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ORIGINAL ARTICLE

# Leptin regulates leukocyte recruitment into the brain following systemic LPS-induced inflammation

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**The appetite suppressing hormone leptin has emerged as an important modulator of immune function and is now considered to be a critical link between energy balance and host defense responses to pathogens. These 'adaptive' responses can, in situations of severe and sustained systemic inflammation, lead to adverse effects including brain damage that is partly mediated by neutrophil recruitment into the brain. We examined the contribution of leptin to this process in leptin-deficient (*ob/ob*), -resistant (*db/db*) and wild-type (WT) mice injected intraperitoneally with a septic dose of lipopolysaccharide (LPS). This treatment induced a dramatic increase in the number of neutrophils entering the brain of WT mice, an effect that was almost totally abolished in the mutant mice and correlated with a significant reduction in the mRNA levels of interleukin-1β, intracellular adhesion molecule-1 and neutrophil-specific chemokines. These effects were reversed with leptin replenishment in *ob/ob* mice leading to recovery of neutrophil recruitment into the brain. Moreover, 48 h food deprivation in WT mice, which decreased circulating leptin levels, attenuated the LPS-induced neutrophil recruitment as did a single injection of an anti-leptin antiserum 4 h before LPS treatment in WT mice. These results provide the first demonstration that leptin has a critical role in leukocyte recruitment to the brain following severe systemic inflammation with possible implications for individuals with altered leptin levels such as during obesity or starvation.**
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## Introduction

Leptin is an adipocyte-derived hormone whose primary function is to regulate energy balance by acting on hypothalamic neurons.[1] It is now known that this hormone can influence a multitude of physiological systems including immunity.[2,3] The importance of this cytokine-like mediator in immune function was clearly demonstrated in mice with genetic leptin deficiency (*ob/ob*) or resistance (*db/db*), which exhibit various signs of immunodeficiency,[4,5] are susceptible to bacterial infection[6,7] or resistant to autoimmune diseases.[8,9] The abnormalities exhibited by these mice are analogous to starvation-induced immunosuppression, which is accompanied by a dramatic reduction in circulating leptin levels and is reversible by leptin replenishment.[10,11] These observations led to the proposal that leptin represents a critical signal that links the nutritional status of the host to its ability to effectively respond to a pathogenic challenge. Our previous results[12,13] and those of others[14,15] supported this proposal by demonstrating that leptin acts as an important circulating mediator of systemic infection or inflammation by activating brain mechanisms responsible for sickness behaviors. This was based on results showing an acute rise in circulating leptin levels following systemic bacterial lipopolysaccharide (LPS) or other inflammatory agents,[16–18] as well as its ability to induce fever and appetite loss along with brain interleukin (IL)-1β and prostaglandin synthesis,[12,14,19,20] and the attenuation of LPS-induced sickness behaviors by a neutralizing antiserum against leptin.[13,15] In a recent study,[21] we characterized the sites of leptin's action on the brain during systemic LPS inflammation and found that it acts predominantly on the leptomeningis, cerebral fissures and vasculatures, which collectively form the barrier-related structures of the brain. These structures were shown to express leptin receptors,[22–24] and brain macrophages residing in them were reported to exhibit IL-1β expression following leptin or LPS

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stimulation.[20,25,26] While these results reinforce the proposed link between leptin and brain IL-1β and the ensuing sickness behaviors, 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, the same 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.[27,28] 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.[21] Therefore, in this 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 also involved in circulating leukocyte recruitment, whereby it could potentially contribute to subsequent neuropathological conditions associated with severe systemic inflammation[29,30] and/or psychiatric conditions such as eating disorders that are linked to abnormal leptin levels[31] and brain inflammatory events.[32,33] To this end, we used a mouse model of sepsis induced by systemic injection of a high dose of LPS, which has previously been shown to trigger recruitment of neutrophils into the brain,[34] and examined brain expression of innate inflammatory mediators and neutrophil recruitment in wild–type (WT), *ob/ob* and *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,[10,11] 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.

By demonstrating a link between leptin and leukocyte recruitment, we highlight in this study the importance of this hormone to an individual's ability to respond effectively to a pathogenic challenge, especially in situations of leptin deficiency such as during prolonged inflammation-induced anorexia.

## Materials and methods

*Animals*

Adult male *ob/ob* (B6. V-*Lep*$^{ob}$/J) and *db/db* (B6.Cg-*m* +/+ *Lepr*$^{db}$/J) mice maintained on a C57BL/6J background were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). In all studies 7-week-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;[35,36] 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 0830 hours) 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*

The appropriate conditions for investigating neutrophil recruitment to the brain during LPS-induced systemic inflammation were determined in a pilot study. Detailed protocols and results are available in 'supplementary information' at Molecular Psychiatry's website. On the basis of these results (Supplementary Figure S1), WT, *ob/ob* and *db/db* mice were injected with either LPS (*Escherichia coli* 0111:B4; cat: L-2630; Sigma, Ontario, Canada) at a dose of 2.5 mg kg$^{-1}$ (intraperitoneally, i.p.) or with pyrogen-free saline, and their brains collected 24 h after the treatment. The dose of LPS was adjusted to the weight of the animals to account for possible confounding effects due to the higher body weight of *ob/ob* and *db/db* mice (average of 40 g) compared with their WT counterparts (average of 20 g). A separate group of *ob/ob* mice was treated with either murine leptin (1 μg μl$^{-1}$, 6 μg day$^{-1}$; PeproTec, Rocky Hill, NJ, USA) or saline delivered by Alzet miniosmotic pumps (0.25 μl h$^{-1}$; model 1002, Durect, Cupertino, CA, USA) 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$^{-1}$) using Alzet pumps before 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 (NIBSC) at 0.5 ml kg$^{-1}$ 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.[21]

