

# Functional Circuitry in the Brain of Immune-Challenged Rats: Partial Involvement of Prostaglandins

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## ABSTRACT

This study investigated the role of prostaglandins (PGs) on the neuronal activity and the transcription of corticotropin-releasing factor (CRF) in the brain of conscious immune-challenged rats. Intravenous (i.v.) administration of indomethacin, an inhibitor of PG synthesis, was performed prior to and after the intraperitoneal (i.p.) injection of different doses [250 µg, 25 µg, and 2.5 µg/100 g body weight (b.w.)] of the immune activator lipopolysaccharide (LPS). Systemic administration of the high and middle doses of LPS caused a robust and widespread induction of both immediate-*early* genes (IEGs), *c-fos* and nerve growth factor-inducible gene B (NGFI-B) mRNAs, whereas injection of the low dose selectively triggered *c-fos* expression within the sensorial circumventricular organs. Pretreatment with indomethacin did not prevent *c-fos* transcription in the rat brains challenged with the high dose of LPS at 3 hours postinjection. Inhibition of PG formation was more effective for interruption of the neuronal activation in animals injected with 25 µg LPS/100 g b.w., although the influence depended on the structures and the groups of activated cells. Indeed, PG inhibition significantly altered LPS-induced *c-fos* mRNA expression in the medial preoptic area/organum vasculosum of the lamina terminalis, the periventricular nucleus, the paraventricular nucleus of the hypothalamus (PVN), and the ventrolateral medulla (VLM) but not in many other regions, including the subfornical organ, the central nucleus of the amygdala, the arcuate nucleus/median eminence, the parabrachial nucleus, the choroid plexus, and the nucleus of the solitary tract (NTS). In the hypothalamic PVN, inhibition of both *c-fos* and NGFI-B transcripts by indomethacin was also associated to an abolished influence of the endotoxin on the transcription of neuroendocrine CRF; induction of CRF primary transcript by the middle dose of LPS was selective to the PVN and was completely blocked by pretreatment with indomethacin. Moreover, a large number of tyrosine hydroxylase (TH)-immunoreactive neurons of the VLM (A1/C1) and the NTS (A2/C2) were positive for *c-fos* mRNA in immune-challenged rats, an effect that was largely prevented by indomethacin in the VLM but not in the NTS. These results indicate that the role of PGs in mediating the stimulatory influence of the acute-phase response depends on the severity of the systemic stressful situation, the brain regions, and the cell groups as well as the activated target genes. *J. Comp. Neurol.* 387:307-324, 1997. © 1997 Wiley-Liss, Inc.

**Indexing terms:** corticotropin-releasing factor; lipopolysaccharide; immediate-*early* genes; indomethacin; tyrosine hydroxylase

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Appropriate communication between neuroendocrine and immune systems is essential to maintain homeostasis in the presence of foreign material. Cytokines produced during immune challenge can stimulate the release of endogenous glucocorticoids, which, in counterpart, could reach the inflammatory site to suppress the immune response (Solomon, 1969). The production of cytokines by activation of macrophages and lymphocytes, which occurs rapidly after the contact between organisms and exogenous

pyrogen (Dinarello, 1989; Rabin et al., 1990), represents an essential feature of the early events of immune

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activation that characterize the acute-phase response. Endotoxins are widely used to mimic some of the events that take place during sepsis (Higgins and Olschowka, 1991; Koenig, 1991); lipopolysaccharide (LPS) triggers the synthesis and release of a series of immunoregulatory, cytotoxic, and inflammatory molecules, including tumor necrosis factor (TNF)- $\alpha$ , interleukin-1 (IL-1), and IL-6 (Andersson et al., 1992; Nathan, 1987). Systemic LPS administration causes a strong and prolonged activation of the hypothalamic-pituitary-adrenal (HPA) axis (Rivier and Rivest, 1993) essentially via neuroendocrine corticotropin-releasing factor (CRF)-mediated mechanisms (for review, see Koenig, 1991; Rivest, 1995; Rivest and Rivier, 1995; Rivier and Rivest, 1993). Selective transcriptional activation of CRF was recently reported in the hypothalamic paraventricular nucleus (PVN) of endotoxin-challenged rats (Rivest and Laflamme, 1995), whereas inhibition of CRF abolishes the effects of LPS on the activation of the HPA axis (Rivier and Rivest, 1993).

Systemic administration of the immune activator LPS also causes a profound expression of mRNA encoding the immediate-early genes (IEGs) *c-fos* and nerve growth factor-inducible gene B (NGFI-B) as well as their protein products in numerous structures of the brain, suggesting that a complex neuronal circuitry is involved in triggering the activity of neuroendocrine neurons controlling the HPA axis (Elmqvist and Saper, 1996; Elmqvist et al., 1996; Hare et al., 1995; Nappi et al., 1997; Rivest and Laflamme, 1995). The ventrolateral medulla (VLM) and the nucleus of the solitary tract (NTS) are among the numerous regions of the brain that are responsive to intraperitoneal (i.p.) injection of the bacterial endotoxin (Rivest and Laflamme, 1995). Catecholamine-synthesizing neurons of these brainstem regions (A1-A2/C1-C2) are two groups of cells that provide massive projections to hypophysiotrophic cells of the PVN (Cunningham and Sawchenko, 1988; Cunningham et al., 1990; Sawchenko and Swanson, 1982; Swanson et al., 1983), pathways believed to play a role in mediating the effects of immune challenge and systemic cytokines on hypothalamic neuroendocrine response. It has recently been demonstrated that rats bearing unilateral fiber transection of catecholaminergic inputs had impaired neuronal activation and CRF mRNA expression in the PVN in response to systemic IL-1 challenge (Ericsson et al., 1994) but not following a neurogenic foot-shock stressor (Li et al., 1996). Influence of catecholaminergic innervation on CRF secretion, nevertheless, can be located directly at the level of the PVN; depletion of PVN noradrenergic (NA) contents with 6-hydroxydopamine (6-OHDA) has been shown to reduce the increase in plasma corticosterone concentration by 80–82% following i.p. IL-1 injection (Chuluyan et al., 1992).

Most of the pleiotropic effects of IL-1 are known to take place via the activation of prostaglandins (PGs) synthesized by cyclooxygenase pathways (Cominelli et al., 1989; Hughes et al., 1989; Kerr et al., 1989; Kohan, 1989). Blockage of the eicosanoid cyclooxygenase pathways can prevent the stimulation of CRF release by both IL-1 and IL-6 cytokines from *in vitro* hypothalamic explants (Lyson and McCann, 1992; Navarra et al., 1991) and isolated median eminence (McCoy et al., 1994) and of IL-1-induced adrenocorticotropin (ACTH) release *in vivo* (Katsuura et al., 1988; Rivier and Rivest, 1993; Watanabe et al., 1990). Inhibition of PG production has been reported to prevent IL-1-induced alteration of other neuroendocrine functions,

such as luteinizing hormone (LH)-releasing hormone (LHRH) and LH release (Rivest and Rivier, 1993, 1995) as well as hypothalamic vasopressin (AVP) and oxytocin (OT) secretion (Yasin et al., 1994). On the other hand, systemic LPS administration stimulates the release of PGs in the preoptic/hypothalamic area (Ueno et al., 1982), and arachidonic acid metabolism participates in the endotoxin-induced corticosterone secretion in the rat (Smith et al., 1994).

The exact PG subtype(s) and the site(s) of action within the brain involved in these effects still remain unclear. Although various PGs have the potential to mediate the influence of immune-related factors on neuronal activation and neuroendocrine functions, a large body of evidence indicates that PG of the  $E_2$  type might be involved in several changes observed during immune challenge and treatment with cytokines. Intracerebroventricular (i.c.v.) administration of  $PGE_2$  (Rassnick et al., 1995) or direct injection into the medial preoptic area (MPOA)/organum vasculosum of the lamina terminalis (OVLT; Katsuura et al., 1990) elevates plasma levels of ACTH and corticosterone in rats. Moreover, central injection of  $PGE_2$  induces expression of *c-fos* protein (Scammell et al., 1996) and mRNA (Lacroix et al., 1996) in several structures known to be activated during the acute-phase response of an immune challenge. In a similar manner, central treatment with this PG triggers transcription of CRF and its type 1 receptor essentially in the hypothalamic PVN (Lacroix et al., 1996). Whether the action of  $PGE_2$  takes place directly within the PVN and/or at the level of structures innervating endocrine hypothalamus has yet to be investigated. The fact that systemic injection of IL-1 provokes sharply increased levels of  $PGE_2$  in the PVN (Watanobe and Takebe, 1994) might suggest that local PVN production of the PG participates in the regulation of neuroendocrine functions, such as the HPA axis. On the other hand, strong *in situ* hybridization histochemical signal for mRNA encoding the EP3  $PGE_2$  receptor subtypes was observed over neurons of the NTS and the VLM (Ericsson et al., 1995a). Neuronal populations of the medulla, particularly A1/C1 and A2/C2 cell groups, are likely to be target cells of  $PGE_2$  during the acute-phase response to inform the hypothalamus of the neuroendocrine changes to be accomplished for the restoration of the homeostasis. The purposes of the present study were to investigate the effects of various doses [250  $\mu$ g, 25  $\mu$ g, and 2.5  $\mu$ g LPS/100 g body weight (b.w.)] of the bacterial LPS on the expression of the IEGs *c-fos* and NGFI-B (used as an index of cellular activity) throughout the rat brain, to study the role of eicosanoid cyclooxygenase pathways in mediating brain cellular activation and transcription of neuroendocrine CRF in the hypothalamic PVN of immune-challenged rats, and to verify the possibility that PGs may modulate LPS-induced activation of A1/C1 and A2/C2 groups of brainstem catecholaminergic neurons.

## MATERIALS AND METHODS

### Animals

Adult male Sprague-Dawley rats (~230–260 g) were acclimated to standard laboratory conditions (14-hour light/10-hour dark cycle; lights on at 0600 and off at 2000) with free access to rat chow and water. Each rat was used only once for experimentation, and all protocols were approved by the Laval University Animal Welfare Commit-

tee. A total of 120 rats were assigned to three different protocols (each corresponding to a different dose of LPS: 250 µg, 25 µg, or 2.5 µg/100 g b.w.), which were further subdivided into four treatments [intravenous (i.v.) vehicle + i.p. vehicle, i.v. vehicle + i.p. LPS, i.v. indomethacin + i.p. vehicle, i.v. indomethacin + i.p. LPS] and two postinjection times (3 and 6 hours following LPS administration).

### Surgery

Each rat was anesthetized with an i.p. injection of 0.3 ml of ketamine hydrochloride (91 mg/kg) and xylazine (9.1 mg/kg) mixture. Once they were anesthetized, rats were implanted with two catheters: one into the jugular vein and the other into the peritoneal cavity that was attached to the abdominal muscle. Catheters were made from a piece of silastic tubing (Silastic medical grade tubing; inner diameter 0.020 inches, outer diameter 0.037 inches; Dow Corning, Midland, MI) connected to an intramedic polyethylene tubing (PE-50; Caly Adams, Parsippany, NJ). Outlets of cannulas were placed at the level of the neck, and rats were housed individually in metal cages for a recuperation period of 2 days.

