–D) Images of a neutrophil at high magnification. Neutrophil-IR (B, red) and its segmented nucleus characteristic of granulocyte neutrophils stained with DAPI (C, blue), clearly co-localize (D, merged), supporting the cellular identity of the staining. (E–I) Magnified images of the areas enclosed by white squares in A indicated with * (E–F) and # (G–I). Note that some neutrophil-IR (red) was observed in the abluminal side of vasculature-like structures with ICAM-1-IR*

*(green). Nuclear DAPI staining is shown in blue. Scale bars in A, 50 µm; B, 5 µm; E, 20 µm; G 50 µm.*

**Figure IV-4. LPS-induced neutrophil recruitment to the brain of WT, ob/ob and leptin-treated ob/ob mice**

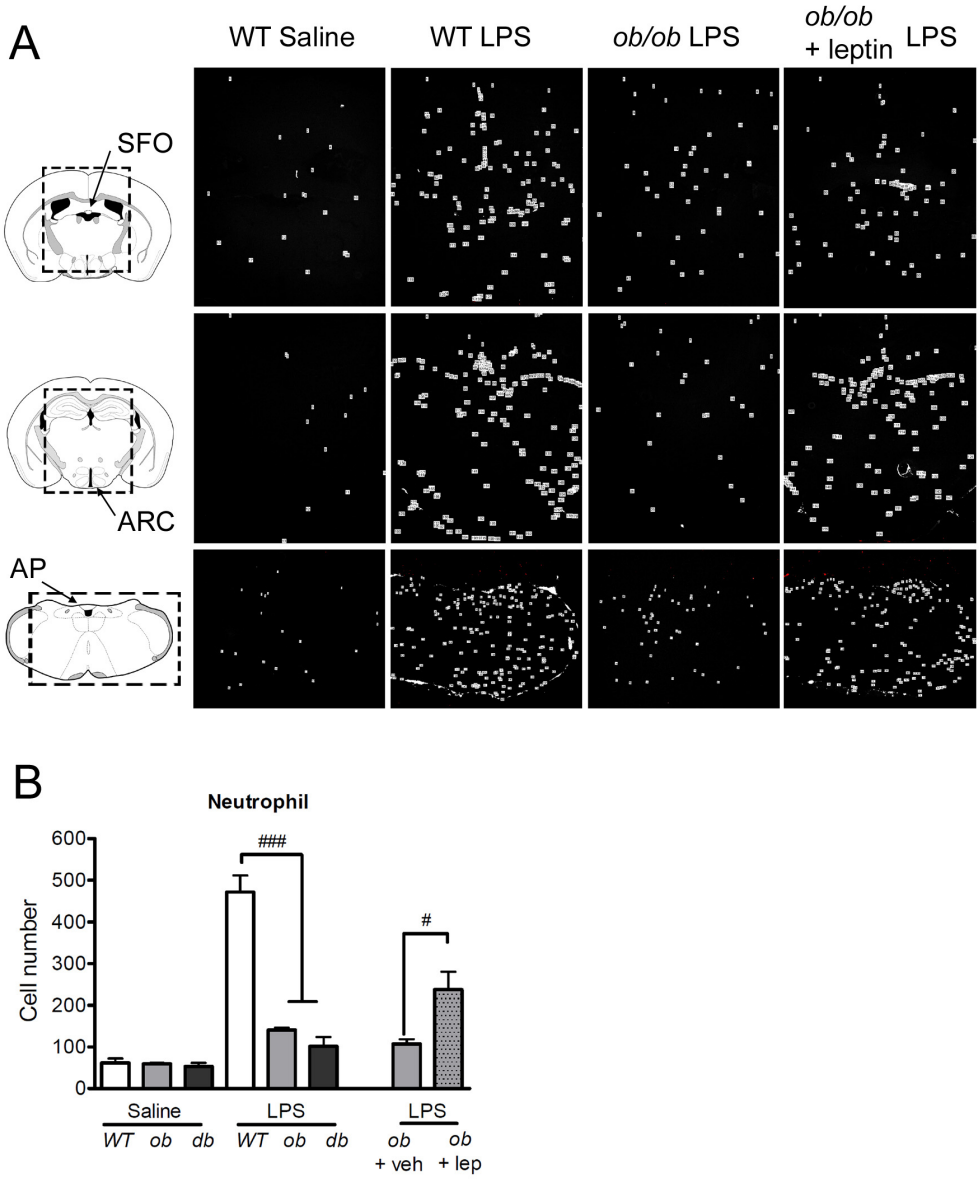


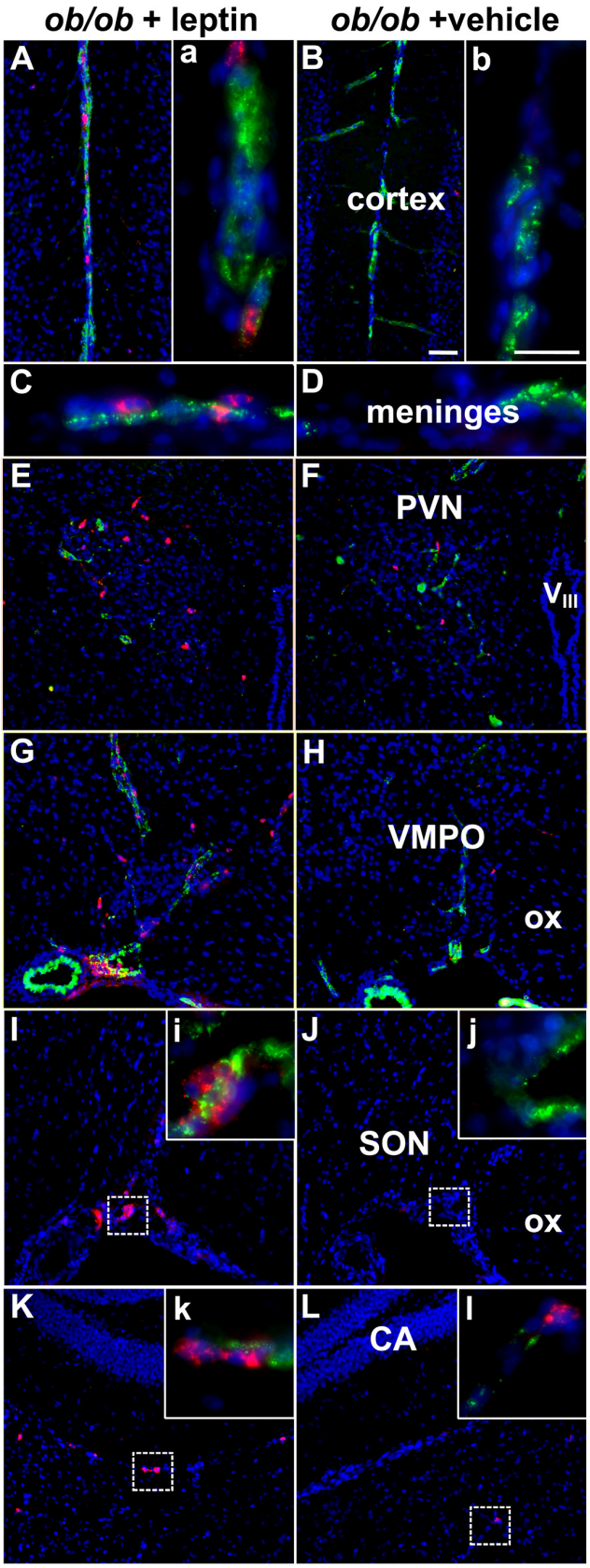

*Figure IV-4. LPS-induced neutrophil recruitment to the brain of WT, ob/ob and leptin-treated ob/ob mice. (A) Representative images depicting neutrophil distribution in coronal sections of the brain at the level of the subfornical organ*

*(SFO), the arcuate nucleus (ARC) and the area postrema (AP). For clarity, the actual neutrophil-IR was superimposed with white squares that were used for automatic quantification of cell numbers. In WT mice, LPS (2.5 mg/kg i.p.) dramatically increased neutrophil accumulation compared with saline treatment 24 h after stimulation. This response was abolished in ob/ob mice and was reversed by leptin treatment. Note that the distribution of neutrophils was prominent along the lateral ventricles, the interhemispheric fissure, the leptomeningis and vasculatures. (B) Quantitative data for neutrophil recruitment. Values are presented as means + S.E.M. P values:* $^{\#} < 0.05$, $^{\#\#\#} < 0.001$.

**Figure IV-5. The distribution of neutrophils in the brain of LPS-treated ob/ob mice with or without leptin replenishment**

*-see next page for Figure IV-5-*

*Figure IV-5. The distribution of neutrophils in the brain of LPS-treated ob/ob mice with or without leptin replenishment. Leptin replenishment in ob/ob mice recovered neutrophil recruitment. The recruited neutrophils were predominantly associated with the barrier-related structures of the brain (A–B) along blood vessels in the interhemispheric fissure, (C–D) in the leptomeningis, (E–F) in the paraventricular nucleus (PVN), (G–H) in the ventromedial preoptic nucleus (VMPO), (I–J) in the supraoptic nucleus (SON) and (K–L) the hippocampal fissures. Neutrophil-IR can be depicted in close vicinity to vW factor-IR endothelial cells (green) as shown at high magnification (a–b, C–D, i–l). DAPI staining was used to label cell nuclei and depict general microanatomy. Scale bars in A, 50 μm; a, 25 μm.*


ob/ob + leptin
ob/ob +vehicle
A
a
B
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cortex
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meninges
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**Figure IV-6. The effects of fasting and leptin replenishment on brain inflammatory gene expression and neutrophil recruitment during systemic LPS inflammation**

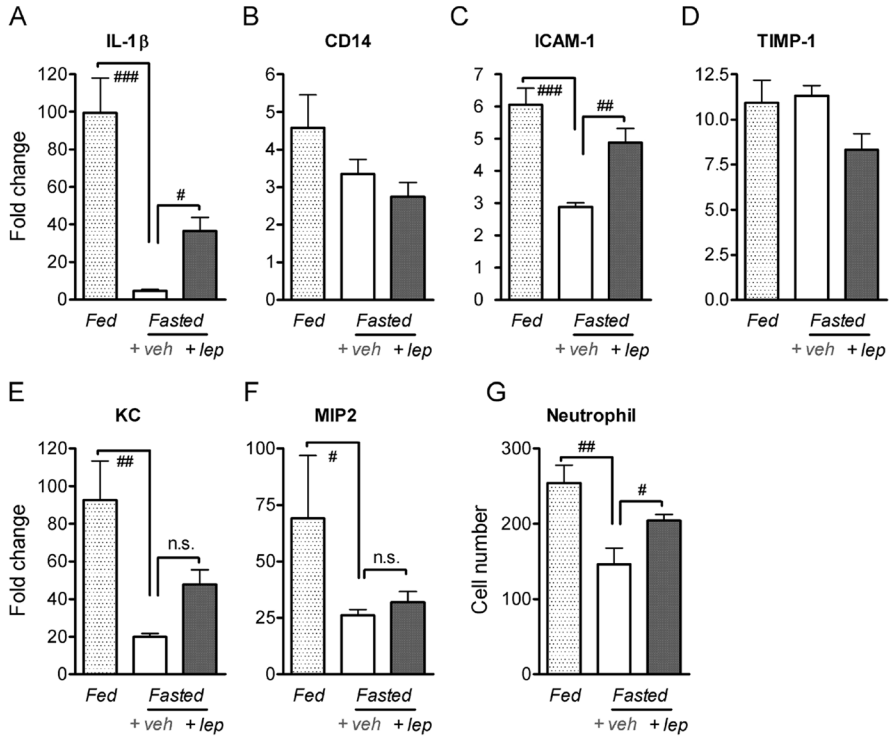


*Figure IV-6. The effects of fasting and leptin replenishment on brain inflammatory gene expression and neutrophil recruitment during systemic LPS inflammation. (A–F) Mice were fed, fasted or fasted with leptin replenishment for 48 h before LPS treatment (2.5 mg/kg i.p.). The levels of mRNA expression in the brain were measured by qRT-PCR 24 h after the LPS challenge. Fasting significantly attenuated the LPS-induced increase in IL-1β, ICAM-1, KC and MIP-2 mRNA levels, whereas it did not affect CD14 and TIMP-1 mRNA levels. Leptin replenishment in fasted mice significantly recovered LPS-induced IL-1β and ICAM-1 mRNA levels. (G) Neutrophil recruitment was quantified in the same animals. Fasting significantly attenuated the LPS-induced neutrophil recruitment, and leptin reversed the effect. Values are presented as means + S.E.M. P values: $^{\#} < 0.05$, $^{\#\#} < 0.01$, $^{\#\#\#} < 0.001$. n.s. not significant*

**Figure IV-7. The effects of acute leptin neutralization on brain inflammatory gene expression and neutrophil recruitment during systemic LPS inflammation**

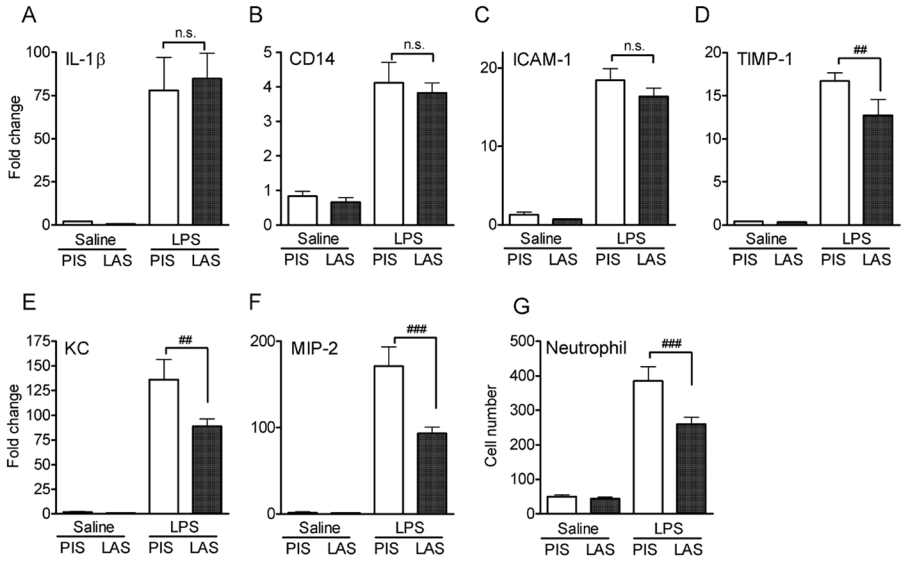


*Figure IV-7. The effects of acute leptin neutralization on brain inflammatory gene expression and neutrophil recruitment during systemic LPS inflammation. Mice were treated with either LAS (0.5 ml/kg, i.p.) or control PIS 4 h before LPS (2.5 mg/kg i.p.) or saline injection. Brains were analyzed 24 h after the LPS/saline challenge. (A–F) The levels of mRNA expression in the brain were measured using qRT-PCR. LAS treatment significantly attenuated the LPS-induced increase in KC, MIP-2 and TIMP-1 mRNA levels, whereas it did not affect the levels of IL-1β, CD14 and ICAM-1 mRNA. (G) Neutrophil recruitment was quantified in the same animals. LAS treatment significantly attenuated LPS-induced neutrophil recruitment. Values are presented as means + S.E.M. P values:* $^{\#\#} < 0.01$, $^{\#\#\#} < 0.001$. *n.s. not significant*

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## IV.3. Supplementary results and discussion

Data from the present manuscript demonstrated that leptin deficiency, resulting from genetic mutation, nutritional deprivation or pharmacological intervention, resulted in a compromised expression of innate inflammatory mediators in the brain and an attenuated recruitment of neutrophils during severe systemic inflammation. Most importantly, food deprivation prior to the introduction of LPS-induced inflammation attenuated the brain's innate inflammatory response in a leptin-dependent manner, thus suggesting a unique role for leptin in linking nutritional status and brain inflammation. Coupled with the known ability of leptin to directly stimulate the synthesis of cytokines in the brain (Chapter II and III), these results strongly support our hypothesis that "leptin is an afferent inflammatory signal to the brain that modulates the brain's innate inflammatory response with relation to the nutritional status of the organism."

Our current working model postulates the importance of the direct actions of leptin on the brain in regulating/modulating brain inflammation. However, the data from the present study does not rule out the possibility that leptin does so indirectly by modulating the peripheral response to LPS (*i.e.*, the peripheral production of other cytokines, such as TNF, IL-1β and IL-6, which in turn act on the brain). In relation to this issue, we determined plasma levels of IL-6 in the animals studied in the current manuscript as a potential alternative afferent inflammatory signal to the brain (Figure IV-8). As expected, in *ad lib* fed wild-type mice, IL-6 concentrations were significantly elevated by LPS treatment as compared to saline control 24 h after injection (Figure IV-8A). The levels of LPS-induced IL-6 were dramatically increased in *ob/ob* and *db/db* mice (samples measured were all above the upper limit of the assay, 2 ng/ml, n = 5 per group), thus indicating that the peripheral inflammatory response in the mutant mice were enhanced (*i.e.*, the opposite of the brain response). This exaggerated production of IL-6 was partly attributable to leptin deficiency as leptin replacement in *ob/ob* mice significantly reversed the elevated IL-6 levels. On the other hand, the reduction in leptin levels induced by fasting or LAS treatment in wild-type mice

had no effect on LPS-induced IL-6 levels (Figure IV-8B-C). The reasons for the dramatic enhancement of IL-6 production in the mutant mice are unknown, but it is possible that permanent leptin deficiency, rather than a transient one, may have different effects on the development of peripheral immune function (*i.e.*, permanent leptin deficiency alters the balance between different subsets of immunocytes etc.). Whatever the mechanisms are, the plasma IL-6 profile can not account for the changes in brain inflammation, thus supporting direct effects of leptin on the brain.

**Figure IV-8. Plasma IL-6 levels**

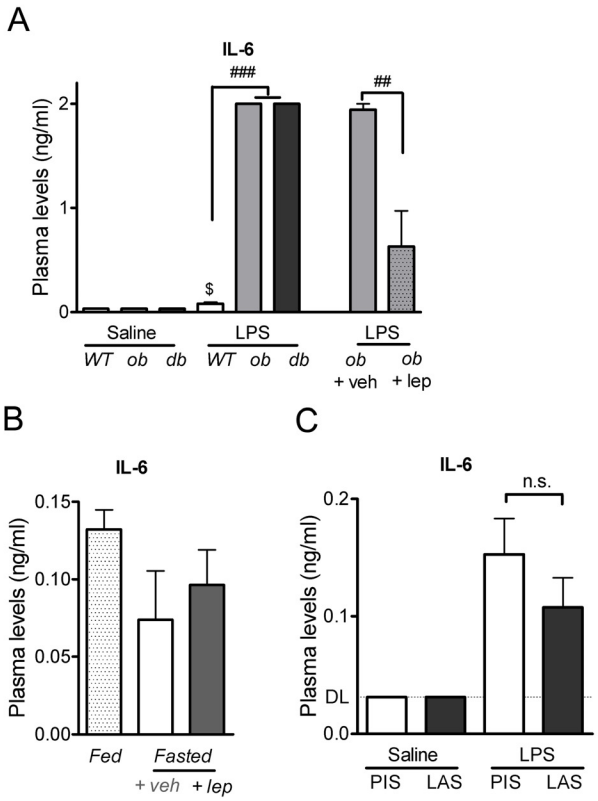


*Figure IV-8. Plasma IL-6 levels after LPS challenge. Mice were treated with an i.p. injection of LPS (2.5 mg/kg) or saline. Blood was collected 24 h after injection. (A) IL-6 levels were below the detection limit of the assay in saline-treated groups regardless of their genotypes (< 0.031 ng/ml). IL-6 levels were significantly higher in LPS-treated groups as compared to their respective saline-*

*treated controls. IL-6 levels were significantly higher in LPS-treated ob/ob and db/db mice as compared to wild-type animals. All samples in LPS-treated ob/ob and db/db (n = 5) and 4 of 5 samples in ob/ob + veh groups exceeded the upper limit of the assay (2 ng/ml). Leptin infusion significantly reduced LPS-induced IL-6 levels in ob/ob mice.* $^{\$}$ *P < 0.05 (vs. saline),* $^{\#\#}$ *P < 0.01 (vs. vehicle),* $^{\#\#\#}$ *P < 0.001 (vs. wild-type). (B–C) IL-6 levels were below the detection limit of the assay in saline-treated groups regardless of the genotypes (< 0.031 ng/ml). IL-6 levels were significantly higher in LPS-treated groups as compared to their respective saline-treated controls, but not different among LPS-treated groups. PIS, pre-immune sheep serum; LAS, sheep anti-leptin antiserum.*

One of the strongest evidence supporting the direct action of leptin on the brain is its reported link with brain IL-1β production. Following systemic LPS challenge, the main sources of brain IL-1β are brain-resident macrophages in the barrier related structures (*i.e.*, the meningis, perivascular spaces and the CVOs) (Van Dam et al., 1995; Quan et al., 1998), which express substantial levels of ObR including ObRb (Mercer et al., 1996; Bjorbaek et al., 1998; Mutze et al., 2006). Administration (*i.c.v.)* of leptin induces IL-1β production in brain macrophages in these structures (Inoue et al., 2006). Further supporting this link, is our recent report that endogenous leptin selectively acts on these barrier-related structures during LPS-induced systemic inflammation (Rummel et al., 2008). In this study, we examined the nuclear translocation of STAT3, an established marker for the activation of ObRb (Hubschle et al., 2001; Inoue et al., 2006; Mutze et al., 2006), after an *i.p.* injection of LPS in mice. As IL-6 (which also signals through the STAT3 pathway, see Figure IV-9) is known to be the major factor contributing to brain STAT3 activation during LPS-induced inflammation (Lebel et al., 2000; Rummel et al., 2006), a potential role of leptin in mediating STAT3 activation was examined in IL-6-deficient mice treated with or without LAS. As shown in Figure IV-10, a substantial amount of IL-6-independent STAT3 activation was still observed in LPS-treated IL-6-deficient mice, which was predominantly localized in the barrier-related structures, such as the cerebral

fissures and the meningis throughout the brain. The IL-6-independent STAT3 activation was almost completely abolished by LAS treatment (Figure IV-10), indicating that leptin directly acts on the barrier-related structures during LPS-induced inflammation. Based on these results we currently hypothesize that leptin's action in these brain areas contributes to the production of IL-1β (and possibly other mediators) and the ensuing brain's innate inflammation including neutrophil recruitment. However, to reinforce the hypothesis it will be important to examine if STAT3 and IL-1β co-localize in the barrier-related structures, and to characterize their cellular identities in LPS-treated animals. On a functional level, it will be interesting to test if blockade of IL-1β action by IL-1RA in wild-type mice or repletion of central IL-1β in *ob/ob* mice affects LPS-induced neutrophil recruitment to the brain in the expected direction.