Animals were killed 24 h after LPS or saline treatment by terminal anesthesia (pentobarbital; 70 mg kg$^{-1}$, i.p.) and blood samples collected by cardiac puncture. Mice were then transcardially perfused with ice-cold saline with 5 IU ml$^{-1}$ 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*

For all studies, brain sections were collected from three levels (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.[37]

To perform immunohistochemistry and reverse transcription (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, quantitative RT-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 is as follows: IL-1β (Mm00434228_m1), CD14 (Mm00438094_g1), intracellular adhesion molecule (ICAM)-1 (Mm00516024_g1), tissue inhibitor of metalloproteinase (TIMP)-1 (Mm00441818_m1), keratinocyte-derived chemokine (KC) (Mm01354329_g1), macrophage inflammatory protein (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 immunoglobulin G (IgG) antibody (1:500 dilution; cat: 712-165-150, Jackson Immuno Research, West Grove, PA, USA). Cell nuclei were stained with 4,6-diamidino-2-phenylindole (1:4000 dilution in phosphate-buffered saline; Molecular Probes, Eugene, OR, USA). All antibodies were diluted in the blocking solution (10% normal donkey serum (Chemicon International, Temecula, CA, USA), 0.2% Triton-X100 in phosphate-buffered saline). 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, USA) was used as a primary antibody.

For the double labeling of neutrophil/ICAM-1, the anti-neutrophil antibody was mixed with a goat anti-mouse ICAM-1 antibody (1:200 dilution; cat: sc-1511, Santa Cruz Biotechnology). All secondary antibodies were multiple labeling-grade, raised in donkey, and used at 1:500 dilution (Jackson Immuno Research): CY3-conjugated anti-rat IgG for neutrophil and anti-goat IgG (cat: 705-095-147) for ICAM-1. 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 and goat IgG for 7/4 and ICAM-1, respectively; Jackson Immuno Research). Inappropriate crossreaction between antibodies was confirmed to be negligible (data not shown).

*Assessment of neutrophil recruitment*

Three different levels of the brain (at the levels of the subfornical organ, the arcuate nucleus and the area postrema) were analyzed. First, for each animal, three to four sections were analyzed at each brain level and the mean value was 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 $\times 2.5$ 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 Supplementary Figure S2. The number of neutrophil-positive labeling as defined by size and light intensity was determined using an automatic counting procedure (Scion Image 4.0.2, Scion, Frederick, MD, USA). Obvious artifacts due to dust or air bubbles were manually excluded from the area of interest.

*Cytokine enzyme-linked immunosorbent assay*

Plasma levels of tumor necrosis factor (TNF), IL-1β and IL-6 were measured in duplicate using a mouse cytokine ELISA kit (Quantikine; R&D systems, Minneapolis, MN, USA) according to the manufacture's instruction. The detection limits of the assay were 10.2, 6.0, 6.0 pg ml$^{-1}$ for TNF, IL-1β and IL-6, respectively. Plasma leptin levels were measured by a mouse leptin enzyme-linked immunosorbent assay (NIBSC), as described previously.[21] The detection limit of the assay was 156.2 pg ml$^{-1}$.

*Data analysis*

All data were presented as mean values ± s.e.m. Data were analyzed using a one-way analysis of variance 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

*Systemic LPS-induced neutrophil recruitment into the brain is leptin dependent*

To examine the role of leptin-ObRb signaling in neutrophil recruitment, we compared the accumulation of neutrophils in the brain in WT, *ob/ob* and *db/db* mice 24 h after the LPS challenge (2.5 mg kg$^{-1}$, i.p.). The robust neutrophil accumulation observed in LPS-treated WT mice was almost completely abolished in *ob/ob* (Figure 1) or *db/db* (not shown) mice. Importantly, leptin replenishment in *ob/ob* mice clearly reversed the recruitment. The neutrophil numbers were quantified in coronal sections collected at three levels of the brain (at the level of the subfornical organ, the arcuate nucleus and the area postrema, see representative images in Supplementary Figure S2). The average total cell number from the three brain levels (3–4 sections per level × 3 levels, $n = 5$–6 per group) are shown in Figure 1b. The neutrophil accumulation in LPS-treated *ob/ob* or *db/db* mice was significantly lower than in WT counterparts ($P < 0.001$). Although LPS tended to increase neutrophil numbers in *ob/ob* and *db/db* mice, this effect was not statistically significant ($P > 0.05$, LPS vs saline). Leptin replenishment in *ob/ob* mice significantly reversed the attenuation of LPS-induced neutrophil accumulation ($P < 0.05$).

*Leptin is involved in cerebral ICAM-1 upregulation during LPS-induced systemic inflammation*

A previous study demonstrated that the adhesion molecule ICAM-1 has a crucial role in neutrophil recruitment into the brain associated with LPS-induced systemic inflammation.[34] Therefore, we next

