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RESEARCH ARTICLE

The Bacterial Endotoxin Lipopolysaccharide has the Ability to Target the Brain in Upregulating Its Membrane CD14 Receptor Within Specific Cellular Populations

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Systemic injection of the bacterial endotoxin lipopolysaccharide (LPS) provides a very good mean for increasing the release of proinflammatory cytokines by circulating monocytes and tissue macrophages. There is now considerable evidence that LPS exerts its action on mononuclear phagocytes via the cell surface receptor CD14. The aim of the present study was to verify the hypothesis that the brain has also the ability to express the gene encoding the LPS receptor, which may allow a direct action of the endotoxin onto specific cellular populations during blood sepsis. Adult male Sprague-Dawley rats were sacrificed 1, 3, 6 and 24 h after systemic (i.v. or i.p.) injection of LPS or the vehicle solution. Brains were cut from the olfactory bulb to the medulla in 30-µm coronal sections and mRNA encoding rat CD14 was assayed by *in situ* hybridization histochemistry using a specific ^{35}S-labeled riboprobe. The results show low levels of CD14 mRNA in the leptomeninges, choroid plexus and along blood vessels of the brain microvasculature under basal conditions. Systemic injection of the bacterial endotoxin caused a profound increase in the expression of the gene encoding CD14 within these same structures as well as in the circumventricular organs (CVOs) the organum vasculosum of the lamina terminalis, subfornical organ, median eminence and area postrema. In most of these structures, the signal for CD14 mRNA was first detected at 1 h, reached a peak at 3 h post-injection, declined at 6 h, and return to basal levels 24 h after LPS treatment. Quite interestingly, a migratory-like pattern of CD14 positive cells was observed from all sensorial CVOs to deeper parenchymal brain 3 and 6 h after LPS injection. At 6 h post-challenge, small positive cells were found throughout the entire parenchymal brain and dual-labeling procedure indicated that different cells of myeloid origin have the ability to express CD14 in response to systemic LPS. These included CVO microglia, choroid plexus and leptomeninge macrophages, parenchymal and perivascular-associated microglial cells, although specific non-myeloid cells were also positive for the LPS receptor. These results provide the very first evidence of a direct role of LPS on specific cell populations of the central nervous system, which is likely to be responsible for the transcription of proinflammatory cytokines; first within accessible structures from the blood and thereafter through scattered parenchymal cells during severe sepsis.

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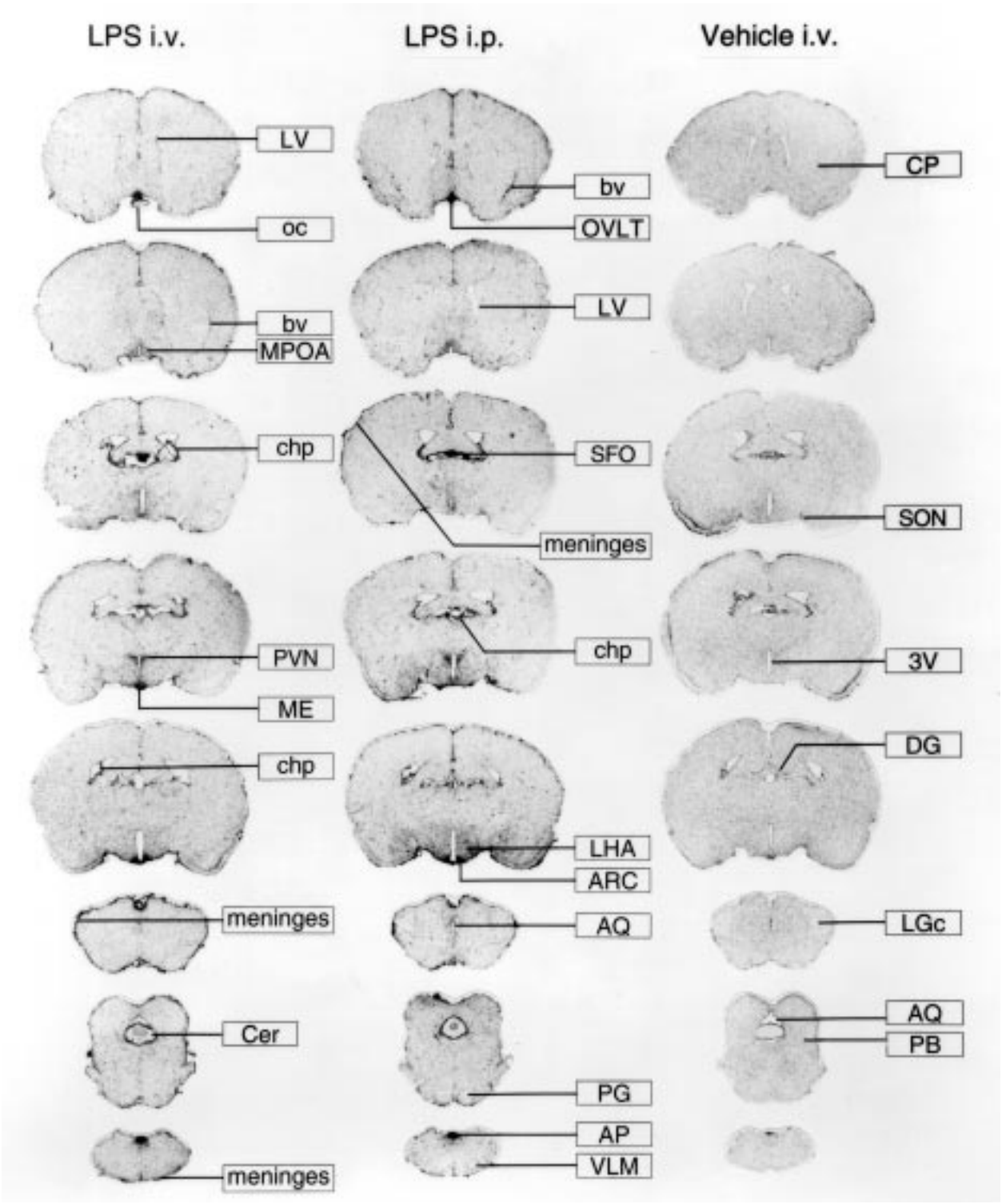
Introduction

Exposure to high levels of the bacterial endotoxin lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, often results in septic shock and death. Since the incidence of Gram-negative sepsis has been reported to be in constant progression during the recent years, numerous groups have begun to use systemic LPS administration as a model of infection and inflammation. Systemic LPS injection has been reported to induce changes in blood pressure, osmolarity, pain, oxygen consumption, fever, energy metabolism, multiple organ failure and different changes in the endocrine system, such as activation of the hypothalamic-pituitary-adrenal (HPA) axis. Indeed, systemic LPS injection has been shown to trigger transcription of corticotropin-releasing factor (CRF) and its type 1 receptor in the paraventricular nucleus of the hypothalamus (PVN) (32, 33) and stimulate the release of the neuropeptide into the hypophyseal-portal vein as well as the adrenocorticotropic hormone (ACTH) into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the general systemic circulation where it can act on distant endocrine organs, such as the adrenal glands. Increased levels of glucocorticoids, in counterpart, are potent immunosuppressors that prevent exaggerated immune response. The importance of a timely release of glucocorticoids is indicated by the high mortality observed in untreated Addisonian patients and in experimentally adrenalectomized animals (8, 36).

Proinflammatory cytokines, including interleukin (IL)-1, IL-6 and tumor-necrosis factor (TNF)-α are considered to be the main mediators of LPS-induced neuroendocrine changes. Although the primary function of cytokines is aimed at expanding the immunologic mass and activity, cytokines also reach the general circulation and thus trigger different functions involved in the neuroendocrine-immune interaction. It is generally believed that the release of proinflammatory cytokines from LPS-stimulated monocytes/macrophages, neutrophils and lymphocytes are directly responsible for the immune acute-phase response (4) and the physiopathological outcomes that take place during sepsis (6, 10). Intravenous (i.v.) injection of IL-1β, IL-6 and TNF-α was shown to reproduce numerous of the events occurring after endotoxaemia, such as the activation of the HPA axis and expression of c-*fos* gene in the rat brain (for review see 4).