**Figure IV-9. STAT3 nuclear translocation**

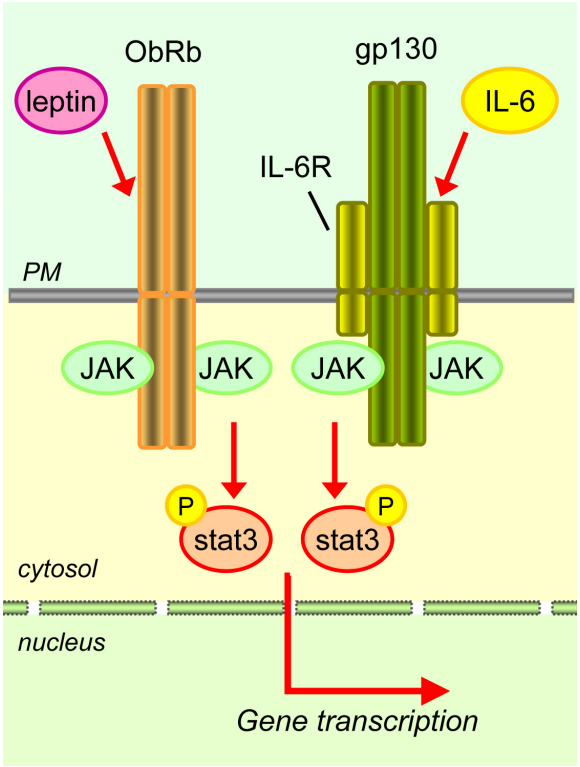


*Figure IV-9. STAT3 nuclear translocation. ObRb and gp130 (the signalling subunit of IL-6 receptor) belong to the class 1 cytokine family. They share a*

*common intracellular signalling pathway, JAK2-STAT3. Phosphorylation of STAT3 leads to nuclear translocation of STAT3, which can be used as a marker for ObRb or gp130 receptor activation.*

**Figure IV-10. Leptin mediates nuclear translocation of STAT3 in the barrier-related structures**

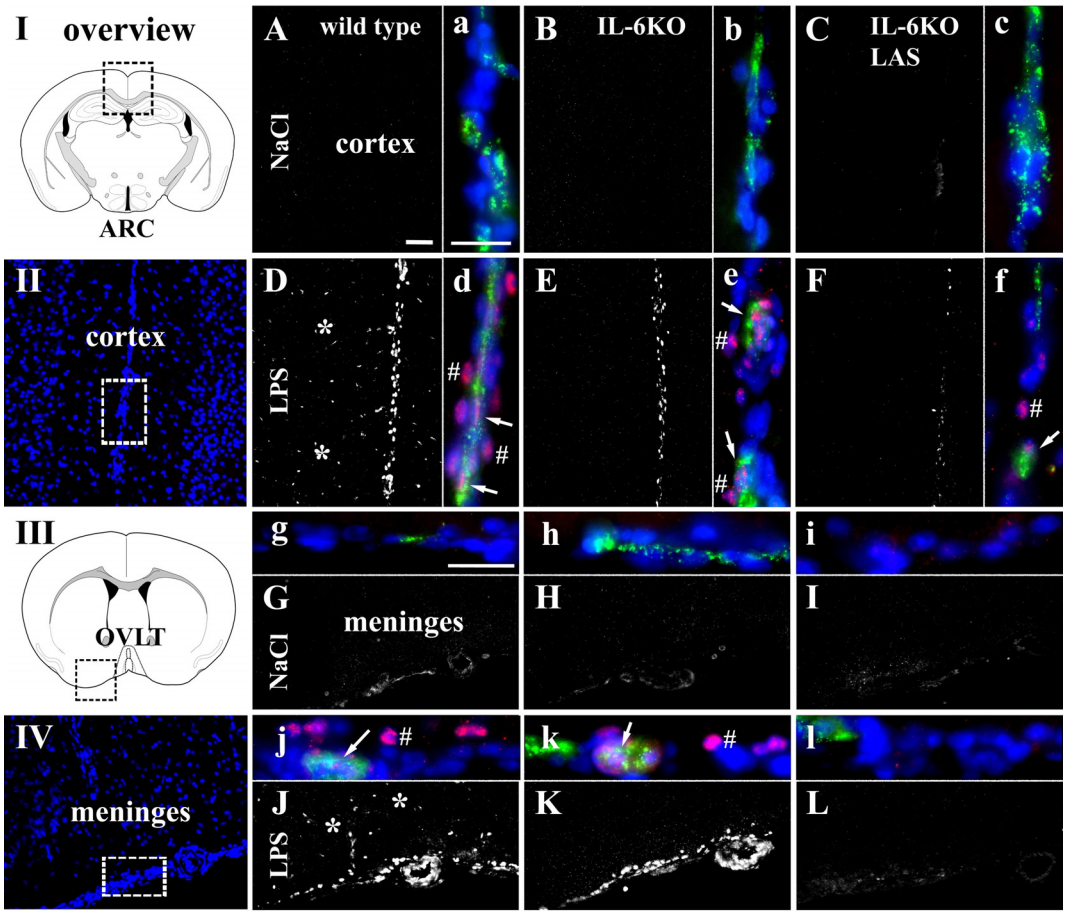


*Figure IV-10. Leptin mediates nuclear translocation of STAT3 in the barrier-related structures. Nuclear translocation of STAT3 in mouse brains was detected by immunohistochemistry 3 h after an i.p. injection of saline (NaCl) or LPS (50 μg/kg). I and III show the areas of the brain examined, and II and IV depict nuclear staining (DAPI) in blue. A–L show black/white pictures of STAT3 immunostaining. A–L are high magnification pictures of field depicting an accumulation of STAT3 (red) in cell nuclei (blue). Some of the STAT3 signals overlap with endothelial cell marker, von Willebrand factor (green). In wild-type*

*mice, LPS clearly induced STAT3 nuclear translocation in the cerebral fissure (A and D) and the meningis (G and J). This STAT3 induction was similar in IL-6-deficient mice (IL-6KO, B, E, H and K). LAS treatment almost completely abolished the STAT3 signal in IL-6KO mice (C, F, I and L). Adopted from Rummel C., Inoue W. Sachot C., Poole S., Hübschle T., Luheshi GN., Journal: The Journal of Comparative Neurology, Volume 511 Issue 3, Pages 373 – 395, Materials presented here used with permission of Willey-Liss, Inc. a subsidiary of John Wiley&Sons, Inc.*

The data from the current manuscript suggested that leptin may regulate neutrophil recruitment independently of IL-1β, in addition to the IL-1β-dependent mechanisms as discussed above. Although IL-1β triggers strong neutrophil recruitment to the brain, available data suggests that this process is indirect via induction of downstream mediators, in particular neutrophil chemokines KC and MIP-2 (Anthony et al., 1998; Shaftel et al., 2007). The data from the present manuscript supports the importance of these neutrophil chemokines, as in all three models tested (mutant *ob/ob* and *db/db* mice, food deprivation and LAS treatment in wild-type mice) the leptin-mediated attenuation of neutrophil recruitment was accompanied by significant decreases in brain KC and MIP-2 mRNA expression. Interestingly, LAS treatment reduced LPS-induced KC and MIP-2 mRNA up-regulation without affecting IL-1β suggesting that leptin may modulate the expression of these chemokines at least partly independently of IL-1β. In this regard, we have obtained a preliminary result that leptin directly potentiates LPS-induced KC and MIP-2 mRNA expression in primary culture of microglia. An application of LPS to the culture increased mRNA levels of KC and MIP-2 approximately 100- and 150-fold, respectively, as compared to the control culture incubated with medium alone (Figure IV-11). Treatment with leptin preceding LPS application significantly potentiated the induction of KC and MIP-2 mRNA, demonstrating the capacity of leptin to directly modulate LPS-induced chemokine production in microglia. Interestingly, leptin treatment alone had no effect on the chemokine levels. These *in vitro* results are in agreement with the current *in vivo*

data that the absence of leptin blunts LPS-induced chemokine expression in the brain. It will be important to examine *in vivo* expression of these chemokines by immunohistochemistry or *in situ* hybridization in order to have an insight into potential targets of leptin in the brain.

**Figure IV-11. Leptin potentiates LPS-induced KC and MIP-2 expression in microglia.**

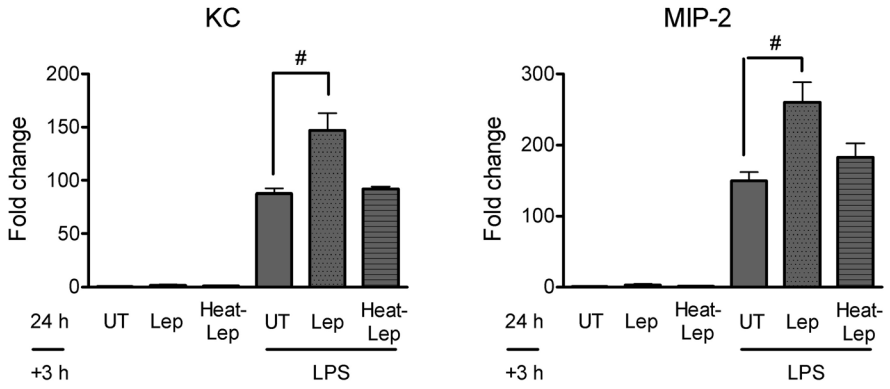


*Figure IV-11. Primary microglia were treated with a serum-free medium alone (UT, untreated), with leptin (Lep, 1 μg/ml) or heat-inactivated leptin (Heat-Lep) for 24 h. The cells were further treated with or without LPS (0.1 μg/ml) for 3 h. mRNA expression levels were examined by qPCR. Values are normalized by 18S RNA levels and are expressed as a fold change from one sample from the control group (UT). Leptin treatment alone did not alter KC or MIP-2 mRNA levels as compared to UT. LPS treatment significantly increased both KC and MIP-2 levels, which were further enhanced by leptin pre-treatment. Heat inactivation abolished the potentiating effects of leptin, excluding the possibility of endotoxin contamination (which is heat resistant) in the leptin preparation.* $^{\#}$ $P < 0.01$

Lastly, the current manuscript demonstrated the key role played by leptin in neutrophil recruitment to the brain and potential mechanisms involved during severe systemic inflammation. However, it is still an open question whether this leptin-mediated process is detrimental or beneficial for the overall disease process. It is generally thought that neutrophil infiltration to the brain contributes to

neuropathologies based on their ability to elicit neurotoxicity and disruption of the BBB through the generation of free radicals and release of proteolytic enzymes, such as matrix metalloproteinases, elastase and serine proteases (Emerich et al., 2002; Dinkel et al., 2004). However, the pathogenic roles of neutrophil recruitment in neuropathologies remain controversial and may depend on the types and contexts of neuroinflammation. For example, Shaftel et al. (Shaftel et al., 2007) reported that sustained transgenic over-expression of IL-1β in the brain caused no sign of neurodegeneration despite a prolonged recruitment of neutrophil that lasted for as long as one year after transgene introduction. Moreover, in experimental models of cerebral ischemia, neutrophil depletion (by administering an anti-neutrophil antibody) had little, if any, protective effects on oedema formation, proteolytic activities and oxidative tissue damages in rodents (Beray-Berthat et al., 2003; Harris et al., 2005), indicating a minor role for neutrophil in the disease process. On the other hand, it was shown that an administration of IL-1β concomitant with experimental stroke in mice exaggerated cortical neutrophil infiltration, which preceded the exacerbation of ischemic brain damage and neurological deficit. Neutropenia (by anti-neutrophil antibody) effectively ameliorated the pathologies (McColl et al., 2007). In this model, it was also shown that neutrophil-derived metalloproteinase-9 mediates a sustained BBB disruption and exaggerated neurological deficits (McColl et al., 2008), demonstrating a pathogenic role of neutrophils. Therefore, it will be important, in future studies, to examine whether leptin-mediated neutrophil infiltration is accompanied by any neurological outcomes, such as BBB disruption and apoptosis of neurons, and if so, to determine the specific contribution of neutrophils. With respect to our current model, there exists an interesting report that a high dose of systemic LPS administration in rats caused long-term behavioural deficits and decreased neuron numbers in the hippocampus after recovery from sepsis (Semmler et al., 2007).

# Chapter V: Immune-to-brain signalling and central prostaglandin E2 synthesis in fasted rats with altered lipopolysaccharide-induced fever

## V.1. Preface

Data obtained from the previous chapter (Chapter IV) demonstrated that leptin plays an important and unique role in linking energy balance (food deprivation) with brain inflammation (neutrophil recruitment) during severe systemic inflammation. In the following two manuscripts, we returned to our original model of sickness behaviour (fever) and tested whether leptin plays a role in the inter-relationship between energy balance and the fever response. Fever is a complex response that involves a series of immunologic, thermoregulatory and metabolic processes. Given that fever is an energy demanding process (immunologic response and thermogenic and metabolic energy expenditure), it would be interesting to know if and how the energy status of the host influences the fever response. Such a regulatory mechanism is of fundamental importance for homeostasis and the survival of organisms in their natural environment where neither food availability nor incidence of infection is under control.

In this chapter, we first aimed to establish an experimental model where food deprivation prior to the induction of inflammation affects the fever response in rats. By using this model, we therefore examined which component of fever (immunologic response, thermoregulation and/or metabolic function) is affected by changes in energy balance. In addition, we examined the influence of gender on the overall interaction between energy balance and fever given that this variable maybe an important factor in the regulation of the two systems (Mouihate et al., 1998; Gayle et al., 2006).

## V.2. Manuscript

**Title: Immune-to-brain signalling and central prostaglandin E2 synthesis in fasted rats with altered lipopolysaccharide-induced fever**

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American Journal of Physiology, Regulatory, integrative and comparative physiology, Vol. 295, pR133-R143, 2008



Keywords: thermoregulation, acute starvation, cyclooxygenase, hypothermia

## Abstract

Acute starvation attenuates the fever response to pathogens in several mammalian species. The underlying mechanisms responsible for this effect are not fully understood, but may involve a compromised immune and/or thermoregulatory function, both of which are prerequisites for fever generation. In the present study, we addressed whether the impaired innate immune response contributes to the reported attenuation of the fever response in fasted rats during lipopolysaccharide (LPS)-induced inflammation. Animals fasted for 48 h exhibited a significant and progressive hypothermia prior to drug treatment. An intraperitoneal injection of LPS (100 μg/kg) resulted in a significantly attenuated fever in the fasted animals when compared to the fed counterparts. This attenuation was accompanied by the diminution in the concentration of some [tumor necrosis factor (TNF) and interleukin (IL)-1 receptor antagonist (IL-1RA)] but not all (IL-1β and IL-6) of the plasma cytokines normally elevated in association with the fever response. Nevertheless, fasting had no effect on the LPS-induced inflammatory responses at the level of the brain as assessed by mRNA expressions of inhibitory factor κB, suppressor of cytokine signalling (SOCS) 3, IL-1β, cyclooxygenase (COX)-2, and microsomal prostaglandin (PG) E synthase (mPGES)-1 in the hypothalamus, as well as by PGE2 elevations in the cerebrospinal fluid. In contrast, fasting significantly attenuated the fever response to central PGE2 injection. These results show that fasting does not alter the febrigenic signalling from the periphery to the brain important for central PGE2 synthesis, but does affect thermoregulatory mechanisms downstream of and/or independent of central PGE2 action.

## Introduction

Fever is a common response to various types of infection and constitutes an important component of an adaptive strategy for fighting disease (Hart, 1988; Kluger, 1991). The development of fever is a finely tuned, complex event that involves both the peripheral immune system and the brain, through which a series of inflammatory and metabolic processes are regulated (Roth et al., 2006). The sum of the changes required to mount an effective fever response is an energy demanding process that can be influenced by the host's energy status. This was clearly shown by numerous studies reporting that acute starvation, such that caused by fasting, negatively affects the fever response to pathogens in various species of experimental animals (Szekely, 1979; Kleitman and Satinoff, 1981; Molnar and Milner, 1983; Shojoony, 1985; Shido et al., 1989). In some of these studies, the underlying mechanisms responsible for the depression of fever were linked to a compromised metabolic function of the starved animals. For example, the impaired fever response to bacterial endotoxin reported in fasted guinea pigs was associated with blunted elevation of metabolic heat production (Shojoony, 1985). In a different study using fasted new born rabbits, the depression of fever was reversed by allowing the animals to freely select warmer ambient temperatures in a thermal gradient (Kleitman and Satinoff, 1981), arguing that the selective attenuation of heat production, rather than the general down-turn of thermoregulatory function, is the more likely mechanism responsible for the depression of fever during acute starvation. This selective ablation of metabolic thermogenesis coexisting with the preserved heat seeking behaviour resembles the thermoregulatory responses to starvation under non-inflammatory conditions where food deprivation in laboratory animals rapidly induces depression of basal metabolic heat production and hypothermia while increasing preference to higher ambient temperature, as a part of an adaptive strategy to conserve energy stores (Sakurada et al., 2000; Yoda et al., 2000).

As well as the depression of metabolism, however, acute starvation is known to trigger a number of other physiological changes including the

suppression of immune function, which in many cases adversely affects the host's defence mechanisms and subsequently decreases its ability to effectively combat infectious disease (Wing et al., 1988; Lord et al., 1998; Mancuso et al., 2006). Given that the initiation of fever is essentially dependent on the innate immune response to exogenous pathogens, accompanied by the production and release of a full compliment of inflammatory immune mediators belonging to the cytokine family (Konsman et al., 2002), the suppression of immunity is likely to contribute to the diminution of fever observed in starved animals (Szekely, 1979; Kleitman and Satinoff, 1981; Molnar and Milner, 1983; Shojoony, 1985; Shido et al., 1989). The link between circulating cytokines and fever is well established with a number of these mediators, most prominently interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF), implicated in triggering the central mechanisms regulating the fever response (Luheshi, 1998; Konsman et al., 2002; Conti et al., 2004). The contribution of these cytokines to the activation of the central mechanisms involved in regulating the fever response has been regularly assessed by measuring the degree of activation of transcription factors such as nuclear factor (NF)κB, which is activated by both TNF and IL-1β, and signal transducer and activator of transcription (STAT)3, which is activated by IL-6 (Laflamme and Rivest, 1999; Lebel et al., 2000). Both of these pathways lead to the transcription and induction of cyclooxygenase (COX)-2 (Xuan et al., 2003; Dawn et al., 2004; Nadjar et al., 2005; Rummel et al., 2006), the rate limiting enzyme for prostaglandin (PG)E2 synthesis, and ultimately fever production (Matsumura et al., 1998b; Scammel et al., 1998; Li et al., 1999; Ivanov and Romanovsky, 2004). Despite the important functional link between these immune mediators and fever, the role of cytokines in the attenuated fever response to exogenous pathogens during acute starvation has not previously been investigated in depth. For example, none of the aforementioned studies reporting the negative effects of acute starvation on the fever response (Szekely, 1979; Kleitman and Satinoff, 1981; Molnar and Milner, 1983; Shojoony, 1985; Shido et al., 1989) evaluated the changes in cytokine production. Nor have any studies investigated whether the central inflammatory mechanisms regulating the fever response (*e.g.* PGE2

production) were affected in these situations as a consequence of a dampened immune response.

The aim of the present study therefore was to address, in detail, if and how the peripheral and central components of the inflammatory response to a single injection of lipopolysaccharide (LPS) is affected in rats food-deprived for 48 h. To achieve this, we measured the following variables in fed and fasted animals: 1) circulating levels of cytokines, 2) the activation of signalling pathways implicated in the cytokine action in the brain during fever (NFκB and STAT3), 3) up-regulation of COX-2 and microsomal PGE synthase (mPGES)-1, two essential enzymes for brain PGE2 production during fever (Li et al., 1999; Ek et al., 2001; Yamagata et al., 2001; Engblom et al., 2003), and 4) levels of PGE2 in the cerebrospinal fluid (CSF). In addition, the thermoregulatory response downstream of central PGE2 action was tested by the direct injection of PGE2 into the brain.

## Methods

*Animals*

Adult female Sprague-Dawley rats with initial body weight 200–225 g (Charles River, Saint Constant, Quebec, Canada) were used in all experiments. They were housed individually in a controlled environment at an ambient temperature of 21 ± 1.5 ˚C on a 12 h light/dark cycle (light on from 08:00 to 20:00 h), with free access to water and standard laboratory chow (Rat chow #5012; Purina Co., St. Louis, MO, USA) unless otherwise indicated. All experimental procedures were approved by the Animal Care Committee of McGill University pursuant to the Canadian Council of Animal Care guidelines.

*Experimental design*

Experiments were performed over four days; one day for baseline measurement (day 0) followed by three days for feeding manipulation (day 1–3). Animals were weighed daily between 09:30 and 10:30 h. On day 0, rats were divided into two weight-matched feeding groups (fed and fasted). On day 1, food was removed from the fasted group immediately after the body weight measurement. Both groups of rats had free access to water throughout the experimental period. On day 3, each feeding group was subdivided into three treatment groups receiving a single injection between 09:30 and 10:30 h (48 h after the start of fasting). For intraperitoneal (*i.p.*) injections, each subgroup (n = 7–8 per group) received either saline (1 ml/kg) or 100 µg/kg LPS (Escherichia coli O111:B4, lot 42k4120; Sigma, Oakville, Ontario, Canada). For intracerebroventricular (*i.c.v.*) injections, each subgroup (n = 7 per group) received vehicle (0.5% ethanol in saline), 10 ng/rat PGE2 (Sigma), or 50 ng/rat PGE2 injection at 5 µl volume.

*Measurement of body temperature using remote biotelemetry*

Pre-calibrated temperature-sensitive radio transmitters (model:TA10TA-F40, Data Sciences, St Paul, MN, USA) were implanted via midline incision into the abdominal cavity of anesthetised rats (intramuscular; 50 mg/kg ketamine

hydrochloride, 5 mg/kg xylazine hydrochloride, 0.5 mg/kg acepromazine maleate; total volume 1 ml/kg). The level of anesthesia was assessed by the withdrawal reflex to a toe pinch. 2% lidocaine (25–50 µl) was applied to the area of incision. Animals were allowed to recover for at least 7 days prior to experimentation. Transmitter output frequency (Hz) was monitored, at 10 min intervals for LPS-induced fever, and 2 min intervals for PGE2-induced fever, by an antenna mounted in a receiver board situated beneath the cage of each animal. The output data from each transmitter were transformed into degrees centigrade using Dataquest A.R.T. software (Data Sciences).