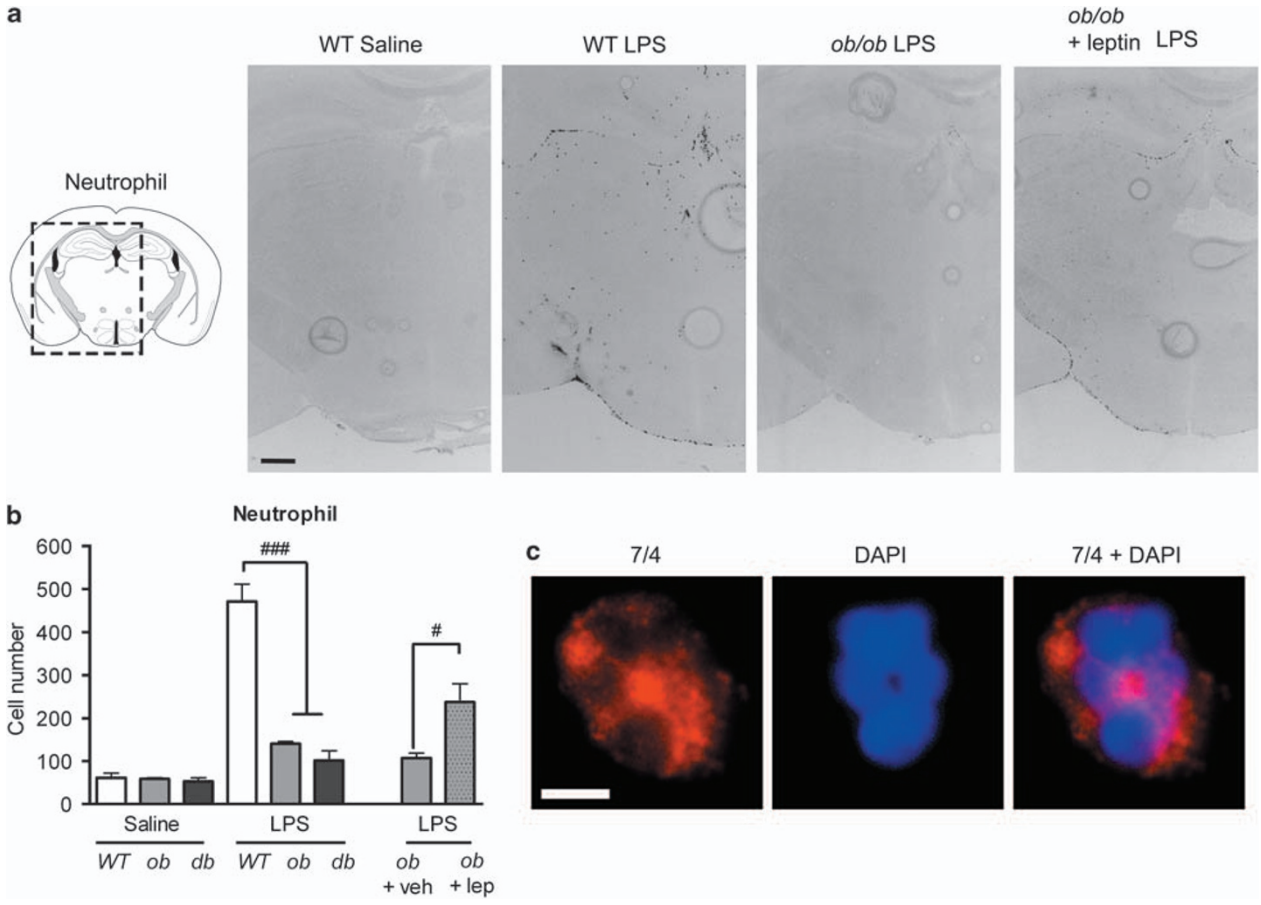


**Figure 1** Lipopolysaccharide (LPS)-induced neutrophil recruitment to the brain of wild-type (WT), ob/ob and leptin-treated ob/ob mice. (**a**) Representative images depicting neutrophil distribution in coronal sections of the brain in the area shown by an atlas template. The fluorescent images are presented in brightfield for clarity of contrast at low magnification. In WT mice, LPS (2.5 mg kg$^{-1}$, intraperitoneally) dramatically increased neutrophil accumulation 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 ventricles, the interhemispheric and hippocampal fissures, the leptomeningis and vasculatures. Scale bar = 400 μm. (**b**) Quantitative data for neutrophil recruitment. The neutrophil numbers were quantified in coronal sections collected at three levels of the brain (at the level of the subfornical organ, the arcuate nucleus and the area postrema). The average total cell number from the three brain levels (3–4 sections per level × 3 levels, $n = 5$–6 per group) are shown here. Values are presented as means ± s.e.m. *P*-values: $^{\#} < 0.05$, $^{\#\#\#} < 0.001$. (**c**) Images of a neutrophil at high magnification. 7/4-immunoreactivity (IR) (red) and its segmented nucleus characteristic of granulocyte neutrophils stained with 4,6-diamidino-2-phenylindole (DAPI) (blue). Scale bar = 5 μm.

asked whether leptin-ObRb signaling is important for cerebral ICAM-1 expression. ICAM-1 protein and mRNA expression were analyzed in the brains of WT, *ob/ob* and *db/db* mice 24 h after the LPS challenge. Figure 2a shows that, in WT mice, LPS treatment strongly increased ICAM-1-immunoreactivity (IR) along vasculature and in the leptomeningis, ependymal linings of the ventricles and the choroid plexus when compared with saline controls. The increase in ICAM-1-IR was clearly attenuated in both *ob/ob* (Figure 2a) and *db/db* mice (data not shown), and this effect was reversed by leptin replenishment in *ob/ob* mice. 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* and *db/db* (not shown) mice, indicating that leptin is not critical for basal ICAM-1 expression in the brain under non-inflammatory conditions. Consistent with the protein expression profile, the LPS treatment induced a 20-fold increase of ICAM-1 mRNA expression in WT mice as measured by quantitative RT-PCR (Figure 2b). In *ob/ob* and *db/db* mice, the LPS-induced ICAM-1 mRNA expression was significantly attenuated ($P<0.001$, approximately 50% of the levels of WT counterparts), and the reduction was nearly completely reversed by leptin replenishment in *ob/ob* mice ($P<0.01$).

Double immunohistochemistry for ICAM-1 and 7/4 revealed that most, if not all, of the accumulating 7/4-IR neutrophils were closely associated with ICAM-1-IR structures (Figure 2c), an observation in line with the reported functional importance of ICAM-1 for neutrophil recruitment.[34] Interestingly, some 7/4-IR cells were found adhering to the abluminal (brain) side of the ICAM-1-IR vasculature-like structures.