### Treatments

On the day of the experiment (~0830 in the morning), the outlet portion of each catheter (i.v. and i.p.) was fixed to a 1.5-cm, 22-gauge needle, which was attached to PE-50 tubing. These connectors were then fixed to a 1-cc syringe, and rats were placed individually in a quiet room for at least 2 hours prior to experimentation. This procedure was used to avoid disturbance of the animals during i.v. and i.p. administrations. Intravenous administration of indomethacin (0.8 mg/100 g b.w.; I-7378, lot, 83H0041; Sigma, Oakville, Ontario, Canada), an inhibitor of PG synthesis, diluted in 300 µl of a sterile saline solution (NaCl 0.9% solution, 5% alcohol) was accomplished 15 minutes prior to a single i.p. injection of various doses of LPS (250 µg, 25 µg, and 2.5 µg/100 g b.w.; from *Escherichia coli*, Serotype 055:B5; L-2880, lot, 122H4025; Sigma) diluted in 300 µl of sterile saline (0.9%). The high dose of LPS used in this study (250 µg/100 g b.w.) caused several visible sickness symptoms in the rats, such as covering themselves, immobility, diarrhea, shivering, and piloerection, but no mortality was observed following this treatment. Twenty-four hours after the i.p. injection of the latter LPS dose, these signs were usually gone in both male and female rats (personal observation). In some animals that were injected i.p. with 25 µg LPS/100 g b.w., few (but at least some) of the physical symptoms described above, especially covering themselves, were observed, whereas the low dose did not cause any apparent signs of sickness. Moreover, the high dose of LPS elicited a robust activation of both IEGs *c-fos* and NGFI-B in multiple regions of the brain of adult male rats (Rivest and Laflamme, 1995). To ensure the complete inhibition of cyclooxygenase pathways, a second and third injection of indomethacin or its vehicle were accomplished 1 and 3 hours after the LPS injection. Similar doses of indomethacin have been shown to reverse the influence of LPS and IL-1 on many neuroendocrine functions (Lacroix and Rivest, 1996; Rivest and Rivier, 1993; Rivier, 1993; Wan et al., 1994). The rats were conscious and were moving freely at all times throughout the experimental procedure. Three or six hours after the i.p. treatment with the bacterial endotoxin or the vehicle solution, the animals were deeply anesthetized with an i.v. injection of 0.1 ml of a

mixture of ketamine hydrochloride and xylazine and were 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 time points were chosen on the basis of previous studies, which showed a strong signal for various IEG mRNAs throughout the rat brain and transcriptional activation of the genes encoding CRF and its type 1 receptor in the endocrine hypothalamus 3 and 6 hours after treatment with the bacterial endotoxin (Rivest and Laflamme, 1995; Rivest et al., 1995). Brains were removed from the skulls, postfixed for 2–8 days, and then placed in a solution containing 10% sucrose mixed 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. The slices were collected in a cold cryoprotectant solution (0.05 M sodium phosphate buffer, pH 7.3, 30% ethylene glycol, 20% glycerol) and stored at -20°C.

### In situ hybridization histochemistry

Hybridization histochemical localization of each transcript was carried out on every sixth section of the whole rostrocaudal extent of each brain (from the olfactory bulb to the end of the medulla) by using [<sup>35</sup>S]-labeled cRNA probes. Protocols for riboprobe synthesis, hybridization, and autoradiographic localization of mRNA signal were adapted from Simmons et al. (1989). All solutions were treated with diethylpyrocarbonate (Depc) and were sterilized to prevent RNA degradation. Tissue sections mounted onto poly-L-lysine-coated slides were desiccated under vacuum overnight, fixed in 4% paraformaldehyde for 30 minutes, and digested by proteinase K (10 µg/ml in 0.1 M Tris HCl, pH 8.0, and 50 mM EDTA, pH 8.0, at 37°C for 25 minutes). Thereafter, the brain sections were rinsed in sterile Depc water followed by a solution of 0.1 M triethanolamine (TEA), pH 8.0; acetylated in 0.25% acetic anhydride in 0.1 M TEA; and dehydrated through graded concentrations of alcohol (50%, 70%, 95%, and 100%). After vacuum drying for a minimum of 2 hours, 90 µl of hybridization mixture (10<sup>7</sup> cpm/ml) were spotted on each slide, sealed under a coverslip, and incubated at 60°C overnight (~15–20 hours) in a slide warmer. Coverslips were then removed, and the slides were rinsed in 4 × standard saline citrate (SSC) at room temperature. Sections were digested by RNase A (20 µg/ml at 37°C for 30 minutes), rinsed in descending concentrations of SSC (2 × SSC, 1 × SSC, and 0.5 × SSC), washed in 0.1 × SSC for 30 minutes at 60°C (1 × SSC = 0.15 M NaCl, 15 mM trisodium citrate buffer, pH 7.0), and dehydrated through graded concentrations of alcohol. After being dried for 2 hours under the vacuum, the sections were exposed at 4°C to x-ray film (Kodak, Rochester, NY) for 18–24 hours (depending of the gene), defatted in xylene, and dipped in NTB2 nuclear emulsion (Kodak; diluted 1:1 with distilled water). Slides were exposed for 8–16 days (NGFI-B and *c-fos* mRNA, 8 days; CRF hnRNA, 16 days), developed in D19 developer (Kodak) for 3.5 minutes at 14–15°C, washed for 15 seconds in water, and fixed in rapid fixer (Kodak) for 5 minutes. Thereafter, tissues were rinsed in running distilled water for 1–2 hours, counterstained with thionin (0.25%), dehydrated through graded concentrations of alcohol, cleared in xylene, and coverslipped with DPX.

### cRNA probe synthesis and preparation

The *c-fos* and NGFI-B cRNA probes were generated from the *EcoR1* fragment of rat *c-fos* cDNA (Dr. I Verma, The Salk Institute) and rat NGFI-B cDNA (Dr. J. Milbrandt; Milbrandt, 1988) subcloned into pBluescript SK-1 (Stratagene, La Jolla, CA) and linearized with *SmaI* and *BamHI*, respectively. pGem3 plasmid containing a pure CRF intronic piece was linearized with *HindIII* (530 bp) specifically to detect CRF heteronuclear (hn) RNA (Dr. S. Watson, The University of Michigan, Ann Arbor; Herman et al., 1992). Radioactive cRNA copies were synthesized by incubation of 250 ng linearized plasmid in 6 mM MgCl<sub>2</sub>; 40 mM Tris, pH 7.9; 2 mM spermidine; 10 mM NaCl; 10 mM dithiothreitol, 0.2 mM ATP/GTP/CTP; [ $\alpha$ -<sup>35</sup>S]UTP; 40 U RNAsin (Promega, Madison, WI); and 20 U of either T7 (*c-fos* mRNA and CRF hnRNA antisense probes) or T3 (NGFI-B mRNA antisense probe) RNA polymerase for 60 minutes at 37°C. Unincorporated nucleotides were removed by using the ammonium-acetate method; 100  $\mu$ l of DNase solution (1  $\mu$ l DNase, 5  $\mu$ l of 5 mg/ml tRNA, 94  $\mu$ l of 10 mM Tris/10 mM MgCl<sub>2</sub>) were added, and, 10 minutes later, an extraction was accomplished by using a phenol-chloroform solution. The cRNA was precipitated with 80  $\mu$ l of 5 M ammonium acetate and 500  $\mu$ l of 100% ethanol for 20 minutes on dry ice. The pellet was washed with 500  $\mu$ l 70% ethanol, dried, and resuspended in 100  $\mu$ l of 10 mM Tris/1 mM EDTA. A concentration of 10<sup>7</sup> cpm probe was mixed into 1 ml of hybridization solution [500  $\mu$ l formamide; 60  $\mu$ l 5 M NaCl; 10  $\mu$ l 1 M Tris, pH 8.0; 2  $\mu$ l 0.5 M EDTA, pH 8.0; 50  $\mu$ l 20 $\times$  Denhart's solution; 200  $\mu$ l 50% dextran sulfate; 50  $\mu$ l 10 mg/ml tRNA; 10  $\mu$ l 1 M dithiothreitol (DTT); (118  $\mu$ l Depc water – volume of probe used)]. This solution was mixed and heated for 5 minutes at 65°C prior to being spotted on slides.

### Combination of immunocytochemistry with in situ hybridization

Immunocytochemistry [tyrosine hydroxylase (TH)-immunoreactive (ir) neurons] was combined with the in situ hybridization histochemistry protocol (*c-fos* mRNA) to determine the number of TH-activated cells after systemic treatment with LPS and to investigate the role of the PGs in the activation of catecholaminergic neurons during immune challenge, particularly in the NTS and the VLM. Every sixth tissue slice 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 were incubated at 4°C with TH 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). Antisera raised in mouse against TH (catalog no. 22941; Incstar Science, Technology and Research, Stillwater, MN) was used at a concentration of 1:5,000. Approximately 18 hours after incubation at 4°C with the primary antibody (TH), the brain slices were rinsed in sterile KPBS and were incubated with a mixture of KPBS + Triton X-100 + heparin + biotinylated goat anti-mouse immunoglobulin (IgG; 1:1,500 dilution; Vector Laboratories, Burlingame, CA) for 90 minutes. Sections were then rinsed with KPBS and incubated at room temperature for 60 minutes with an avidin-biotin-peroxidase complex (Vectastain ABC elite kit; Vector Laboratories). The peroxidase

complex was amplified by means of a 10-minute incubation with a 70-nM solution of biotin [sulfo succinimidyl 6-(biotinamido) hexanoate; 21335; Pierce, Rockford, IL]-tyramine HCl (4-hydroxyphenethylamine hydrochloride; T-2879; Sigma)-H<sub>2</sub>O<sub>2</sub> (0.01%), followed by a second incubation of 30 minutes with the ABC elite solution. After several rinses in sterile KPBS, the brain slices were reacted in a mixture containing sterile KPBS, the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB; 0.05%), and 0.003% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>).

Thereafter, tissues were rinsed in sterile KPBS, mounted onto poly-L-lysine-coated slides, desiccated under vacuum overnight, fixed in 4% PFA for 30 minutes, and digested by proteinase K (10  $\mu$ g/ml in 100 mM Tris HCl, pH 8.0, and 50 mM EDTA, pH 8.0, at 37°C for 25 minutes). Prehybridization, hybridization, and posthybridization steps were performed as described above, although dehydration steps (alcohol 50%, 70%, 95%, 100%) were shortened to avoid decoloration of TH cells (brown staining). After drying for 2 hours under vacuum, sections were exposed at 4°C to x-ray film (Kodak) overnight, defatted in xylene, and dipped in NTB2 nuclear emulsion (Kodak; diluted 1:1 with distilled water). Slides were exposed for 8 days, developed in D19 developer (Kodak) for 3.5 minutes at 15°C, and fixed in rapid fixer (Kodak) for 5 minutes. Thereafter, tissues were rinsed in running distilled water for 1–2 hours, rapidly dehydrated through graded concentrations of alcohol, cleared in xylene, and coverslipped with DPX. The presence of *c-fos* transcript was evident as silver grains, which were clearly visible in perikarya, and as TH immunoreactivity within the cell cytoplasm, which was stained brown.

### Quantitative analysis

The *c-fos* mRNA, NGFI-B mRNA, and CRF hnRNA signals revealed on dipped, NTB2 nuclear emulsion slides were analyzed and quantified (relative levels) with an Olympus Optical System (BX-50, BMax) coupled to a Macintosh computer (PowerPC 7100/66) and Image software (version 1.59, non-FPU; W. Rasband, NIH). The refraction density (R.D. in arbitrary units) of the hybridization signal was measured under darkfield illumination at a magnification of  $\times 10$ . Sections from the experimental and control animals were matched for rostrocaudal level. Because of the lack of basal expression of *c-fos* mRNA, NGFI-B mRNA, and CRF hnRNA in the hypothalamic medial PVN and supraoptic nucleus (SON), the whole nuclei were digitized under brightfield illumination and then subjected to densitometric analysis under darkfield, yielding measurements of integrated R.D. (area of nucleus  $\times$  average R.D.). The R.D. of each specific region was then corrected for the average background signal, which was determined by sampling cells immediately outside the cell group of interest (McCabe and Pfaff, 1989). The procedure used to quantify the relative levels of refraction density in arbitrary units on nuclear emulsion-dipped slides is based on the work published by Ericsson and colleagues (1994).