*Intracerebroventricular cannula implantation*

Following the transmitter implantation described above, the animals designated for *i.c.v.* injection were implanted with 21G guide cannulae (Plastics One, Roanoke, VA, USA) as previously described (Inoue et al., 2006). Briefly, rats were secured in a stereotaxic frame, the skull exposed by mid line incision, and 2 % lidocaine (25–50 µl) applied to the area of incision. The coordinates using bregma as a reference point were the following: 0.8 mm posterior, 1.5 mm lateral and 3.0 mm ventral (from scull surface), resulting in the tip of guide cannula being situated 1 mm above the right lateral ventricle. The guide cannula was then fixed to the skull with dental cement (Lang Dental Mfg. Co. Inc., Wheeling, IL, USA) holding two supporting screws with a diameter of 1.2 mm secured on the skull, and was sealed with a 'dummy' cannula designed to reach the tip of the guide. Rats were allowed to recover for at least 7 days post-surgery. Injections were performed in conscious free moving animals, with a 26G injection cannula which extended 2 mm beyond the tip of the guide cannula and connected to a 10 µl Hamilton syringe via P20 tubing. Accuracy of the cannula placement was verified by an injection of black ink (5 µl). The dye was visible in the 4th ventricle in all animals examined.

*Repeated blood collection via a jugular vein catheter*

Separate sets of rats were prepared for repeated blood collection. Animals received the anesthesia mixture described above, and the depth of anesthesia

assessed by the withdrawal reflex to a toe pinch. A single dose of ampicillin (subcutaneous; 150 mg/kg; Nanopharm, Ontario, Canada) was administered 10 min before the surgery, and 2% lidocaine (25–50 µl) applied to the area of incision. The external jugular vein was exposed through a small skin incision, and the linguofacial vein branching from the jugular vein was isolated and ligated 5 mm rostral from the bifurcation. A sterile Silastic catheter (0.97 mm OD; Dow Corning, Midland, MI, USA) filled with heparinized (50 IU/ml) sterile saline was inserted 4 cm toward the jugular vein until the tip of the catheter reached the level of the right atrium; at which point it was secured. The other end of the catheter was tunnelled under the skin, exteriorized at the nape and sealed with a stainless steel cap. Animals were allowed to recover from surgery for two days before experimentation. Blood was collected from conscious free moving animals, by connecting the catheter to a 1 ml syringe containing 5 IU of heparin via PE50 tubing with 22G blunted needle tip. The sample volume (150 µl/collection) collected was replaced with sterile saline pre-warmed to 37 °C.

In order to compare the effect of fasting on cytokine production during LPS-induced inflammation, the fed and fasted rats were treated with a single *i.p.* injection of either saline or LPS 100 µg/kg (n = 4 per group). Food was removed from the fasted groups 48 h before the injection and blood collected at –48, –24, 1, 2, 4, 8, and 24 h with respect to the injection time at 0 h. The samples were immediately placed on ice and centrifuged at 3000 g for 5 min, plasma collected, aliquoted and stored at –80°C until assays were performed.

*Cerebrospinal fluid and brain collection*

In order to assess the levels of PGs in the CSF, and to measure the expression levels of inflammatory genes in the hypothalamus, CSF and brains were collected from the fed and fasted rats treated with either saline (*i.p.*; 1 ml/kg) or LPS (*i.p.*; 100 µg/kg) (n = 5–6 per group). Animals were sacrificed 2 h after the injection under deep anesthesia with a terminal dose of pentobarbital sodium (*i.p.*; 60 mg/kg). CSF was sampled from the cisterna magna with a 27G needle connected to a microsyringe (250 µl) via PE20 tubing. The sampled CSF was immediately frozen on dry ice and kept at –80 °C. Blood was collected from the

same animals via cardiac puncture using sterile heparinized syringes and plasma samples prepared for additional cytokine assay. Animals were then perfused with ice-cold saline, the brains removed, and the hypothalami dissected, frozen on dry ice and kept at –80 °C until use.

*Cytokine and prostaglandin measurement*

Sandwich enzyme-linked immunosorbant assays (ELISA) for TNF, IL-1β, IL-6, IL-1 receptor antagonist (RA) and leptin (NIBSC, Potters Bar, UK) were performed as previously described (Rees et al., 1999a), except that plasma samples and biotinylated detection antibodies were diluted in a buffer containing 0.5 M NaCl, 10 mM phosphate, 0.1% Tween 20, pH 7.4. All plasma samples were diluted 1:10 except for IL-1β (1:5 dilution). Intra-assay and inter-assay variations were below 10%. The sensitivities of the assays were: 3.9 pg/ml for TNF and IL-1RA, 7.8 pg/ml for IL-1β, IL-6 and leptin. All samples were assayed in duplicate.

PGE2 and 15-deoxy-Δ12,14-PGJ2 (15-d-PGJ2) levels in CSF were measured using an enzyme immuno assay (EIA) kit (PGE2 EIA kit-Monoclonal, Cayman Chemical, Ann Arbor, MI, USA; 15-deoxy-Δ12,14-PGJ2 EIA kit, Assay designs, Ann Arbor, MI, USA) according to the manufacturer's protocol. The CSF samples were diluted 1:5 in an assay buffer provided as part of the kit. The sensitivity of the assay for PGE2 and 15-d-PGJ2 was 7.8 pg/ml and 36.8 pg/ml, respectively. All samples were assayed in duplicate.

*RNA extraction and RT-PCR*

Total RNA was extracted from the hypothalamus in 1 ml of TRIzol (Invitrogen, Burlington, Ontario, Canada), according to the manufacturer's protocol. The first-strand cDNA was synthesized from 1 µg of total RNA using 200 units of Molony murine leukemia virus reverse transcriptase (Invitrogen), 5 µM of random hexamers (Applied Bioscience, Streetsville, Ontario, Canada), and 1 mM of dNTP mix (Sigma) in a total reaction volume of 20 µl. The cDNA product (0.9 µl) was added to 15 µl PCR reaction mix containing ReadyMix Taq PCR (Sigma) and 6 pmol of gene-specific primer sets for inhibitory factor (I)κBα, suppressor of cytokine signalling (SOCS)3, IL-1β, COX-2, mPGES-1and β-actin

(Alpha DNA, Montreal, Quebec, Canada) using a Gene Amp PCR system 9700 Thermocycler (Applied Biosystems, Foster City, CA, USA). The following parameters were used: (1) denaturing; 95 ºC for 5 min, (2) amplification cycle; 95 ºC for 30 sec, annealing temperature for 30 sec and 72 ºC for 1 min (3) final extension; 72 ºC for 10 min. Primers were designed to span a sequence derived from different exons (separated by intron(s) in the genomic DNA sequence) in order to minimize amplification from non-mRNA-derived templates. Inappropriate amplification from genomic DNA was negligible when amplification was performed with a template without reverse transcription. The gene accession numbers, primer sequences, annealing temperatures and cycle numbers used are listed as following: IκBα (NM_01105720; forward, 5'-AACAACCTGCAGCAGACTCC-3', reverse, 5'-GTGTGGCCGTTGTAGTTGG-3'; 60 °C; 28 cycles), SOCS3 (NM_053565; forward, 5'-CCAGCGCCACTTCTTCAC-3', reverse: 5'-GTGGAGCATCATACTGGTCC-3'; 60 °C; 36 cycles), IL-1β (NM_031512; forward, 5'-CCCAAGCACCTTCTTTTCCTTCATCTT-3', reverse, 5'-CAGGGTGGGTGTGCCGTCTTTC-3'; 60 ˚C; 36 cycle), COX-2 (NM_017232; forward, 5'-TGATAGGAGAGACGATCAAGA-3', reverse, 5'-ATGGTAGAGGGCTTTCAACT-3'; 57 ˚C; 32 cycles), mPGES-1 (NM_022415; forward, 5'-TTTCTGCTCTGCAGCACACT-3', reverse, 5'-CATGGAGAAACAGGTGAACT-3'; 57 °C, 36 cycles), β-actin (NM_031144; forward, 5'-GCCGTCTTCCCCTCCATCGTG-3', reverse, 5'-TACGACCAGAGGCATACAGGGACAAC-3'; 60 ˚C; 20 cycles). PCR products were separated by gel electrophoresis (1.5% agarose) and band densities were obtained using GeneTool image analysis software (Syngene, Frederick, MD, USA). To normalize the expression level of genes between different samples, the levels were estimated as the ratio of geneX/β-actin. In a pilot experiment, the amount of PCR product (on a log scale) *vs.* the number of cycles was plotted, and the linear range of template amplification was determined for 2 samples from each treatment group. The cycle numbers were determined within the exponential phase of amplification for all treatment groups.

*Data analysis*

*Measurements of body temperature*

In order to evaluate the changes of core body temperature ($T_{core}$) over the course of fasting, the average $T_{core}$ of the dark (between 24:00–06:00h) and light (between 12:00–18:00h) periods were calculated for each animal. This 6 h interval was chosen to exclude the temperature change caused by body weight measurement performed daily at 10:00h in the light period ($T_{core}$ returned to normal level by 12:00h). A corresponding interval was chosen for the dark period (starting from 24:00h for 6 h).

Two measures have been used to assess the fever response: absolute $T_{core}$ and the rise in $T_{core}$ ($\Delta T_{core}$: $\Delta T_{core} = T_{core} -$ pre-injection $T_{core}$). However, these two measures are not interchangeable when animals have different pre-injection $T_{core}$ (*e.g.* day/night variation and different feeding conditions as in the present study). Satinoff and colleagues (Feng et al., 1989) demonstrated that absolute $T_{core}$, rather than $\Delta T_{core}$, provides a better representation of the physiological characteristic of fever because the former is solely determined by the dose of pyrogen (PGE2) and is largely unaffected by day/night variation in pre-injection $T_{core}$. The validity of this definition was supported by several other studies investigating the fever response in animals with different pre-injection $T_{core}$ (Kent et al., 1988; Malkinson et al., 1988; Ivanov and Romanovsky, 2002). Therefore, in the present study, we used absolute $T_{core}$ rather than $\Delta T_{core}$ to assess the fever response.

In order to evaluate the overall change in $T_{core}$ by pyrogenic stimuli in the fed and fasted animals, pre-injection $T_{core}$ and post-injection $T_{core}$ were calculated. The pre-injection $T_{core}$ was defined as the average $T_{core}$ during the period of –30 to 0 min prior to the injection. The post-injection $T_{core}$ was defined as the average $T_{core}$ between 0–60 min in the case of *i.c.v.* PGE2 injection, and, in the case of *i.p.* LPS injection, the average between 120–480 min to exclude stress-induced hyperthermia.

*Statistics*

All data are presented as mean values ± S.E.M. and were analyzed using StatView software 4.57 (Abacus Concepts Inc., Berkeley, CA, USA). Two-way repeated ANOVA (time x treatment) was used to analyze the data from body weight change, temperature course and cytokine time course studies. Two-way ANOVA (feeding x inflammation) was used to analyze the data from mRNA expression and PG measurements at a single time point. Newman-Keuls multiple comparison test was performed as post-hoc analysis when applicable. In all cases, P values less than 0.05 were deemed statistically significant.

## Results

*Changes in body weight during fasting and inflammation*

The body weight changes of the animals in all treatment groups are shown in Figure V-1A. The rats in the fasted groups were deprived of food immediately after the body weight measurement on day 1 between 09:30–10:30 h until the end of the experiment, while the fed groups were fed *ad libitum*. All groups were injected *i.p.* with either saline or LPS 100 μg/kg on day 3, at the time point corresponding to 48 h after the start of food deprivation in the fasted groups. The initial body weight on day 1 was similar across all groups (243.8 ± 7.4 g, 254.1 ± 6.2 g, 247.2 ± 5.9 g, and 253.0 ± 7.5 g for fed-saline, fed-LPS, fasted-saline, and fasted-LPS) and decreased significantly following food deprivation (Ps < 0.001; fed *vs.* fasted for days 2, 3, and 4). LPS injection resulted in a significant loss of body weight 24 h after treatment in the fed groups (P < 0.01; saline *vs.* LPS). In contrast, the LPS injection in the fasted groups caused no further loss of body weight compared with the saline-treated counterpart.

*The effects of fasting on basal body temperature*

Figure V-1 B and C illustrate the daily cycle of $T_{core}$ of the fed and fasted rats that were treated with saline on day 3 as an example of $T_{core}$ cycle under non-inflammatory conditions. The $T_{core}$ was higher in the active (dark) and lower in the inactive (light) phase displaying clear day/night variations in the fed condition. This daily rhythm was still sustained during fasting. However, as reported previously (Severinsen and Munch, 1999; Yoda et al., 2000), the fasted groups showed a progressive decline in the $T_{core}$ (P < 0.01, time x feeding). In order to evaluate the change of $T_{core}$ over the course of fasting, the average $T_{core}$ in the active and inactive phases are shown in Figure V-1C. In the active phase, the average $T_{core}$ of fasted rats tended to be lower (P = 0.07) on day 2 and became significantly lower (P < 0.001) on day 3 compared to its control value on day 0 (Figure V-1C). The decline of the average $T_{core}$ in the inactive phase was more

prominent than that in the active phase, reaching significance by day 2 ($P < 0.001$, day 0 *vs.* day 2) and was further decreased on day 3 ($P < 0.05$, day 2 *vs.* day 3).

*The effect of fasting on lipopolysaccharide-induced fever*

The fever response to a single *i.p.* injection of LPS was examined on day 3. The average $T_{core}$ of the fasted groups prior to the injection was significantly lower than those of the fed counterparts (Figure V-2). The saline injection caused a transient, stress-associated hyperthermia in both groups reaching a similar peak (Figure V-2A). Thereafter, the $T_{core}$ of the fasted rats returned to a lower level than that of the fed group (170-600 min, $P < 0.05$, fed-saline *vs.* fasted-saline). LPS injection at the dose of 100 μg/kg caused a clear thermal response in the fed group (Romanovsky et al., 2005), which was characterized by an initial mild hypothermia occurring immediately after the injection-associated hyperthermia, followed by a biphasic temperature increase (Figure V-2C). The fasted rats also showed a clear polyphasic fever response to LPS, however, the $T_{core}$ was consistently lower as compared to the fed counterparts (100-200 min and 250-390 min, $P < 0.05$, fed-LPS *vs.* fasted-LPS). In addition, the first fever peak was significantly attenuated ($P < 0.05$, 38.5 ± 0.2 °C *vs.* 37.9 ± 0.2 °C, fed-LPS *vs.* fasted-LPS) and delayed in the fasted rats ($P < 0.001$, 171.4 ± 5.1 min *vs.* 215.7 ± 8.4 min, fed-LPS *vs.* fasted-LPS). The second peak was also significantly attenuated in the fasted rats ($P < 0.05$, 39.1 ± 0.2 °C *vs.* 38.4 ± 0.1 °C, fed-LPS 100 *vs.* fasted-LPS), but this reached maximum at the same time in both groups (360.0 ± 11.1 min *vs.* 375.7 ± 18.1 min, fed-LPS *vs.* fasted-LPS).

The fact that the fasted rats had a smaller fever response may be a factor of the smaller amount of LPS given which was corrected for body weight (pre-injection body weight, 260.6 ± 6.8 g *vs.* 226.6 ± 7.0 g, fed-LPS *vs.* fasted-LPS). In order to eliminate this possibility, an extra set of fed rats (pre-injection body weight 264.5 ± 3.7 g) were injected with a fixed amount of LPS (23 μg/rat) that is equivalent to the absolute amount given to the fasted group at the dose of 100 μg/kg. The fever response induced by this amount of LPS was almost identical to the one seen in the fed rats treated with the fixed LPS dose, confirming that the

attenuation of fever response in the fasted rats was not due to the smaller amount of LPS injected (Figure V-2C).

Figure V-2B, and D illustrate pre-injection $T_{core}$ and the average post-injection $T_{core}$ in different treatment groups. It is clear from these graphs that, in the LPS-treated groups, both pre-injection and post-injection $T_{core}$ were similarly attenuated by fasting (two-way ANOVA, Ps < 0.001 for feeding and injection, P > 0.05 for feeding x injection interaction). Saline injection had no effect on the $T_{core}$. The same experiments were also conducted in male rats for comparison with a similar outcome, thus negating the effect of gender on the fever response (data not shown).

*Circulating cytokines*

We next examined if the attenuation of fever response to LPS was attributable to the alteration in immune-to-brain signalling involved in the development of fever (Luheshi, 1998; Konsman et al., 2002; Conti et al., 2004). To this end, we characterized the plasma levels of pro- and anti-inflammatory cytokines, mRNA levels of the genes involved in PGE2 synthesis in the hypothalamus and PGE2 levels in the CSF after an *i.p.* LPS (100 μg/kg) injection. Figure V-3 illustrates the time course of plasma TNF, IL-6, IL-1RA and leptin, as well as the IL-1β levels at 2 h after injection in the four groups of rats. LPS treatment dramatically increased the plasma levels of TNF, IL-6 and IL-1RA in the fed rats. The same cytokines were also increased by LPS in the fasted group, however, the TNF and IL-1RA elevations were significantly attenuated. The LPS-induced TNF was significantly lower at 2 h (P < 0.05), and the IL-1RA levels were lower at 2, 4 and 8 h (Ps < 0.01) in the fasted than fed rats. On the other hand, the increase in IL-6 was almost identical between the fed and fasted rats. The fasted rats treated with saline, however, had significantly higher IL-6 levels at 2 h after the injection as compared to the fed counterpart (P < 0.01). The present study did not determine the time course of IL-1β levels because of the limitation of the amount of sample available from the repetitive blood collection. Alternatively, its plasma levels were measured in rats sacrificed at 2 h after LPS or saline treatment (from the same animals used for CSF PGs measurement

presented below). Plasma IL-1β was found to be similar between the fed and fasted rats ($1.4 \times 10^{-1} \pm 0.2 \times 10^{-1}$ ng/ml *vs.* $2.0 \times 10^{-1} \pm 0.6 \times 10^{-1}$ ng/ml, fed-LPS *vs.* fast-LPS), while in the in the same samples, IL-1RA levels were confirmed to be lower in the fasted than in fed rats ($P < 0.01$, $4.5 \pm 0.1$ ng/ml *vs.* $3.3 \pm 0.1$ ng/ml, fed-LPS *vs.* fast-LPS). The relative ratio between these two interlinked cytokines was calculated as a predictor of net IL-1 signalling (IL-1β/IL-1RA ratio) and was found to be significantly higher in the fasted than fed rats ($P < 0.01$, $3.1 \times 10^{-2} \pm 0.4 \times 10^{-2}$ *vs.* $6.1 \times 10^{-2} \pm 1.2 \times 10^{-2}$, fed-LPS *vs.* fast-LPS). Leptin was also measured in these studies. As reported previously (Ahima et al., 1996), fasting dramatically reduced the plasma levels of this appetite suppressing hormone. The treatments, on the other hand, did not have a significant effect on the leptin levels in either feeding condition. However, the time x treatment interaction was significant ($P < 0.001$) and post-hoc test revealed that the LPS-treated fed animals tended to have higher leptin levels as compared to saline-treated controls at 8 h after injection although the analysis did not reach statistical significance ($P = 0.05$). More specifically, the leptin levels in the saline-treated controls showed a profile of circadian change (Dallman et al., 1999); its levels were higher in the earlier stage of the inactive phase (at 1, 2 and 24 h) and lower in the later stage (4 and 8 h). After LPS injection, the decline of leptin levels toward the end of the inactive phase was abolished, and indeed reversed. Accordingly, the fed-LPS group had a tendency to have higher leptin levels than the fed-saline counterparts. In the fasted groups, however, the circadian change of leptin levels was no longer apparent, and LPS did not induce any further change. Interestingly, there was a relatively large inter-animal variability of leptin levels within the same treatment groups, but an animal with higher leptin level at one time point consistently showed higher levels at other time points as compared to those with lower levels. Therefore, in order to reduce the inter-animal variability, the leptin levels of each animal were normalized to its baseline levels measured 48 h before the injection on day 1 (just prior to the start of food restriction). The leptin levels at the baseline were similar across all the treatment groups ($1.6 \pm 0.4$ ng/ml, $1.3 \pm 0.2$ ng/ml, $1.2 \pm 0.2$ ng/ml and $1.0 \pm 0.2$ ng/ml for fed-saline, fed-

LPS, fasted-saline and fasted-LPS, respectively). This analysis revealed that LPS treatment in the fed group significantly increased leptin levels at 4 and 8 h after the injection as compared to saline treatment in the counterpart controls (Ps < 0.01).

*Inflammatory gene expression in the brain*

Figure V-4 shows mRNA expression levels of IκB, SOCS3, IL-1β, COX-2 and mPGES-1 in the hypothalamus studied 2 h after LPS (*i.p.*, 100 μg/kg) injection. Despite the altered cytokine levels in the circulation, the feeding condition of the animals did not have any major effect on the mRNA expressions during LPS inflammation. Two-way ANOVA (feeding x LPS) revealed that LPS significantly (P < 0.001) increased mRNA levels of all the genes studied. The overall effect of feeding was significant only for the IκB mRNA levels which were increased in the fasted compared to the fed animals. The levels of all the other genes tested were unaffected by the feeding condition with no significant interaction apparent.

*Prostaglandin levels in the cerebrospinal fluid*

Consistent with the expression levels of COX-2 and mPGES-1, LPS treatment significantly increased PGE2 levels (P < 0.001) in the CSF which was similar between the fed and fasted groups 2 h after injection (Figure V-5). It has recently been shown that 15-d-PGJ2, another PG family member derived from the COX metabolite PGH2, exerts antipyretic actions during LPS fever (Mouihate et al., 2004). Therefore we also measured its CSF levels in the same groups of animals. Neither LPS nor feeding condition had any effect on the 15-d-PGJ2 concentrations at the time point studied.