Secretion of cytokines by circulating monocytes and tissue macrophages following LPS treatment requires a series of mechanisms in cascade. The endotoxin must first reach the bloodstream to bind with the serum proteins LPS binding protein (LBP) or septins (35, 40). The new formed complex binds to CD14 receptor attached to the cell membrane of differentiated myeloid cells (principally monocytes/macrophages and also, to a lesser degree, neutrophils) and therefore induces the release of proinflammatory cytokines (41). Using different neutralizing monoclonal antibodies specific to CD14, many cellular responses to LPS concentrations sufficient to induce sepsis have been shown to be prevented *in vitro* (21, 25, 41). It has recently been reported that pretreatment of primates with antibodies directed against CD14 significantly prevented tissue damage, hypotension and the increase in plasma levels of TNF-α, IL-1β and IL-6 (26). Transgenic mice expressing the membrane-associated human CD14 on their mononuclear phagocytic cells surface have been found to be hypersensitive to LPS, as demonstrated by their susceptibility to endotoxic shock and their mortality rate (14). In addition, transfection of human CD14 into Chinese hamster ovary fibroblasts was capable of transforming the cells from LPS nonresponders to LPS responders (18). It is not yet well understood how cell activation is triggered after binding between the LPS-LBP complex and the glycosyl-phosphatidylinositol (GPI)-anchored membrane CD14 (mCD14).

Recent studies on cytokine-related knock-out mice have clearly demonstrated that endogenous pyrogens, such as IL-1 and IL-6, are dispensable for LPS to activate the hypothalamus and HPA axis. In fact, IL-1β -/- and IL-6 -/- mice injected with the bacterial endotoxin LPS exhibit normal levels of plasma corticosterone in comparison to their corresponding LPS-treated wild-type mice (11, 12, 42). Moreover, we have recently found a robust expression of IL-6 and TNF-α mRNA in the sensorial circumventricular organs (CVOs) and the choroid plexus of rats that received a single systemic endotoxin injection, whereas localized inflammation of the rat hindlimb and i.v. injection of different proinflammatory cytokines failed to mimic these effects (38, S. Nadeau and S. Rivest, *in preparation*). This clearly suggests that LPS has, by itself, the ability to stimulate cytokine production within the central nervous system and that activation of phagocytic cells and cytokine release of systemic origin may be a distinct and independent response. Whether the gene encoding CD14 is expressed within specific cellular populations of the brain that may allow a direct action of the bacterial endotoxin when circulating into the bloodstream has yet to be determined *in vivo*. The purpose of the present study was therefore to determine the distribution of the gene encoding CD14 and investigate the effects of systemically-injected LPS on the transcriptional regulation of its own receptor throughout the rat brain. We provide here the very first *in vivo* evidence that the bacterial

Figure 1. (Opposing page) Representative example of the influence of endotoxin lipopolysaccharide (LPS) injection on the distribution of the mRNA encoding CD14 in the rat brain. Animals were sacrificed 3 h after intravenous (i.v.; 100 μg/kg b.w.) or intraperitoneal (i.p.; 500 μg/kg b.w.) treatment with LPS or the vehicle solution. These rostro-caudal coronal sections (30 μm) of LPS-treated rats exhibit a positive signal on X-ray films (Biomax) for CD14 mRNA in various parenchymal and non-parenchymal structures of the brain. 3V, third ventricle; AP, area postrema; AQ, aqueduct; ARC, arcuate nucleus; bv, blood vessels; Cer, cerebellum; chp, choroid plexus; CP, caudate putamen; DG, dentate gyrus; LGc, lateral geniculate complex; LHA, lateral hypothalamic area; LV, lateral ventricle; ME, median eminence; MPOA, medial preoptic area; oc, optic chiasm; OVLT, organum vasculosum of the lamina terminalis; PB, parabrachial nucleus; PG, pontine gray; PVN, paraventricular nucleus of the hypothalamus; SFO, subfornical organ; SON, supraoptic nucleus; VLM, ventrolateral medulla.

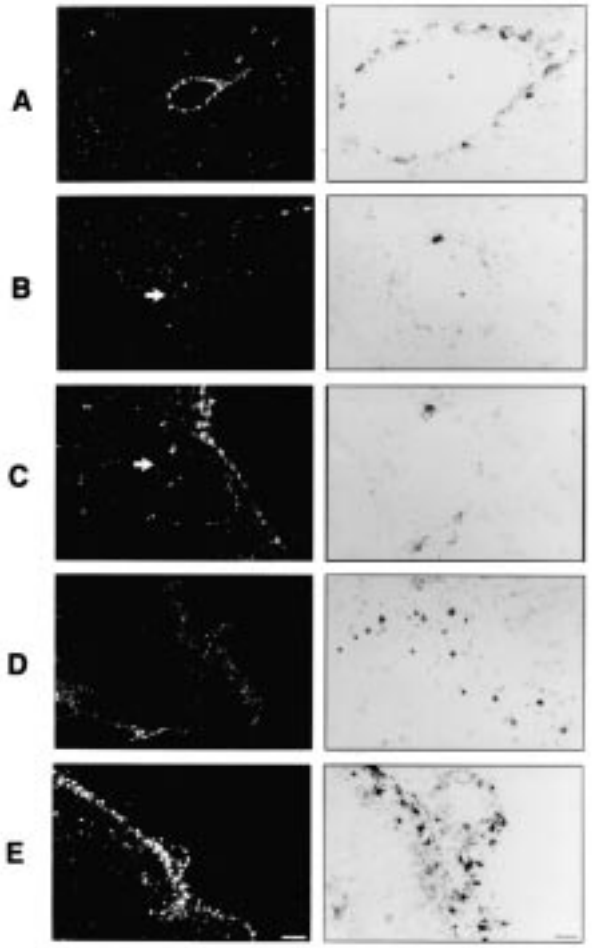

Figure 2. Low-power darkfield (left column) and high-power brightfield (right column) photomicrographs showing the expression of CD14 mRNA in blood vessels (**A**, **B** and **C**), choroid plexus (**D**), and leptomeninges (**E**) of rats sacrificed 3 h after intravenous (i.v.) injection of the bacterial endotoxin lipopolysaccharide (100 μg of LPS/kg b.w.). Note the robust hybridization signal within non-parenchymal cells surrounding the blood vessels, choroid plexus and leptomeninges 3 h following the LPS challenge. Note also that CD14-positive cells in the blood vessels of LPS treated rats are round in contrast to the fusiform shape of endothelial cells and not uniformly distributed around the internal lumen of the microvasculature suggesting that they are most likely perivascular microglial-associated cells. The arrows in **B** and **C** point the blood vessels observed under brightfield at high magnification. Magnification panels **A**, **B**, **C** and **E** (left column), X25, Scale bar = 100 μm; panel D (left column), X10, Scale bar = 250 μm; right column, X100, Scale bar = 25 μm.

endotoxin has a profound stimulatory influence on CD14 expression in both parenchymal and non-parenchymal elements of the brain, which may subserve a direct binding ability of the endotoxin to modulate different brain functions under severe blood endotoxaemia. The physiological relevance of such phenomenon is presented and discussed.

Materials and Methods

Animals. Adult male Sprague-Dawley rats (~230-260 g b.w.) were acclimated to standard laboratory conditions (14-h light, 10-h dark cycle; lights on at 0600 and off at 2000) with free access to rat chow and water. Each rat was only used once for experimentation, and all protocols were approved by the Laval University's Animal Welfare Committee. A total of 64 rats were assigned to different protocols (each corresponding to a different route of administration and a different dose of LPS; 20 and 100 μg of LPS i.v./kg of b.w. or 25 and 500 μg of LPS i.p./kg of b.w.), which were further subdivided into four post-injection times (1, 3, 6 and 24 hours following LPS administration). Paired vehicle-treated rats were also sacrificed at corresponding times after the systemic injection.

Surgeries. Animals receiving i.v. or i.p. injections were implanted with sterile cannulas. Rats were anesthetized with an i.p. injection of a mixture (1 ml/kg b.w.) of ketamine hydrochloride (91 mg/ml) and xylazine (9 mg/ml) and implanted with a catheter into the jugular vein or into the peritoneal cavity. Catheters were made from a piece of silastic tubing (Silastic medical grade tubing, ID 0.020 in., OD 0.037 in.; Dow Corning, Midland, MI) connected to an intramedic polyethylene tubing (PE-50, Caly Adams, Parsippany, N.J.). Outlets of cannulas were placed at the level of the neck and rats were housed individually in metal cages for a recuperation period of two to four days.