Measurements of TH-ir cells and double-labeled cells (TH-ir neurons expressing *c-fos* mRNA) were accomplished at a magnification of  $\times 100$  under brightfield illumination for the whole rostrocaudal NTS (A2/C2) and the VLM (A1/C1). For each rat, an average of 14–16 and 36–44 bilateral sections were quantified for the NTS and the VLM, respectively. Determination of the double-labeled cells was performed visually for each cell exhibiting clear brown cytoplasm (TH) and numbers of silver

grains (*c-fos* mRNA) within the cell body that were at least five times higher than background.

**Statistical analysis**

Data from Figures 3–5, 7, and 9 were analyzed by a 2 × 2 analysis of variance (ANOVA), followed by a Bonferroni/Dunn test procedure as post-hoc comparisons for each time postinjection (Statview 4.01). Factors were identified as follows: intraperitoneal treatment, which was composed of two levels (i.p. vehicle or i.p. LPS) and intravenous treatment, which was also divided into two levels (i.v. control or i.v. indomethacin).

**RESULTS**

**Induction of *c-fos* transcript throughout the brains of immune-challenged rats**

Table 1 describes the qualitative analysis of hybridization signal on x-ray films for *c-fos* mRNA in the brain of male rats killed 3 hours after i.p. vehicle or LPS administration for each dose used in this study. Note that *c-fos* was analyzed and presented in detail because it appears to be a more sensitive index of postsynaptic neuronal activation than NGFI-B throughout the brain. In fact, all structures that expressed NGFI-B transcript in response to LPS were also positive for *c-fos*, but not vice versa; compared with the IEG *c-fos* mRNA, positive hybridization signal for NGFI-B transcript appeared limited to fewer LPS-responsive nuclei.

Positive hybridization signal for *c-fos* transcript, as previously described by several studies, was detected in different regions of the brain under basal conditions. Low-to-moderate basal signals were generally observed in the suprachiasmatic nucleus, in various divisions of the thalamus including the anterodorsal (AD) and anteroventral (AV) nuclei, the lateral geniculate complex (LGC), the cochlear nucleus (CN), layer 2 of the piriform area (Pir), and in multiple layers of the cerebral cortex including layer 2, 3, 4, and 6a. However, vehicle injection did not provoke expression of the IEG in nuclei that are generally stress-responsive, because experiences were accomplished in chronically implanted animals in absolute-quiet conditions. The endotoxin injected i.p. induced expression of *c-fos* in a wide variety of nuclei and areas, an effect that was dependent on the dose. Representative examples of *c-fos* distribution throughout the brain of animals administered with increasing i.p. doses of the bacterial endotoxin LPS are depicted by Figure 1.

**Forebrain.** The high dose of LPS (250 µg/100 g b.w.) that was used here as a model of severe induction of the acute-phase response, as recently reported (Rivest and Laflamme, 1995) and confirmed by this study, caused a strong and prolonged (up to 6 hours after the injection) hybridization signal for *c-fos* mRNA in several (but selective) regions of the diencephalon: the SON, the PVN, the paraventricular nucleus of the thalamus (PVT), and the arcuate nucleus (ARC). A moderate-to-low signal was induced in several regions, including the subependymal zone (SEZ), the bed nucleus of the stria terminalis (BnST), the periventricular nucleus of the hypothalamus (PV), the central nucleus of the amygdala (CeA), and the dorsomedial nucleus of the hypothalamus (DMH) of rats injected with this high dose. Administration of the middle dose of endotoxin (25 µg LPS/100 g b.w.) produced a similar

TABLE 1. Qualitative Analysis of Hybridization Signal for *c-fos* mRNA in the Brain of Immune-Challenged Rats 3 Hours After i.p. Lipopolysaccharide Administration<sup>1</sup>

| Brain region                                 | Dose of lipopolysaccharide |                      |                       | Vehicle |
|----------------------------------------------|----------------------------|----------------------|-----------------------|---------|
|                                              | 250 µg/<br>100 g b.w.      | 25 µg/<br>100 g b.w. | 2.5 µg/<br>100 g b.w. |         |
| Clastrum                                     | 0/+                        | 0/+                  | 0/+                   | 0/+     |
| Subependymal zone                            | +/++                       | +/++                 | 0/+                   | —       |
| Lateral septal nucleus                       | 0/+                        | 0/+                  | 0/+                   | 0/+     |
| Bed nucleus of the stria terminalis          | ++                         | +/++                 | 0/+                   | 0/+     |
| Suprachiasmatic nucleus                      | +/++                       | +/++                 | +/++                  | +/++    |
| Medial preoptic area OVL                     | +++/>++++                  | +++/>++++            | +/++                  | 0/+     |
| Periventricular nucleus of the hypothalamus  | +                          | +/++                 | —                     | —       |
| Supraoptic nucleus                           | +++/>++++                  | +++/>++++            | +                     | 0/+     |
| Anterior hypothalamic nucleus                | 0/+                        | 0/+                  | 0/+                   | 0/+     |
| Subfornical organ                            | ++                         | +/+++                | +/++                  | 0/+     |
| Hypothalamic paraventricular nucleus         | +++/>++++                  | +++/>++++            | 0/+                   | 0/+     |
| Paraventricular nucleus of the thalamus      | +/+++                      | +/+++                | +/++                  | 0/+     |
| Thalamus (anterodorsal and anteroventral)    | +                          | +                    | +                     | +       |
| Central nucleus of the amygdala              | +/++                       | +/++                 | 0/+                   | 0/+     |
| Arcuate nucleus                              | +/+++                      | +/+++                | 0/+                   | 0/+     |
| Median eminence                              | +/++                       | +/+++                | 0/+                   | 0/+     |
| Dorsomedial nucleus of the hypothalamus      | +/++                       | +/++                 | 0/+                   | 0/+     |
| Subthalamic nucleus                          | 0/+                        | 0/+                  | —                     | —       |
| Lateral geniculate complex                   | +                          | +                    | +                     | +       |
| Laterodorsal tegmental nucleus               | +/++                       | +/++                 | 0/+                   | 0/+     |
| Parabrachial nucleus (external lateral part) | +/+++                      | +/+++                | 0/+                   | 0/+     |
| Locus coeruleus                              | ++                         | ++                   | 0/+                   | 0/+     |
| Cochlear nucleus                             | +                          | +                    | +/+++                 | +/+++   |
| Spinal nucleus of the trigeminal             | 0/+                        | 0/+                  | 0/+                   | 0/+     |
| Nucleus of the solitary tract                | +++/>++++                  | +++/>++++            | 0/+                   | 0/+     |
| Lateral reticular nucleus/ambiguous nucleus  | +/+++                      | +/+++                | 0/+                   | 0/+     |
| Area postrema                                | ++                         | +/++                 | +                     | —       |
| Cortex (general)                             | +                          | +                    | +                     | +       |
| Choroid plexus                               | ++                         | +/+++                | 0/+                   | 0/+     |
| Ependymal cells of ventricles                | +/++                       | +/++                 | 0/+                   | —       |
| Meninges (lepto)                             | +/+++                      | +/+++                | 0/+                   | —       |

<sup>1</sup>In situ hybridization histochemistry was accomplished by using a [<sup>35</sup>S]-labeled cRNA probe encoding the immediate-early gene *c-fos*. The cDNA-encoding the rat *c-fos* was generously provided by Dr. I. Verma (The Salk Institute, La Jolla, CA). +++++, Very strong signal; +++, strong signal; ++, moderate signal; +, low but positive signal; —, undetectable signal; b.w., body weight; OVL, organum vasculosum of the lamina terminalis.

pattern in terms of intensity and expressing sites, although the signal for *c-fos* mRNA declined in all of these structures 6 hours after injection. In contrast, injection of the low dose (2.5 µg LPS/100 g b.w.) caused a modest expression of the IEG *c-fos* in very few regions of the forebrain. Low but significant expression of *c-fos* transcript was indeed detected in the SON and the PVT at 3 hours, whereas no positive message was present at 6 hours postinjection.

**Circumventricular organs, meninges, and ependymal layer.** Injection of the high dose (250 µg LPS/100 g b.w.) of the bacterial endotoxin caused a profound expression of *c-fos* mRNA in the MPOA/OVL and in the lepto-

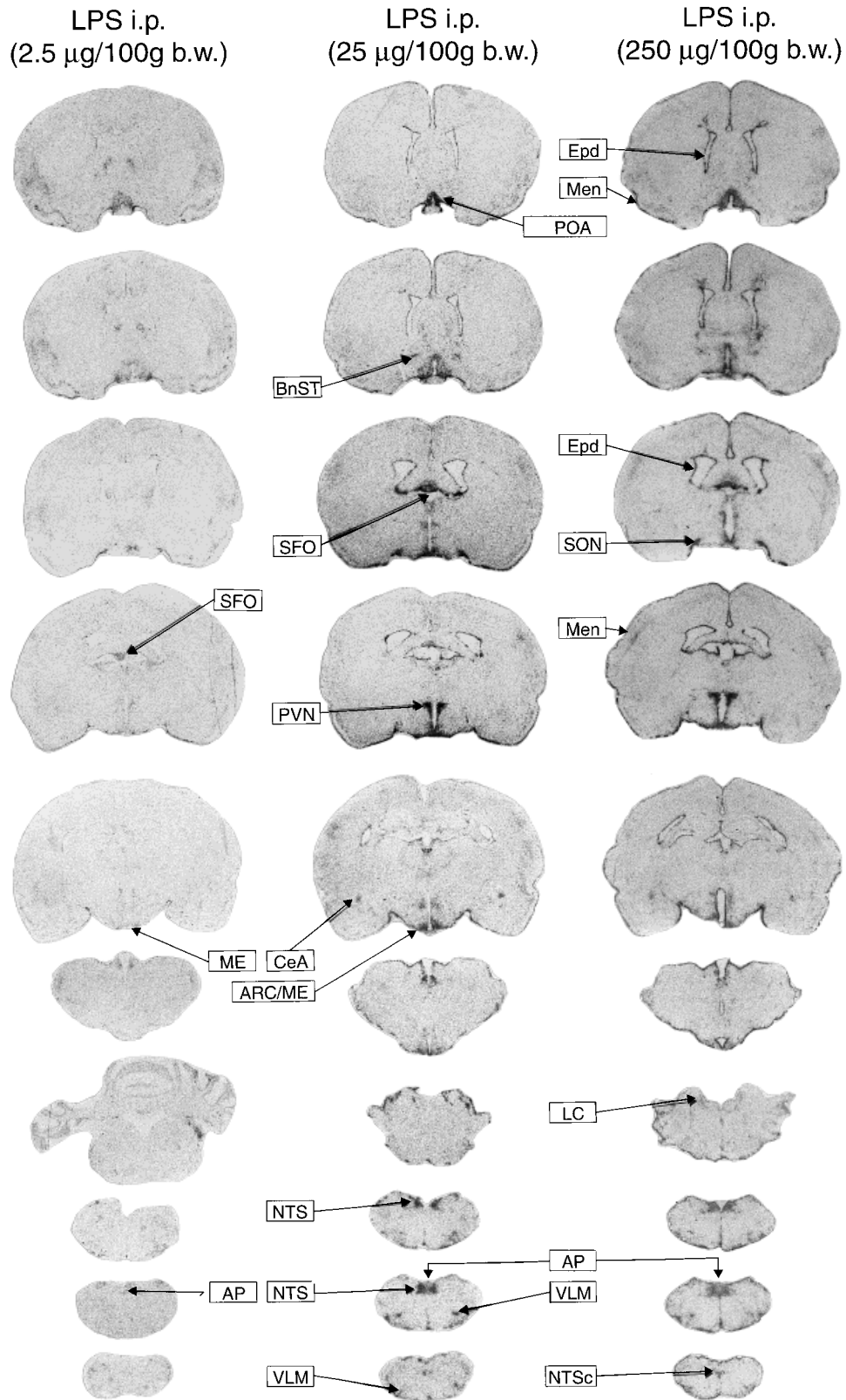


Fig. 1. Representative example of the distribution of the mRNA encoding the immediate-early gene *c-fos* in the rat brain after intraperitoneal (i.p.) administration of three different doses of the endotoxin lipopolysaccharide (LPS). Animals were deeply anesthetized and rapidly perfused with 4% paraformaldehyde 3 hours after treatment with LPS [low dose, 2.5 µg/100 g body weight (b.w.); middle dose, 25 µg/100 g b.w.; high dose, 250 µg/100 g b.w.]. These rostrocaudal coronal sections (30 µm) of LPS-treated rat exhibited a positive signal on x-ray film (Biomax, Rochester, NY) for *c-fos* transcript in various structures

throughout the brain, a phenomenon dependent on the dose administered. AP, area postrema; ARC, arcuate nucleus; BnST, bed nucleus of the lamina terminalis; CeA, central nucleus of the amygdala; Epd, ependymal lining cells of lateral ventricle; LC, locus coeruleus; ME, median eminence; Men, meninges (lepto); MPOA, medial preoptic area; NTS, nucleus of the solitary tract; NTSc, nucleus of the solitary tract, caudal part; PVN, paraventricular nucleus of the hypothalamus; SFO, subfornical organ; SON, supraoptic nucleus of the hypothalamus; VLM, ventrolateral medulla.