*Fever response to central prostaglandin E2 injection*

The fact that fasting did not affect the LPS-induced synthesis of PGE2 in the brain while it attenuated the fever response to LPS indicated that the attenuation is due to the altered thermal response either downstream or independent of PGE2 action in the fasted rats. To test this hypothesis, both the fed and fasted rats were injected with two doses of PGE2 (10 and 50 ng/rat) into the

right lateral ventricle. Vehicle containing 0.5% ethanol in sterile saline was injected as a control. As expected, the average pre-injection $T_{core}$ were significantly lower in the fasted groups in comparison to the respective fed counterparts (37.0 ± 0.1 °C, 37.0 ± 0.1 °C, 37.0 ± 0.0 °C, 36.3 ± 0.1 °C, 36.1 ± 0.2 °C, and 36.3 ± 0.2 °C for fed-vehicle, fed- PGE210, fed- PGE250, fasted-vehicle, fast- PGE210, and fasted- PGE250). In the vehicle-injected groups, the $T_{core}$ of the fasted group was consistently lower than that of the fed group (Figure V-6A; 0–60 min, $P < 0.05$). Both doses of PGE2 caused a fever response in both the fed and fasted groups. The major difference between the fed and fasted rats was observed at the lower dose (10 ng/rat) of PGE2 treatment (Figure V-6C). The $T_{core}$ course was significantly attenuated in the fasted groups (0–22 min, $P < 0.05$). The peak $T_{core}$ was lower in the fasted than the fed group ($P < 0.01$, 38.1 ± 0.1 °C *vs.* 37.6 ± 0.2 °C, fed-PGE210 *vs.* fasted-PGE210) while the time to reach this peak was similar between the groups (17.4 ± 1.4 min *vs.* 24.3 ± 4.4 min, fed-PGE210 *vs.* fasted-PGE210). The attenuation of fever was less prominent after the higher dose (50 ng/rat) of PGE2 treatment (Figure V-6E). By 10 min after the injection, the $T_{core}$ of fasted rats reached levels similar to those of the fed group. The peak $T_{core}$ value and timings were similar between the groups ($T_{core}$, 38.6 ± 0.1 °C *vs.* 38.4 ± 0.2 °C; timing, 21.4 ± 2.0 min *vs.* 21.4 ± 1.1 min; fed-PGE250 *vs.* fasted-PGE250).

Figure V-6B, D and F illustrate the pre-injection $T_{core}$ and the average post-injection $T_{core}$ in three different treatment groups. At 10 ng of PGE2 injection, both the pre- and post-injection $T_{core}$ were similarly attenuated by fasting (two-way ANOVA, Ps $< 0.001$ for feeding and injection, $P > 0.05$ for feeding x injection interaction). At 50 ng dose, two-way ANOVA yielded significant effect of both feeding ($P < 0.05$) and injection ($P < 0.001$), and a significant interaction ($P < 0.05$). Post-hoc analysis revealed no significant difference between the fed and fasted groups on either pre-injection $T_{core}$ or post-injection $T_{core}$.

## Discussion

Previous studies have reported that acute starvation prior to inflammatory events attenuates the fever response in various species of experimental animals (Szekely, 1979; Kleitman and Satinoff, 1981; Molnar and Milner, 1983; Shojoony, 1985; Shido et al., 1989). The present study tested if the attenuation of fever is due to diminished inflammatory responses to pathogen, resulting in reduced immune-to-brain pyrogenic signalling. Our results demonstrated that 48 h pre-inflammation fasting clearly lowered the $T_{core}$ during the fever response to a systemic injection of LPS. Characterization of the LPS-induced inflammatory response revealed that pre-inflammation fasting diminished the elevations of circulating levels of TNF and IL-1RA, while the rise of IL-1β and IL-6 levels were unchanged. Fasting affected neither the mRNA up-regulation of genes important for PGE2 synthesis in the brain, nor the elevation of PGE2 levels in the CSF. These results indicate that, despite the changes in some cytokine levels, the pyrogenic signalling to/within the brain is well preserved during acute starvation, and that a mechanism(s) independent of this cascade is (are) responsible for the attenuation of the LPS-induced fever. With this in mind, the final experiment in the present study examined the fever response to central PGE2 injection and demonstrated that the pre-inflammation fasting also the $T_{core}$ during the PGE2-induced fever. With these results, we conclude that the alteration in the thermoregulatory mechanisms downstream and/or independent of PGE2 action are responsible for the attenuation of LPS-induced fever during acute starvation.

It is widely known that malnutrition impairs various aspects of the immune function and ultimately affects the host's susceptibility to infection (Wing et al., 1988; Chandra, 1992; Grimble, 1998; Lord et al., 1998; Mancuso et al., 2006). In line with this, the present study found that pre-inflammation fasting blunted the induction of plasma levels of TNF and IL-1RA by systemic LPS injection. On the other hand, LPS-induced elevation of IL-1β and IL-6, two important pyrogenic cytokines besides TNF, was largely unaffected by the fasting. Interestingly, the IL-1β/IL-1RA ratio, an indicator of net IL-1β action, was significantly higher in the fasted than the fed animals, because of the diminished IL-1RA levels in the

fasted group. These results imply that pre-inflammation fasting alters the balance between pro- and anti-inflammatory cytokines and thereby could enhance certain aspects of the inflammatory response and resistance to infection (Wing and Young, 1980). Interestingly and unexpectedly, saline injection alone increased plasma IL-6 levels in fasted rats but not *ad lib* fed animals. The reason for this difference is not clear, however it could partly be a reflection of a heightened stress response (to injection/handling) in these animals resulting from the food deprivation, thus rendering them more susceptible to stress- induced IL-6 release (LeMay et al., 1990a) when compared to *ad lib* fed rats.

We initially predicted that the inflammatory response in the brain after LPS treatment would be altered as a consequence of the blunted peripheral cytokine production in the fasted rats. However, the LPS-induced levels of IκB and SOCS3 mRNAs, two commonly used markers for assessing the magnitude of the inflammatory response to afferent cytokine signals in the brain (Laflamme and Rivest, 1999; Lebel et al., 2000), were unimpaired in fasted animals. Similarly, mRNA up-regulations of IL-1β, COX-2 and mPGES-1 in the brain as well as PGE2 elevation in the CSF, critical components of fever development, were similar between the fed and fasted rats. Among the classical pyrogenic cytokines, namely TNF, IL-1β and IL-6, both TNF and IL-1β actions are linked to NFκB activation (assessed by IκB mRNA up-regulation in the present study) (Laflamme and Rivest, 1999), while IL-6 is linked to STAT3 activation (assessed by SOCS3 mRNA up-regulation) (Lebel et al., 2000). Therefore, the slight enhancement of the LPS-induced IκB levels in the fasted rats, despite the diminished TNF levels, can be explained by the enhanced net IL-1β action (IL-1β/IL-1RA ratio). This explanation also applies to the intact up-regulation of COX-2 and mPGES-1 (and consequently PGE2 production) in the TNF-diminished fasted rats since NFκB is known to be critical for the transcription of these genes (Kojima et al., 2004; Nadjar et al., 2005). An alternative, but not mutually exclusive, explanation is that circulating IL-6, the levels of which were unaffected by fasting, plays a major role in central PGE2 production. Among the three major pyrogenic cytokines, IL-6 was demonstrated to consistently increase in the circulation after various types of

pyrogenic challenges including LPS, and the circulating levels of this cytokine were shown to correlate with the magnitude of fever more closely to those of TNF and IL-1β (LeMay et al., 1990b; Roth et al., 1993; Kozak et al., 1997; Cartmell et al., 2000; Aguilar-Valles et al., 2007). In support of the significant role of this cytokine in the fever response, studies from our laboratory and those of others demonstrated that IL-6 deficiency or anti-IL-6 antiserum treatment strongly attenuates LPS-induced fever (Chai et al., 1996; Cartmell et al., 2000; Rummel et al., 2006). In addition, we have recently reported that IL-6 directly induces the expression of COX-2 in the brain (Rummel et al., 2006). The present study further supports the role of IL-6 in regulating central PGE2 synthesis during fever.

Other than IL-6, we and others reported previously that neutralization of leptin, another circulating cytokine, with anti-leptin antiserum attenuated the fever response to LPS in *ad lib* fed rats. The fever attenuation was accompanied by blunted up-regulations of brain IL-1β and COX-2, suggesting a role for leptin as a circulating pyrogen (Sachot et al., 2004; Harden et al., 2006; Inoue et al., 2006). However, in the present study, the reduction of leptin levels after fasting had no effect on the LPS-induced up-regulation of IL-1β or COX-2 mRNA in the brain arguing against its pro-inflammatory roles. Moreover, in another study using obese Koletsky rats that lack leptin receptor, Steiner et al. (Steiner et al., 2004) found that the deficiency of leptin signalling caused a prolonged hypothermia (indicative of a more severe systemic inflammation than fever) after LPS injection, indicating possible anti-inflammatory effects of leptin. In the same study, the hypothermia exhibited by the obese rats was accompanied by an exaggerated increase of plasma TNF, which is opposite to the diminished TNF rise in the fasted low-leptin rats in the current study. The basis of these discrepancies is currently unclear, however, both the fasted and obese animals have a number of hormonal and metabolic abnormalities due to the change in the nutritional status besides the direct effects of change in leptin signalling, and it is possible that these differences may have contributed to the different outcomes. Acute leptin neutralization with anti-leptin antiserum in *ad lib* fed animals would help to delineate the role of leptin in mediating the CNS component of the inflammatory

response since this model of transient leptin deficiency would circumvent starvation- or obesity-associated abnormalities.

The results from the present study did not show a causal link between the attenuation of fever and alteration in the pyrogenic inflammatory response during acute starvation. This is in contrast to the case of chronic protein-calorie malnutrition which is a major common cause of secondary immune deficiency (Chandra, 1992). The attenuation of fever by chronic protein deficiency was ascribed to a significant attenuation in the cytokine production by monocytes and macrophages responding to exogenous pathogens in experimental animals (Hoffman-Goetz and Kluger, 1979b, a) as well as malnourished patients (Kauffman et al., 1986). Interestingly, the protein-deficient animals, as opposed to the fasted rats manifesting hypothermia in our study, were able to maintain normal body temperature under non-inflammatory conditions, even when exposed to a cold environment down to 5 °C (Hoffman-Goetz and Kluger, 1979b), indicating that chronic protein deficiency has little effects on thermoregulatory function. The mechanisms of how nutritional deficiency affects the immune and thermoregulatory function are currently unclear and potential mechanisms are numerous (general shortage of energy, deficiency of specific macro- or micro-nutrients such as protein, zinc, iron, vitamin A, C, and E, and an adaptive hormonal response to starvation such as increased glucocorticoid levels). However, these different forms of malnutrition, both of which attenuate the fever response but in a different manner, will serve as useful models to investigate the precise mechanisms that specifically affect immune or thermoregulatory function.

Fasted rats showed significantly lower $T_{core}$ at the time of injection as compared to their fed counterparts (Figure V-2 and 6). These data support previous studies that acute starvation alone alters thermoregulatory mechanisms leading to hypothermia under non-inflammatory condition (Severinsen and Munch, 1999; Yoda et al., 2000). Interestingly, it was shown that the fasted rats remained hypothermic even when they were allowed to select (and indeed moved to) warmer ambient temperatures in a thermal gradient (Sakurada et al., 2000). Moreover, the hypothermia was accompanied by a fall in the threshold $T_{core}$ for

cold-induced thermogenesis, and once the critical temperature was reached, the thermogenic abilities of these animals per se were unimpaired (Sakurada et al., 2000). Therefore, it was argued that the fasting-induced hypothermia is not simply due to a debilitation of thermogenesis but rather a regulated decline of body temperature. In the present study, both LPS and PGE2 injections induced a clear rise in $T_{core}$ in the fasted rats. In fact, the $\Delta T_{core}$ rise of the fasted rats was equivalent to, or even greater than (at 50 ng of PGE2), that of their fed counterparts (Figure V-2D, 6D, and 6F). These results indicate that the febrigenic capacity of PGE2-driven thermoeffectors remains largely intact in the fasted rats.

In analyzing the results from our fever studies we chose to use absolute $T_{core}$, rather than $\Delta T_{core}$ rise, because the former was deemed to represent a more accurate reflection of the upward shift of threshold temperature (the underlying thermoregulatory response of fever; see Data analysis). The elevated absolute $T_{core}$ following pyrogen administration (LPS and 10 ng of PGE2) was clearly and consistently lower in the fasted rats when compared to the fed counterparts (Figure V-2 and 6). Based on these observations we described the fever response as "attenuated". Nevertheless, the present data demonstrated that fasting did not per se attenuate the "febrigenic response" of animals, namely LPS-induced central PGE2 production as well as LPS- and PGE2-induced thermogenesis (or heat gain) when judged by $\Delta T_{core}$ elevation (Figure V-2D, 6D, and 6F). This is obviously counterintuitive and somewhat difficult to explain. An additional, yet very important, point here is that the $T_{core}$ is not regulated by a single unified system (previously known as the "set point" theory) but is controlled by multiple thermoeffector loops that defend different threshold temperatures and work relatively independently of each other (Romanovsky, 2007). By taking this into account, our interpretation of the data is that the fever response (absolute $T_{core}$) is a combination of both PGE2-dependent and -independent thermoreffector functions (each defending a unique threshold temperature), with the former remaining relatively intact, or even enhanced, during fasting whilst the latter process is largely responsible for the lowered $T_{core}$ in the absence of, as well as, during fever. Based on this, we conclude that fasting does not attenuate fever, in a

physiological sense, but it does diminish the absolute $T_{core}$ during fever via a thermoregulatory function independent of the PGE2-driven fever response.

*Perspectives and Significance*

The present study further highlights the complexity of the thermoregulatory system, especially when linked to the nutritional status of the organism. Whilst it was clear that fasting altered the mechanisms regulating body temperature control, it did not affect the fever response. The fasted animals exhibited a remarkable resilience, given their diminished caloric status, to respond to exogenous pyrogens. This was to a large extent, reflected in all aspects of the 'fever-pathways' tested both in the periphery and the brain. The main objective of this study was to examine the effect of fasting on immune-to-brain signalling which remained more or less intact. We did however only study the contribution of the established 'pyrogenic' cytokines, and it maybe that other mediators especially those linked with energy balance regulation, play an important part. Leptin could be a likely candidate, given that the decrease in the levels of this hormone is known to mediate a number of the neuroendocrine responses to starvation. In the 48 h-fasted rats used in the present study, we confirmed that the leptin levels in the circulation were dramatically reduced when compared to *ad lib* fed rats. The role of this cytokine-like hormone in fasting-induced hypothermia and alteration of the fever response to exogenous pathogens warrants further investigation.

**Acknowledgements**

This work was supported by the Canadian Institutes of Health Research (CIHR) and the National Sciences and Engineering Research Council (NSERC) of Canada. W.I. is a recipient of a PhD scholarship from the Nakajima Foundation, Japan.

## Figures and Tables

**Figure V-1. Changes in body weight and body temperature**

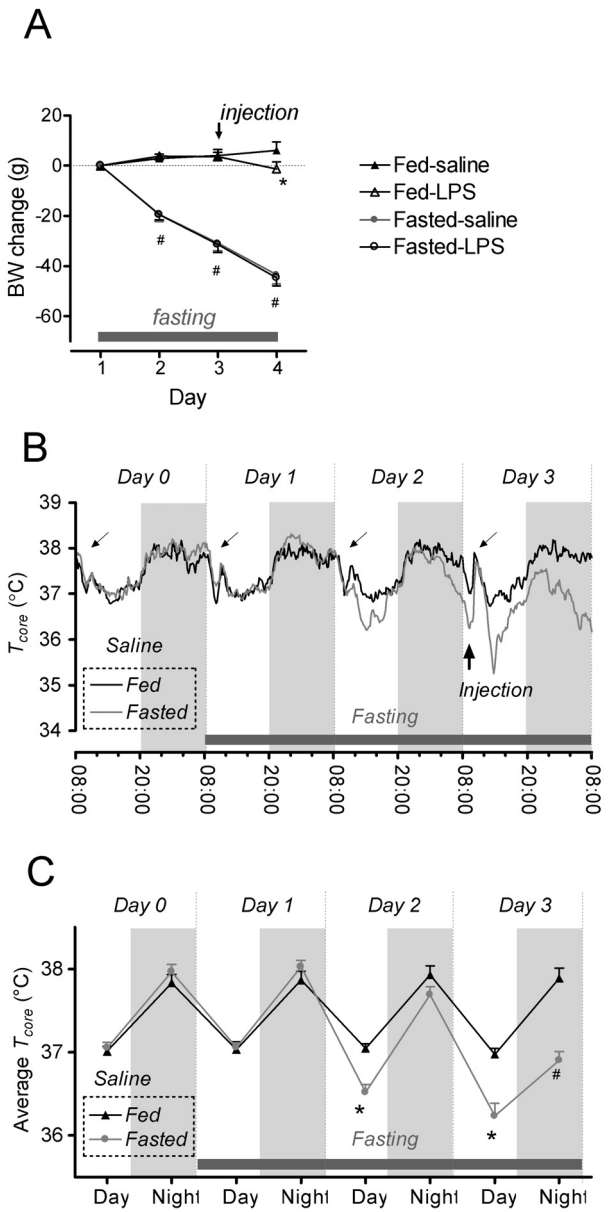


*Figure V-1. (A), Body weight change. Animals were weighted between 09:30 and 10:30 h. Food was removed from the fasted groups after the body weight measurement on day 1. All groups of rats received an i.p. injection of the respective drug on day 3 immediately after the body weight measurement. Values*

*are means ± S.E.M.. Fed-saline and fasted-saline (n = 8), fed-LPS and fasted-LPS (n = 7).* * *significantly different from saline-treated controls, P < 0.05;* # *significantly different from fed counterpart, P < 0.05. (B), Daily cycles of* $T_{core}$*. The time of injection is indicated by a thick arrow, and small thin-angled arrows indicate body weight measurement. Note that the handling of animals caused a transient hyperthermia in both the fed and fasted groups. The grey background represents the dark period (20:00–08:00 h). (C), The average* $T_{core}$ *in the active and inactive phases.* * *Significantly different from the inactive phase on day 0, P < 0.05.* # *Significantly different from the active phase on day 0, P < 0.05.*

**Figure V-2.** $\mathbf{T_{core}}$ **change after LPS challenge**

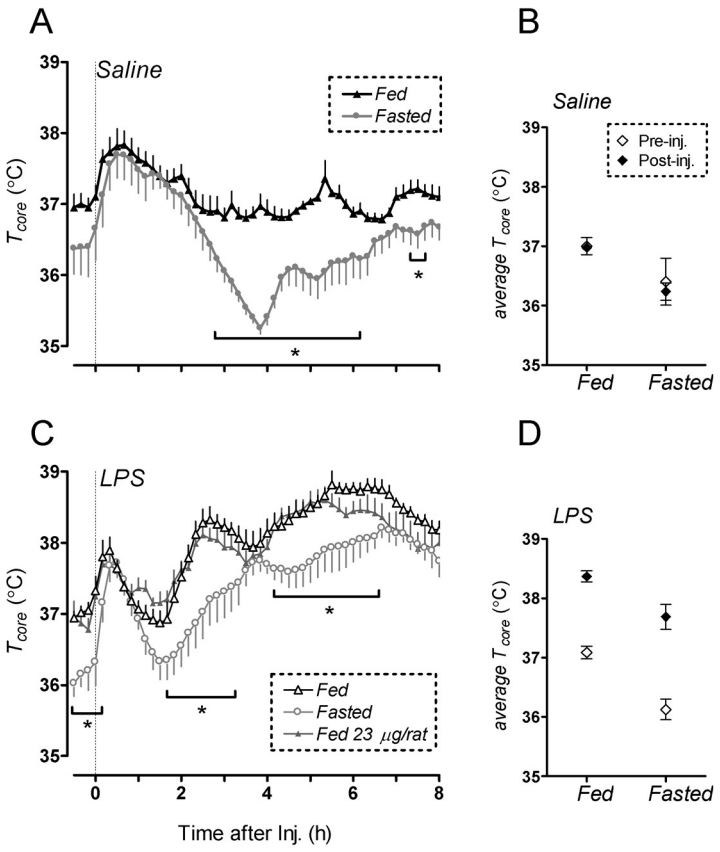


*Figure V-2.* $T_{core}$ *course of rats treated with an i.p. injection of saline (A) or LPS 100 μg/kg (C) at time point 0 h. An additional fed rats received a fixed amount of LPS (23 μg/rat) equivalent to the amount given to the fasted group (100 μg/kg) as*

*a control. Values are means ± S.E.M.. Fed and fasted saline (n = 8), fed and fasted LPS (n = 7), and fed (23 μg/rat) (n = 5).* * *significantly different from fed counterpart, P < 0.05. Pre- and post-injection* $T_{core}$ *of saline- (B) and LPS- (D) treated rats. Two-way (feeding x injection) ANOVA; (B), interaction P > 0.05, feeding P < 0.05, injection P > 0.05; (D), interaction P > 0.05, feeding P < 0.001, injection P < 0.001.*

**Figure V-3. Time course of circulating cytokine levels after LPS challenge**

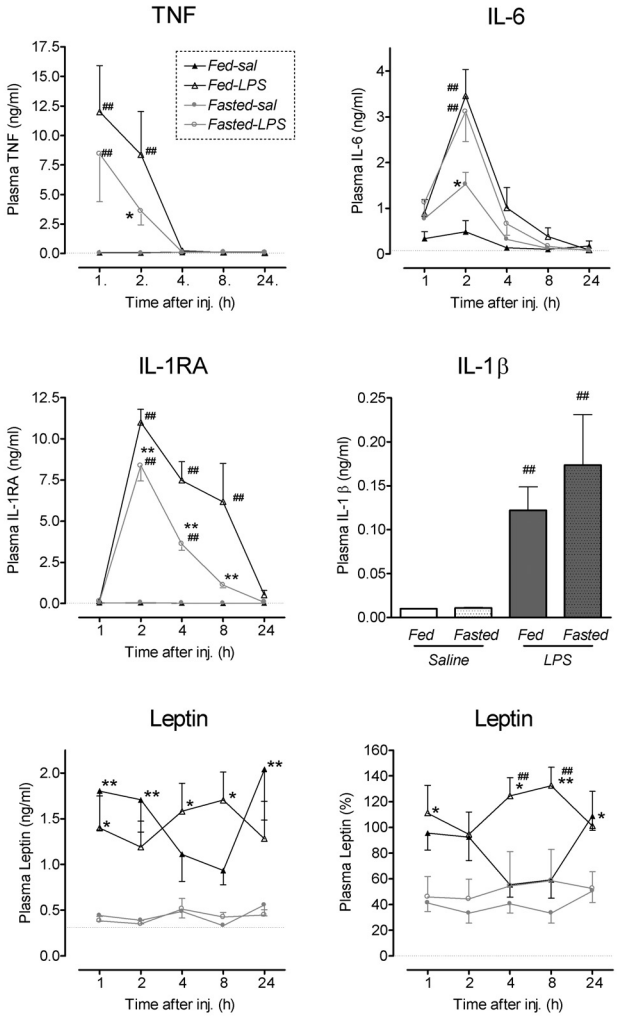


*Figure V-3. The fed and fasted rats were treated with an i.p. injection of saline or LPS (100 μg/kg) at time point 0 h. Blood was sampled repeatedly starting at pre-fasting (48 h before injection) and from then at the time points indicated. Leptin*

*levels are expressed as % change from pre-fasting baseline levels of each animal in an additional graph. Values are means ± S.E.M. for (n = 4 per group). Plasma IL-1β levels were determined in a separate set of animals sacrificed 2 h after injection (n = 5-6 per group)* $^{*}$ *Significantly different from fed counterparts,* $^{*}$ $P < 0.05$, $^{**}$ $P < 0.01$. $^{\#}$ *Significantly different from saline-treated counterparts,* $^{\#\#}$ $P < 0.01$.