Experimental protocols. On the day of the experiment (~0830 in the morning), the outlet portion of each catheter (i.v. or i.p.) was fixed to a truncated 27 g needle which was attached to a PE-50 tubing. These connectors were then fixed to a 1cc syringe and rats were placed individually in a quiet room for at least 2 hours before the injections. This allows injections without disturbing the animals and induction of genes that may be activated following the stress of handling. Intravenous (20 and 100 μg/kg diluted in 200 μl of sterile pyrogen-free saline) and intraperitoneal (25 and 500 μg/kg in 300 μl of saline) administration of LPS (from *Escherichia coli*, Serotype 055:B5, Sigma, L-2880, lot #122H4025) or the vehicle solution was performed into the right jugular vein and through the chronically implanted cannula into the peritoneal cavity, respectively. The animals were

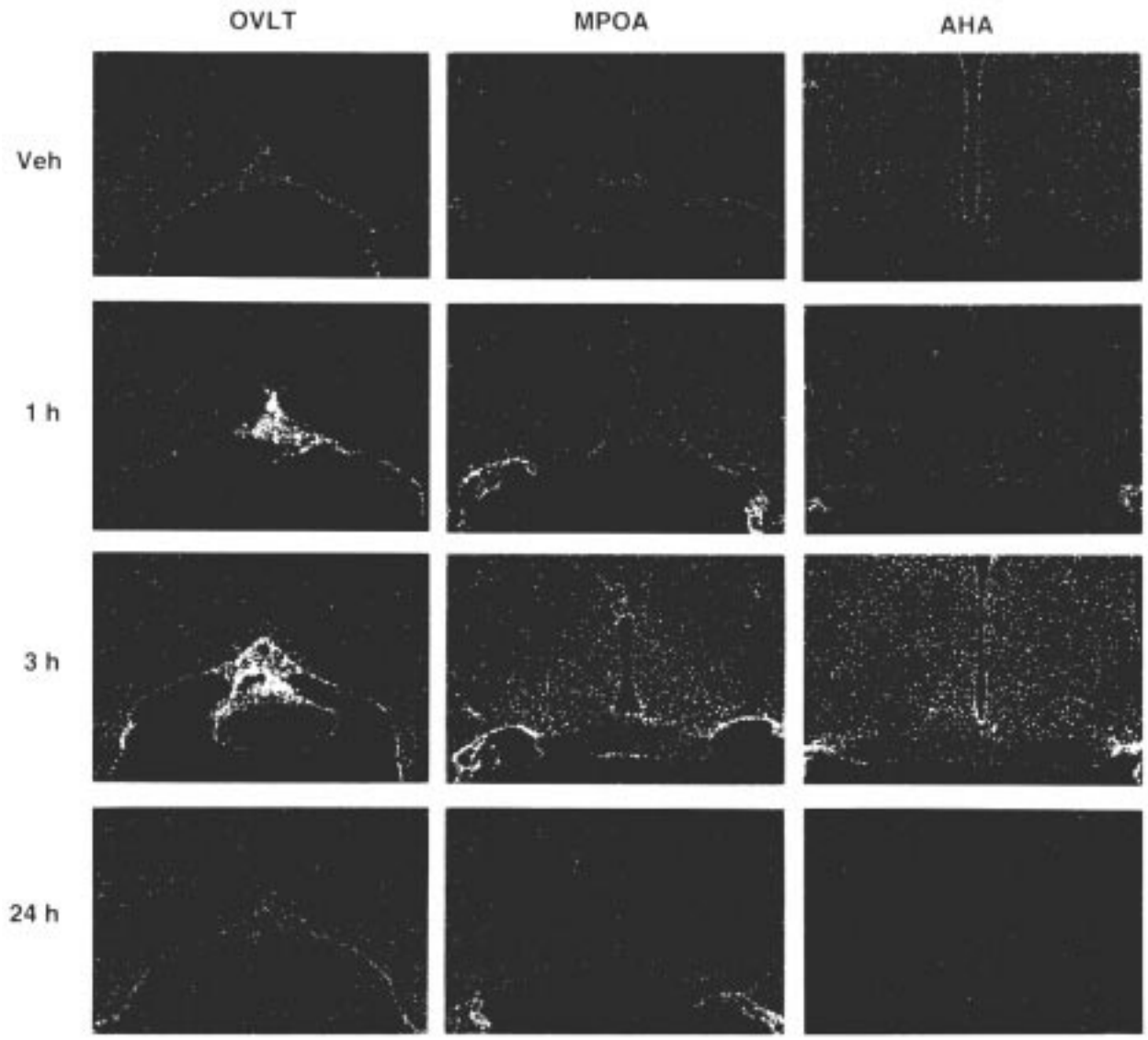

Figure 3. Intravenous (i.v.) lipopolysaccharide (LPS; 100 μg/kg b.w.) caused a migratory-like pattern of CD14 expression from the organum vasculosum of the lamina terminalis (OVLT) to the medial preoptic area (MPOA) and anterior hypothalamic area (AHA). These darkfield photomicrographs were taken from dipped nuclear emulsion sections of animals sacrificed 1, 3 and 24 h after i.v. injection of LPS or vehicle solution (top panels). Note the positive hybridization signal in the OVLT as early as 1 h after LPS treatment, whereas the MPOA and AHA did not show detectable CD14 signal at this post-injection time. Three hours after LPS treatment, numerous scattered cells expressing CD14 mRNA were found in these structures, suggesting a migratory-like pattern of CD14-positive cells from the OVLT to the surrounding parenchymal brain. Magnification, X10, Scale bar = 250 μm.

conscious and freely moving at all times throughout the procedure. One, 3, 6 and 24 hours after treatment, animals were deeply anesthetized with an i.v. (100 μl) or i.p. (400 μl) injection of a mixture of ketamine hydrochloride and xylazine and then rapidly perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M borax buffer (pH 9.5 at 4°C). The dosage and time points were determined on the basis of previous studies demonstrating strong neuronal activation and transcriptional induction of the genes encoding CRF in the rat brain following such treatments (22). For the combination of immunocytochemistry (especially for OX-42-immunoreactive cells) to *in situ* hybridization, rats were perfused with saline followed by 4% paraformaldehyde in 0.1 M sodium phosphate (pH: 7.2). Brains were removed from the

skull, postfixed for 2 h and then placed in 20% sucrose diluted in 4% paraformaldehyde-sodium phosphate buffer for 12-15 h.

Single* in situ *hybridization histochemistry. Rapidly after the transcardiac perfusions, brains were removed from the skulls, postfixed for 2 to 8 days, and then placed in a solution containing 10% sucrose diluted in 4% paraformaldehyde-borax buffer overnight at 4°C. The frozen brains were mounted on a microtome (Reichert-Jung, Cambridge Instruments Company, Deerfield, IL) and cut into 30-μm coronal sections from the olfactory bulb to the end of the medulla. The slices were collected in a cold cryoprotectant solution (0.05M sodium phosphate buffer, pH 7.3, 30% ethylene glycol, 20% glycerol) and stored at -20°C. Hybridization histochemical procedure was carried out as described previously (22, 23, 33, 38). The hybridized sections with the ^{35}S-labeled cRNA probe were exposed at 4°C to X-ray films (Kodak) for 3 days, defatted in xylene, and dipped in NTB2 nuclear emulsion (Kodak; diluted 1:1 with distilled water). Slides were exposed for 12 to 16 days, developed in D19 developer (Kodak) for 3.5 min at 14-15°C, washed 15 sec in water, and fixed in rapid fixer (Kodak) for 5 min. Tissues were then rinsed in running distilled water for 1-2 h, counterstained with thionin (0.25 %), dehydrated through graded concentrations of alcohol, cleared in xylene, and coverslipped with DPX.

cRNA probe synthesis and preparation. The pBlueScript SK minus plasmids containing a rat CD14 cDNA fragment of 1528 base pairs was linearized with *Sac* I and *Kpn* I for the antisense and sense riboprobes, respectively. Radioactive cRNA copies were synthesized by incubation of 250 ng linearized plasmid in 6 mM $MgCl_2$, 40 mM Tris (pH 7.9), 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol (DTT), 0.2 mM ATP/GTP/CTP, 200 μCi of α-^{35}S-UTP (Dupont NEN, #NEG 039H), 40U RNAsin (Promega, Madison, WI) and 20U of either T7 (CD14 antisense probe) or T3 (CD14 sense probe) RNA polymerase for 60 min at 37°C. Unincorporated nucleotides were removed using ammonium-acetate method; 100 μl of DNAse solution (1 μl DNAse, 5 μl of 5 mg/ml tRNA, 94 μl of 10 mM tris/10 mM $MgCl_2$) was added, and 10 min later, a phenol-chloroform extraction was performed. The cRNA was precipitated with 80 μl of 5M ammonium acetate and 500 μl of 100% ethanol for 20 min on dry ice. The pellet was washed with 500 μl 70% ethanol, dried, and resuspended in 100 μl of 10 mM Tris/1 mM EDTA. A concentration of 10^7 cpm probe was mixed into 1 ml of hybridization solution (500 μl formamide, 60 μl 5 M NaCl, 10 μl 1 M Tris [pH 8.0], 2 μl 0.5 M EDTA [pH 8.0], 50 μl 20x Denhart's solution, 200 μl 50% dextran sulfate, 50 μl 10 mg/ml tRNA, 10 μl 1M DTT, [118 μl Depc water - volume of probe used]). This solution was mixed and heated for 5 min at 65°C before being spotted on slides.