TABLE 2. Hybridization Signal for *c-fos* mRNA in the Brain of Immune-Challenged Rats 3 Hours After i.p. Lipopolysaccharide Administration: Role of Prostaglandins<sup>1</sup>

| Brain region                                 | Dose of lipopolysaccharide |         |                  |         |                   |         | IND | VEH |
|----------------------------------------------|----------------------------|---------|------------------|---------|-------------------|---------|-----|-----|
|                                              | 250 µg/100 g b.w.          |         | 25 µg/100 g b.w. |         | 2.5 µg/100 g b.w. |         |     |     |
|                                              | LPS                        | LPS-IND | LPS              | LPS-IND | LPS               | LPS-IND |     |     |
| Clastrum                                     | 0/+                        | 0/+     | 0/+              | 0/+     | 0/+               | 0/+     | 0/+ | 0/+ |
| Subependymal zone                            | +++                        | +++     | +++              | +++     | 0/+               | 0/+     | —   | —   |
| Lateral septal nucleus                       | 0/+                        | 0/+     | 0/+              | 0/+     | 0/+               | 0/+     | 0/+ | 0/+ |
| Bed nucleus of the stria terminalis          | ++                         | ++      | +++              | +       | 0/+               | 0/+     | 0/+ | 0/+ |
| Suprachiasmatic nucleus                      | +++                        | +++     | +++              | +++     | +++               | +++     | +++ | +++ |
| Medial preoptic area/OVLT                    | ++++                       | ++++    | ++++             | +++     | +++               | 0/+     | 0/+ | 0/+ |
| Periventricular nucleus of the hypothalamus  | +                          | 0/+     | +++              | 0/+     | —                 | —       | —   | —   |
| Supraoptic nucleus                           | ++++                       | ++++    | ++++             | +++     | +                 | 0/+     | 0/+ | 0/+ |
| Anterior hypothalamic nucleus                | 0/+                        | 0/+     | 0/+              | 0/+     | 0/+               | 0/+     | 0/+ | 0/+ |
| Subfornical organ                            | ++                         | ++      | +++              | +++     | +++               | +++     | 0/+ | 0/+ |
| Hypothalamic paraventricular nucleus         | ++++                       | +++     | ++++             | +       | 0/+               | 0/+     | 0/+ | 0/+ |
| Paraventricular nucleus of the thalamus      | +++                        | +++     | +++              | +++     | +++               | +       | 0/+ | 0/+ |
| Thalamus (anterodorsal and anteroventral)    | +                          | +       | +                | +       | +                 | +       | +   | +   |
| Central nucleus of the amygdala              | +++                        | +++     | +++              | +++     | 0/+               | 0/+     | 0/+ | 0/+ |
| Arcuate nucleus                              | +++                        | +++     | +++              | +++     | 0/+               | 0/+     | 0/+ | 0/+ |
| Median eminence                              | +++                        | +++     | +++              | +++     | 0/+               | 0/+     | 0/+ | 0/+ |
| Dorsomedial nucleus of the hypothalamus      | +++                        | +++     | +++              | +       | 0/+               | 0/+     | 0/+ | 0/+ |
| Subthalamic nucleus                          | 0/+                        | 0/+     | 0/+              | +       | —                 | —       | —   | —   |
| Lateral geniculate complex                   | +                          | +       | +                | +       | +                 | +       | +   | +   |
| Laterodorsal tegmental nucleus               | +++                        | +++     | +++              | +       | 0/+               | 0/+     | 0/+ | 0/+ |
| Parabrachial nucleus (external lateral part) | +++                        | +++     | +++              | +++     | 0/+               | 0/+     | 0/+ | 0/+ |
| Locus coeruleus                              | ++                         | +++     | ++               | +++     | 0/+               | 0/+     | 0/+ | 0/+ |
| Cochlear nucleus                             | +                          | +       | +                | +       | +++               | +       | +   | +++ |
| Spinal nucleus of the trigeminal             | 0/+                        | 0/+     | 0/+              | 0/+     | 0/+               | 0/+     | 0/+ | 0/+ |
| Nucleus of the solitary tract                | ++++                       | ++++    | ++++             | ++++    | 0/+               | 0/+     | 0/+ | 0/+ |
| Lateral reticular nucleus/ambiguous nucleus  | +++                        | +++     | +++              | +       | 0/+               | 0/+     | 0/+ | 0/+ |
| Area postrema                                | ++                         | +++     | +++              | +++     | +                 | +       | —   | —   |
| Cortex (general)                             | +                          | +++     | +                | +++     | +                 | +++     | +   | +   |
| Choroid plexus                               | ++                         | ++      | +++              | +++     | 0/+               | 0/+     | 0/+ | 0/+ |
| Ependymal cells of ventricles                | +++                        | +++     | +++              | +       | 0/+               | 0/+     | —   | —   |
| Meninges (lepto)                             | +++                        | +++     | +++              | +++     | 0/+               | 0/+     | —   | —   |

<sup>1</sup> +++++, Very strong signal; +++, strong signal; ++, moderate signal; +, low but positive signal; —, undetectable signal; b.w., body weight; IND, indomethacin pretreatment; LPS, lipopolysaccharide; OVLT, organum vasculosum of the lamina terminalis; PGs, prostaglandins; VEH, vehicle treatment.

meninges. Moreover, numerous other structures, including the subfornical organ (SFO), the median eminence (ME; principally in the internal lamina), the area postrema (AP), the choroid plexus (ChP) and the cuboidal ependymal lining cells of the ventricles, exhibited moderate-to-low signal in rats injected with the high dose. For the forebrain sites, as mentioned above, mRNA encoding the IEG remained as long as 6 hours after the treatment.

Injection of the middle dose (25 µg LPS/100 g b.w.) activated the same brain regions with similar intensity, except for the SFO and the ME, which exhibited strong hybridization signal. In contrast to the high dose, in which the signal was still detectable 6 hours after treatment in most of the above described areas, signal for the IEG largely declined 6 hours after administration of the middle dose, except for the sensorial CVOs (OVLT, SFO, ME, and AP), in which strong hybridization signal was still detected.

Interestingly, the low dose (2.5 µg LPS/100 g b.w.) of the endotoxin activated transcription of *c-fos* in few regions, including the CVOs, the SON, and the PVT. Indeed, the MPOA/OVLT, the SFO and the AP of rats injected i.p. with the low dose displayed moderate-to-low hybridization signal at 3 hours after administration, whereas the message essentially vanished 6 hours after the administration.

**Brainstem.** Strong hybridization signal for *c-fos* mRNA was observed in the parabrachial nucleus (PB) and throughout the entire NTS, including the dorsal motor nucleus of the vagus (X) and the caudal ventrolateral medulla (cVLM) at the level of lateral reticular nucleus (LRN)/ambiguous nucleus (AMB) of rats injected with the high and middle doses of the bacterial endotoxin. The laterodorsal tegmen-

tal nucleus (LDT), the locus coeruleus (LC), and, in some instances, the gracile nucleus (GR) exhibited moderate-to-low signals in response to both high and moderate doses of LPS. The only noticeable difference in the effects of both doses (250 µg and 25 µg LPS/100 g b.w.) on *c-fos* expression in brainstem regions was detected at 6 hours postinjection; animals treated with the high dose exhibited persistent expression of the IEG, whereas the message was clearly diminished in rats treated with the middle dose. Note that no clear induction of either IEG was detected in any regions of the brainstem of scarcely challenged rats (2.5 µg LPS/100 g b.w.), except for the AP, as described above.

### Effects of indomethacin in the brains of immune-challenged rats

Table 2 shows that inhibition of PG synthesis had limited influence on LPS-induced *c-fos* expression throughout the rat brain. In fact, 3 hours after injection with the high dose of endotoxin, the hybridization signal for the IEG was similar (in terms of distribution and intensity) in the brain of rats either pretreated or not treated with indomethacin. However, at 6 hours post-LPS injection, some structures exhibited a reduced *c-fos* mRNA signal following blockage of cyclooxygenase pathways. Indeed, a lower expression of the gene encoding *c-fos* was observed in the PVN, SON, and LRN/AMBd of severely immune-challenged rats that received indomethacin compared with animals that were i.v. injected with the vehicle solution. Inhibition of PG was more efficient in attenuating *c-fos* expression in different brain nuclei of rats that received the middle dose of LPS; the PV, PVN, LRN/AMBd, and leptomeninges displayed a notable attenuated hybridiza-

tion signal for *c-fos* transcript 3 hours after systemic injections with both indomethacin and LPS. Representative examples of such phenomenon are depicted in Figure 2, which illustrates darkfield photomicrographs of the right PVN hybridized with *c-fos* riboprobe and dipped in NTB2 nuclear emulsion to reveal the silver grains. Although it was significantly reduced, transcription of *c-fos* was not completely abolished by indomethacin 3 hours after injection with the middle dose of 25 µg LPS/100 g b.w. (Figs. 2, 3, middle), indicating a partial influence of PGs in mediating the effects of LPS on brain neuronal activation. Image analysis was performed on the SON and PVN, because both hypothalamic nuclei are extremely well-defined structures, so that drawing inaccuracies between brains were avoided. Moreover, different neuropeptidergic cell groups located in these nuclei were found to be activated by systemic i.p. LPS (Rivest and Laflamme, 1995).

Indomethacin had a modest influence in preventing *c-fos* expression in few regions of the brain, such as the SON, even in animals treated with the dose of 25 µg LPS/100 g b.w. The *c-fos* signal intensity in the SON was similar in rats that were killed 3 hours after injection of the high and middle doses of LPS, whereas pretreatment with indomethacin did not significantly modulate this effect (Fig. 4, top and middle). On the other hand, inhibition of cyclooxygenase pathways largely abolished the increase in the average R.D. for *c-fos* mRNA 6 hours after treatment with the high dose of endotoxin (Fig. 4, top). Pretreatment with indomethacin did not modify the hybridization signal for *c-fos* mRNA in several responsive structures, including the SFO, PVT, CeA, ARC/ME, PB, LC, NTS, AP, and choroid plexus in animals injected with the middle dose of LPS (Table 2).