### *Leptin-mediated neutrophil recruitment is associated with cerebral IL-1β and neutrophil chemokine upregulation*

Systemic inflammation triggers *de novo* synthesis of various inflammatory mediators in the brain, some of which, in addition to ICAM-1, have key roles in

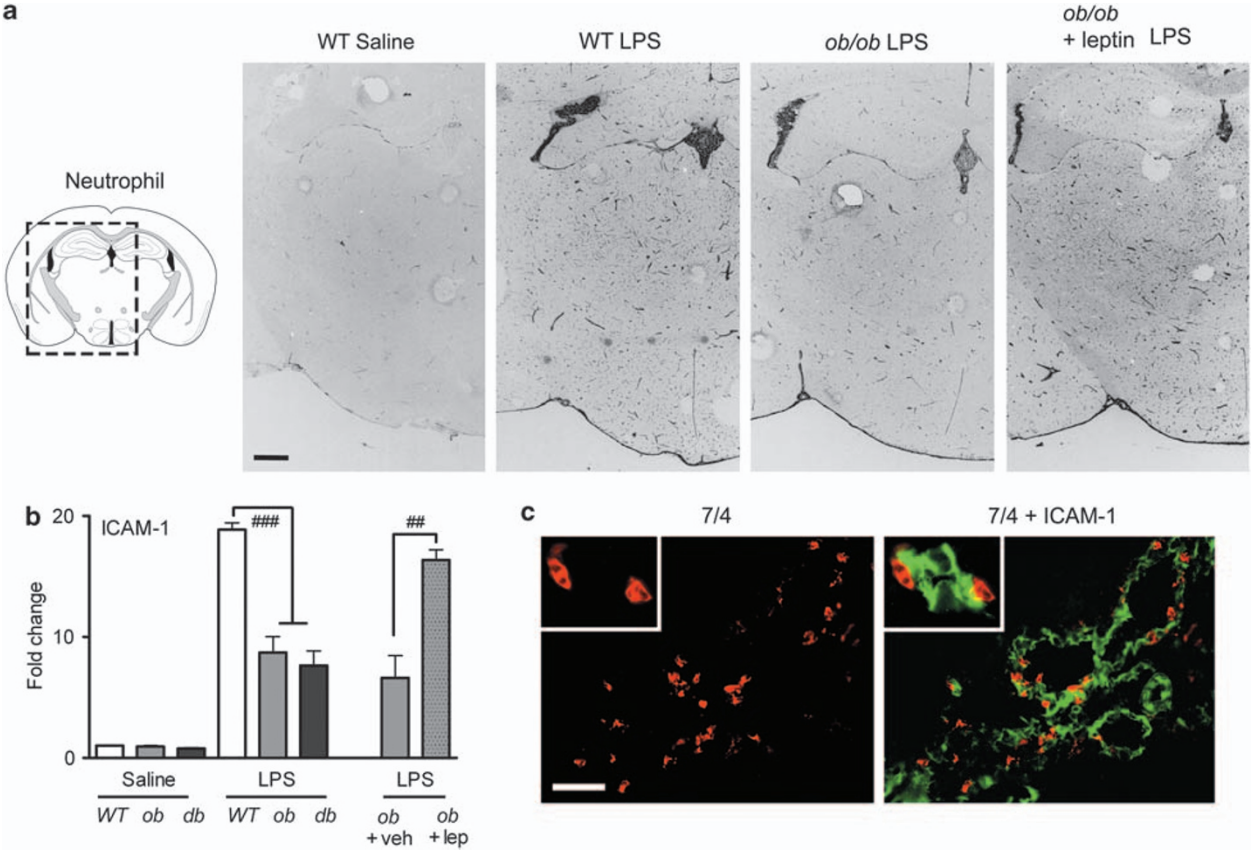


**Figure 2** Lipopolysaccharide (LPS)-induced intracellular adhesion molecule-1 (ICAM-1) expression in the brain of wild-type (WT), ob/ob and leptin-treated ob/ob mice. (**a**) Representative images depicting ICAM-1 protein expression in coronal sections of the brain in the area shown by an atlas template. The fluorescent images are presented in brightfield for clarity of contrast at low magnification. LPS treatment (2.5 mg kg$^{-1}$, intraperitoneally) dramatically increased ICAM-1-immunoreactivity (IR) in the leptomeningis, cerebral vasculatures and fissures, and the choroid plexus in WT mice 24 h after the challenge. The increase of ICAM-1-IR was clearly attenuated in *ob/ob* mice and was reversed by leptin treatment in these animals. Scale bar = 400 μm. (**b**) ICAM-1 mRNA levels in the brain analyzed by using quantitative reverse transcription-PCR. Values are presented as means + s.e.m. ($n=5$–6). *P*-values: $^{\#\#}<0.01$, $^{\#\#\#}<0.001$. (**c**) Double immunohistochemistry for ICAM-1 and neutrophils (7/4 antigen) in the brain of LPS-treated WT mice. 7/4-IR (red) was closely associated with ICAM-1-IR (green). Scale bar = 50 μm.