Combination of immunocytochemistry with* in situ *hybridization. Immunocytochemistry was combined with the *in situ* hybridization histochemistry protocol to determine the types of cells that express CD14 transcript in the rat brain after systemic treatment with the bacterial endotoxin LPS. Among the antibodies selected for this study, OX-42 was used to stain the rat complement receptor type 3 on phagocytes (CD11b/c), such as monocytes, macrophages and microglia. Polyclonal antibody raised against CRF was also used to identify the cells expressing CD14 mRNA within parenchymal elements of the parvocellular division of the PVN; numerous CD14 positive cells of large diameter were observed in this division of the PVN where CRF cells are predominant. Fos immunostaining was used to determine if cells activated following acute immune challenge were able to synthesize the mCD14 receptor. Every sixth brain section was processed by using the avidin-biotin amplification bridge method with peroxidase as a substrate. Briefly, slices were washed in sterile Depc-treated 50 mM potassium phosphate-buffered saline (KPBS) and incubated at room temperature with either OX-42, CRF or Fos antibody mixed in sterile KPBS, 0.4% Triton X-100, 0.25% heparin sodium salt USP (ICN Biomedicals Inc., Aurora, OH) and 1% bovine serum albumin (fraction V, Sigma, St. Louis, MO). OX-42 monoclonal antibody raised in mouse (Cedarlane Lab. Limited, Hornby, Ont., Canada, Cat # CL042B) was diluted 1:2 500 in the solution described above. Rabbit antihuman/rat CRF serum (code PBL rc 70, 8/9/83 bleed), a generous gift from Dr. Wylie Vale (Peptide Biology Laboratory, The Salk Institute, La Jolla, CA), was used at a concentration of 1:20 000. Antiserum raised in rabbit against proto-oncogene Fos [Ab-5] (Oncogene Research Products, Cambridge, MA, Cat # PC38) was used at a concentration of 1:10 000. One to two hours after incubation with the primary antibodies (OX-42, CRF or Fos), the brain slices were rinsed in sterile KPBS and incubated with a mixture of KPBS + triton-X + heparin + biotinylated secondary antibodies (horse anti-mouse IgG for OX-42 and goat anti-rabbit IgG for CRF and Fos; 1:1 500 dilution;

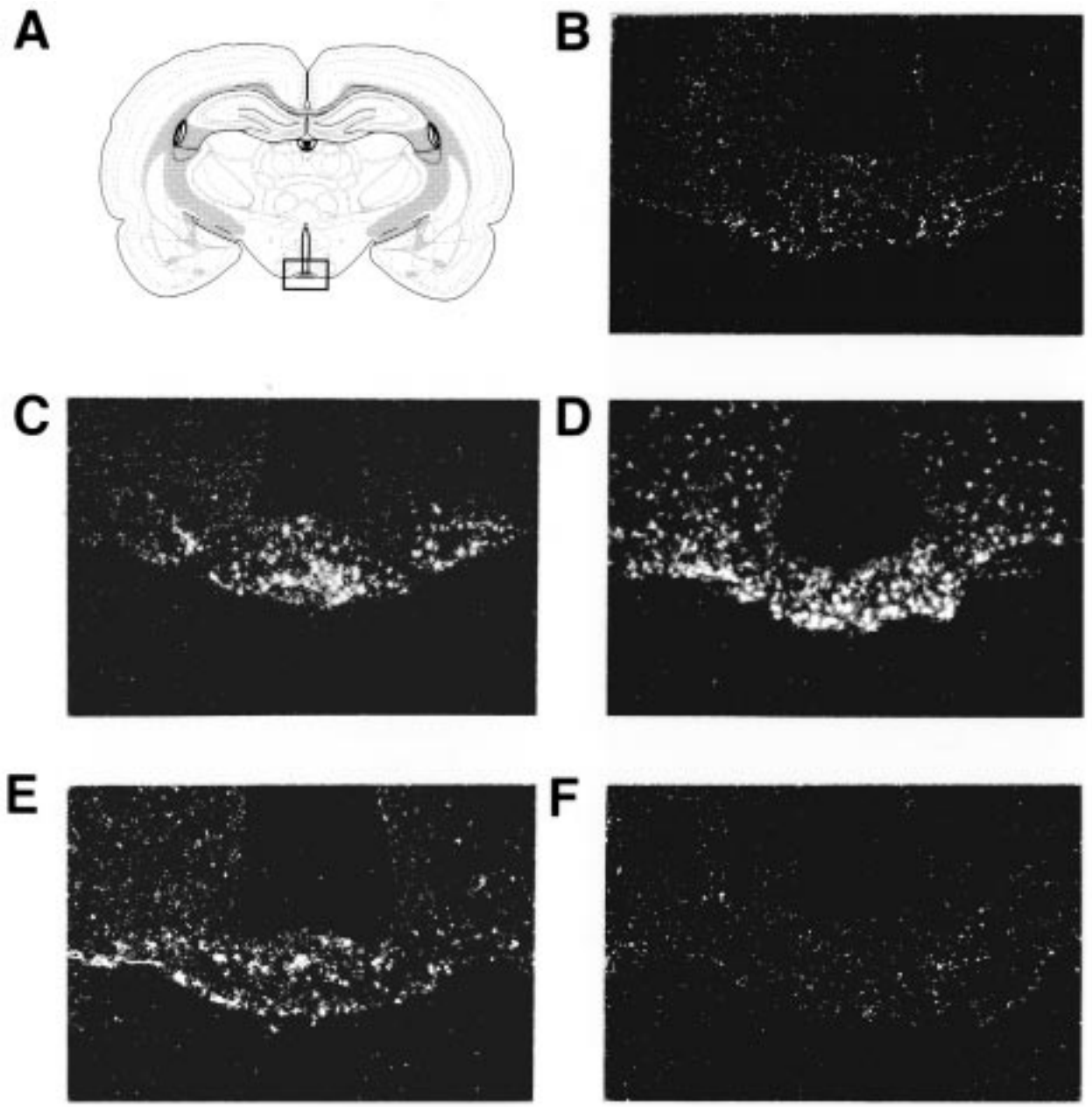

Figure 4. Effects of systemic i.v. injection of lipopolysaccharide (LPS; 100 μg/kg b.w.) on the gene encoding CD14 in the median eminence (ME) and the arcuate nuclei (ARC) of the hypothalamus. These darkfield photomicrographs of dipped NTB-2 emulsion slides depict CD14 mRNA hybridization signal in rats sacrificed after vehicle injection (**B**) or 1 h (**C**), 3 h (**D**), 6 h (**E**) and 24 h (**F**) following i.v. LPS-treatment. The signal for CD14 mRNA in the ME was first detected at 1 h, reached a peak at 3 h post-injection, declined at 6 h and returned to basal levels 24 h after LPS treatment. Note that CD14-positive cells became apparent in the ARC at 3 h. The schema from panel A was taken from the atlas of Swanson (37) . Magnification, X25, Scale bar = 100 μm.