Figure 5 depicts the average R.D. of the hybridization signal for the IEG NGFI-B in the PVN and SON after i.p. administration of the middle dose of the bacterial endotoxin LPS (25 µg LPS/100 g b.w.). The signal intensity, as previously described for *c-fos* mRNA, was very strong 3 hours after a single i.p. administration with this dose of LPS and largely vanished at 6 hours postinjection. Inhibition of cyclooxygenase pathways significantly ( $P < 0.05$ ) prevented LPS-induced expression of NGFI-B mRNA in these hypothalamic regions at 3 hours postinjection; 1.7- and 0.8-fold increases were detected in rats that received the endotoxin alone compared with the rats that were submitted to a treatment combining both i.v. indomethacin and i.p. LPS in the PVN (Fig. 5, top) and SON (Fig. 5, bottom), respectively.

### Effects of indomethacin on LPS-induced CRF gene transcription in the endocrine hypothalamus

Because indomethacin is capable of attenuating PVN neuronal activation in response to systemic LPS (middle dose), it was of interest to investigate the effect of both treatments on neuroendocrine CRF gene transcription by using an intronic probe as a tool to detect the CRF primary transcript (CRF hnRNA). We have previously reported that i.p. LPS administration triggered CRF transcription selectively within the parvocellular division of the rat PVN (Rivest and Laflamme, 1995). Although it is not expressed under basal conditions, i.p. injection of 25 µg LPS/100 g b.w. caused a selective expression of CRF primary transcript within the parvocellular division of the PVN (Fig. 6)

without inducing detectable signals in other areas (results not shown). Positive hybridization signal for CRF hnRNA was detected in the PVN at both times post-LPS administration but was more intense at 3 hours than at 6 hours after systemic injection with the endotoxin. Inhibition of cyclooxygenase pathways interrupted transcription of CRF hnRNA in animals injected with the middle dose of LPS, indicating that PGs can influence the expression of the gene encoding the neuropeptide that is directly responsible for the control of the HPA axis during the acute-phase response.

The average R.D. of the hybridization signal for CRF primary transcript in the PVN after i.p. administration of the high dose (Fig. 7, top), the middle dose (Fig. 7, middle), or the low dose (Fig. 7, bottom) of the bacterial endotoxin is presented in Figure 7. In contrast to the results obtained with the middle dose of endotoxin, inhibition of PG synthesis did not interfere with the signal intensity of CRF hnRNA in the PVN of rats injected with the high dose and killed 3 hours postinjection. However, similar to *c-fos* mRNA, pretreatment with indomethacin tended to inhibit, at least in part, the expression of PVN CRF hnRNA at 6 hours postinjection. A low signal was detected in the endocrine hypothalamus 3 and 6 hours after treatment with the low dose of the bacterial endotoxin (Fig. 7, bottom).

### Effect of indomethacin on the activation of catecholaminergic neurons during immune challenge

Systemic LPS injection, as described previously, provoked a robust induction of the IEG *c-fos* in the NTS and the VLM, suggesting that noradrenergic (A1, A2) and adrenergic (C1, C2) pathways arising from these two brainstem nuclei could be activated during acute-phase response. To investigate this possibility, the percentage of TH-ir cells displaying *c-fos* mRNA in the NTS and VLM was determined by combining immunocytochemistry with *in situ* hybridization histochemistry in the same brain sections. Measurements of TH-ir cells and double-labeled cells (TH-ir neurons expressing *c-fos* mRNA) were accomplished at a magnification of  $\times 100$  under brightfield illumination on the entire NTS (A2/C2) and the full rostrocaudal extent of the VLM (A1/C1). Figure 8 shows high-magnification photomicrographs of TH-ir neurons expressing *c-fos* transcript in the NTS (Fig. 8, top) and the VLM (Fig. 8, bottom) of rats killed 3 hours postinjection of the middle dose of LPS. Darkfield photomicrographs illustrate the exact location from which high-magnification brightfield photomicrographs of the NTS (Fig. 8, top) and the VLM (Fig. 8, bottom) were taken. In the NTS, the number of TH-ir cells displaying *c-fos* mRNA (Fig. 8, solid arrows) was almost similar in animals either treated or not treated with indomethacin prior to receiving the bacterial endotoxin at a dose of 25 µg LPS/100 g b.w. In the VLM of immune-challenged rats, agglomerations of silver grains delineating *c-fos* mRNA<sup>+</sup> cells were largely colocalized within TH-ir neurons (Fig. 8, solid arrows), whereas inhibition of the cyclooxygenase pathways largely prevented expression of the IEG *c-fos* within TH-ir cells located in this brainstem region.

Of the catecholaminergic neurons of the NTS, 23.89%  $\pm$  4.67 and 5.25%  $\pm$  1.80, respectively, were positive for *c-fos* mRNA 3 and 6 hours after injection of the middle dose of LPS (Fig. 9, top). Interestingly, pretreatment with indo-

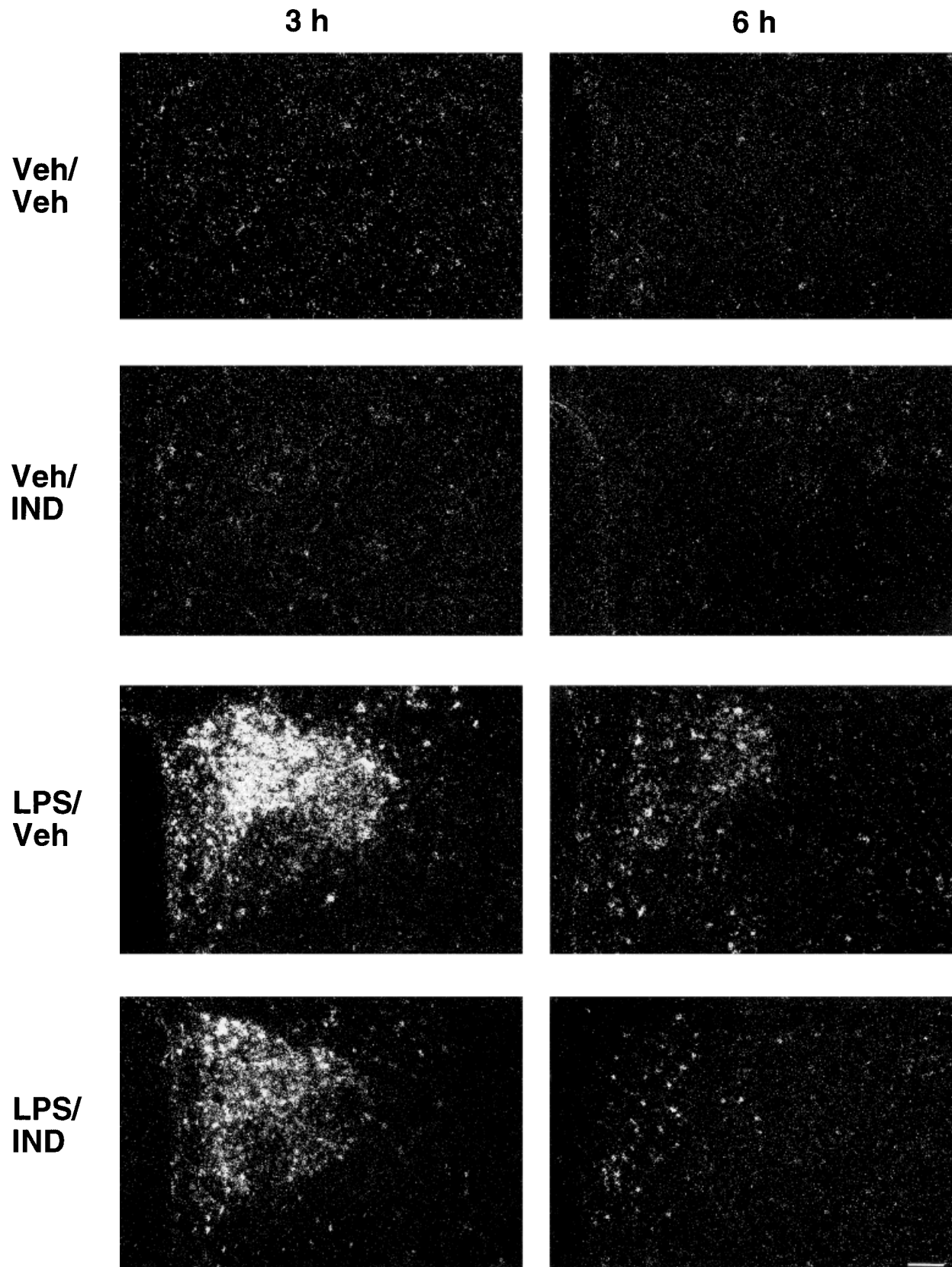


Fig. 2. Effect of indomethacin (IND) intravenous (i.v.) injection on the expression of *c-fos* mRNA in the hypothalamic paraventricular nucleus (PVN) 3 and 6 hours after i.p. administration of the middle dose of the bacterial endotoxin LPS (25  $\mu$ g LPS/100 g b.w.). These darkfield photomicrographs show in situ hybridization signals for the mRNA encoding the immediate-early gene throughout similar areas of

the right PVN. Note that inhibition of cyclooxygenase pathways attenuated *c-fos* transcription in animals injected i.p. with this dose of LPS at both postinjection times (3 and 6 hours). In addition, a stronger expression of the mRNA encoding Fos was detected selectively in the parvocellular PVN. Veh, vehicle. Magnification  $\times 25$ . Scale bar = 100  $\mu$ m.

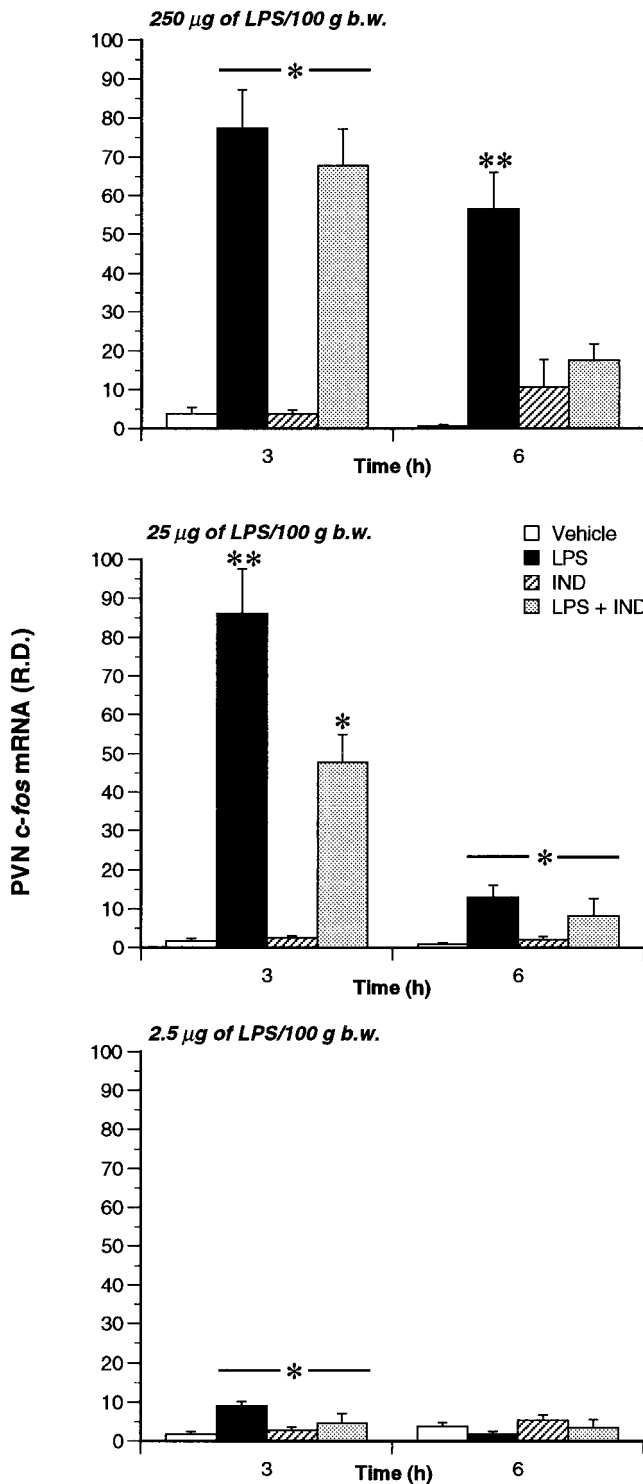


Fig. 3. Average refraction density (R.D.; in arbitrary units) of the hybridization signal for the relative levels of *c-fos* transcript in the hypothalamic PVN after i.p. administration of the high (**top**), middle (**middle**), or low (**bottom**) dose of the bacterial endotoxin LPS. Results represent means  $\pm$  S.E.M. of four rats; an average of two medial PVNs were digitized for each rat. Statistical analysis was performed by using a  $2 \times 2$  analysis of variance (ANOVA) followed by a Bonferroni/Dunn post-hoc test for each posttreatment time (Statview 4.01). Asterisk between lines indicates that the main effect of the LPS treatment was without significant interaction between i.p. LPS and i.v. IND treatments. Significant interaction between i.p. and i.v. treatments: single asterisk, significantly different ( $P < 0.05$ ) from the appropriate control group; double asterisk, significantly different ( $P < 0.05$ ) from all other groups. For more information on image analysis, see Materials and Methods. For abbreviations, see Figure 1 and 2.