**Figure V-4. Gene expression levels in the brain after LPS challenge**

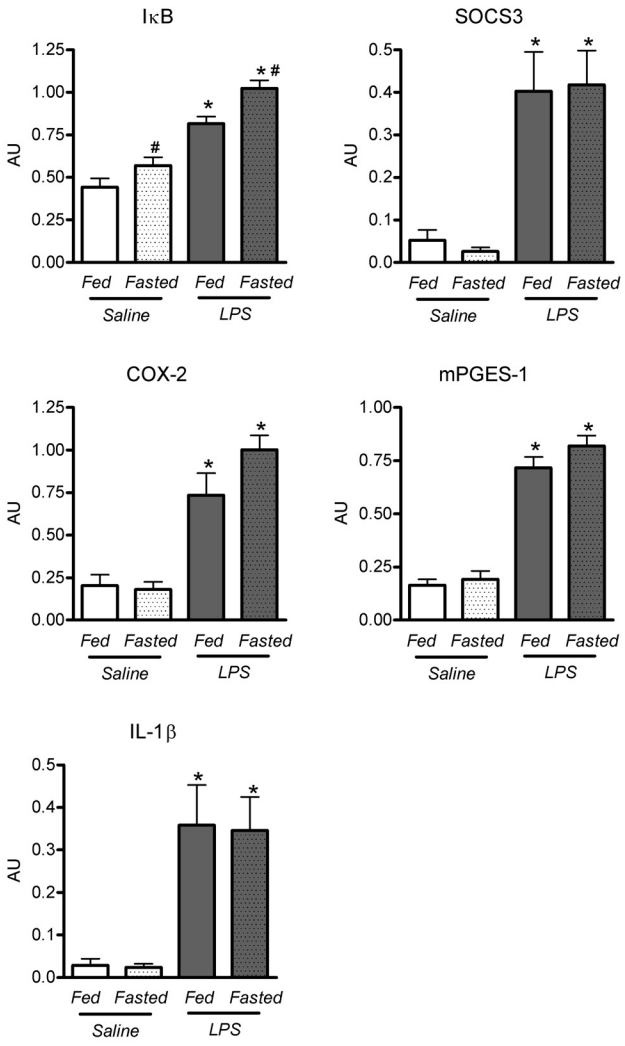


*Figure V-4. Semi-quantitative RT-PCR analysis of SOCS3, IκB, IL-1β, COX-2 and mPGES-1 mRNA levels in the hypothalamus 2 h after injection. Values are mean*

*± S.E.M. for 5–6 animals. Two-way ANOVA (feeding x LPS) revealed significant main effect of LPS (Ps < 0.001) for all genes studied. The effect of fasting was significant only for IκB. There was no significant interaction (feeding x LPS) on any gene tested. AU, arbitrary unit.* * *Significant effect of LPS,* # *significant effect of feeding.*

**Figure V-5. PGE2 and 15-d-PGJ2 levels in the brain after LPS challenge**

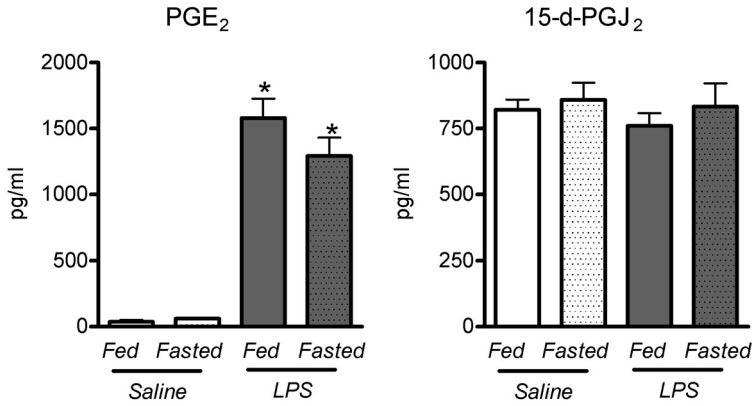


*Figure V-5. PGE2 and 15-d-PGJ2 levels in CSF 2 h after injection were measured by EIA. Values are mean ± S.E.M. for 5–6 animals. Two-way ANOVA (feeding x LPS) revealed significant main effect of LPS (P < 0.001) on PGE2 levels, while feeding had no effect. Interaction (feeding x LPS) was not significant. None of the parameter (feeding, LPS, interaction) was significant in 15-d-PGJ2.* * *Significant effect of LPS.*

**Figure V-6. $T_{core}$ change after central PGE2 challenge**

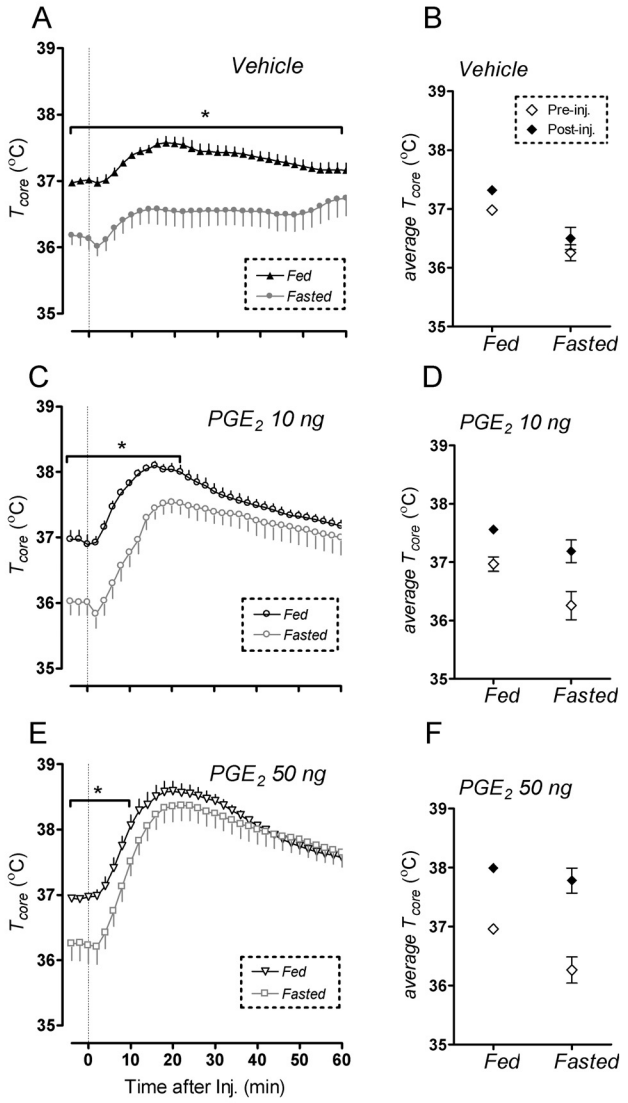


*Figure V-6. $T_{core}$ course of rats treated with an i.c.v. injection of vehicle (A), PGE2 10 ng/rat (C) or PGE2 50 ng/rat (E) at time point 0 min. Values are means ± S.E.M. for 7 animals. * Significantly different from fed counterpart, $P < 0.05$. Pre- and post-injection $T_{core}$ of vehicle- (B), PGE2 10 ng/rat- (D) or PGE2 50 ng/rat- (F) treated rats. Two-way (feeding x injection) ANOVA; (B), interaction $P > 0.05$, feeding $P < 0.001$, injection $P < 0.05$; (D), interaction $P > 0.05$, feeding $P < 0.05$, injection $P < 0.001$; (F) interaction $P < 0.05$, feeding $P < 0.05$, injection $P < 0.001$.*

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## V.3. Supplementary results and discussion

As mentioned in the manuscript, we have preformed another set of experiments in male rats to address potential gender influences on the fasting-induced fever alteration. The results negated any significant effects of gender on the fever response (Figure V-7) and febrigenic immune-to-brain signalling (Figure V-8) in the current starvation paradigm. It should be noted, however, that the female rats used in the current experiment were randomly cycling and that the phase of the estrous cycle may have some impact on the interaction between fasting and febrigenic inflammatory response given the reported influence of ovarian hormone levels on fever (Mouihate et al., 1998).

**Figure V-7. $T_{core}$ changes after LPS challenge in male rats**

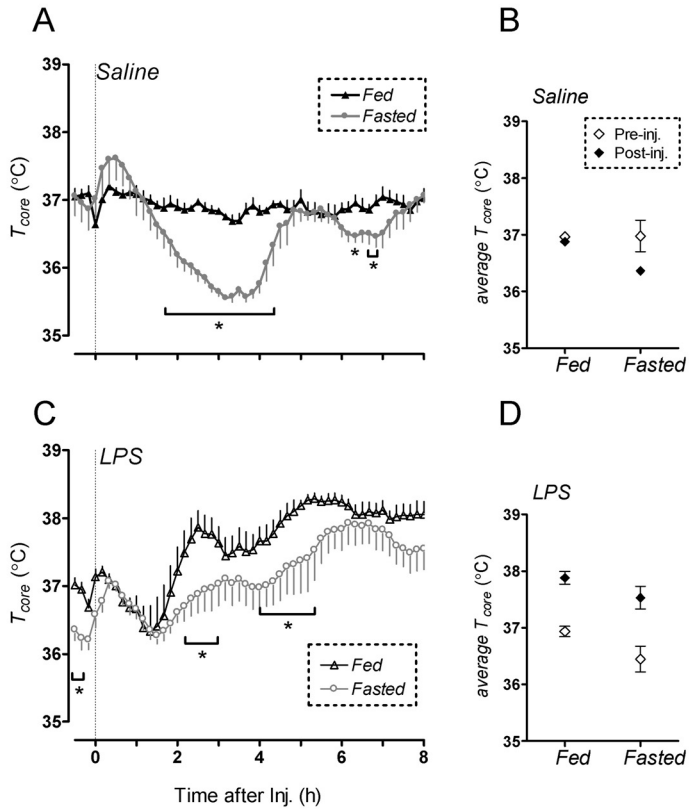


*Figure V-7. $T_{core}$ course of male rats treated with an i.p. injection of saline (A) or LPS 100 μg/kg (C) at time point 0 h. Values are means ± S.E.M. Fed and fasted*

*saline (n = 8) and fed and fasted LPS (n = 7).* * *Significantly different from fed counterparts, P < 0.05. Pre- and post-injection* $T_{core}$ *of saline- (B) and LPS- (D) treated rats. Two-way (feeding x injection) ANOVA; (B), interaction P > 0.05, feeding P < 0.05, injection P > 0.05; (D), interaction P > 0.05, feeding P < 0.001, injection P < 0.001.*

**Figure V-8. Gene expression levels in the brain after LPS challenge in male rats**

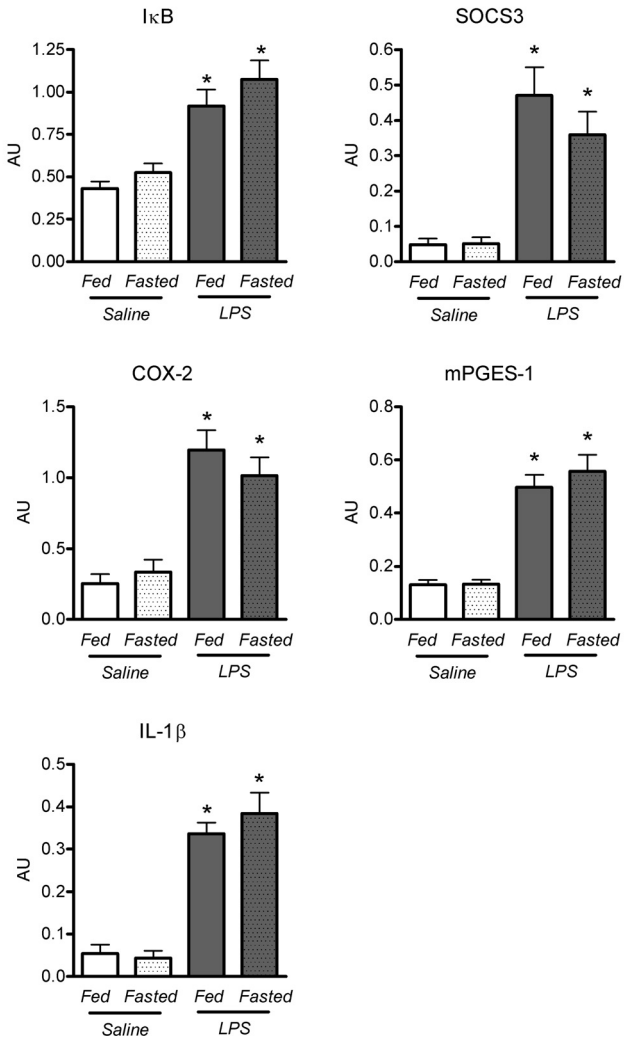


*Figure V-8. Semi-quantitative RT-PCR analysis of SOCS3, IκB, IL-1β, COX-2 and mPGES-1 mRNA levels in the hypothalamus 2 h after injection. Values are mean ± S.E.M. for 5–6 animals. Two-way ANOVA (feeding x LPS) revealed significant*

*main effect of LPS (Ps < 0.001) for all genes studied. The effect of fasting was significant only for IκB. There was no significant interaction (feeding x LPS) on any gene tested. AU, arbitrary unit.* * *Significant effect of LPS,* # *significant effect of feeding.*

The results from this manuscript unexpectedly demonstrated that the brain's innate inflammatory responses relevant to fever generation are remarkably resilient during nutritional deprivation. This is in contrast with the observation made in Chapter IV showing that pre-inflammation fasting in mice strongly blunted the induction of innate inflammatory mediators in the brain. For example, the LPS-induced IL-1β mRNA were comparable between fed and fasted rats in the present study (Figure V-4), whereas it was strongly attenuated in fasted mice examined in Chapter IV (Figure IV-6). The species difference (rat *vs.* mouse) is certainly an issue to be considered, however there are several other important possibilities that could account for the discrepancy. Firstly, the degrees of nutritional deprivation are different between the two studies. In both studies, animals were food-deprived for 48 h prior to LPS injection. However, 48 h-fasting is likely to have a greater negative impact on mice than on rats given the difference in body size. Thus, it is possible that a longer period of pre-inflammation fasting in rats may be required to significantly attenuate the brain's innate inflammatory response to LPS. Secondly, the two studies employed different magnitudes of inflammation. In the rat study, the dose of LPS was moderate (100 μg/kg, *i.p.*) which was chosen to be sufficient to induce a clear fever response but does not cause any detectable neuronal damage or immune cell recruitment to the brain (Mouihate and Pittman, 1998; Bohatschek et al., 2001). On the other hand, a high LPS dose (2.5 mg/kg, *i.p.*) was chosen for the mouse study because the study aimed to investigate neutrophil recruitment associated with severe systemic inflammation. Thus, it is possible that the difference in the severity of inflammation may be an important factor leading to the different outcomes: the effects of pre-inflammation fasting may only be evident in the case of severe forms of neuroinflammation such as those that involve neurotoxicity or

immune cell recruitment. Lastly, the two studies examined different time points after LPS injection. The rat study focused on an earlier time point (2 h) whereas the mouse study investigated a later time point (24 h). It is therefore possible that the effects of pre-inflammation fasting on the indices of neuroinflammation may be more evident at the later time point.

In summary, the results from the present manuscript revealed a complex relationship between nutritional deprivation and the brain's innate inflammatory response, which may be sensitive to the magnitude of nutritional deprivation, the severity of inflammation, and/or the time point investigated. Further studies are warranted to gain a more complete understanding of this interaction.

# Chapter VI: Leptin reduction diminishes long-lasting fever following lipopolysaccharide injection in fasted rats independent of central prostaglandin E2 synthesis.

## VI.1. Preface

In the previous manuscript (Chapter V), we established a rat model of fever in which pre-inflammation fasting clearly altered the magnitude of the fever response. Therefore, this model serves as a useful tool to investigate the interaction between energy balance and sickness behaviour. It remains to be determined whether leptin plays a role in this interaction. Therefore, the main aim of the present manuscript is to test if the decrease in leptin levels associated with acute starvation contributes to the alteration of the fever response provoked by LPS-induced inflammation.

## VI.2. Manuscript

**Title: Leptin reduction diminishes long-lasting fever following lipopolysaccharide injection in fasted rats independent of central prostaglandin E2 synthesis.**

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To be submitted

Keywords: thermoregulation, acute starvation, cyclooxygenase, hypothermia, prostaglandin

## Abstract

A decrease in circulating leptin levels with the onset of starvation triggers a myriad of adaptive responses to starvation including immunosuppression and hypometabolism/hypothermia in several mammalian species. Acute starvation attenuates the fever response to pathogens, in part because of a compromised immune and/or thermoregulatory function both of which are prerequisites for fever generation. However, the potential link between leptin and fever in the starvation paradigm remains unexplored. In the present study, we addressed whether the decrease in leptin contributes to the reported alteration of the fever response in fasted rats. Animals were fasted for 48 h with continuous infusion of either vehicle or leptin (12 μg/day) prior to lipopolysaccharide (LPS) injection. The pre-inflammation fasting alone caused a progressive hypothermia: leptin repletion almost completely reversed this decrease in body temperature. An intraperitoneal injection of LPS (100 μg/kg) resulted in an attenuated fever in the fasted animals, which was evident 2–6 h after the injection when compared to the fed counterparts. Leptin replacement did not reverse the fever attenuation during the rising phase. A higher dose of LPS (1000 μg/kg) induced a long-lasting fever in fed rats which remained unabated until 36 h after the injection. The fasted rats failed to sustain fever and their body temperature progressively decreased from 18 h after the injection. Leptin repletion almost completely restored the long-lasting fever in the fasted rats. In the fed rats, leptin infusion had no effect on body temperature regardless of LPS injection. The leptin-mediated sustenance of fever was not due to the enhanced febrigenic inflammatory responses in the brain as leptin repletion affected neither mRNA levels involved in prostaglandin (PG)E2 synthesis nor PGE2 elevations in the cerebrospinal fluid (CSF). In contrast mRNA levels of pro-opiomelanocortin (POMC), a pro-hormone for thermogenic β-endorphin, was decreased by fasting and was reversed by leptin repletion: POMC levels significantly correlated with the magnitude of fever in the fasted rats treated with or without leptin. These results are the first to describe that acute starvation strongly diminishes the prolonged fever associated with relatively

severe inflammation, and that this fever attenuation is leptin-dependent. This response is dissociated from central PGE2 synthesis but appears to involve POMC products such as β-endorphin.

## Introduction

Fever is a common response to various types of infection and constitutes an important component of an adaptive strategy for fighting disease (Hart, 1988; Kluger, 1991). The development of fever is a finely tuned, complex event that involves both the immune and the central nervous systems, through which a series of inflammatory, thermoregulatory and metabolic processes are regulated (Roth et al., 2006). The sum of the changes required to mount an effective fever response is an energy-demanding process that can be influenced by the energy status of the host. This was clearly shown by a number of studies reporting that malnutrition prior to the induction of inflammation compromises the fever response in various species of experimental animals (Hoffman-Goetz and Kluger, 1979b, a; Szekely, 1979; Kleitman and Satinoff, 1981; Shojoony, 1985; Shido et al., 1989; Inoue et al., 2008). The suggested explanations for this observation include suppression of inflammatory responses (Hoffman-Goetz and Kluger, 1979b, a; Shido et al., 1989), an alteration of thermoregulatory functions (Inoue et al., 2008) and an inhibition of metabolic thermogenesis (Kleitman and Satinoff, 1981; Shojoony, 1985). However, it remained unclear how the insufficiency of the host's energy balance is signalled to any of the aforementioned fever-related functions and subsequently alters the overall response.

Among the multiple metabolic and hormonal changes that occur during malnutrition is a reduction in leptin levels (Dallman et al., 1999; Ahima and Osei, 2004). This adipose tissue-derived hormone was originally identified as an important satiety factor that prevents excessive weight gain by signalling the presence of sufficient energy storage (*i.e.*, fat mass) (Friedman and Halaas, 1998). However, it is now well established that leptin also signals the transition from energy balance sufficiency to insufficiency. The levels of this hormone decrease rapidly with the onset of caloric deprivation and the prevention of this reduction by administering exogenous leptin blunts a number of physiological changes known to occur during starvation (Ahima et al., 1996; Flier, 1998). The reported roles of leptin in the adaptation to starvation are broad, affecting a wide variety of

functions including neuroendocrine (Ahima et al., 1996), immune (Lord et al., 1998; Howard et al., 1999), behavioural (Exner et al., 2000; Hillebrand et al., 2005) and metabolic (Doring et al., 1998; Overton et al., 2001) adaptations. Some of these functions serve as important prerequisites for an effective fever response during inflammation. For example, a decrease in leptin levels is one of the major contributors to immunosuppression during starvation (Lord et al., 1998; Howard et al., 1999). Administration of exogenous leptin in food deprived mice improves the host defence response against bacterial infection, in part by recovering the impaired phagocytic activity of macrophages, neutrophil mobilization and cytokine production (Mancuso et al., 2006). In the brain, leptin regulates expression and release of various neuropeptides involved in energy balance regulation (Inui, 1999). The reduction of leptin signalling shifts the balance between catabolic [corticotrophin releasing factor (CRF), α-melanocyte stimulating hormone (MSH), β-endorphin and cocaine an amphetamine related transcript (CART)] and anabolic [neuropeptide Y (NPY) and agouti-related peptide (AgRP)] neuropeptide signalling towards the latter (Inui, 1999). Although, such a shift in neuropeptide balance has been repeatedly linked to the enhanced appetite of starved animals, it can also account for the occurrence of a regulated hypometabolism/hypothermia associated with starvation (Rothwell et al., 1983; Sakurada et al., 2000; Yoda et al., 2000; Nagashima et al., 2003). The peripheral mechanisms, the starvation-induced hypometabolism/hypothermia, involve an inhibition of the activity of brown adipose tissue (BAT) (Hayashi and Nagasaka, 1983; Trayhurn and Jennings, 1988), a major site of metabolic heat production in rodents (Cannon et al., 1998). Preventing the fall in leptin levels during starvation effectively disinhibits metabolic thermogenesis in BAT (Sivitz et al., 1999). Together, the available data indicates that leptin serves as a permissive signal for each of the aforementioned physiological functions when energy balance is sufficient. Despite the strong functional link between leptin and these important prerequisites for effective fever response, the question of whether the decrease in leptin levels during starvation contributes to the alteration of the fever response has not been previously addressed. The aim of the present study is

to address if and how the decline in leptin levels affects the fever response resulting from a single systemic injection of lipopolysaccharide (LPS) in rats which have been food deprived for 48 h with and without leptin replacement.