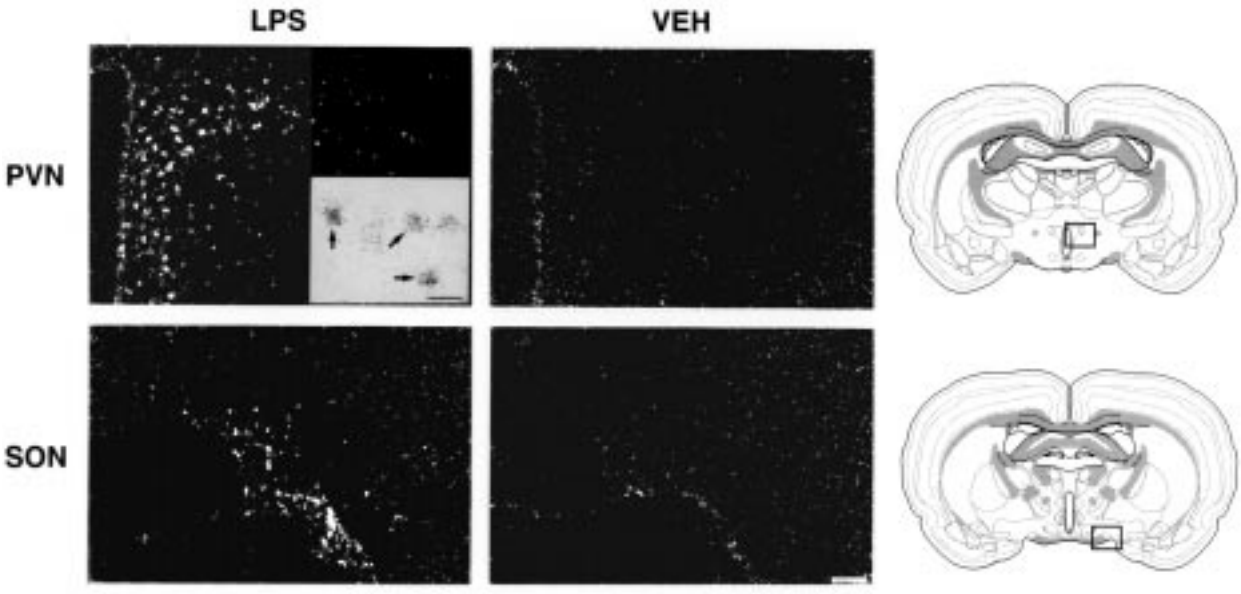

Figure 5. Representative example of the effect of intravenous injection of LPS or vehicle solution on the expression of CD14 mRNA in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the rat hypothalamus. These darkfield photomicrographs of dipped NTB-2 emulsion slides depict positive hybridization signal for CD14 mRNA over the parvocellular division of the PVN and SON 3 h after i.v. injection of LPS (100 μg/kg b.w.). The brightfield photomicrograph inserted adjacent to the PVN of LPS-treated rats depicts examples of dual-labeled neurons immunoreactive for corticotropin-releasing factor (CRF) and expressing CD14 transcript. Note that few double-labeled neurons were found, while numerous CD14- and CRF-containing cells were not colocalized together in the hypothalamic PVN. For more details on the dual-labeling procedure, please see the Material and Methods. The schema of the right column were taken from the atlas of Swanson (37) . Magnification, X25, Scale bar = 100 μm; brightfield photomicrograph insert, X250, Scale bar = 19 μm.

Vector Laboratories, CA) for 60 min. Sections were then rinsed with KPBS and incubated at room temperature for 60 min with an avidin-biotin-peroxidase complex (Vectastain ABC elite kit, Vector Laboratories, CA). After several rinses in sterile KPBS, the brain slices were reacted in a mixture containing sterile KPBS, the chromagen 3,3'-diaminobenzidine tetrahydrochloride (DAB, 0.05%), and 0.003 % hydrogen peroxide (H_2O_2).

Thereafter, tissues were rinsed in sterile KPBS, immediately mounted onto poly-L-lysine-coated slides, desiccated under vacuum for 30 min, fixed in 4% paraformaldehyde [pH 7.2] for 30 min, and digested by proteinase K (10 μg/ml in 100 mM tris HCl [pH 8.0] and 50 mM EDTA [pH 8.0]), at 37°C for 25 min. Prehybridization, hybridization, and posthybridization steps were performed according to the above description with the difference of dehydration (alcohol 50, 70, 95, 100%), which was shortened to avoid decoloration of OX-42-, CRF-, and Fos-immunoreactive cells (brown staining). After being dried for 2 h under the vacuum, sections were exposed at 4°C to X-ray film (Kodak) for 3 days, defatted in xylene, and dipped in NTB2 nuclear emulsion (Kodak; diluted 1:1 with distilled water). Slides were exposed for 14 to 21 days, developed in D19 developer (Kodak) for 3.5 min at 15°C, and fixed in rapid fixer (Kodak) for 5 min. Tissues were then rinsed in running distilled water for 1 to 2 h, rapidly dehydrated through graded concentrations of alcohol, cleared in xylene, and coverslipped with DPX. The presence of CD14 transcript was detected by the agglomeration of silver grains in perikarya, and OX-42, CRF or Fos immunoreactivity within the cell cytoplasm or nucleus (Fos-ir) was indicated by a brown homogeneous coloration.

Results

Low to moderate basal expression of CD14 transcript was generally detected in various non-parenchymal structures of the brain, such as the choroid plexus (chp), the leptomeninges and along blood vessels (bv) of the brain (Fig. 1, right column). Of interest is the fact that positive signal was not detected throughout the entire microvasculature of the brain, but within some bv irrigating particularly the forebrain. However, the signal in cells associated to bv increased throughout the entire

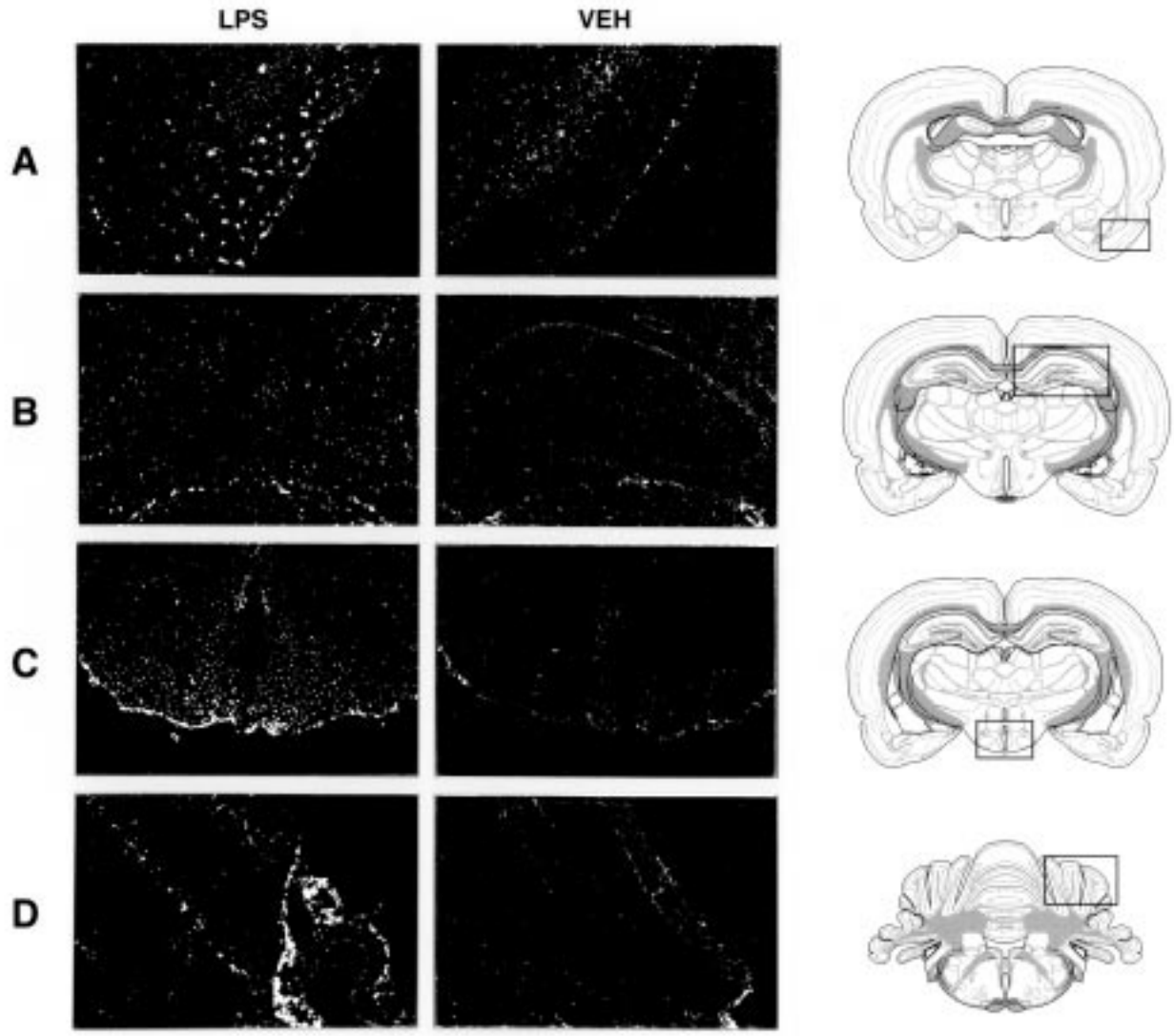

Figure 6. Representative examples of the ubiquitous-like CD14 signal within different areas of the brain of rats sacrificed 3 (panels **A**, **C** and **D**) and 6 (panel B) h after intravenous injection of LPS (100 μg/kg b.w.) or vehicle solution. These darkfield photomicrographs of nuclear emulsion-dipped sections show positive hybridization signal for CD14 mRNA in the cerebral isocortex (**A**), hippocampus (**B**), tuberomammillary area (**C**) and within the leptomeninges, blood vessel-associated cells and over the granular cell layer of the cerebellum (**D**). The schema of the right column were taken from the atlas of Swanson (37) . Magnification panel A, X25, Scale bar = 100 μm; other panels, X10, Scale bar = 250 μm.

brain of LPS-administered rats. Microscopic analysis of emulsion-dipped slides indicated that these non-parenchymal CD14 positive cells are likely to be of perivascular microglial-associated type; the round shape of their perikarya (in contrast to the fusiform shape of endothelial cells) is characteristic of perivascular microglia along the bv (Fig. 2, panels A, B and C). Moreover, the irregular signal across the bv does not support the hypothesis that the endothelial line of the blood-brain barrier is positive for the gene encoding CD14. Numerous studies have also reported the incapacity of the endothelial cells to express mCD14 under basal and immune-challenged conditions, while perivascular microglial-associated cells are known to display both the mCD14 protein and transcript in response to LPS. The positive signal observed across the brain microvasculature was increased 1 h after i.v. LPS, but peaked at time 3 h and declined progressively 6 and 24 h following the LPS-treatment.