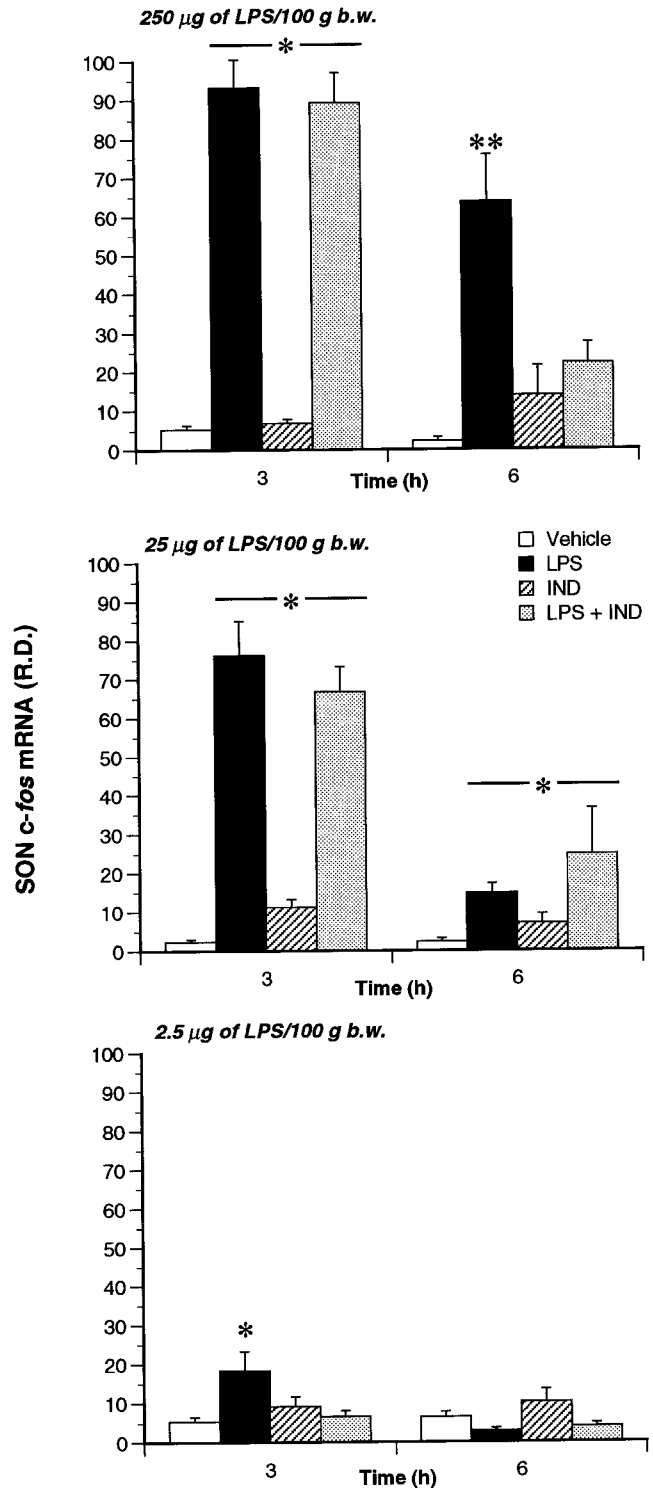


Fig. 4. Effect of i.p. injection of the endotoxin LPS on the average R.D. (in arbitrary units) of the hybridization signal for *c-fos* transcript in the supraoptic nucleus of the hypothalamus (SON) of rats either pretreated with or without the cyclooxygenase inhibitor IND. Results represent means  $\pm$  S.E.M. of four rats; an average of four medial SONs were digitized for each rat. Statistical analysis was performed by using a  $2 \times 2$  ANOVA followed by a Bonferroni/Dunn post-hoc test for each postinjection time (Statview 4.01). Asterisk between lines indicates that the main effect of the LPS treatment was without significant interaction between i.p. LPS and i.v. IND treatments. Significant interaction between i.p. and i.v. treatments: single asterisk, significantly different ( $P < 0.05$ ) from the appropriate control group; double asterisk, significantly different ( $P < 0.05$ ) from all other groups. For more information on image analysis, see Materials and Methods.

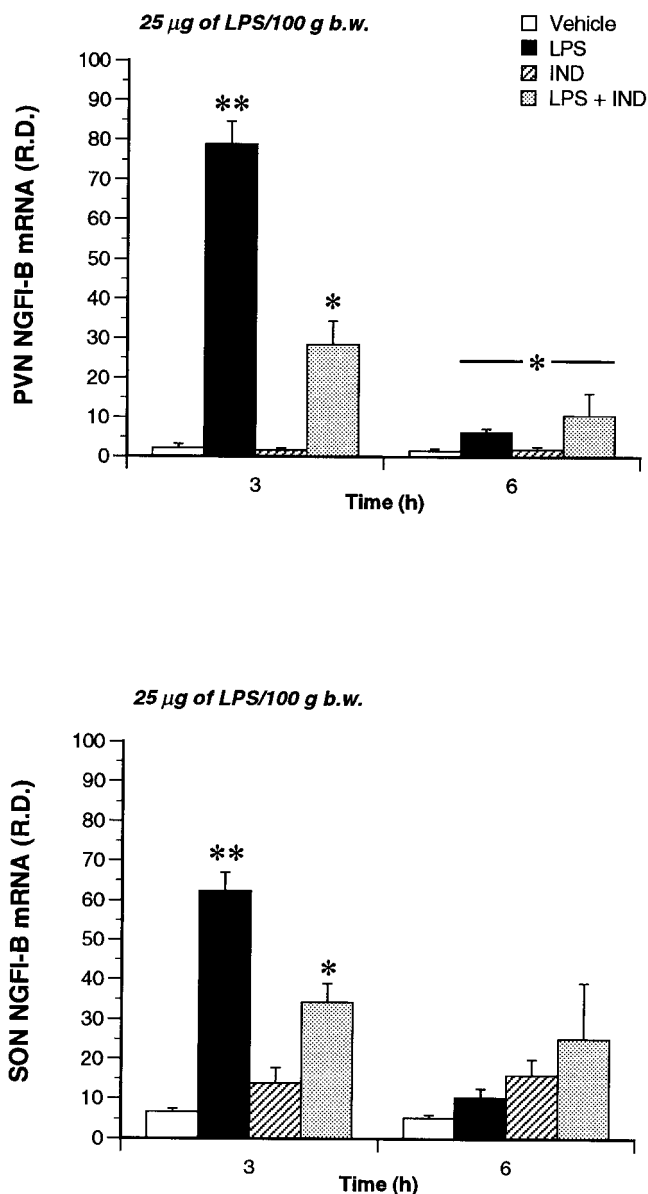


Fig. 5. Average R.D. (in arbitrary units) of the hybridization signal for the immediate-early gene nerve growth factor-inducible gene B (NGFI-B) in the PVN and SON after i.p. administration of the middle dose of the bacterial endotoxin (25 µg LPS/100 g b.w.). Results represent means ± S.E.M. of four rats; an average of two medial PVNs and four medial SONs were digitized for each rat. Statistical analysis was performed by using a 2 × 2 ANOVA followed by a Bonferroni/Dunn post-hoc test for each postinjection time (Statview 4.01). Asterisk between lines indicates that the main effect of the LPS treatment was without significant interaction between i.p. LPS and i.v. IND treatments. Significant interaction between i.p. and i.v. treatments: single asterisk, significantly different ( $P < 0.05$ ) from the appropriate control group; double asterisk, significantly different ( $P < 0.05$ ) from all other groups. For more information on image analysis, see Materials and Methods. For abbreviations, see Figure 1 and 2.

methacin did not interfere significantly with the number of double-labeled cells of the NTS 3 hours after LPS administration. However, Bonferroni/Dunn post-hoc test revealed significant differences between vehicle- and LPS-injected rats killed 6 hours after immunogenic challenge but not

between both indomethacin-treated groups. In the VLM, the number of TH-ir cells expressing *c-fos* mRNA peaked 3 hours after a single i.p. administration of 25 µg LPS/100 g b.w. ( $22.58\% \pm 6.73$ ), whereas this value dropped to  $3.42\% \pm 0.74$  6 hours after the i.p. injection. In contrast to the results obtained previously for the NTS, inhibition of cyclooxygenase pathways significantly ( $P < 0.05$ ) attenuated the number of activated TH cells in the VLM (Fig. 9, bottom); the number of double-labeled cells fell to  $6.47\% \pm 3.20$  at 3 hours postinjection and was almost undetectable in this brainstem region ( $0.25\% \pm 0.10\%$ ) 6 hours after combined LPS and indomethacin treatments.

DISCUSSION

The present study demonstrates that the three doses of the bacterial endotoxin LPS have different effects on the brain cellular activity, as illustrated by the selective and transient expression of *c-fos* transcript. Systemic administration of the high dose of LPS leads to a robust, widespread, and prolonged induction of both *c-fos* and NGFI-B mRNAs throughout the brain. The middle dose of endotoxin causes similar effects at 3 hours postinjection, but hybridization signal for *c-fos* mRNA vanishes rapidly 3 hours later, except for the sensorial CVOs, in which strong signal was still detectable. On the other hand, injection of the low dose selectively triggered *c-fos* expression within the CVOs at 3 hours, whereas the message returned to basal expression at 6 hours. Pretreatment with indomethacin did not significantly influence *c-fos* transcription in rats challenged with the high dose of LPS at 3 hours postinjection. Inhibition of PG formation was more effective in interrupting the neuronal activation in animals injected with the middle dose of LPS, although the influence depended on the structures and the groups of activated cells. Indeed, PG inhibition significantly altered LPS-induced *c-fos* mRNA expression in the PV, PVN, cVLM (LRN/AMBd), and leptomeninges. In the hypothalamic PVN, inhibition of both *c-fos* and NGFI-B transcripts by indomethacin was also associated to a diminished expression of CRF hnRNA. In fact, induction of CRF primary transcript by 25 µg LPS/100 g b.w. was selective to the PVN and was completely blocked by pretreatment with indomethacin. Moreover, a large number of TH-ir neurons of the VLM (A1/C1) and NTS (A2/C2) were positive for *c-fos* mRNA in immune-challenged rats, an effect that was essentially abolished by indomethacin in the VLM, but not in the NTS. The role of PGs as mediators of the stimulatory influence of the acute-phase response, therefore, seems to depend on the severity of the systemic stressful situation, the brain regions, and the cell groups as well as the activated target genes.