## Methods

*Animals*

Adult female Sprague-Dawley rats with initial body weight of 200–225 g (Charles River, Saint Constant, Quebec, Canada) were used in all experiments. The animals were housed individually in a controlled environment at an ambient temperature of 21 ± 1.5 ˚C on a 12 h light/dark cycle (lights on from 0800 to 2000), with free access to water and standard laboratory chow (Rat chow #5012; Purina Co., St. Louis, MO, USA) unless otherwise indicated. All experimental procedures were approved by the Animal Care Committee of McGill University pursuant to the Canadian Council of Animal Care guidelines.

*Experimental design*

Experiments were performed over five days; one day for baseline measurement (day 0) followed by four days of feeding manipulation (day 1–4). Animals were weighed daily between 0930 and 1030. Rats were initially divided into four weight-matched feeding x drug groups (fed-vehicle, fed-leptin, fasted-vehicle and fasted-leptin). On day 1, Alzet miniosmotic pumps (0.5 µl/h; model 1007D, Durect Corp., CA) containing either saline or recombinant rat leptin (12 µg/day; PeproTec Inc, Rocky Hill, NJ) were implanted subcutaneously in the dorsal midline caudal to the scapulae of the animals under isoflurane anaesthesia, and 2 % lidocaine (25–50 µl) was applied to the area of incision. Surgeries were performed between 1000 and 1200, and thereafter food was removed from the fasted groups. All groups of rats had free access to water throughout the experimental period. On day 3, each group was subdivided into three treatment groups (n = 7–8 per group) receiving a single intraperitoneal (*i.p.*) injection of either 100 or 1000 µg/kg of LPS (Escherichia coli O111:B4, lot 42k4120; Sigma, Oakville, Ontario, Canada) or saline (1 ml/kg) as a control between 0930 and 1030 (approximately 48 h after the start of fasting/drug infusion).

*Measurement of body temperature using remote biotelemetry*

Pre-calibrated temperature-sensitive radio transmitters (model:TA10TA-F40, Data Sciences, St Paul, MN, USA) were implanted via midline incision into the abdominal cavity of anesthetised rats (intramuscular; 50 mg/kg ketamine hydrochloride, 5 mg/kg xylazine hydrochloride, 0.5 mg/kg acepromazine maleate; total volume 1 ml/kg). The level of anaesthesia was assessed by the withdrawal reflex to a toe pinch. 2 % lidocaine (25–50 µl) was applied to the area of incision. Animals were allowed to recover for at least 7 days prior to experimentation. Transmitter output frequency (Hz) was monitored at 10 min intervals for LPS-induced fever by an antenna mounted in a receiver board situated beneath the cage of each animal. The output data from each transmitter were transformed into degrees centigrade using Dataquest A.R.T. software (Data Sciences).

*Cerebrospinal fluid and brain collection*

To assess the levels of PGs in the cerebrospinal fluid (CSF), and to measure the expression levels of inflammatory genes in the hypothalamus, CSF and brains were collected from the fed and fasted rats treated with either saline (1 ml/kg, *i.p.*) or LPS (100 or 1000 µg/kg, *i.p.*) (n = 5–6 per group). Animals were sacrificed 30 h after the LPS or saline injection under deep anaesthesia with a terminal dose of pentobarbital sodium (60 mg/kg, *i.p.*). CSF was sampled from the cisterna magna with a 27G needle connected to a microsyringe (250 µl) via PE20 tubing. The sampled CSF was immediately frozen on dry ice and kept at –80 °C. Blood was collected from the same animals via cardiac puncture using sterile heparinized syringes and plasma samples were prepared for the hormone assay. Animals were then perfused with ice-cold saline, the brains removed, and the hypothalami dissected, frozen on dry ice and kept at –80 °C until use.

*Cytokine and prostaglandin measurement*

Sandwich enzyme-linked immunosorbant assays (ELISA) for leptin (NIBSC, Potters Bar, UK) were performed as previously described (Inoue et al., 2008). All plasma samples were diluted 1:10. Intra-assay and inter-assay variations were below 10 %. The sensitivity of the assay was 15.6 pg/ml. All samples were assayed in duplicate.

PGE2 and 15-deoxy-Δ12,14-PGJ2 (15-d-PGJ2) levels in CSF were measured using an enzyme immuno assay (EIA) kit (PGE2 EIA kit-Monoclonal, Cayman Chemical, Ann Arbor, MI, USA; 15-deoxy-Δ12,14-PGJ2 EIA kit, Assay designs, Ann Arbor, MI, USA) according to the manufacturer's protocol. The CSF samples were diluted 1:5 in an assay buffer provided as part of the kit. The sensitivity of the assay for PGE2 and 15-d-PGJ2 was 7.8 pg/ml and 36.8 pg/ml, respectively. All samples were assayed in duplicate.

*RNA extraction and RT-PCR*

Total RNA was extracted from the hypothalamus in 1 ml of TRIzol (Invitrogen, Burlington, Ontario, Canada), according to the manufacturer's protocol. The first-strand cDNA was synthesized from 1 μg of total RNA using 200 units of Molony murine leukemia virus reverse transcriptase (Invitrogen), 5 μM of random hexamers (Applied Bioscience, Streetsville, Ontario, Canada), and 1 mM of dNTP mix (Sigma) in a total reaction volume of 20 μl. The cDNA product was diluted with autoclaved distilled water for a final volume of 200 μl and stored at –20 °C until use. qRT-PCR was carried out in duplicate using pre-optimized primer/probe mixture (TaqMan® Gene Expression Assays; Applied Biosystems, Ontario, Canada) and TaqMan® universal PCR master mix (Applied Biosystems) on a 7500 Real-Time PCR System (Applied Biosystems). The cDNA quantities between different reactions were normalized by using a housekeeping gene β-actin (Cat: 4352640E, Applied Biosystems) as a reference. The sample values represent X-fold differences from a control sample (given a designated value of 1) within the same experiment. The assay ID for each gene are as follows: interleukin (IL)-1β (Rn00580432_m1); IL-1 receptor antagonist (IL-1RA) (Rn00573488_m1); cyclooxygenase (COX)-2 (Rn01483828_m1); microsomal PGE synthase (mPGES)-1 (Rn00572047_m1); macrophage inflammatory protein (MIP)-1β (Rn00587826_m1); NPY (Rn01410145_m1); Pro-opiomelanocortin (POMC) (Rn00595020_m1); AgRP (Rn01431703_g1); CART (Rn00567382_m1); CRF (Rn01462137_m1); iodothyronine deiodinase-2 (DIO2) (Rn00581867_m1); uncoupling protein (UCP)-1 (Rn00562126_m1); UCP-3 (Rn00565874_m1).

*Data analysis*

*Measurement of body temperature*

To evaluate the changes in core body temperature ($T_{core}$) over the course of fasting, the average $T_{core}$ of the dark (between 2400–0600) and light (between 1200–1800) periods were calculated for each animal. This 6 h interval was chosen to exclude the temperature change caused by body weight measurement performed daily at 1000 during the light period ($T_{core}$ returned to a normal level by 1200). A corresponding interval was chosen for the dark period (starting from 2400 for 6 h).

Two measures have been used to assess the fever response: absolute $T_{core}$ and the rise in $T_{core}$ ($\Delta T_{core}$: $\Delta T_{core} = T_{core} -$ pre-injection $T_{core}$). However, these two measures are not interchangeable when animals have different pre-injection $T_{core}$ (*e.g.*, day/night variation and different feeding conditions as in the present study). Satinoff and colleagues (Feng et al., 1989) demonstrated that absolute $T_{core}$, rather than $\Delta T_{core}$, provides a better representation of the physiological characteristic of fever because the former is solely determined by the dose of pyrogen (PGE2) and is largely unaffected by day/night variation in pre-injection $T_{core}$. The validity of this definition was supported by several other studies investigating the fever response in animals with different pre-injection $T_{core}$ (Kent et al., 1988; Malkinson et al., 1988; Ivanov and Romanovsky, 2002). Therefore, in the present study we used absolute $T_{core}$ rather than $\Delta T_{core}$ to assess the fever response.

To evaluate the overall change in $T_{core}$ by pyrogenic stimuli in the fed and fasted animals, pre-injection $T_{core}$ and post-injection $T_{core}$ were calculated. The pre-injection $T_{core}$ was defined as the average $T_{core}$ during the 30 min prior to the injection. The post-injection $T_{core}$ was defined as the average $T_{core}$ 120–480 min after the injection, which should exclude stress-induced hyperthermia.

*Statistics*

All data are presented as mean values ± S.E.M. and were analyzed using StatView software 4.57 (Abacus Concepts Inc., Berkeley, CA, USA). Two-way

repeated ANOVA (time x treatment) was used to analyze the data from body weight change, temperature course and cytokine time course studies. Two-way ANOVA was used to analyze the data related to mRNA expression and PG measurements at a single time point. Newman-Keuls multiple comparison test was performed as post-hoc analysis when applicable. In all cases, P values less than 0.05 were deemed statistically significant.

## Results

*Changes in body weight during fasting and inflammation*

Table VI-1 shows the baseline body weight of the animals (day 1) and that at the time of injection (day 3), as well as % body weight changes during 24 h after injection. All rats were implanted with an osmotic pump for vehicle or leptin (12 μg/day) infusion on day 1 immediately after body weight measurement, and thereafter the rats in the fasted groups were deprived of food until the end of the experiment. The fed groups received free access to food and water. All groups were injected *i.p.* with either saline or two different doses of LPS (100 or 1000 μg/kg) on day 3, at the time point corresponding to approximately 48 h after the start of drug infusion/feeding manipulation. The initial body weight on day 1 was similar across all groups. In fed groups, leptin infusion caused a slight, but significant, decrease in body weight by day 3 [two-way (time x leptin) ANOVA, $P < 0.01$ for interaction; post hoc $P < 0.05$ for within leptin day 1 *vs.* day 3]. In the fasted groups, food deprivation significantly decreased body weight ($P < 0.001$ for the main effect of time) whereas leptin infusion did not cause further reduction in body weight ($P > 0.05$ for interaction or leptin). LPS injection caused a dose-dependent loss of body weight within 24 h after the injection in the fed groups [two-way (leptin x LPS) ANOVA, $P < 0.001$ for LPS], whereas leptin infusion had no further effect ($P > 0.05$ for interaction or leptin). In contrast, neither the LPS injection nor leptin infusion in the fasted groups contributed to the loss of body weight ($P > 0.05$ for interaction, LPS or leptin).

*The effect of leptin on basal body temperature*

Figure VI-1 shows the daily cycle of $T_{core}$ of the animals treated with saline on day 3 as an example of the $T_{core}$ cycles under non-inflammatory conditions. The fed and fasted rats were infused with either vehicle or leptin. The animals in all treatment groups displayed clear day/night variations of $T_{core}$ that were higher in the active (dark) and lower in the inactive (light) phase. Leptin infusion had no effect on either the baseline $T_{core}$ or its daily rhythm under *ad lib* fed conditions

(Figure VI-1A). On the other hand, in the fasted groups leptin infusion effectively prevented the development of hypothermia which was evident in vehicle-infused rats on day 2 and 3 (Figure VI-1B). To evaluate the changes in $T_{core}$ over the course of fasting/drug infusion, the average $T_{core}$ in the active and inactive phases is shown in Figure VI-1C. In the active phase, the fasting caused a significant hypothermia on day 3 [two-way (time x treatment) ANOVA, $P < 0.05$ for interaction; post-hoc within day 3 $P < 0.001$ for fed-vehicle *vs.* fasted-vehicle]. Leptin treatment partially but significantly reversed the hypothermia in this period ($P < 0.01$, fasted-vehicle *vs.* fasted-leptin; $P < 0.001$, fed-vehicle *vs.* fasted-leptin). The fasting-induced hypothermia was more prominent in the inactive phase, reaching significance by day 2 and remained at a similar level on day 3 in the vehicle-infused group ($P < 0.001$ for fed-vehicle *vs.* fasted-vehicle). Leptin infusion almost completely reversed the hypothermia in the inactive phase ($P < 0.001$ for fasted-vehicle *vs.* fast-leptin; $P > 0.05$ for fed-vehicle *vs.* fasted-leptin). Together, these results indicate that the fasting-induced hypothermia is largely mediated by the reduction of leptin resulting from the food deprivation. After the $T_{core}$ recording, all animals were sacrificed and blood leptin levels verified. Fasting strongly reduced and leptin infusion restored plasma levels of leptin [0.84 ± 0.42, 2.53 ± 0.45, below detection limit of the assay (0.16), 1.27 ± 0.33 ng/ml for fed-vehicle, fed-leptin, fasted-vehicle and fasted-leptin, respectively].

*Food deprivation alters fever through a leptin-independent mechanism*

We then examined whether the repletion of leptin during fasting affects the fever response. On day 3, the animals received a single *i.p.* injection of saline or two different doses of LPS (100 or 1000 μg/kg) between 1000 and 1030, a time point approximately 48 h after the start of drug infusion/feeding manipulation. As described above, leptin repletion effectively prevented the fasting-induced hypothermia in saline-injected groups, reversing the lowered $T_{core}$ to levels similar to those of the fed animals (Figure VI-2A). Following the injection of the lower dose of LPS (100 μg/kg), the $T_{core}$ of the fasted rats was consistently lower than those of the fed counterparts (Figure VI-2C; 60–340 min, $P < 0.05$ for fed-vehicle *vs.* fasted-vehicle), confirming our recent study using the same protocol (Inoue et

al., 2008). However, leptin repletion in the fasted rats did not reverse the alteration of fever. In response to the higher dose of LPS (1000 μg/kg), fasting also attenuated the elevation of $T_{core}$, but it was less prominent than with the lower dose (Figure VI-2E). The $T_{core}$ of the fasted rats was significantly attenuated (relative to fed rats) only at the beginning of the fever response (60–160 min, $P < 0.05$, fed-vehicle *vs.* fasted-vehicle), thereafter the $T_{core}$ elevated to a level similar to those of fed counterparts. The leptin treatment in the fasted rats did not affect the $T_{core}$ course during fever.

Figure VI-2B, D and F show the average $T_{core}$ before (−1–0 h) and after injection (2–8 h, during the rising phase of fever). These graphs help to clarify two important points. First, the repletion of leptin in the fasted rats prevented the pre-injection hypothermia ($P < 0.01$, fasted-vehicle *vs.* fasted-leptin; $P > 0.05$ fed-vehicle *vs.* fasted-leptin), indicating a role for leptin in this process. Despite this observation, leptin treatment had no effect on the lowered post-injection $T_{core}$ ($P > 0.05$, fasted-vehicle *vs.* fasted-leptin; $P < 0.01$ fed-vehicle *vs.* fasted-leptin), suggesting that the fever alteration is a leptin-independent process. Second, although the post-injection $T_{core}$ was attenuated by fasting (in the case of the lower dose), the net elevation of $T_{core}$ ($\Delta T_{core}$ = pre-injection $T_{core}$ − post-injection $T_{core}$) was by no means attenuated, rather it was exaggerated in the fasted rats as compared to fed counterparts.

*Food deprivation alters fever through a leptin-dependent mechanism*

Previous studies reporting an altered fever response in fasted animals (including our own) all focused their observation on the rising phase of fever, within a few hours (~6 h) of pyrogen administration (Szekely, 1979; Kleitman and Satinoff, 1981; Shojoony, 1985; Shido et al., 1989; Inoue et al., 2008). Therefore, it remains unknown whether fasting and the fall in leptin levels affects the course of fever beyond the rising phase. The present study found that food deprivation strongly attenuated fever at a later stage and that this fever alteration was leptin-dependent (Figure VI-3). In response to the higher dose of LPS, the fed rats showed a long-lasting fever which remained unabated until the end of the recording period (~36 h). During this sustained fever phase, the $T_{core}$ of the fasted

rats progressively decreased and deviated from that of the fed counterparts (Figure VI-3A–B; 17–36 h, $P < 0.05$, fed-vehicle *vs.* fasted-vehicle). Leptin repletion in the fasted group almost completely prevented the decline of $T_{core}$ (20–27 h and 29–36 h, $P < 0.05$, fasted-vehicle *vs.* fasted-leptin). Figure VI-3C clarifies that all three groups of rats still maintained fever during this period as the post-injection $T_{core}$ is significantly higher than the respective pre-injection $T_{core}$ ($P < 0.001$ for pre-injection *vs.* post-injection). Fasting significantly lowered the post-injection $T_{core}$ ($P < 0.001$ for fed-vehicle *vs.* fasted-vehicle), and leptin repletion almost completely reversed the fasting-induced attenuation ($P < 0.001$ for fasted-vehicle *vs.* fasted-leptin, $P > 0.05$ for fed-vehicle *vs.* fasted-leptin). These results indicate that fasting diminishes the sustained phase of fever through a leptin-dependent mechanism during relatively severe inflammation.

With the lower dose of LPS, the fever response was more or less resolved within 12 h after injection in the fed group: the post-injection $T_{core}$ during the sustained phase (of the higher LPS dose) was only modestly, albeit significantly, elevated. In the fasted rats, the elevation of post-injection $T_{core}$ was not evident regardless of whether leptin was replenished or not (data not shown).

*The effects of food deprivation and leptin on immune-to-brain signalling*

We next sought to understand how fasting and the reduction in leptin levels alter the LPS-induced fever during the sustained phase. To this end, four groups of rats [(fed or fasted) x (vehicle or leptin)] were injected with LPS (1000 μg/kg, *i.p.*). An additional fed-vehicle group received an *i.p.* saline injection as a control. All animals were sacrificed 30 h after injection, a time point when leptin repletion significantly reversed fever attenuation in fasted rats. The leptin-dependent alteration of fever during fasting was confirmed in this cohort of animals (Figure VI-4A). Plasma leptin levels were confirmed to be reduced by fasting and were increased by leptin infusion (Figure VI-4B). We first examined if the fever alteration was attributable to the changes in febrigenic inflammatory response at the level of the brain. In the fed groups, LPS injection significantly up-regulated the mRNA levels of genes involved in PGE2 synthesis (IL-1β, COX-2 and mPGES-1) and increased the concentration of PGE2 in the CSF (Figure VI-4C–F;

$P < 0.05$, saline *vs.* LPS). Among the LPS-injected groups, a two-way (feeding x leptin) ANOVA revealed that fasting significantly reduced the CSF PGE2 levels ($P < 0.05$) and marginally attenuated IL-1β mRNA levels ($P = 0.08$). However, leptin did not affect any factors involved in PGE2 synthesis ($P > 0.05$ for leptin and interaction), suggesting that the leptin-mediated alteration of fever is dissociated from brain PGE2 synthesis. In addition, we studied other inflammatory mediators linked to fever that are independent of PGE2 (Minano et al., 1991b; Mouihate et al., 2004). Leptin affected neither the levels of MIP-1β mRNA in the hypothalamus nor 15-d-PGJ2 in the CSF during LPS-induced inflammation (Figure VI-4G–H).

*The effects of food deprivation and leptin on neuropeptides expression in the hypothalamus*

To examine the potential changes in thermoregulatory functions of the CNS, the mRNA levels of neuropeptides known to be thermogenic (POMC, CRH and CART) or anti-thermogenic (NPY and AgRP) were examined in the hypothalamus. A two-way (feeding x leptin) ANOVA found a marginal interaction in the levels of POMC, a prohormone from which β-endorphin is derived (Figure VI-5A, $P = 0.09$). A post-hoc analysis revealed a significant effect of leptin within the fasted groups ($P < 0.05$, fasted-vehicle *vs.* fasted-leptin). Moreover, POMC mRNA levels were found to be positively correlated with the magnitude of fever during the sustained phase in the fasted group (Figure VI-5B, $r^2 = 0.33$ and $P < 0.05$) but not in the fed groups ($r^2 = 0.27$, $P > 0.05$), suggesting that POMC (*i.e.*, its derivative β-endorphin) may be a downstream factor mediating leptin effects on fever during fasting. Fasting significantly reduced thermogenic CRH and increased anti-thermogenic NPY and AgRP mRNA levels (Figure VI-5C–F, $P < 0.05$). However, leptin repletion did not significantly affect these fasting-mediated changes ($P > 0.05$ for leptin and interaction).

*The effects of food deprivation and leptin on brown adipose tissue*

As a peripheral effecter mechanism, BAT comprises a major site of metabolic thermogenesis (Foster and Frydman, 1979) and has repeatedly been

linked to the fever response in rats (Jepson et al., 1988; Dascombe et al., 1989b; Morrison, 2003). Therefore, we examined whether leptin repletion in the fasted rats causes any change in the capacity of BAT thermogenesis. LPS injection in the fed rats unexpectedly reduced the mRNA levels of UCP-1 and DIO-2 ($P < 0.001$), two important molecules involved in BAT thermogenesis (Cannon et al., 1998), implying that the BAT thermogenesis per se may not significantly contribute to the fever response (at least in the sustained phase). In the LPS-treated groups, fasting dramatically reduced the levels of both UCP-1 and DIO-2 [two-way (feeding x leptin) ANOVA, $P < 0.01$ for feeding), an observation in agreement with the suppression of BAT thermogenesis during fasting. However, leptin repletion did not significantly reverse the reduction in UCP-1 and DIO-2 levels ($P > 0.05$ for interaction or leptin). UCP-3 levels were not affected by any of the experimental manipulations.

## Discussion

Previous studies have reported that acute starvation prior to inflammatory events attenuates the fever response in various species of experimental animals (Szekely, 1979; Kleitman and Satinoff, 1981; Molnar and Milner, 1983; Shojoony, 1985; Shido et al., 1989; Inoue et al., 2008). The present study examined if the changes in leptin levels, which decline during acute starvation (Ahima and Osei, 2004; Gautron et al., 2005; Inoue et al., 2008), are responsible for the alteration of the LPS-induced fever response in fasted rats. Our results demonstrated a complex interaction between the energy balance, leptin and the thermoregulatory systems involved in the fever response. It was clear that fasting altered the fever response and did so though two different, leptin-dependent and -independent, mechanisms depending on the phase of the fever. The fasting-induced alteration of fever was independent of leptin during the rising phase. However, the present study demonstrated, for the first time, that fasting reduced the severity of not only the rising phase but also the maintenance of a long-lasting fever caused by a relatively high dose of LPS. The diminution of sustained fever was reversed by leptin repletion in the fasted rats.