I.v. and i.p. injection of the bacterial endotoxin also

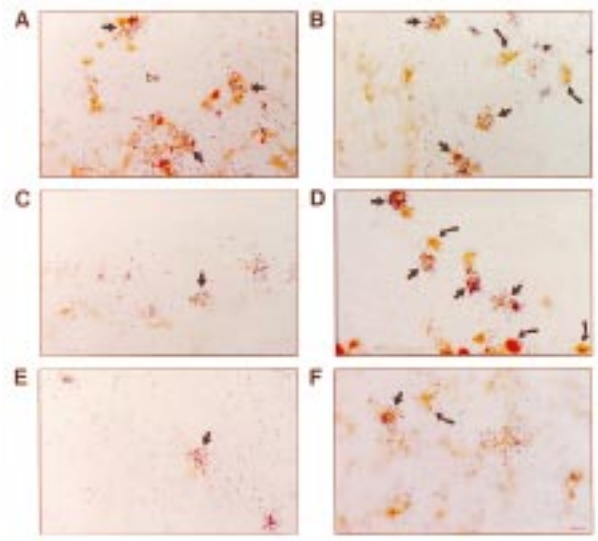

Figure 7. Expression of CD14 mRNA within OX-42-immunoreactive (ir) cells associated to brain microvasculature (**A**) or found in the choroid plexus (chp; **B**), leptomeninges (**C**), median eminence/arcuate nucleus (ME/ARC; **E**) and area postrema/nucleus of the solitary tract (AP/NTS; **F**) of LPS-challenged rats. Colocalization of Fos-ir nucleus within CD14-expressing cells in the leptomeninges (particularly into the subarachnoid space) of rats treated with the bacterial endotoxin is also depicted (**D**). Around the blood vessels (bv), OX-42-labeled cells (most likely perivascular-associated microglia) were positive for the gene encoding the CD14 receptor. Numerous OX-42-ir macrophages expressing the LPS receptor were observed in the chp and leptomeninges of endotoxin-challenged animals. In the ME/ARC and AP/NTS, several CD14 positive cells were identified as parenchymal microglia. Note that some CD14 positive cells were not co-localized within OX-42-ir perikarya, suggesting that other parenchymal cell types may have the ability to express the LPS receptor. Filled arrowheads, OX-42-ir or Fos-ir cells expressing CD14 mRNA; curved arrowheads, OX-42-ir or Fos-ir (**D**) cells alone. Magnification, X250, Scale bar = 10 μm.

increased the expression of CD14 transcript in the chp and leptomeninges (Fig. 1, left and middle column). This increase of CD14 mRNA levels was apparent 1 h after the i.v. treatment, reached a maximum at 3 h post-injection (Fig. 2, panels D and E) and persisted until 24 h after LPS treatment. Meticulous analysis of emulsion-dipped slides revealed that some positive cells of the chp and leptomeninges were found surrounding bv and are likely to be perivascular microglial-associated cells. However, the high power brightfield photomicrograph (Fig. 2, panels D and E, right column) also exhibit positive CD14-hybridized cells that did not seem to be associated to the microvasculature irrigating the chp and leptomeninges. Because of the heterogeneity of the cell populations characterizing these non parenchymal structures (epithelial, ependymal, macrophage, ...) it was difficult without double-labeling procedure to clearly establish the cell type(s) that has (have) the ability to express the LPS receptor in the chp and leptomeninges of systemically endotoxin-challenged rats.

Systemic injection of LPS stimulated the expression of CD14 mRNA in a wide variety of nuclei and brain areas, an effect that was dependent on the dose and time following the LPS administration. In addition to the previously described non-parenchymal structures (chp, leptomeninges and bv), transcription of CD14 was rapidly induced in most of the CVOs, including the organum vasculosum of the lamina terminalis (OVLT), subfornical organ (SFO), median eminence (ME), and area postrema (AP), 1 h after i.v. LPS administration. The intensity of the signal increased to its maximal level of expression at 3 h, whereas all the CVOs exhibited a reduced CD14 mRNA message at 6 h post-LPS injection. One day following the i.v. and i.p. injection of the endotoxin, the signal in the CVOs returned to the levels of control animals. A delayed response to LPS treatment was detected within different structures; a convincing expression of the mRNA encoding CD14 was observed for the first time 3 h after i.v. LPS administration in the medial preoptic area (MPOA), anterior hypothalamic area (AHA), supraoptic nucleus (SON), paraventricular nucleus of the hypothalamus (PVN), arcuate nucleus (ARC), and the nucleus of the solitary tract (NTS) (Fig. 1). Qualitative analysis also revealed that the hybridization signal for CD14 mRNA was still highly detectable 6 h after the treatment with the endotoxin in most of these areas, but completely vanished at 24 h. No clear hybridization signal was observed in these parenchymal structures under basal conditions and with the sense probe.

Examples of this time-related influence of the bacterial endotoxin on the expression of CD14 transcript is depicted by the Fig. 3, which exhibits darkfield photomicrographs of the OVLT, MPOA and AHA. The intensity of the signal for CD14 mRNA was moderate to high in the OVLT 1 h after the i.v. injection, strong and maximum at 3 h, moderate at 6 h and almost totally vanished at 24 h (Fig. 3, left column). In contrast, minimal to undetectable signal was observed in the MPOA and AHA of animals sacrificed 1 h following the i.v. administration of the endotoxin (Fig. 3, middle and right columns), respectively. Three hours after the LPS treatment, a moderate signal was found in the MPOA and the AHA. At that time, numerous scattered cells expressing CD14 mRNA were confined to those structures, while the message seemed to spread out the anatomical

boundaries of the MPOA and AHA 3 h later. This was particularly obvious in animals treated with the highest doses of LPS i.v. and i.p. Animals sacrificed 24 h after the LPS injection displayed undetectable CD14 signal. These results strongly suggest that the bacterial endotoxin LPS first reaches the OVLT (an organ devoid of the blood brain barrier) to induce transcription of the gene encoding CD14 mRNA and thereafter increases CD14 mRNA levels in the regions surrounding the vascular organ.

It is very interesting to note that such phenomenon of migratory-like pattern was not only seen from the OVLT, but from all the other sensorial CVOs. In the SFO and AP, low to moderate levels of CD14 expression was noted 1 h after the i.v. injection with LPS. The signal peaked at 3 h, declined to low-to-moderate levels at 6 h and finally returned to baseline level 24 h following the systemic treatment. In the parenchymal structures adjacent to the SFO and AP, no message was observed 1 h after injection of LPS, whereas a widespread of CD14-expressing cells became apparent in the fimbria and NTS at 3 h, two structures closely related to the SFO and AP, respectively. Another clear example of migratory-like pattern is presented in figure 4; strong signal was detected in the ME 1 h after LPS administration, but at that time no labeling was observed in the adjacent ARC nuclei (C). Two h later, the ARC now exhibited moderate-to-strong CD14 levels, while the ME was entirely covered of CD14-expressing cells (D). The hybridization signal declined 6 h after the single i.v. LPS injection (E) and essentially vanished 1 day later (F).

Intravenous injection of the endotoxin significantly increased the transcription of the gene encoding CD14 in the endocrine hypothalamus, more specifically in the PVN and SON. Interestingly, the effect of LPS injection on the stimulation of CD14 transcription within the PVN and SON was slow and transient; CD14 transcript was not detected in the PVN and SON of animals sacrificed 1 h after LPS treatment (either i.v. or i.p.), whereas maximal expression of this gene took place at 3 h and essentially vanished at 6 h post i.v. injection time. Figure 5 (left column) depicts a representative example of the distribution of the mRNA encoding CD14 in these hypothalamic structures 3 h after i.v. administration of LPS. In the PVN, the signal was particularly intense within parenchymal elements of the parvocellular division beside the wall of the ventral third ventricle, which is directly in contact with the cerebrospinal fluid secreted by the chp. The high power brightfield photomicrograph of the Fig. 5 (see "insert" PVN/LPS) depicts CRF-immunoreactive (ir) perikarya of the parvocellular PVN that are positive for the LPS receptor. Few, but some, double-labeled CRF neurons were indeed found 3 h after i.v. LPS treatment (filled arrowheads), although this hypothalamic nucleus exhibited numerous single CRF-ir neurons and CD14-positive cells. In the SON, the distribution of cells expressing CD14 mRNA was more heterogeneous and several positive cells were located near the leptomeninges (Fig. 5, SON LPS). Although it is difficult to evaluate whether LPS may reach directly the endocrine hypothalamus, the possibility remains that endotoxin particles find their way through chp, leptomeninges, bv and CVOs. No constitutive expression of CD14 transcript was detected in these hypothalamic nuclei (Fig. 5, right column).