cerebral recruitment of leukocytes. To examine the contributions of leptin-ObRb signaling to this brain response, mRNA levels of several innate inflammatory mediators were measured by using quantitative RT-PCR. The LPS treatment resulted in a 120-fold increase in IL-1β mRNA levels ($P<0.001$) in WT mice when compared with saline controls (Figure 3). This increase was dramatically attenuated in both *ob/ob* and *db/db* mice ($P<0.001$), and replenishment of leptin in *ob/ob* mice significantly reversed the attenuated IL-1β mRNA levels ($P<0.05$). Similarly, LPS-induced TNF mRNA levels were significantly reduced in *ob/ob* ($P<0.05$) and *db/db* ($P<0.05$) mice. However, unlike IL-1β, leptin replenishment in *ob/ob* mice did not reverse the attenuation of TNF. We in addition detected significant effects of leptin-ObRb signaling on the LPS-induced increases of the key neutrophil chemokines KC and MIP-2 (Figure 3). In contrast, the increase of CD14 mRNA, another LPS-inducible innate inflammatory gene,[38] was comparable between WT, *ob/ob* and *db/db* mice, and the increase of TIMP-1, an endogenous metalloproteinase inhibitor also implicated in leukocyte infiltration into the brain,[39] was enhanced in *ob/ob* ($P<0.05$) but not

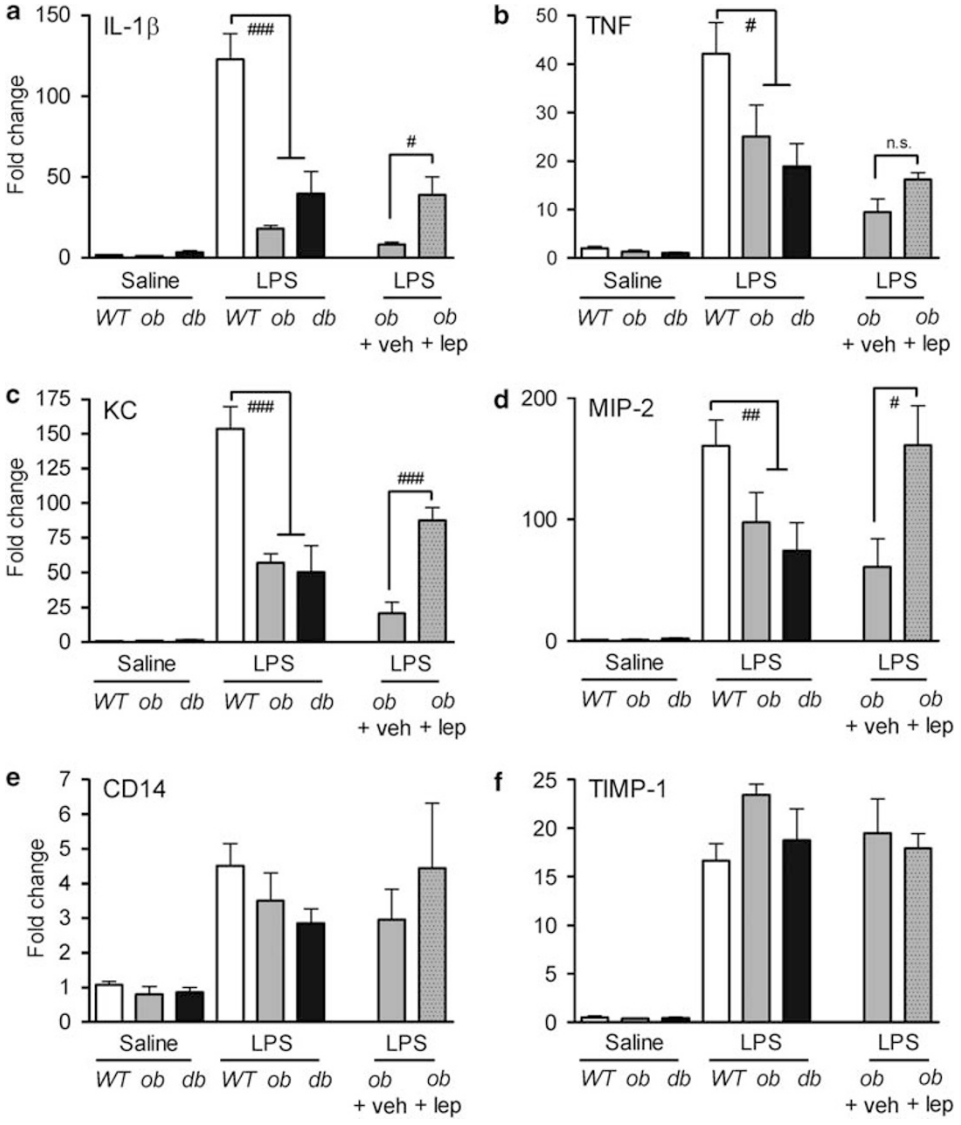


**Figure 3** mRNA expression of innate inflammatory mediators in the brain following systemic lipopolysaccharide (LPS) challenge in wild-type (WT), ob/ob and db/db mice. mRNA levels in the brain were analyzed 24 h after LPS challenge (2.5 mg kg$^{-1}$, intraperitoneally) using quantitative RT-PCR. (**a–d**) LPS-induced mRNA levels of interleukin (IL)-1β, tumor necrosis factor (TNF), keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (MIP)-2 were dramatically reduced in *ob/ob* and *db/db* mice when compared with WT controls. (**e** and **f**) The LPS-induced CD14 and tissue inhibitor of metalloproteinase (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, KC and MIP-2, whereas it had no effect on TNF, CD14 and TIMP-1 mRNA increase, as compared with vehicle infusion. Values are presented as means + s.e.m. ($n=5$–6). *P*-values: $^{\#}<0.05$, $^{\#\#}<0.01$, $^{\#\#\#}<0.001$; NS, not significant.

in *db/db* mice. These results indicate that the impaired mRNA induction of ICAM-1, IL-1β, KC and MIP-2 were 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-ObRb signaling to their gene regulation in the brain.