The second part of the study aimed to delineate the underlying mechanisms of the leptin-mediated fever alteration in the sustained phase. Characterization of febrigenic inflammatory responses in the brain clarified that leptin affected neither PGE2 synthesis nor the up-regulation of other inflammatory mediators linked to fever which function independently of PGE2 (*i.e.*, MIP-1β and 15-d-PGJ2). On the other hand, POMC mRNA levels were altered in accordance with the leptin-mediated changes in $T_{core}$ and were significantly correlated with the magnitude of the fever response in the fasted animals. The thermogenic capacities of BAT were not significantly altered by leptin as estimated by the mRNA levels of the genes important for BAT thermogenic activities (UCP-1 and DIO-2). Based on these results, we conclude that a decrease in leptin levels during acute starvation attenuates long-lasting fever during relatively severe inflammation. The leptin regulation of fever in the starvation paradigm is dissociated from the

febrigenic inflammatory response in the brain. Our data suggested that the changes in thermoregulatory mechanisms of the CNS appear to play an important role. One potential mechanism may be the reduced β-endorphin (a derivative of the prohormone POMC) tone due to decreased leptin signalling.

The main finding of the present study is that leptin plays a critical role in sustaining the fever response over a long period. In response to a relatively high dose of LPS (1000 μg/kg), the *ad lib* fed rats demonstrated a long-lasting fever that remained unabated until the end of the recording period (~36 h). Although the fasted counterparts initially mounted a fever response that was more or less equivalent to the fed rats, they failed to sustain $T_{core}$ elevation. The $T_{core}$ of the fasted rats progressively deviated from those of the fed counterparts from approximately 18 h after LPS injection. Leptin repletion in the fasted rats almost completely reversed the attenuation of the sustained fever response. To the best of our knowledge, the present study is the first to report 1) nutritional deprivation compromises long-lasting fever during the sustained phase, and 2) the contribution of leptin to such an alteration of fever. These results indicate that leptin serves as a link between the influence of nutritional status and the magnitude of fever. Such regulations are of fundamental importance to the survival of organisms given the substantial impact of fever (especially when it is long-lasting) on energy balance. However, it is important to note that the initial part of fever was, albeit moderately attenuated by fasting, independent of leptin. Therefore it appears that fever (an energy costly process) in its rising phase overrides a series of energy saving processes driven by the reduction of leptin including hypometabolism and hypothermia (Stehling et al., 1996; Doring et al., 1998; Geiser et al., 1998; Overton et al., 2001). These observations suggest that there exists a rather complex inter-relationship between energy balance regulation and the host defence response against infection, two important homeostatic mechanisms.

During the rising phase of fever, the pre-inflammation fasting clearly lowered the $T_{core}$, an observation which is in accordance with a number of previous studies (Szekely, 1979; Kleitman and Satinoff, 1981; Molnar and Milner,

1983; Shojoony, 1985; Shido et al., 1989; Inoue et al., 2008). However, it is important to note that fasting alone, under non-inflammatory conditions, caused hypothermia as was clearly seen in the saline-injected group. This raises the possibility that the thermoregulatory alteration (which is independent of fever) may be contributing to the overall fever response during inflammation. By taking this into account, the LPS-induced thermogenesis (or heat gain), when judged by the $\Delta T_{core}$ rise (post-injection $T_{core}$ − pre-injection $T_{core}$), was not attenuated and was even enhanced in the fasted groups (Figure VI-2D, F). The exaggeration of $\Delta T_{core}$ rise was greater when animals received the higher dose of LPS. In a recent study (Inoue et al., 2008), we reported a similar, dose-dependent enhancement of $\Delta T_{core}$ rise following an intracerebroventricular PGE2 injection in fasted rats. Thus, these results collectively suggest that the febrigenic capacity of the fasted rats remains largely intact and that the apparent suppression of fever may be attributable to changes in the thermoregulatory systems responding to the insufficient energy balance.

The repletion of leptin during fasting effectively prevented the development of hypothermia in the saline-injected animals (Figure VI-2A–B). This observation is in agreement with the reported roles of leptin, that a decrease in levels of this hormone initiates various physiological responses to starvation including hypothermia and suppression of metabolism under non-inflammatory conditions (Stehling et al., 1996; Doring et al., 1998; Geiser et al., 1998; Overton et al., 2001). Along with the resilience of the "febrigenic capacity" to fasting discussed above, we initially predicted that the leptin treatment, which effectively prevented fasting-induced hypothermia, would reverse the attenuation of fever. However, our results showed that leptin repletion did not in fact do this during the rising phase of fever. These results indicate that the alteration of the rising phase of fever is due to a mechanism(s) other than the one involved in the fasting-induced hypothermia under non-inflammatory conditions. In *ad lib* fed rats, absence of leptin signalling, due to genetic mutations or pharmacological blockade with a specific antiserum against leptin, blunts the rising phase of fever induced by LPS (Rosenthal et al., 1996; Ivanov and Romanovsky, 2002; Sachot et al., 2004;

Steiner et al., 2004). The basis of this apparent discrepancy is currently unclear; however, fasting certainly involves, in addition to the reduction in leptin levels, numerous hormonal and metabolic abnormalities. It is therefore possible that leptin is essential for normal fever in fed rats (and possibly in fasted rats), whereas it is not sufficient to reverse the altered fever response in the fasted rats during the rising phase.

To understand the mechanisms which use leptin as a permissive signal for the occurrence of a long-lasting fever during severe inflammation, we explored several possibilities. It is well established that nutritional deprivation compromises immune function via a reduction in leptin levels (Lord et al., 1998; Howard et al., 1999; Faggioni et al., 2000a; Mancuso et al., 2006). Therefore, we first addressed whether pre-inflammation fasting attenuates and leptin repletion recovers the febrigenic inflammatory signalling in the brain, an important prerequisite for fever response. Neither the mRNA levels of the genes involved in PGE2 synthesis (IL-1β, COX-2 and mPGES-1) nor the concentration of PGE2 in the CSF were altered by leptin treatment in the fasted rats. Likewise, leptin treatment had no effect on MIP-1β and 15-d-PGJ2 levels, inflammatory mediators that were reported to modulate fever independently of PGE2 (Minano et al., 1991b; Li et al., 2003; Mouihate et al., 2004). Therefore these results suggest that the leptin-mediated alteration of fever by fasting is dissociated from the pyrogenic inflammatory responses in the brain.

Indeed the only brain signal tested in the present study that was significantly altered by fasting was POMC. The hypothalamic mRNA levels of this neuropeptide were down-regulated by fasting in a leptin-dependent manner during LPS-induced fever. This observation is in accordance with a previous study showing that pre-inflammation food restriction reduced hypothalamic POMC mRNA levels as compared to fed counterparts during inflammation (Lennie et al., 2001), although contradictory results were also reported (Gautron et al., 2005). In the present study, the POMC mRNA levels changed in a way that reflects the fever alteration, and were positively correlated with $T_{core}$ elevation during the sustained phase of fever in the fasted groups (Figure VI-4B), implying that this

catabolic factor is a downstream mediator of leptin's actions. Leptin is known to act directly on POMC expressing neurons in the arcuate nucleus of the hypothalamus and stimulates its expression (Schwartz et al., 1997; Elmquist et al., 1999). POMC peptide is a precursor for several biologically active neuropeptides including α-MSH, β-endorphin and adrenal corticotrophic hormone (ACTH). Of particular interest is β-endorphin, as this endogenous opioid has been strongly linked to thermogenesis, including the fever response. An injection of β-endorphin into the preoptic anterior hypothalamus, a thermoregulatory centre, evokes hyperthermia similar to that induced by pyrogenic cytokines (Xin et al., 1997). Conversely, a blockade of μ-opioid receptor, one of the receptors for β-endorphin, either by specific antagonists or genetic mutation abrogates the fever response induced by classical pyrogens including LPS (Benamar et al., 2000; Tsai et al., 2003; Benamar et al., 2005; Fraga et al., 2008). Therefore it is conceivable that fasting attenuates β-endorphin tone in a leptin-mediated manner and thereby compromises the sustained fever response.

In summary, the present study further highlights the complexity of the thermoregulatory functions, especially when it is coupled with immune and nutritional challenges. The fasting clearly altered the overall fever response to LPS, by attenuating the $T_{core}$ during the course of fever. However, the underlying mechanisms for this alteration in the fever response involved different mechanisms during different fever phases. The leptin system, as a signal of sufficient energy balance, was found to be essential (and sufficient in fasted rats) for the occurrence of a long-lasting fever during severe inflammation. The leptin-dependent maintenance of fever was independent of febrigenic inflammatory responses in the brain, but it was associated with changes in levels of neuropeptides known to be involved in thermoregulation. Our preliminary data suggest that β-endorphin may be an important mediator of leptin fever regulation.

## Acknowledgements

This work was supported by the Canadian Institutes of Health Research (CIHR) and the National Sciences and Engineering Research Council (NSERC) of Canada. W.I. is a recipient of a PhD scholarship from the Nakajima Foundation, Japan.

## Figures and Tables

**Figure VI-1. Daily cycle of body temperature under non-inflammatory conditions.**

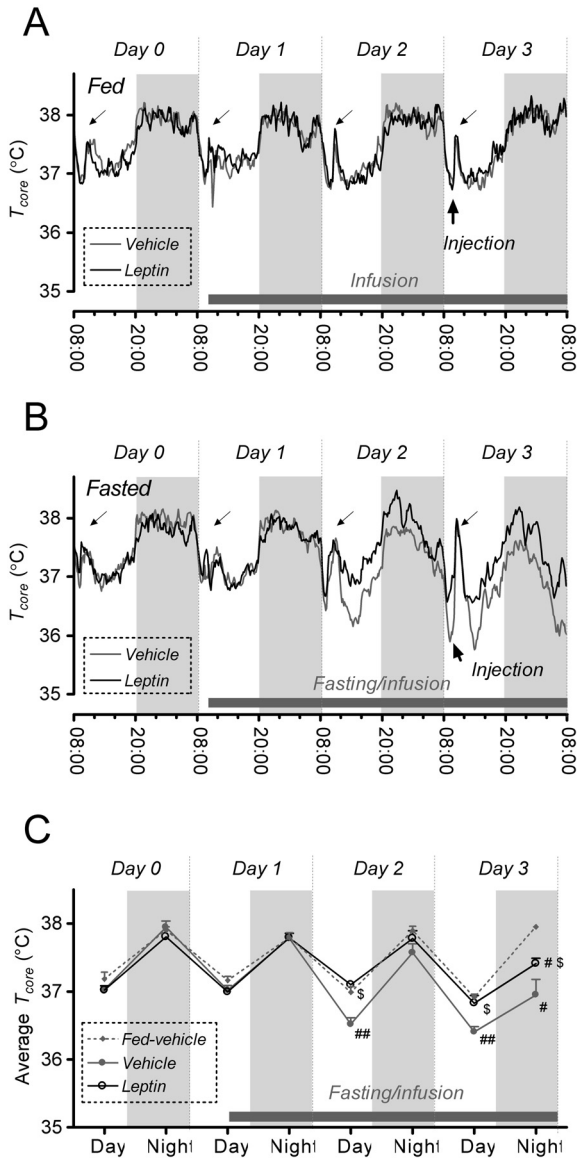


*Figure VI-1. (A) The $T_{core}$ of ad lib fed rats infused with vehicle or leptin. The time of injection (with saline) is indicated by a thick arrow, and small thin-angled arrows indicate body weight measurement. Note that the handling of animals caused a transient hyperthermia. The grey background represents the dark period (2000–0800). (B) The $T_{core}$ of fasted rats infused with vehicle or leptin. (C) The*

*average $T_{core}$ in the active and inactive phases. Values are mean + S.E.M. for 5–8 rats. # P < 0.05, ## P < 0.01, fed-vehicle vs. fasted-vehicle; $ P < 0.05, fasted-vehicle vs. fasted-leptin within the same day.*

**Figure VI-2. $T_{core}$ change after LPS injection–the rising phase.**

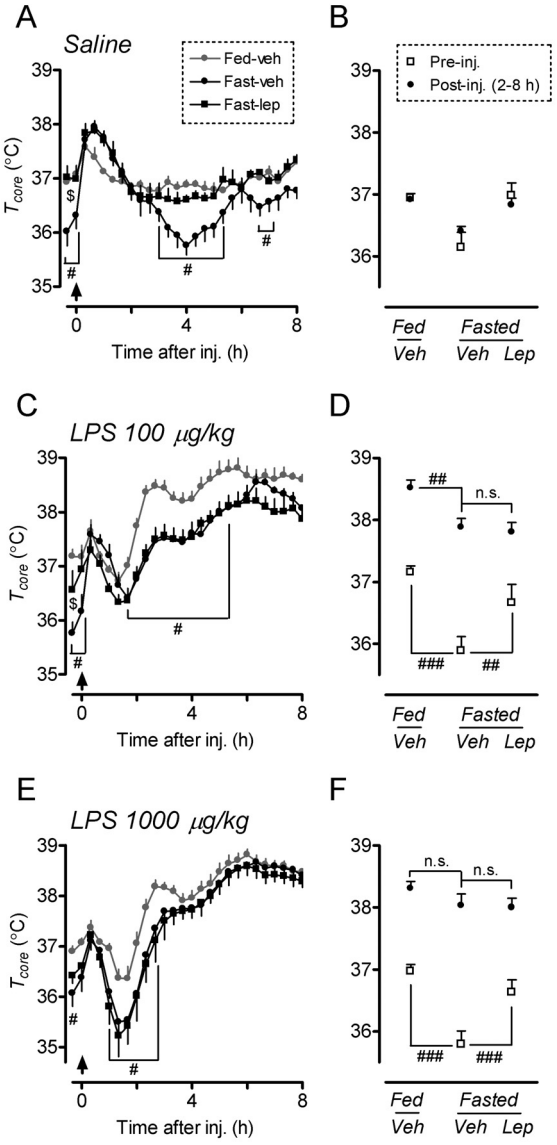


*Figure VI-2. $T_{core}$ course of rats treated with an i.p. injection of saline (A), LPS 100 μg/kg (C) or LPS 1000 μg/kg (E) at time point 0 h. Values are means ± S.E.M. for 5–8 rats. # P < 0.05, fed-vehicle vs. fasted-vehicle; $ P < 0.05, fasted-vehicle*

*vs. fasted-leptin. The average pre- and post-injection $T_{core}$ of rats treated with saline (B), LPS 100 μg/kg (D) or LPS 1000 μg/kg (F). Values are means ± S.E.M. for 5–8 rats. Two-way (treatment x time) ANOVA, interaction and time P > 0.05, treatment P < 0.001 for (B); interaction, treatment and time P < 0.001 for (D); interaction P < 0.01, treatment and time P < 0.001 for (F). Pair wise comparisons, $^{\#}$ P < 0.05, $^{\#\#}$ P < 0.01, $^{\#\#\#}$ P < 0.001.*

**Figure VI-3. $T_{core}$ change after LPS injection–the sustained phase.**

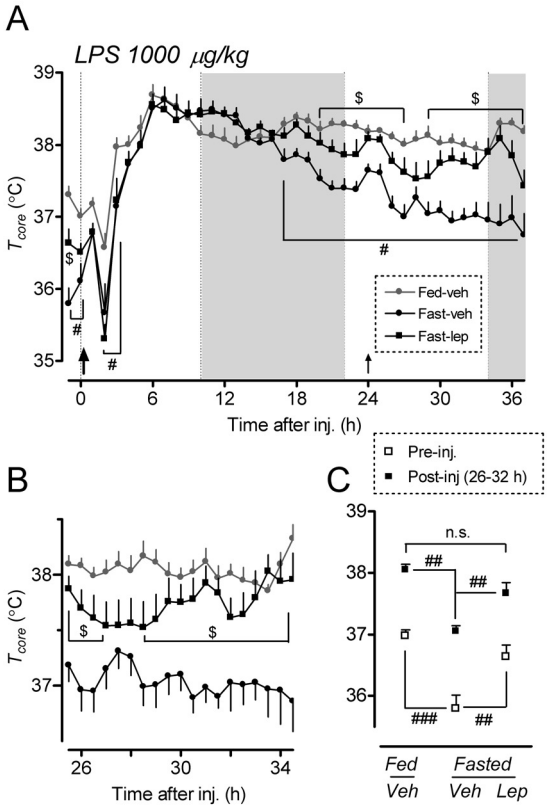


*Figure VI-3. (A) Course of rat $T_{core}$ over 36 h after an i.p. injection of LPS 1000 μg/kg. (B) The graph between 26 and 34 h is enlarged. Values are means ± S.E.M. for 8 rats. $^{\#}$ P < 0.05, fed-vehicle vs. fasted-vehicle; $^{\$}$ P < 0.05, fasted-vehicle vs. fasted-leptin. (C) The average pre- and post-injection $T_{core}$. $^{\#\#}$ P < 0.01.*

**Figure VI-4. $T_{core}$, plasma leptin and hypothalamic gene expression during LPS-induced fever.**

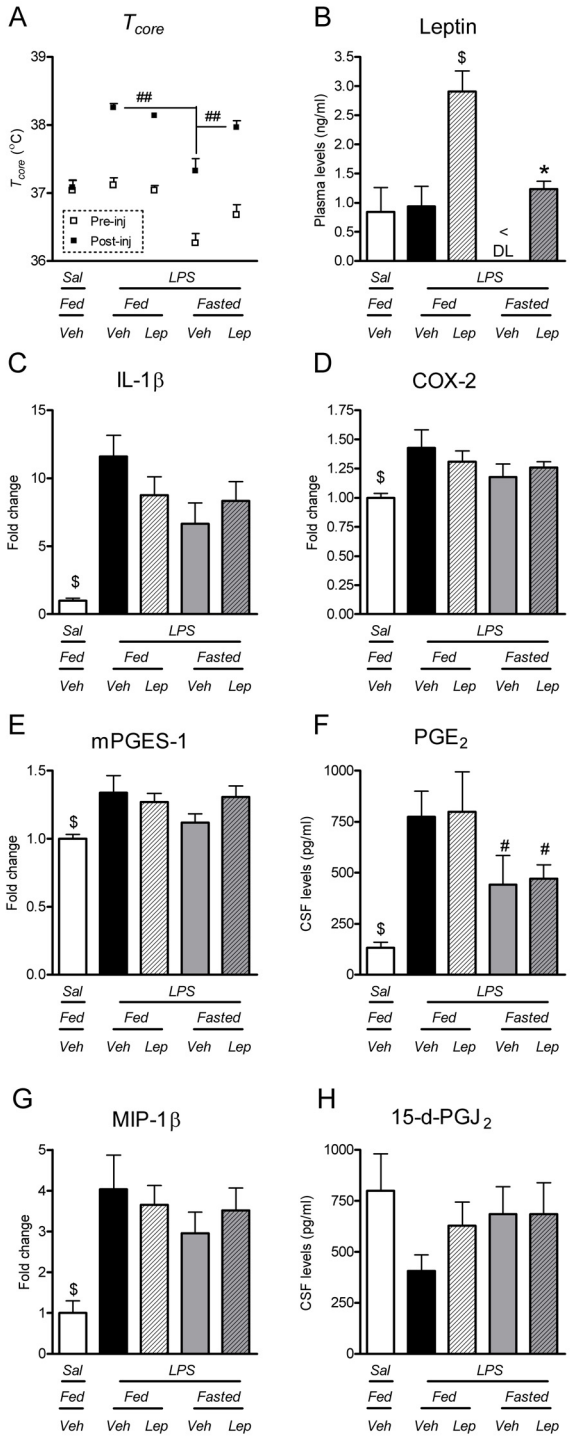


*Figure VI-4. (A) The average pre- and post-injection $T_{core}$.* $^{\#\#}$ *$P < 0.01$. (B) Plasma concentration of leptin 30 h after LPS injection (1000 μg/kg, i.p.).* $^{\$}$ *$P <$*

*0.05, fed-vehicle-LPS vs. fed-leptin-LPS; P < 0.05 fasted-vehicle-LPS vs. fasted-leptin-LPS. (C–H) qRT-PCR analysis of mRNA levels in the hypothalamus 30 h after injection. $ P < 0.05 fed-vehicle-saline vs. fed-vehicle-LPS. Two-way (feeding x LPS) ANOVA within LPS groups, #P < 0.05 for the main effect of fasting. Values are mean ± S.E.M. for 5–6 rats.*

**Figure VI-5. Hypothalamic gene expression during LPS-induced fever.**

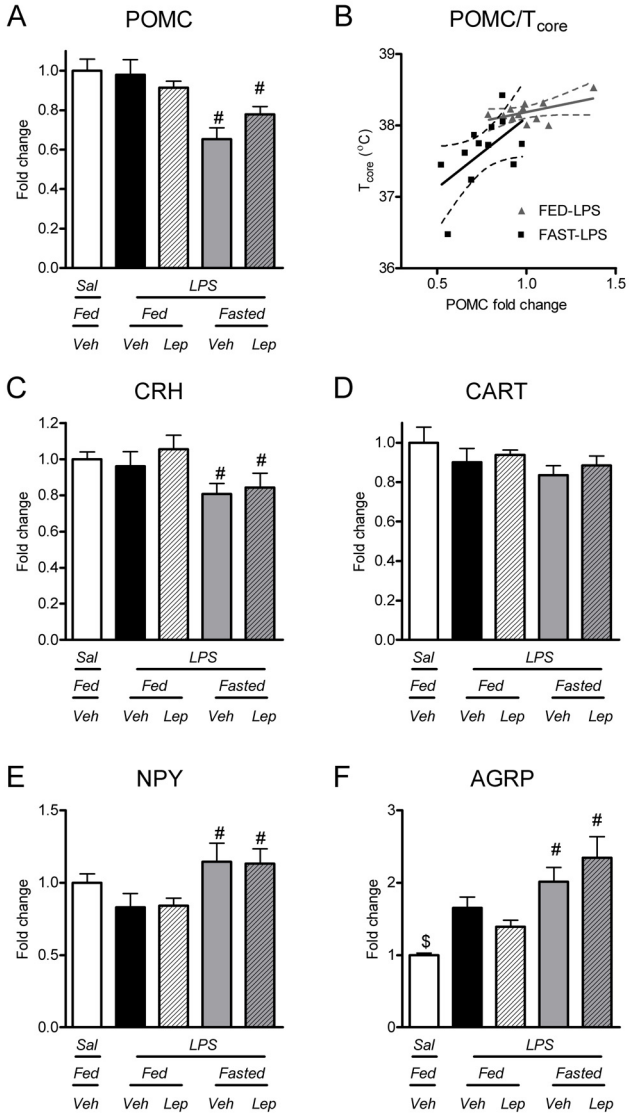


*Figure VI-5. (A, C–F) qRT-PCR analysis of mRNA levels in the hypothalamus 30 h after injection. Values are mean ± S.E.M. for 5–6 rats. $ P < 0.05 fed-vehicle-*