Figure 6 shows darkfield photomicrographs of an ubiquitous-like type of CD14 signal within different areas of the brain of LPS-challenged rats. Indeed, i.v. LPS treatment stimulated transcription of CD14 within parenchymal cells of the cerebral isocortex (Fig. 6A), the hippocampus (B), the tuberomammillary area (C) and the cerebellum (D). In this latter region, the signal was particularly intense over the leptomeninges and the bv, while scattered cells were found over the parenchymal granular cell layer. As described, this induction across the parenchymal brain was late, transient and dependent on the dose of the endotoxin. The lowest doses of the bacterial endotoxin injected i.v. (20 μg/kg) or i.p. (25 μg/kg) caused expression of CD14 in a more restricted pattern; *i.e.* bv, leptomeninges, CVOs and surrounding structures, whereas the highest doses (500 μg i.p./kg; 100 μg i.v./kg) induced the LPS receptor in numerous but clearly defined parenchymal regions, such as those depicted in the Figs. 1 and 6. Of interest is the fact that i.p. injection of a very high dose of LPS (2.5 g/kg) was able to provoke a robust CD14 expression across the whole brain without any localized pattern 6 h after the treatment (data not shown).

Dual-labeling data. To determine the cell types that express the gene encoding CD14 following systemic injection of LPS or vehicle solution, a combination of immunocytochemistry and *in situ* hybridization histochemistry was performed. Sections were immunoreacted against OX-42 to stain phagocytic cells (macrophages and microglia) and hybridized immediately after with an antisense rat CD14 isotopic riboprobe. OX-42-ir cells associated to bv, most likely perivascular microglia, were positive for the LPS receptor (Fig. 7A), whereas macrophages expressing CD14 mRNA were found in the chp and leptomeninges of LPS treated rats (Fig. 7B and C). As depicted in the panel D,

very few (if any) Fos-ir epithelial lining cells were positive for CD14 in the leptomeninges, but double-labeled cells were detected into the subarachnoid space where fibroblasts, macrophages and endothelial cells are abundant. Since there is no clear evidence that endothelial cells are able to express CD14 receptor on their membrane surfaces and that some CD14-expressing cells were not immunoreactive to OX-42, fibroblasts are likely to be another cell type that express the LPS receptor within the subarachnoid space. It is interesting that fibroblasts has previously been shown to display high levels of CD14 and this event was shown to mediate the LPS-induced stimulation of AP-1 transcription factor (39).

The profile of distribution of the hybridized signal across the brain parenchyma of endotoxin-challenged animals along with the small size of the cells led us to believe that these CD14-positive cells were of glial type. Dual labeling with a specific antisera against OX-42 provided strong evidence that a large number of the cells that express the gene encoding LPS receptor during severe blood sepsis are of parenchymal microglial type. The Fig. 7 (panels E and F) shows examples of such phenomenon within different structures of the brain, including the ME/ARC and AP/NTS where numerous OX-42-ir cells exhibited CD14 transcript. However, not all CD14 positive cells were colocalized within OX-42-ir perikarya, suggesting that more than one type of parenchymal cells could express mCD14 during endotoxaemia. Expression of the LPS receptor has recently been reported in rat primary astrocytes (15), although we faced some technical difficulties to unravel these glial cells throughout the brain using different antisera against GFAP rending double-labeling difficult. No conclusion can therefore be reached at the moment for astrocytes.

Discussion

The present study shows that specific cellular populations of the central nervous system (CNS) have the ability to express the gene encoding the membrane (m) form of CD14 LPS receptor. Under basal conditions, low levels of CD14 mRNA were detected in various non-parenchymal structures of the brain, such as the leptomeninges, the chp and within blood vessels (bv) of the brain microvasculature. Systemic injection of the bacterial endotoxin induced strong expression of CD14 transcript within these same structures as well as in many other regions, including the OVLT/MPOA, SFO, PVN, ME/ARC and the AP/NTS. In the non-parenchymal structures enumerated above and all the sensorial CVOs, the CD14 mRNA signal was rapidly detected at 1 h, reached a peak at 3 h post-injection, declined at 6 h and returned to basal levels 24 h after LPS treatment. A delayed response to LPS treatment was detected in the brain parenchyma within numerous nuclei where robust expression of the mRNA encoding CD14 was observed 3 and 6 h after the systemic challenge. Interestingly, microscopic analysis of emulsion-dipped slides revealed that CD14 positive cells spread over the anatomical boundaries of these parenchymal structures in a migratory-like pattern 6 h following i.v. LPS administration. Taken together, these results strongly suggest that the bacterial endotoxin LPS first reaches organs devoid of the blood brain barrier (BBB) to induce the transcription of its own receptor and thereafter increased CD14 biosynthesis within parenchymal structures surrounding the CVOs and then the entire brain of severely challenged animals. Using immunocytochemistry/*in situ* hybridization double labeling, CD14 mRNA was identified within various cells of the brain, including CVO resident macrophages, parenchymal and perivascular-associated microglia as well as few CRF neurons of the hypothalamic PVN. The direct action of LPS on myeloid- and non-myeloid-derived parenchymal cells expressing mCD14 could be responsible for the modulation of numerous responses occurring during endotoxaemia, such as the production of proinflammatory cytokines of central origin and activation of the corticotroph axis for the appropriate release of glucocorticoids. Moreover, it is possible that cells expressing CD14 mRNA play a key role in the defense of the neural parenchyma against invading LPS particles penetrating the CNS via altered BBB during blood sepsis or unselective diffusion through available target structures, such as the CVOs and chp.

Systemic LPS administration is a well accepted model of infection and inflammation that activates phagocytes and stimulates the synthesis and release of cytokines into the circulation. It is believed that the secretion of these proinflammatory molecules by stimulated systemic monocytes/macrophages, neutrophils and lymphocytes is directly responsible for most of the effects of the endotoxin in the brain. Among many others, stimulation of CRF neurons and the consequent activation of the HPA axis reflected by elevated plasma glucocorticoid levels is a classical LPS response; a phenomenon of great importance in the immunosuppressive processes. Although systemic injections of IL-1β, IL-6 or TNF-α are capable of mimicking these neuroendocrine events, proinflammatory cytokines of systemic origin may not be essential for LPS-induced activation

of the HPA axis. Systemic administration of IL-1-receptor antagonist protein failed to prevent the increase of CRF mRNA in the PVN and circulating corticosterone levels (20), whereas mice receiving TNF-α antibody, either alone or in combination with an IL-1-receptor antagonist protein, still exhibited robust activation of the HPA axis in response to LPS (7). It has also been demonstrated that the surge of plasma ACTH and corticosterone levels obtained after intra-arterial infusion of LPS preceded by at least 30 min measurable proinflammatory cytokines into the bloodstream (17). These results clearly indicate that cytokines of systemic origin secreted in response to LPS administration are dispensable in the early stages of HPA axis stimulation. However, the possibility remains that these cytokines contribute in the prolonged and sustained neuroendocrine responses to systemic endotoxaemia. Pretreatment with antibodies specific for either IL-1 receptor or TNF-α was indeed able to prevent LPS-induced ACTH release, but at times not earlier than 4 h post LPS challenge, whereas simultaneous administration of both antibodies diminished, but did not eliminate, the ACTH release at 2 h (28). In consequence, systemic production of cytokines may not be an essential step in the early neuroendocrine changes provoked by the bacterial endotoxin, a fact that has been further supported in proinflammatory cytokine-deficient mice (for review, see 11).