Genetic deficiency of leptin or ObRb may cause an abnormal systemic inflammatory response to LPS,[40,41] which in turn affects brain responses. To examine this possibility, plasma levels of cytokines (TNF, IL-1β and IL-6) were measured in the same groups of mice. The results are summarized in Supplementary Table S1, which shows that the levels of all cytokines were below the detection limit of the assay in saline-treated animals regardless of the genotypes. In WT mice, TNF and IL-6 levels were significantly elevated by LPS, whereas IL-1β levels, although detectable, were not statistically higher than the saline controls (which were assigned the value of the detection limit of the assay) 24 h after injection. In contrast to the apparent attenuation of brain inflammation (that is, neutrophil recruitment and inflammatory gene expressions), the LPS-induced elevations of TNF and IL-6 levels were significantly enhanced in *ob/ob* ($P<0.001$) and *db/db* ($P<0.001$) mice as compared with WT counterparts. Leptin replenishment in *ob/ob* mice significantly reversed the exaggerated IL-6 ($P<0.01$) but not TNF levels. IL-1β levels were not affected by leptin or ObRb signaling. Plasma leptin levels were not altered by LPS treatment in WT mice but were significantly increased in *db/db* animals ($P<0.001$). As expected, leptin was not detectable in *ob/ob* mice in any of the treatment groups and leptin infusion in *ob/ob* mice increased leptin levels ($P<0.001$).

### *Acute starvation suppresses the brain innate inflammatory response by a decrease in circulating leptin levels*

Given leptin's role in 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 before LPS administration (2.5 mg kg$^{-1}$, i.p.), and the inflammatory responses in the brain were assessed 24 h after the injection. The LPS-induced neutrophil accumulation was significantly attenuated by fasting without leptin infusion as compared with *ad lib* feeding ($P<0.01$), and leptin replenishment significantly reversed neutrophil accumulation ($P<0.05$, Figure 4a). Fasting impaired the LPS-induced innate inflammatory gene expression in the brain in a manner almost identical to leptin or leptin receptor genetic deficiency (Figures 4b–h). Briefly, fasted mice with vehicle infusion showed impaired LPS-induced increase in IL-1β ($P<0.001$), KC ($P<0.01$), MIP-2 ($P<0.05$) and ICAM-1 ($P<0.001$) mRNA levels while having comparable CD14 and TIMP-1 mRNA increases when compared with *ad lib*-fed counterparts. Leptin replenishment during fasting reversed the

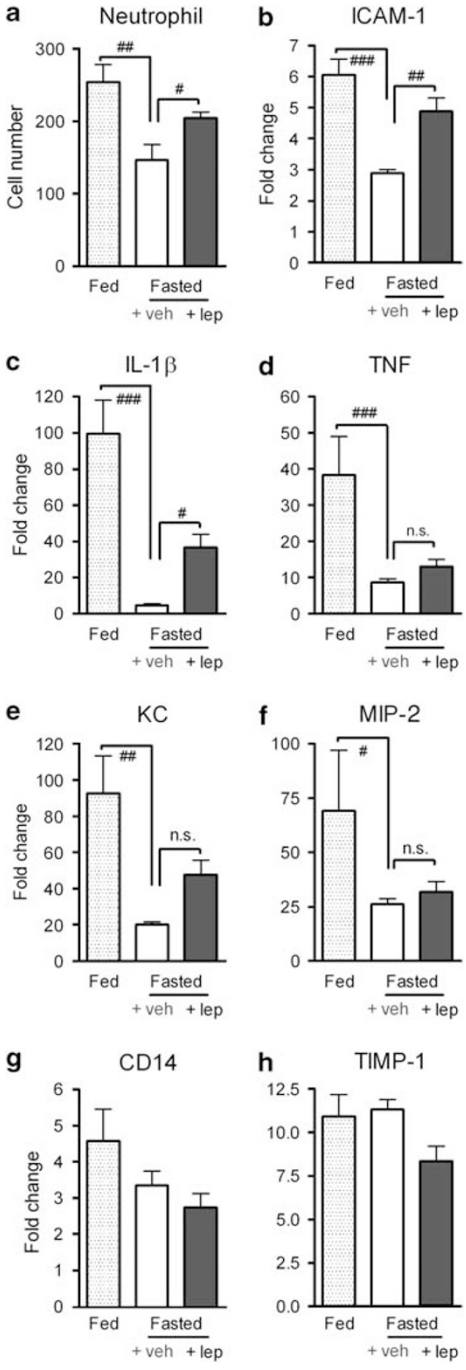


**Figure 4** The effects of fasting and leptin replenishment on neutrophil recruitment and brain inflammatory gene expression during systemic LPS inflammation. (**a**) Mice were fed, fasted or fasted with leptin replenishment for 48 h before LPS treatment (2.5 mg kg$^{-1}$, intraperitoneally). Neutrophil recruitment was quantified 24 h after the LPS challenge. (**b–h**) The levels of mRNA expression in the brain were measured by quantitative reverse transcription (RT)-PCR in the same animals. Values are presented as means + s.e.m. ($n=5$). *P*-values: $^{\#}<0.05$, $^{\#\#}<0.01$, $^{\#\#\#}<0.001$. NS, not significant.

induction of IL-1β ($P<0.05$) and ICAM-1 ($P<0.01$) mRNA levels. Although there was a tendency for KC mRNA levels to recover, this did not reach statistical

significance. MIP-2 mRNA levels were not affected by leptin replacement. In saline-treated groups, fasting with or without leptin did not alter neutrophil numbers ($44.3 \pm 3.7$ and $44.0 \pm 2.6$ for vehicle and leptin, respectively) and inflammatory gene expressions in the brain (data not shown).

Supplementary Table S1 shows that neither fasting nor leptin infusion affected the plasma levels of TNF, IL-1β or IL-6, an observation in contrast to the case of genetic leptin deficiency. Fasting reduced plasma leptin levels below the detection limit of the assay, and leptin infusion in fasted mice increased leptin levels.