Table 1 and Figure 1 show that the effects of systemic injection of LPS on *c-fos* mRNA expression in the brain of male rats depended on the dose used during the experimentation. Injection of the low dose triggered transcription of *c-fos* in very selective sites, including the sensorial CVOs, at 3 hours postinjection. The exact mechanisms involved in the influence of the low dose of the bacterial endotoxin on neuronal activity are unknown, and whether production of specific cytokines by LPS is responsible for induction of *c-fos* mRNA within the CVOs remains to be established. Berkenbosch et al. (1991) reported that a similar low dose of LPS caused an increase in plasma IL-6 levels without

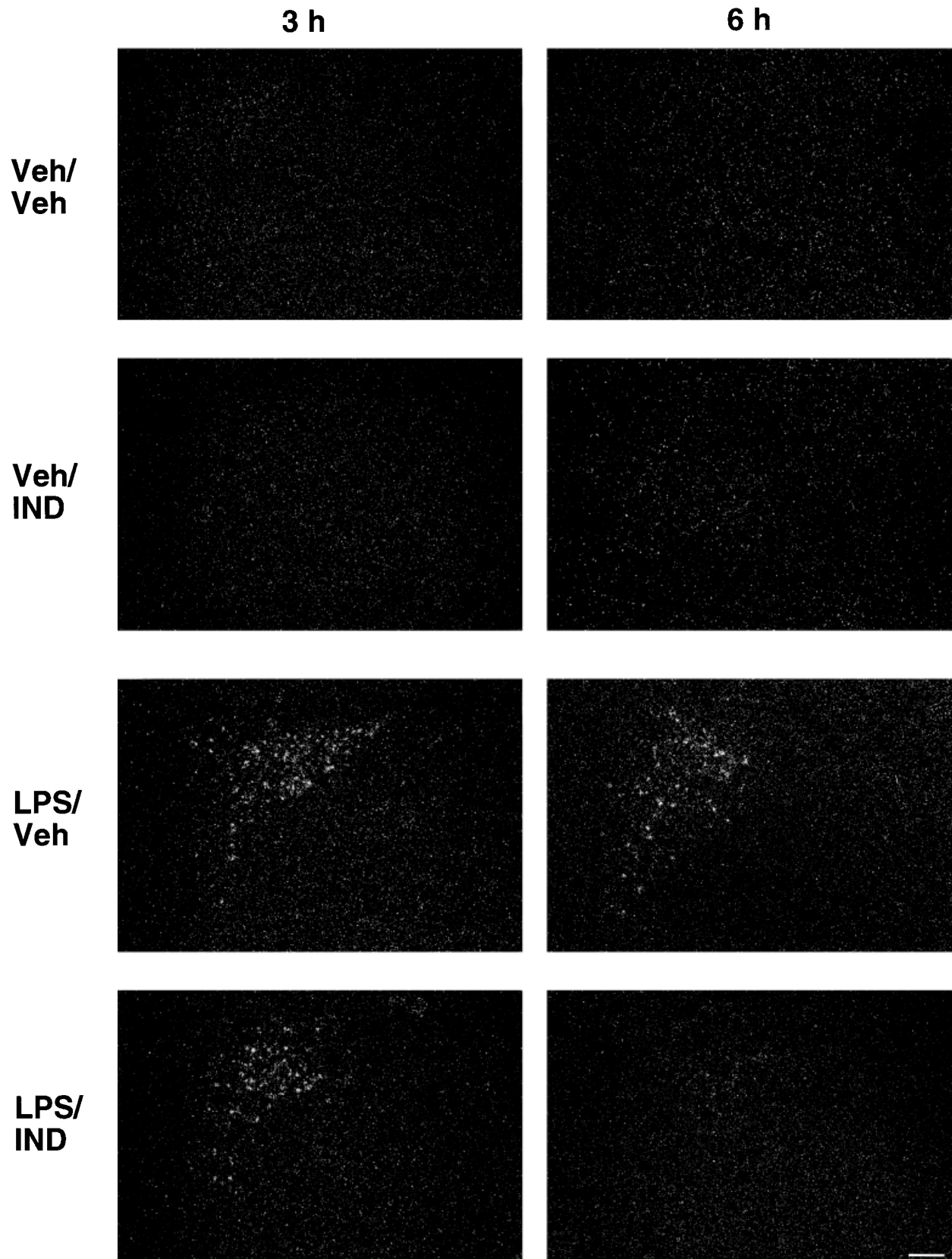


Fig. 6. Influence of cyclooxygenase pathways on the expression of corticotropin-releasing factor (CRF) primary transcript in the hypothalamic PVN of rats treated i.p. with the middle dose of LPS (25  $\mu$ g/100 g b.w.). These darkfield photomicrographs show in situ hybridization signals for CRF heteronuclear (hn) RNA through similar right medial

PVNs. Note that inhibition of prostaglandin production by intravenous IND administration decreased expression of CRF hnRNA (index of transcriptional activity) in the PVN of animals injected i.p. with this dose of LPS at both postinjection times (3 and 6 hours). Magnification  $\times 25$ . Scale bar = 100  $\mu$ m.

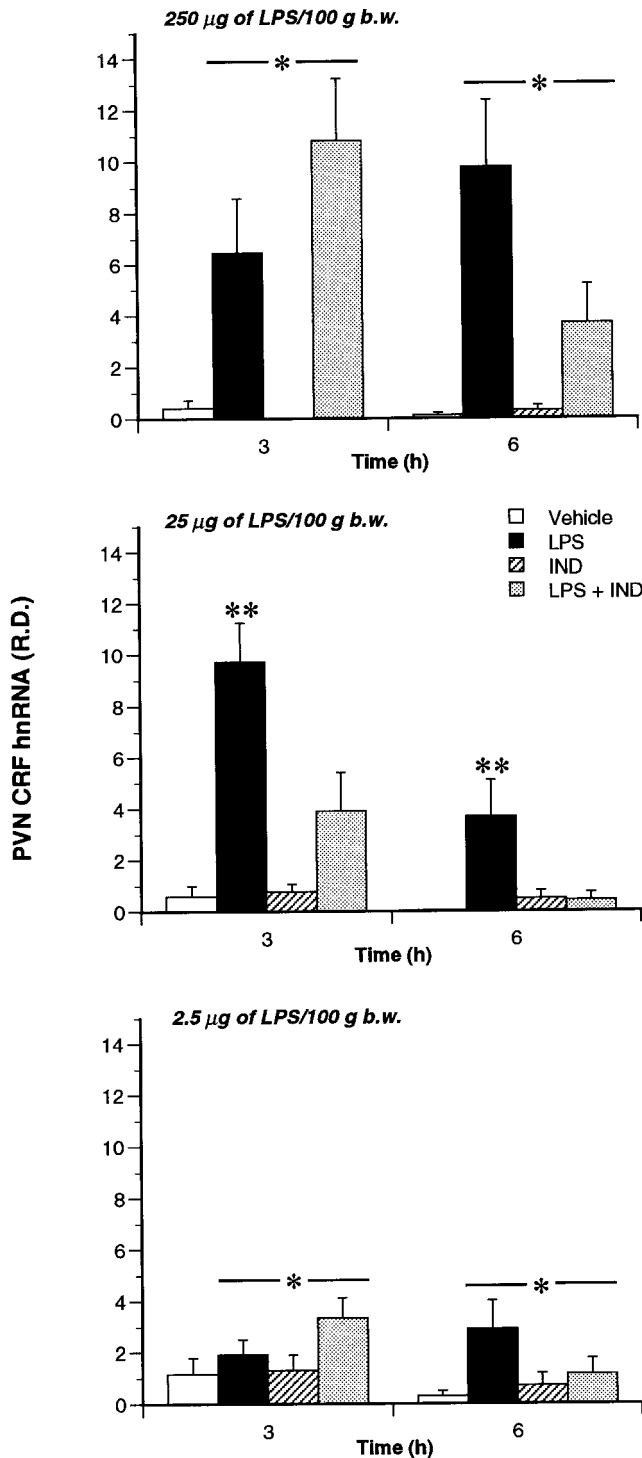


Fig. 7. Average R.D. (in arbitrary units) of the hybridization signal for the relative levels CRF primary transcript in the PVN after i.p. administration of 250 µg (top), 25 µg (middle), or 2.5 µg (bottom) of the bacterial endotoxin LPS/100 g b.w. Results represent means ± S.E.M. of four rats; an average of two medial PVNs were digitized for each rat. Statistical analysis was performed by using a 2 × 2 ANOVA followed by a Bonferroni/Dunn post-hoc test for each time postinjection (Statview 4.01). Asterisks between lines indicate that the main effect of the LPS treatment was without significant interaction between i.p. LPS and i.v. IND treatments. Significant interaction between i.p. and i.v. treatments: double asterisk, significantly different ( $P < 0.05$ ) from all other groups. For more information on image analysis, see Materials and Methods.

affecting the levels of circulating IL-1. Another group also reported that circulating IL-1β was hardly noticeable until 8 hours after the administration of a low dose of the endotoxin, whereas a significant increase in the concentrations of IL-6 was detected (Givalois et al., 1994). Moreover, we recently reported that systemic i.v. injection of IL-6 induced a robust expression of *c-fos* in most of the sensorial CVOs, including the OVL, SFO, ME, and AP (Vallières et al., 1997). It is therefore tempting to speculate that IL-6 may mediate the influence of a low dose of LPS on the activity of CVOs during modest activation of the acute-phase response.

Systemic i.p. administration of the high and middle doses of LPS induced a widespread neuronal activity throughout the rat brain. In fact, similar patterns of intensity for *c-fos* mRNA signal were observed at 3 hours postinjection in various nuclei of the forebrain, CVOs, and brainstem regions of animals treated with both doses of LPS. Circulating IL-1 can be detected rapidly at similar doses of LPS (Berkenbosch et al., 1991), and i.v. injected IL-1 mimics quite well the endotoxin-induced distribution of *c-fos* mRNA throughout the brain, except for most of the CVOs (Ericsson et al., 1994; Rivest and Laflamme, 1995). Whether circulating IL-1 is responsible for triggering brain cellular activity in animals treated with such doses of the bacterial endotoxin has yet to be confirmed, but is a likely mechanism of action. It is also possible that IL-1 and IL-6 act in parallel and at different levels of the brain to stimulate neuronal activity during acute-phase response.

Animals injected with the high dose (250 µg LPS/100 g b.w.) and killed 6 hours later still exhibited strong hybridization signal for the IEGs throughout the brain, whereas, at that time, the message returned to basal expression in the brains of rats treated with the middle dose (25 µg LPS/100 g b.w.), except for the sensorial CVOs. This phenomenon could be explained in part by the dose- and time-dependent influence of LPS on circulating cytokines (Berkenbosch et al., 1991). Moreover, *in vivo* macrophage depletion is able to prevent the increase in plasma ACTH and corticosterone levels in animals injected with a low dose of LPS, but not following high dose of the endotoxin (Derijk et al., 1991). These results are in agreement with the hypothesis that macrophage-independent mechanisms are involved in the activation of HPA axis during severe immune challenge (Derijk et al., 1991). It is possible that many other pathways related to changes in blood pressure and osmolarity, pain, oxygen consumption, fever, and energy metabolism participate to restore homeostasis in response to profound emergency situations, such as those provoked by the high dose of endotoxin. It is worth reminding here that rats receiving the high dose of LPS displayed signs of sickness (covering themselves, immobility, shivering, and piloerection; some had diarrhea), whereas barely any visible sickness symptoms were observed in animals administered with the middle dose. However, *c-fos* was still highly expressed throughout the brain of this latter group, which also exhibited activation of the sensorial CVOs up to 6 hours postinjection. These results, once again, support the hypothesis of a dose- and time-dependent influence of the endotoxin on the production of selective cytokines that have the ability to produce these events.