As mentioned, significant increase of circulating levels of ACTH and corticosterone takes place as early as 15 to 30 min after systemic injection of LPS, while IL-1, IL-6 and TNF-α were not yet detected in the plasma (17). Although it has been suggested that lymphocyte-derived ACTH can play a role in triggering corticosterone release in immune-challenged animals (5), this hypothesis remains highly controversial and many studies do support the concept of neuroendocrine CRF-mediated mechanisms (31, 34). Few, but some, CRF neurons of the PVN exhibited positive CD14 signal, indicating that LPS may directly target the neurons controlling the HPA axis. However, the induction was quite late and whether systemic LPS may reach these cells has yet be to confirmed. On the other hand, it is possible that LPS stimulates accessible structures to the bloodstream, which play key roles in the activation of the neurons controlling the corticotroph axis. The ME is a good candidate and is rather well positioned to be a direct target of circulating endotoxin to modulate the HPA axis, because the region is devoid of BBB, exhibits a robust induced expression of mCD14 mRNA and contains the nerve terminals of the neurons that secrete into the infundibular system. Moreover, qualitative analysis of autoradiographic films and emulsion-dipped slides revealed that the ME is the structure that expressed the highest levels of CD14 mRNA at 1 h post-injection time. The present manuscript does not show the effects of LPS on CD14 transcription at times preceding 1 h, although it is highly possible that LPS stimulates CD14 expression in the ME within few minutes following the i.v. treatment. Circulating LPS into the bloodstream could therefore bind to its own receptor in the ME and among different functions, trigger infundibular CRF release and corticotroph axis-related hormone secretion.

Rapid induction of CD14 mRNA was also observed in the OVLT, SFO, AP, chp, leptomeninges and bv of the whole brain microvasculature 1 h after systemic (both i.v. and i.p.) LPS injection; no CD14 mRNA signal was detected in any other brain parenchymal cells at that time. These results can be explained by the fact that almost all these structures have been shown to be accessible to large molecules circulating into the bloodstream. The sensorial CVOs contain a rich vascular plexus with specialized arrangements of the bv. The tight junctions normally present between the endothelial cells are shifted in part to the ventricular surface and partly to the boundary between the CVOs and the adjacent structures explaining the diffusion of large molecules into the perivascular region. It is very likely that LPS injected into the general circulation penetrates the OVLT, SFO, ME and AP tissues, which then allow the endotoxin to trigger locally the synthesis of its own receptor. The chp and leptomeninges are also recognized as being highly vascularized regions and agglomeration of silver grains forming CD14 positive cells were detected along bv irrigating these structures, although numerous isolated positive cells were also found. Since it is virtually impossible to cut through these structures without isolating cells of the microvasculature, the possibility remains that a large proportion of the cells expressing CD14 mRNA in the chp and the leptomeninges at early post-challenge times are perivascular-associated cells or macrophages and therefore in direct contact with molecules of the blood.

At fairly low doses of LPS, CD14 receptor is recognized to be essential for the release of proinflammatory cytokines (21, 24, 26, 41) and recent studies indicate that Nuclear factor Kappa B (NF-κB) is likely to be the key mediator through which the endotoxin provokes such cellular response. NF-κB is normally present into the cytoplasm forming an inactive complex with an inhibitor known as I-κBα. Following extracellular stimulation by growth factors, mitogens, cellular activators,

and activators of second messengers, I-κBα is phosphorylated by intracellular kinases and degraded by cytoplasmic protease releasing, by the same token, active NF-κB (16, 19). Free active NF-κB is then translocated into the nucleus where it will be able to regulate transcription of various genes, including proinflammatory cytokines. Using detection of I-κBα transcript as a marker of NF-κB activity, Quan and coworkers have recently shown a dramatic increase of the I-κBα mRNA levels in the CVOs, chp and throughout the whole brain microvasculature 1 and 2 h after i.p. LPS administration (30). In the present study, we were able to detect a rapid induction of CD14 in the CVOs, chp and bv, indicating that LPS binding to CD14 may be responsible for the transcription of I-κBα in the structures devoid of the BBB.

Interestingly, systemic administration of the bacterial endotoxin LPS was shown to induce transcription of the genes encoding the proinflammatory cytokines in the regions of the rat brain that display rapid induction of CD14 and I-κBα. Using *in situ* hybridization histochemistry, maximal intensity of IL-1β mRNA were observed in the CVOs, chp, leptomeninges and bv at 2 h post-injection time (29), while cells expressing IL-6 transcript were detected 3 and 6 h after i.p. administration of the bacterial LPS (38). The proinflammatory cytokine IL-1β is recognized to be an important stimulator of IL-6 production in peripheral organs (1), although i.v. injection of recombinant rat IL-1β did not activate expression of IL-6 mRNA in the CNS (38). A direct action of the bacterial endotoxin on cells forming the CVOs and chp may therefore be responsible for the production of IL-6 within the brain, but the possibility remains that the IL-1β of central origin may increase the production of IL-6. We have also recently found that systemic LPS treatment stimulates TNF-α transcription in a time frame and pattern quite similar to CD14 and I-κBα mRNA expression in the rat brain (27). It is permit to believe that the effects of LPS on the activation of CD14/NF-κB and synthesis of proinflammatory cytokines are related phenomena within specific cellular population of the brain, such as macrophages, perivascular-associated microglia and parenchymal microglial cells.

The chp and cerebral microvasculature are in very good position to transduce the information received from systemic circulation to the brain parenchymal cells. We report here that the CD14 signal increased rapidly in cells associated to these structures after LPS treatment and microscopic analysis of emulsion-dipped slides indicated that most of the non-parenchymal CD14 positive cells are likely to be of perivascular microglial-associated and macrophage type. Indeed, the gene encoding CD14 was not uniformly expressed across the fusiform endothelium forming the internal lumen of the microvasculature and several bv exhibited only one or two CD14 positive cells, although a more uniform pattern of expressing cells were detected in other capillaries (see Fig. 2A). The cells were nevertheless not forming the internal edge of the microvasculature, a phenomenon that does not support the endothelia as being positive for the transcript. Moreover, double labeling with OX-42 provided clear evidence that perivascular-microglial associated cells have the capacity to express the gene encoding CD14 in response to systemic endotoxin challenge.

In agreement with our data, numerous studies have reported the incapacity of the endothelium to express mCD14, although these cells were shown to play a major role in the pathogenesis of gram-negative bacterial sepsis via free soluble CD14. In fact, LPS can trigger tyrosine phosphorylation of mitogen-activated protein kinases within endothelial cells despite the lack of mCD14 (2) and this event was reported to be associated to NF-κB and cyclooxygenase enzyme 2 (COX-2) activation (23, 30). The bacterial endotoxin-induced release of proinflammatory cytokines from systemic phagocytes may also contribute to activate the endothelium of brain capillaries; IL-1 type 1 receptor mRNA is predominantly expressed in cerebral vascular endothelial cells, leptomeninges and chp (9), whereas mRNAs encoding TNF-α receptors were detected in cerebrovascular endothelial cells under basal conditions (3). Moreover, we have recently reported that LPS, IL-1β and TNF-α i.v. injection caused COX-2 gene transcription in the chp, leptomeninges and along the entire brain microvasculature (23). It is conceivable that binding of proinflammatory cytokines to their receptors or a direct effect of LPS on the endothelium may induce prostaglandin synthesis that may diffuse through parenchymal elements, which in turn, modulate different brain functions occurring during endotoxaemia.

Apart from its recognized role as a glycosyl-phosphatidylinositol (GPI)-anchored membrane protein that induces, in presence of LPS, activation of tyrosine kinase leading to transduction signal and cytokine gene transcription, CD14 has also been suggested to serve as an opsonic receptor for LPS-coated particles resulting in an uptake and clearance of LPS by the activated cells (13). In this regard, our data showing the biphasic regulation of CD14 in the rat brain are quite fascinating; the transcript was rapidly detected in the CVOs and the chp

and dual labeling procedure indicated that most of these expressing cells were of myeloid type, including macrophages (chp) and microglial (CVOs). However, a migratory-like pattern was observed and 6 h after injection of high doses of LPS, positive cells were found over the entire brain. Although we found several parenchymal microglial cells that were positive for one of the LPS receptor, numerous CD14-expressing cells were not OX-42 immunoreactive. Non-myeloid parenchymal cells of epithelial origin, such as astrocytes and neurons, should therefore be considered and the fact that CD14 was expressed within few neuroendocrine CRF neurons supports this concept. In agreement with these data is the elegant study showing that LPS can stimulate the transcription of CD14 in non-myeloid epithelial cells of various peripheral tissues (13), a phenomenon that seems dependent on the production of cytokines from phagocytes. In the present case, it is possible that LPS targets first the myeloid cells of the CVOs and chp, which in turn may produce proinflammatory cytokines and upregulate parenchymal CD14 expression in a dose- and time-dependent manner as presented in this study. It will be crucial to determine if such mechanism is indeed taking place in the brain and to investigate its physiological relevance. One hypothesis is that CD14 expression in parenchymal cells of the CNS primes the activated cells to produce cytokines and/or prepare them to increase the clearance of the endotoxin particles that may reach the CSF and be toxic for the neurons. This complex machinery may be of great importance to maintain the body homeostasis and protect neurons during infectious diseases and severe blood sepsis.

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