*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 could be used as a potential tool for therapeutic intervention. To test this, WT mice received a single injection of LAS ($0.5\ ml\ kg^{-1}$, i.p.) or a control preimmune sheep serum 4 h before the administration of LPS ($2.5\ mg\ kg^{-1}$, i.p.) or saline, and the brains examined 24 h after LPS treatment. Figure 5a shows that LAS treatment significantly attenuated the LPS-induced neutrophil accumulation in the brain ($P<0.001$). 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 ($P<0.01$) and MIP-2 ($P<0.001$) mRNA induction, whereas it did not affect the induction of ICAM-1 and IL-1β mRNA (Figures 5b–h). In addition, LPS-induced TIMP-1 mRNA expression was attenuated by LAS ($P<0.01$).

Analysis of circulating cytokine levels in this experiment showed that LPS-induced increases in plasma TNF and IL-6 levels ($P<0.01$) were not affected by LAS treatment (Supplementary Table S1). Neither LPS not leptin altered IL-1β levels. The leptin levels both in the LAS–saline and LAS–LPS groups were below the detection limit of the assay, confirming the effectiveness of LAS treatment.

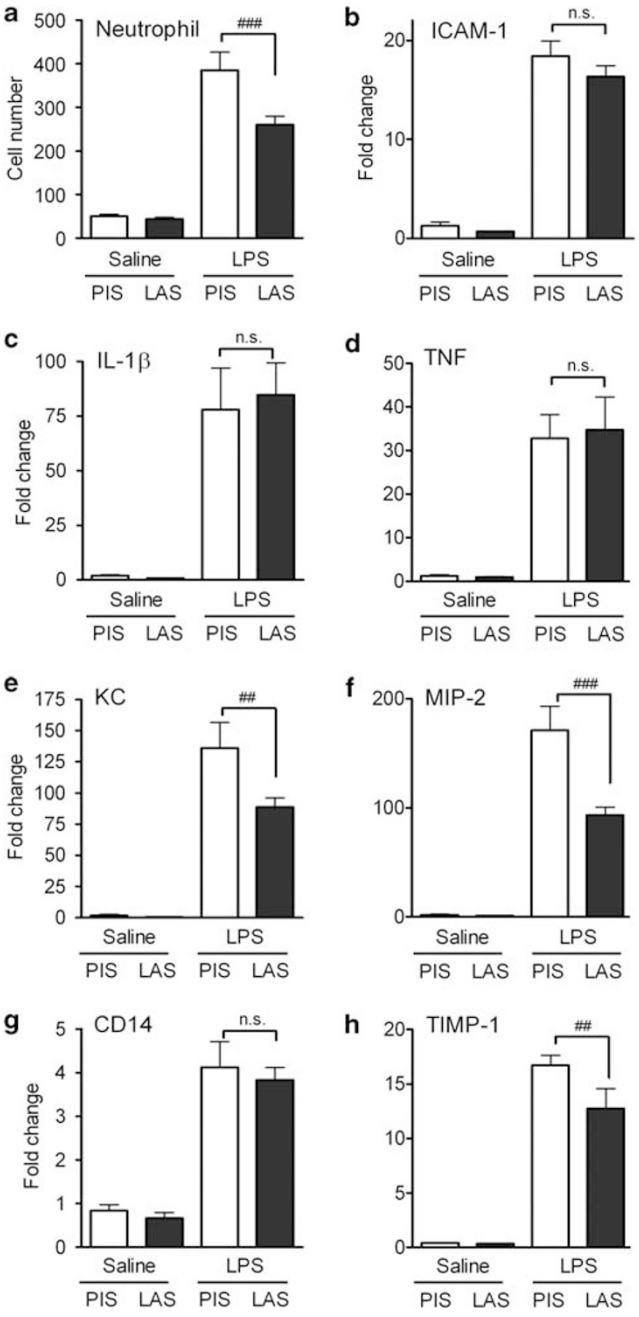


**Figure 5** The effects of acute leptin neutralization on brain inflammatory gene expression and neutrophil recruitment during systemic lipopolysaccharide (LPS) inflammation. Mice were treated with either LAS ($0.5\ ml\ kg^{-1}$, intraperitoneally (i.p.)) or control preimmune sheep serum (PIS) 4 h before LPS ($2.5\ mg\ kg^{-1}$ i.p.) or saline injection. Brains were analyzed 24 h after the LPS/saline challenge. (**a**) Neutrophil recruitment. (**b–h**) mRNA levels measured by using quantitative reverse transcription (RT)-PCR. Values are presented as means + s.e.m. ($n=5$). *P*-values: $^{\#\#}<0.01$, $^{\#\#\#}<0.001$. NS, not significant.

## Discussion

The main finding of this study is that leptin mediates the recruitment of neutrophils to the brain during severe systemic LPS inflammation by acting at the level of barrier-related structures that are involved in immune cell trafficking into the brain. Interestingly, the pattern of leptin activity demonstrated by us previously[21] exhibited remarkable similarity to the distribution of leptin-mediated neutrophil accumulation observed in this study (Figure 1 and Supplementary Figure S1).

The attenuation of LPS-induced neutrophil recruitment, as well as the accompanying gene expression, was 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), in which this hormone has been shown to stimulate chemotaxis[42] or inhibit spontaneous apoptosis,[43] suggesting that its action on alternative targets including the brain is the major contributing factor to the leptin-mediated processes observed in this study.