*saline vs. fed-vehicle-LPS. Two-way (feeding x LPS) ANOVA within LPS groups, # P < 0.05 for the main effect of fasting. (B) Correlation between POMC mRNA levels and $T_{core}$.*

**Figure VI-6. BAT gene expression during LPS-induced fever.**

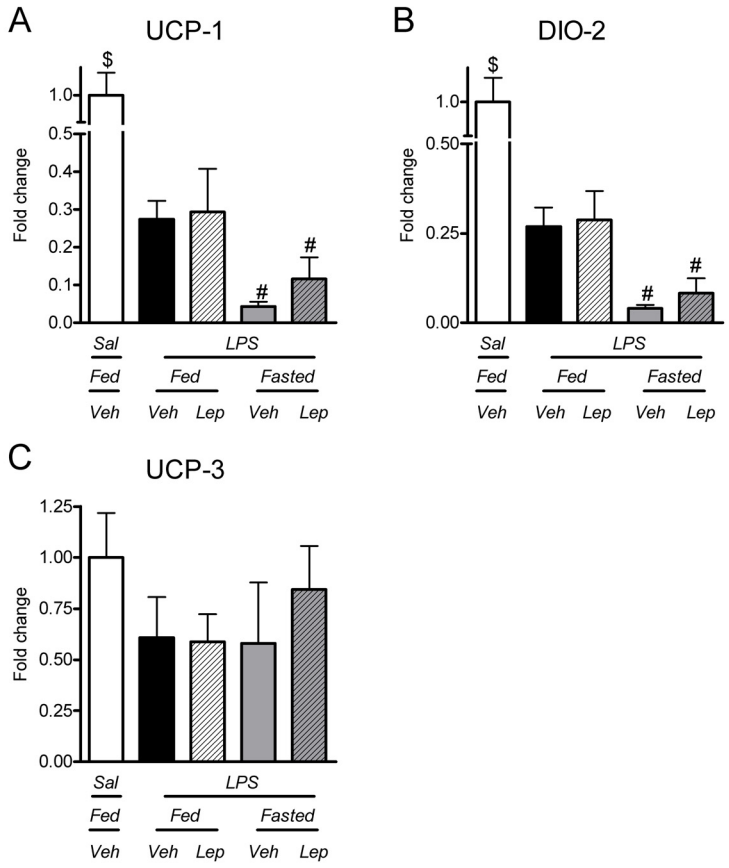


*Figure VI-6. (A–C) qRT-PCR analysis of mRNA levels in brown adipose tissue 30 h after injection. Values are mean ± S.E.M. for 5-6 rats. $ P < 0.05 fed-vehicle-saline vs. fed-vehicle-LPS. Two-way (feeding x LPS) ANOVA within LPS groups, # P < 0.05 for the main effect of fasting.*

**Table VI-1. The effects of fasting, leptin and LPS on body weight**

| | Fed | | | | | |
|---|---|---|---|---|---|---|
| | Saline | | LPS 100 µg/kg | | LPS 1000 µg/kg | |
| | vehicle (6) | leptin (6) | vehicle (7) | leptin (8) | vehicle (8) | leptin (8) |
| Baseline BW (g) | 273.7 ± 17.8 | 269.4 ± 14.3 | 258.1 ± 6 | 262.1 ± 19 | 266.3 ± 10.7 | 265.4 ± 8.7 |
| BW at inj. (g) | 273.5 ± 15.7 | 267.3 ± 14.1[a] | 260.3 ± 4.8 | 255.5 ± 15.6 [a] | 269.9 ± 14 | 263.9 ± 12.8 [a] |
| 24 h BW change after inj. (%) | 0.2 ± 2.1 | 0.1 ± 1.3 | -2.9 ± 1.8 [c] | -2.7 ± 2.2 [c] | -5.1 ± 2.4 [c, d] | -5.3 ± 2.4 [c, d] |

| | Fast | | | | | |
|---|---|---|---|---|---|---|
| | Saline | | LPS 100 µg/kg | | LPS 1000 µg/kg | |
| | vehicle (7) | leptin (8) | vehicle (8) | leptin (6) | vehicle (8) | leptin (7) |
| Baseline BW (g) | 258.5 ± 8.5 | 257.3 ± 12.3 | 257.1 ± 9.7 | 263.1 ± 7.1 | 261.3 ± 10.6 | 271.2 ± 9.3 |
| BW at inj. (g) | 221.3 ± 10 [b] | 223 ± 9.9 [b] | 222.1 ± 9.1 [b] | 229.2 ± 5.4 [b] | 225 ± 8.1 [b] | 238.5 ± 10.6 [b] |
| 24 h BW change after inj. (%) | -5.8 ± 2.2 | -5.7 ± 1.5 | -6 ± 1.3 | -6.6 ± 1.6 | -5.2 ± 1.3 | -6.1 ± 1 |

[a], Two-way ANOVA (leptin x time); $P < 0.01$ for drug x time; post hoc, $P < 0.05$ within leptin, BW at inj. *vs.* Baseline

[b], Two-way ANOVA (leptin x time); $P < 0.001$ for time

[c], Two-way ANOVA (drug x injection); $P < 0.001$ for injection; host hoc $P < 0.001$, *vs.* Saline

[d], Two-way ANOVA (drug x injection); $P < 0.001$ for injection; host hoc, $P < 0.01$, *vs.* LPS 100 µg/kg

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## VI.3. Supplementary results and discussion

The main finding of the present manuscript is that leptin contributes to the maintenance of long-lasting fever that occurs during relatively severe inflammation. This observation supports the general hypothesis of this thesis that leptin links energy balance (food deprivation) and sickness behaviour (fever). However, the mechanisms by which leptin links these two responses are different from what we originally anticipated: the recovery of fever by leptin in the fasted rats was not attributable to the inflammatory component of fever (Figure VI-4). We show that leptin prevented fasting-induced hypothermia under non-inflammatory conditions (*i.e.*, in saline-injected rats, Figure VI-1) and increased POMC levels in the hypothalamus of the fasted rats during LPS-induced inflammation (Figure VI-5). Therefore, it is reasonable to think that the leptin-mediated fever recovery in the current starvation paradigm was mainly attributable to its role in thermoregulatory functions. At present, how leptin affects thermoregulatory functions is largely unknown, especially when it is combined with fever. This issue highlights an interesting physiological question, how organisms prioritize two opposing drives, energy saving to cope with starvation and energy expenditure to cope with infection.

In the present manuscript, we failed to detect a significant effect of leptin on CSF PGE2 levels during LPS-induced inflammation, negating its role in brain inflammation in the current experimental setting. However, this does not fully exclude a role for leptin in the innate inflammatory response in the brain. It is still possible that the technique used in the present study (qPCR for mRNA levels and EIA for CSF PGE2 levels) may not be sensitive enough to reveal potential leptin-dependent changes in the inflammatory mediators. More stringent methods, such as *in situ* hybridization or immunohistochemistry to examine region specific gene expression, or microdialysis to study local PGE2 concentration/turnover, may help to detect leptin-dependent inflammatory processes that can account for the changes in fever. In addition, it is important to examine whether a blockade of PG

synthesis by a specific COX-2 inhibitor attenuates the fever response in the presence of leptin in fasted animals.

It is also possible that aspects of innate inflammation other than those directly involved in fever may be regulated by leptin in the current rat model. We recently obtained preliminary data suggesting that leptin may contribute to BBB disruption during a sustained fever response. Figure VI-7 shows immunohistochemical detection of endogenous rat IgG in the brain. The brains examined were well perfused with heparinized-saline to flush out blood from the cerebrovasculature. The detection of IgG in the brain sections indicates an entrance of IgG into the brain across the BBB, thus a disruption of the BBB (as IgG proteins cannot cross an intact BBB). The experiment was performed on the same groups of animals studied in the present manuscript (Figure VI-4). In the saline-injected groups, IgGs were barely detectable except for in the meningis and the choroid plexus and along large blood vessels regardless of feeding conditions (fed or fasted) and leptin infusion (vehicle or leptin) (data not shown). The pattern and intensity of the staining were similar in the fed rats following LPS injection regardless of leptin treatment (Figure VI-7). In contrast, leptin dramatically increased IgG staining when repleted in the fasted rats during LPS-induced inflammation, indicating its role in BBB disruption during inflammation. However, this phenomenon appears to involve a complex mechanism; the combination of LPS and leptin under fed conditions did not affect BBB integrity, whereas it did so dramatically under fasted conditions (see Figure VI-4 for leptin levels). Therefore it is likely that the interaction between leptin and factor(s) associated with fasting plays an important role in this potentially neuroinflammatory process. At present, specific mechanisms involved in this phenomenon are unclear. In a mouse model of sepsis (Chapter IV), we have shown that leptin promotes neutrophil recruitment into the brain by increasing cytokine, chemokine and adhesion molecule expression in the brain. The neutrophil recruitment process is closely linked with BBB breakdown (Papadopoulos et al., 2000; Bohatschek et al., 2001; McColl et al., 2008). Therefore it will be interesting to examine whether leptin stimulates neutrophil

recruitment and expression of the genes involved in this process (*e.g.*, neutrophil chemokines and adhesion molecules) during a sustained fever response in rats.

**Figure VI-7. Immunohistochemical detection of rat IgG in rat brains**

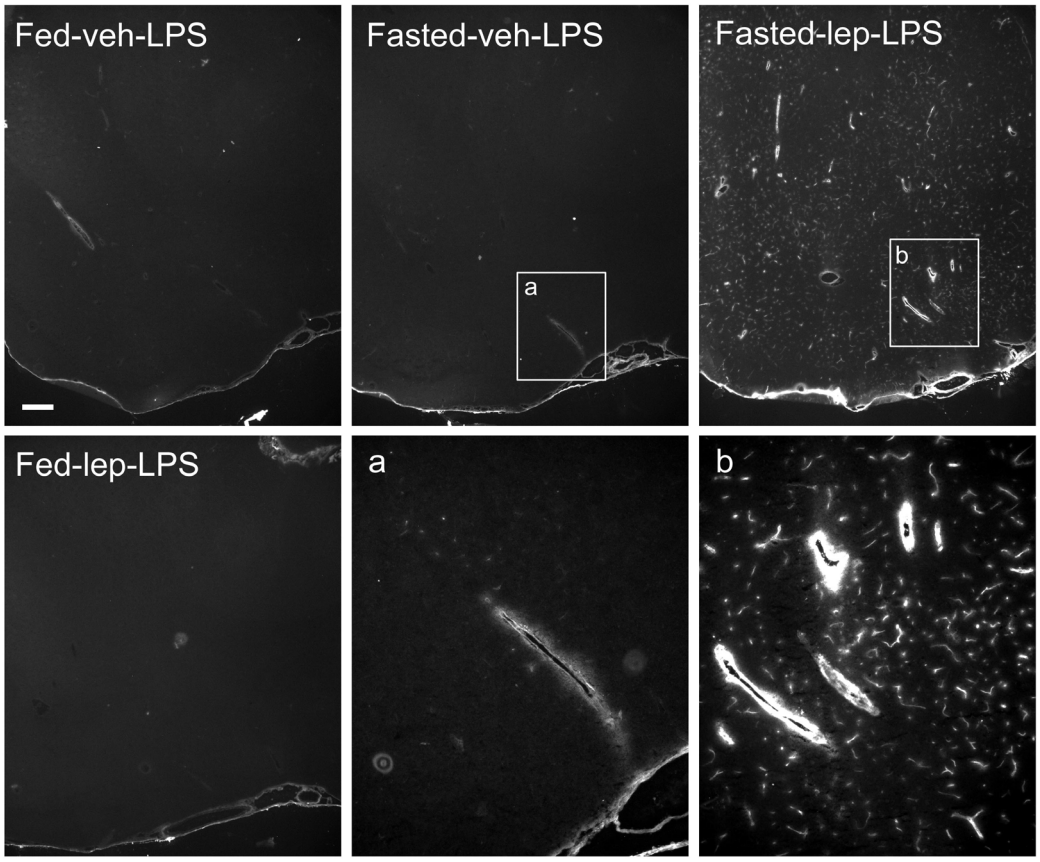


*Figure VI-7. Immunohistochemical detection of rat IgG in rat brains. The IgG immunoreactivity was increased in leptin-treated, LPS-injected fasted rats. (a and b) Areas enclosed by white rectangles are magnified. Scale bar 400 µm (100 µm for a and b)*

# General discussion and conclusion

Sickness behaviour is an evolutionally conserved, homeostatic mechanism that facilitates the survival of the host at the time of illness (Dantzer, 2001; Konsman et al., 2002). The behavioural and metabolic responses associated with sickness behaviour are energy demanding processes and are elaborately linked with the nutritional status of the host (Kleitman and Satinoff, 1981; Shido et al., 1989; Lennie et al., 1995; Gautron et al., 2005). A previous study from our laboratory demonstrated that leptin, a body weight regulating hormone, is able to induce fever by activating an inflammatory-type response in the brain (Luheshi et al., 1999). This result signalled the starting point of our investigation into the complex inter-relationship between energy balance and sickness behaviour. The present thesis focused on the role of leptin in activating the brain's innate inflammatory response, a key brain mechanism contributing to the development of sickness behaviour, and addressed three main questions 1) Does leptin activate innate inflammatory response in the brain? 2) Does leptin link the brain's innate inflammatory response with energy balance? 3) Does leptin link fever with energy balance? The conclusions derived from a series of experiments are presented below and can be summarized in Figure 1.

### *Does leptin activate the innate inflammatory response in the brain?*

The findings from Chapter II demonstrated that an administration of exogenous leptin induces IL-1β and PG synthesis, the classical innate inflammatory mediators, in the brain *in vivo*. Moreover, pharmacological intervention against endogenous leptin (by using LAS) during LPS-induced systemic inflammation significantly blunted the synthesis of IL-1β and PG in the brain. The induction of IL-1β and PG is a key step for immune-to-brain signalling and ensuing sickness behaviour (*e.g.*, fever and anorexia) (Chapter I1.4−1.5). Therefore, the results strongly support a role for leptin as an afferent inflammatory signal to the brain during inflammation (Figure 1). In addition, the results from this chapter demonstrated, for the first time, that leptin activates non-

neuronal cells of the brain, namely brain-resident macrophages and endothelial cells. Because leptin is known to directly target neurons to regulate body weight homeostasis (Friedman and Halaas, 1998), the findings presented in this thesis provide an anatomical basis for different actions of leptin (body weight regulation *vs.* inflammation) mediated by distinct cell groups (neurons *vs.* non-neurons).

In Chapter III, we examined the molecular mechanisms underlying the inflammatory actions of leptin by using a primary culture of rat microglia. This *in vitro* study demonstrated that leptin stimulates microglial IL-1β release via a STAT3-dependent mechanism. Perhaps most interestingly, the leptin-induced IL-1β release was independent of caspase-1, a protease known to mediate IL-1β release following a number of inflammatory stimuli including LPS (Dinarello, 1998; Schonbeck et al., 1998). This is an important finding, as it highlighted the difference in the mode of action of leptin with classical inflammatory stimuli, such as LPS. An atypical inflammatory action of leptin was further supported by the subsequent experiments demonstrating that leptin alone did not induce TNF and IL-6 (LPS induced both cytokines), whereas leptin significantly potentiated the release of these cytokines when combined with LPS. These observations suggested that leptin may be a modulator/enhancer rather than a *bona fide* stimulator of inflammation. As a hormone regulating body weight homeostasis, leptin constitutively exists in the general circulation and CSF in a ng/ml concentration range (Ishihara et al., 2004). Collectively, the results from Chapter III supported a dual role of leptin in energy balance and brain inflammation, and led to the second part of our hypothesis that it may be an important homeostatic factor for optimal microglial function (and possibly other brain cells involved in inflammation). In a physiological context, whilst leptin alone may not be a strong inflammatory stimulus, its decrease/deficiency may compromise microglial function and their response to other inflammatory stimuli. One obvious example of such a physiological condition is during starvation which dramatically reduces leptin levels (Ahima et al., 1996) and compromises immunity, at least in the periphery (Wing et al., 1988; Chandra, 1992).

**Figure 1. Conclusion**

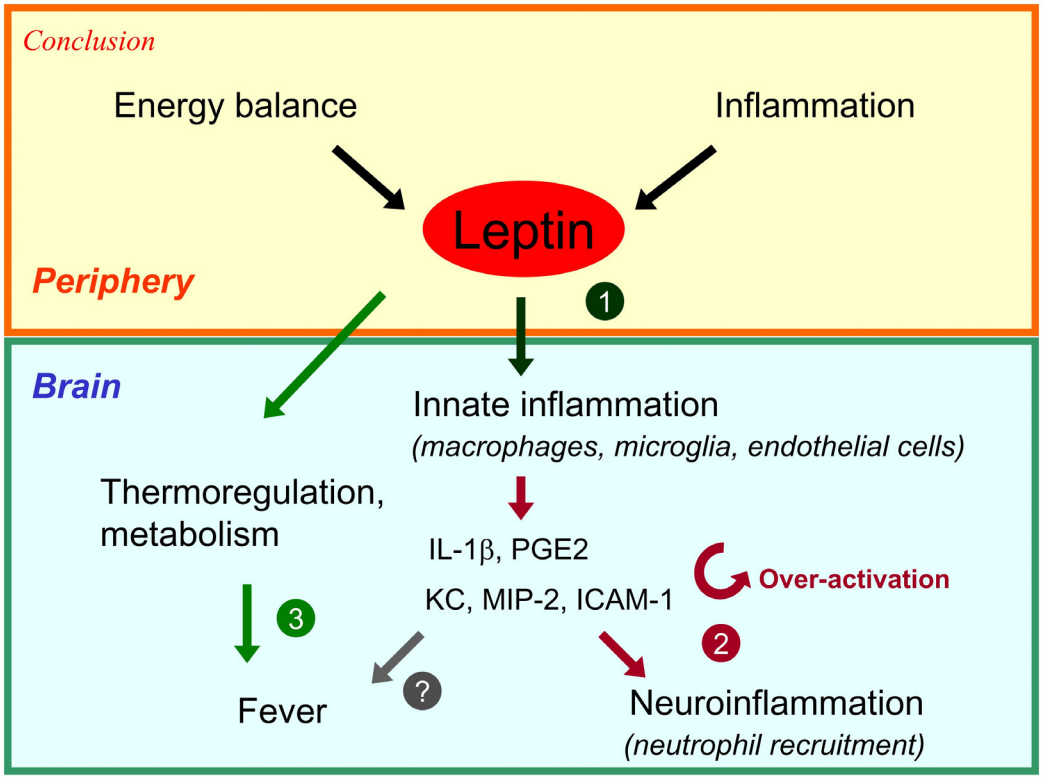


*Figure 1. Conclusion. (1) The data presented in chapters II and III demonstrated that leptin acts on non-neuronal cells and activated the brain's innate inflammatory response. (2) Chapter IV showed that leptin links energy balance and neuroinflammation (neutrophil recruitment) via the modulation of the brain's innate inflammation (cytokine, chemokine and adhesion molecule expression). (3) Chapter V and VI revealed that leptin links energy balance and the fever response. Leptin regulation of fever was associated with its effects on thermoregulation/metabolism and was dissociated from the brain's innate inflammation (indicated by ?).*

***Does leptin link the brain's innate inflammatory response with energy balance?***

The conclusions made based on the aforementioned observations were examined further in the second part of the thesis. In Chapter IV we investigated whether leptin links the nutritional status of the host with its innate inflammatory

response in the brain. The results from these studies demonstrated that food deprivation significantly attenuates the induction of cytokines, chemokines and adhesion molecules as well as neutrophil recruitment to the brain caused by severe systemic inflammation (LPS-induced sepsis). Replenishment of leptin in fasted animals effectively reversed all the indices of neuroinflammation, supporting a role for leptin in linking energy balance with brain inflammation. The importance of leptin in this neuroinflammatory process was further confirmed by using two additional models of leptin deficiency, genetic mutation (*ob/ob* and *db/db* mice) and pharmacological intervention, with the same outcome seen in the food deprived animals. The results from this chapter added substantially to our understanding of the physiological significance of leptin, by highlighting a dual role for this hormone whereby linking energy balance and neuroinflammation following a systemic pathogenic stimulus (Figure 1).

***Does leptin link fever with energy balance?***

In the final part of this thesis (Chapters V and VI), we tested whether leptin plays a role in the inter-relationship between energy balance and the fever response as a recognized outcome of inflammatory mechanisms in the brain (Chapter I1.6). This was particularly relevant to the current thesis given that fever is an energy demanding process and will likely be influenced by the changes in the energy status of the animals. We first established a rat model of food restriction, in which we demonstrated a negative effect on the fever response to a pyrogenic dose of LPS (Chapter V). Repletion of leptin during acute starvation effectively prevented, at least part of, the fever attenuation (Chapter VI), supporting a role for this cytokine-like hormone in linking energy balance and the fever response. However, how leptin does this remains to be identified. What is clear is that the leptin regulation of the fever response, in the current starvation paradigm, is dissociated from central PGE2 production (Chapters V and VI), indicating that the brain's febrigenic inflammatory pathway is, to our surprise, resistant to nutritional perturbation. On the other hand, the results from Chapter VI suggested that leptin contributes to the fever response most likely via the

activation of thermoregulatory functions, based on it ability to increase hypothalamic POMC levels (a prohormone for thermogenic β-endorphin) during LPS-induced inflammation and the leptin-mediated reversal of the fasting-induced hypothermia under non-inflammatory conditions. The examination of the contribution of leptin to fever in the starvation paradigm highlighted the pleiotropic actions of leptin, implicated in not only body weight and inflammation but also thermoregulation (Figure 1). In addition, it is obvious from these observations and those made in earlier chapters (Chapters II–IV) that the involvement of leptin in the brain's inflammatory response is complex and depends to some degree on the level of the pathogenic challenge.

In summary, the findings presented in this thesis demonstrated the interaction between energy balance and host defence responses to infection. An adipose tissue-derived cytokine, leptin, plays a critical role in this relationship in part by linking energy balance with sickness behaviour and the brain's innate inflammatory response. Emerging evidence indicates that the brain's innate immune response contributes to the development and/or progression of various brain pathologies (Chapter I1.6), and that some of these brain diseases are under the influence of the nutritional status of the individuals (Chapter I4.2). By adding significantly to our knowledge of the role of leptin, this thesis will hopefully contribute to a better understanding of the physiology of brain diseases that may involve the interaction between nutritional status, immunity and brain.

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