Parallel to the suppression of the neutrophil recruitment, LPS-induced IL-1β mRNA upregulation was strongly blunted in the brains of *ob/ob*, *db/db* or fasted WT mice when compared with (*ad lib* fed) WT counterparts. IL-1β is rapidly induced in the brain by various inflammatory stimuli, and is a potent contributor to subsequent brain innate inflammatory responses.[44] IL-1β alone, when acting centrally, is sufficient to trigger recruitment of neutrophils to the brain,[27,28,45,46] whereas blockade of IL-1β actions by using IL-1 receptor antagonist or genetic deletion of its functional receptor IL-1R1 prevents the neutrophil recruitment associated with neuroinflammation.[47,48] In the context of this 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,[12,14,19,20] which localized mainly in macrophages in the meningis and perivascular spaces.[20] Interestingly, this is very similar to the pattern of expression induced by systemic injection of LPS where brain IL-1β production is known to emanate largely from brain macrophages primarily residing in perivascular spaces, as well as from parenchymal microglia.[25,26,49,50] These data favor our speculation that defective brain IL-1β production resulting from leptin (or ObRb) deficiency is one of the underlying mechanisms responsible for reduced neutrophil recruitment during systemic inflammation observed in this study.

Neutrophil attachment to brain endothelium is the prerequisite step for their eventual recruitment into the central nervous system. In this study, the replenishment of leptin in leptin-deficient *ob/ob* or fasted WT mice almost completely restored the attenuated ICAM-1 mRNA upregulation 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 (Figures 1 and 2). In fact, double-labeling 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 2c). As the brains examined in this study were well perfused with heparinized saline, we presume that this close contact may represent an integrin-mediated neutrophil arrest to the endothelium, the essential step for eventual neutrophil infiltration into the parenchyma. In fact, Bohatschek *et al*.[34] reported that neutrophil infiltration into the brain following peripheral LPS challenge was strongly reduced in ICAM-1-deficient mice to the 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 (for example, vasculatures, the leptomeningis and the choroid plexus),[51,52] and IL-1β readily induces ICAM-1 in these structures.[46] Given that leptin induces IL-1β in perivascular and meningeal macrophages *in vivo*,[20] it is conceivable that leptin-induced IL-1β acts on the barrier-related structures in a paracrine manner and regulates leukocyte recruitment through ICAM-1.

A transient neutralization of leptin with LAS effectively attenuated neutrophil recruitment during LPS inflammation. This is an important observation with therapeutic potential for combating certain neuropathologies, such as cerebral ischemia, in which both neutrophil recruitment and systemic inflammation are linked with detrimental outcomes.[53,54] 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 the 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 signaling preceding, rather than in the course of inflammation, 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.[55] This indicates that although IL-1β has been demonstrated to be a powerful inducer of KC and MIP-2,[28,45] 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.[56] As these chemokines are essential for neutrophil recruitment to the brain even in the presence of IL-1β and ICAM-1,[28,55] their LAS-mediated attenuation explains the reduced neutrophil recruitment by LAS treatment without affecting the expression of either IL-1β or ICAM-1.

A number of studies have shown that replenishment of leptin in starved animals restores their abnormal host defense responses in various experimental models of inflammation,[10,57,58] highlighting the physiological importance of this hormone. This study demonstrated that acute starvation attenuates brain production of innate inflammatory mediators including IL-1β, ICAM-1 and KC in a leptin-dependent

manner, which was paralleled by the reduction in neutrophil recruitment during LPS-induced systemic inflammation. Importantly, these changes are specific to a certain facet of the brain inflammatory response and are not a result of a generalized insensitivity to LPS, as the expression of CD14 and TIMP-1 mRNA in the brain, which were also induced by LPS, were unaffected by fasting or leptin levels. Similarly, the reduction of leptin levels by fasting (as well as by LAS treatment) did not significantly affect plasma levels of TNF, IL-1β and IL-6 (measured as indicators for systemic inflammation) during LPS-induced inflammation, although a previous study[58] reported an exaggeration of plasma TNF in LPS-treated fasted mice (by using a different LPS dose and time points for cytokine measurements). Interestingly, the mutant *ob/ob* and *db/db* mice demonstrated exaggerated plasma TNF and IL-6 elevations in response to LPS. These results indicate that factor(s) unique to mutant mice (for example, a permanent leptin deficiency and/or excessive fat storage), rather than a transient leptin deficiency, have a significant impact on peripheral TNF and IL-6 production. Importantly, the increase in the levels of these cytokines in the circulation was dissociated from the attenuated brain inflammation observed in our studies, and therefore negates their influence on the observed changes in neutrophil recruitment and favors the direct action of leptin on the brain as reported in this study. Interestingly, leptin levels were not affected by the LPS treatment in our experiments (Supplementary Table S1), in agreement with some[59,60] but contrary to other[16–18] studies. The reason for this apparent discrepancy is currently unclear, but the relatively late time point chosen in this study (24 h) may have not been ideal for the detection of LPS-induced leptin increase. Regardless, our results favor the scenario that the deficiency of leptin, rather than the absence of its increase from baseline, impairs the inflammatory response in the brain during systemic LPS-induced inflammation.

In addition to the direct role of leptin in the recruitment of neutrophils, other facets of its actions need to be considered as possible contributors to the response observed in our study. In particular, this includes its role in mediating the temperature response to LPS reported previously by us,[13] and others.[15,61,62] Given the relatively high dose of LPS used in our study, it is likely that a mixture of hypothermia followed by a fever ensues following the injection as shown previously.[63,64] This temperature response could conceivably affect the integrity of the blood–brain barrier,[65] which in turn could potentially influence leukocyte entry into the brain.

In conclusion, this study, by demonstrating a significant contribution 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 leukocyte-mediated pathogenesis[34,53,66] and/or immune-to-brain communication by leukocytes involved in the generation of sickness behavior.[67] Our finding that leptin is integral to the brain's innate inflammatory response may have broad implications on how the abnormal nutritional status during starvation, obesity or eating disorders (for example, anorexia nervosa or binge-eating), all of which are associated with changes in circulating levels of leptin,[31] can impact the etiology of brain disease.

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## Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)