Pretreatment with indomethacin did not prevent transcription of *c-fos* in the brains of rats challenged with the high dose of LPS at 3 hours postinjection. The possibility

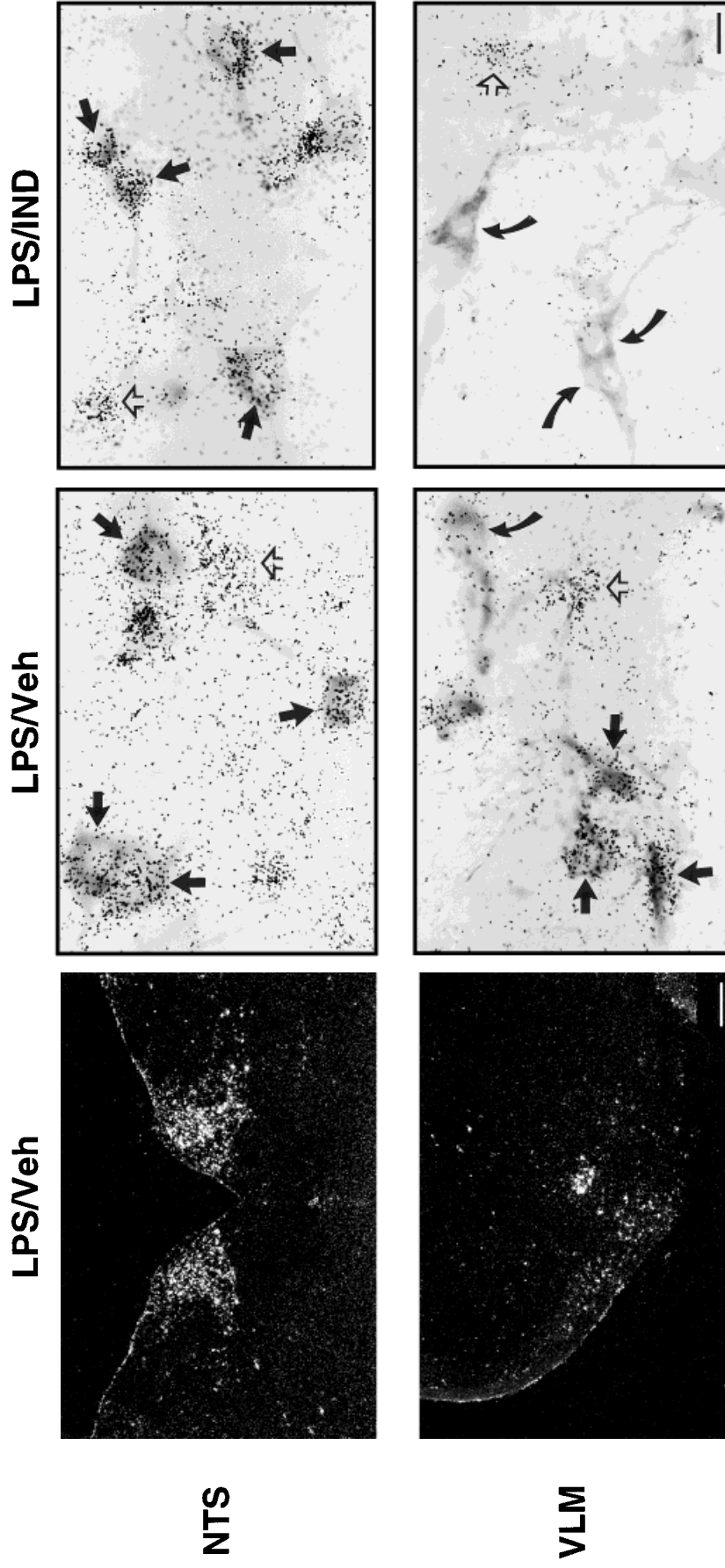


Fig. 8. High-power brightfield photomicrographs representing tyrosine hydroxylase (TH)-immunoreactive (ir) neurons expressing c-fos transcript in the nucleus of the solitary tract (NTS; **top**) and the ventrolateral medulla (VLM; **bottom**) of rats killed humanely 3 hours after the injection of the middle dose of LPS (25  $\mu$ g/100 g b.w.). Immunocytochemistry (TH protein, stained cytoplasm, and fibers) was performed on the same brain sections (30  $\mu$ m) prior to in situ hybridization histochemistry (c-fos mRNA, silver grains). Darkfield photomicrographs (left) of the NTS (top) and VLM (bottom) of a representative LPS-treated rat illustrate the

exact level of the nucleus from which the high-magnification brightfield photomicrographs were taken. LPS/Veh, animal that received 25  $\mu$ g LPS i.p./100 g b.w. and vehicle solution i.v.; LPS/IND, animal injected i.p. with the same dose of LPS and pretreated i.v. with IND. Solid arrows, TH-ir neurons expressing the mRNA encoding c-fos; open arrows, c-fos positive neurons alone; curved arrows, TH-ir neurons alone. Magnification  $\times 10$  on left,  $\times 250$  on middle and right. Scale bars = 250  $\mu$ m on left, 10  $\mu$ m on middle and right.

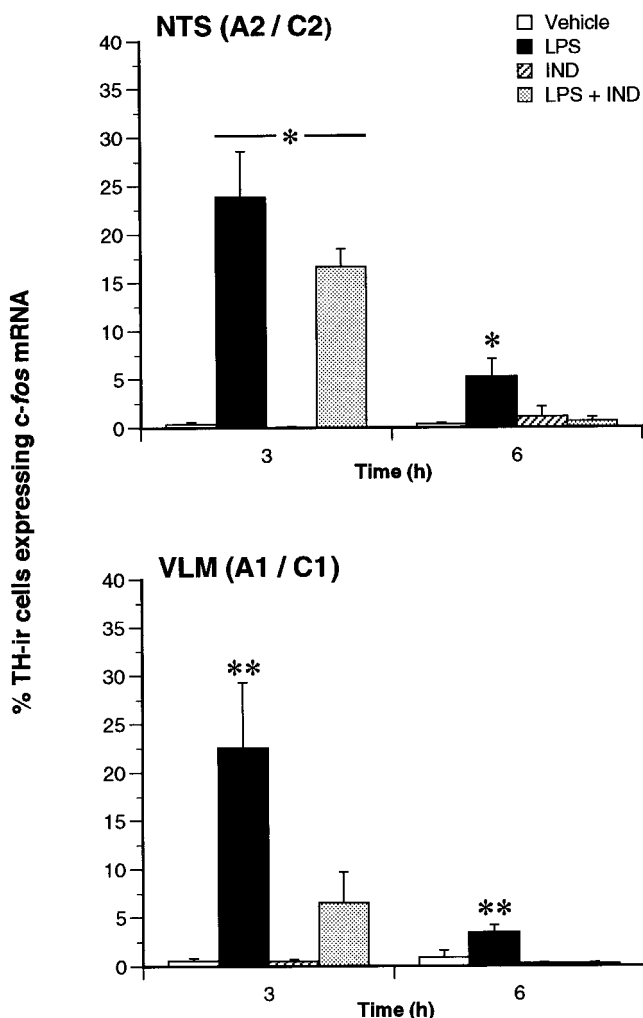


Fig. 9. Percentage of TH-ir cells exhibiting positive signal for *c-fos* mRNA in the NTS and the VLM of endotoxin-challenged rats (LPS i.p., 25 µg/100 g b.w.) that received either i.v. vehicle or IND injections. Immunocytochemistry was performed prior to in situ hybridization histochemistry on the same brain sections, and measurements of TH-ir cells and double-labeled cells (TH-ir neurons expressing *c-fos* mRNA) were performed at a magnification of  $\times 100$  under brightfield illumination for the entire rostrocaudal structures delineating the NTS (A2/C2) and the VLM (A1/C1). Results represent means  $\pm$  S.E.M. of four rats for an average of 14–16 and 36–44 bilateral sections corresponding to the NTS and the VLM, respectively, for each rat. Statistical analysis was performed by using a  $2 \times 2$  ANOVA followed by a Bonferroni/Dunn post-hoc test for each postinjection time (Statview 4.01). Asterisk between lines indicates that the main effect of the LPS treatment was without significant interaction between i.p. LPS and i.v. IND treatments. Significant interaction between i.p. and i.v. treatments: single asterisk, significantly different ( $P < 0.05$ ) from the appropriate control group; double asterisk, significantly different ( $P < 0.05$ ) from all other groups.

that the dose of indomethacin used in the present study did not completely block the PG formation in LPS-treated rats has to be considered, although this dose has been shown to be effective in preventing the effect of LPS and IL-1 on various neuroendocrine functions. Indeed, the HPA axis response to LPS and IL-1 has been reported to be inhibited by systemic pretreatment with similar or lower doses of indomethacin (Katsuura et al., 1988; McCoy et al.,

1994; Rivier and Vale, 1991; Watanabe et al., 1990). Blockage of PG production with the same dose of the cyclooxygenase inhibitor also prevented the IL-1-induced alteration of neuroendocrine LHRH system and plasma LH levels in female rats (Rivest and Rivier, 1993). Therefore, our results may suggest that the role of PGs in mediating the stimulatory influence of immune challenge on the transcription of the IEGs throughout the brain depends on the severity of this systemic stressful situation.

On the other hand, indomethacin significantly abolished the expression of both IEG *c-fos* and NGFI-B transcripts in the neuroendocrine hypothalamic PVN 3 hours after injection of 25 µg LPS/100 g b.w. These results are in agreement with the known influence of eicosanoid cyclooxygenase pathways in mediating several neuroendocrine responses to immune challenge and acute exposure to cytokines. Indeed, the effect of cytokines on the release of CRF, vasopressin (AVP), and oxytocin (OT) and on the activity of the HPA axis can be antagonized by drugs blocking PG synthesis (Bernardini et al., 1990; Navarra et al., 1991; Yasin et al., 1994). In the hypothalamic PVN, the influence of the endotoxin (middle dose) on the transcription of neuroendocrine CRF (this study) and its type 1 receptor (Lacroix and Rivest, 1996) is inhibited by the administration of indomethacin. Whether the action of PGs takes place directly within the PVN or at the level of the structures innervating the endocrine hypothalamus remains an open question. It has been reported that i.v. administration of IL-1 $\beta$  provokes a sharp increase in the levels of PGE<sub>2</sub> within the PVN (Watanobe and Takebe, 1994), which provides evidence that local PVN production of the PG may participate in the regulation of neuroendocrine functions, such as the HPA axis. However, this may be a simplistic view of this complicated issue, because PGE<sub>2</sub> has also been found to be increased in many other structures, and perfusion of indomethacin within the PVN failed to prevent IL-1-induced activation of the HPA axis (Komaki et al., 1992). Systemic LPS and IL-1 administrations have recently been shown to stimulate the transcription of the gene encoding PG G/H synthase 2 (COX-2, the limiting enzyme for the central PG production) throughout the entire brain microvasculature (Cao et al., 1995, 1996). Moreover, central i.c.v. injection of PGE<sub>2</sub> caused expression of *c-fos* mRNA in numerous structures throughout the brain, including the PVN (Lacroix et al., 1996), although neurons of this hypothalamic region do not seem to express the genes that encode PGE<sub>2</sub> receptors (Ericsson et al., 1995a).

Synthesis of PGs within the MPOA/OVLT could be a determinant mechanism through which immune-related factors trigger the transcription of *c-fos* in the hypothalamic PVN and other hypothalamic nuclei. High density of PGE<sub>2</sub> binding sites (Matsumura et al., 1990, 1992) and positive hybridization signal for the mRNA encoding EP3 PGE<sub>2</sub> receptor (Ericsson et al., 1995a) have been found in the OVLT/MPOA, and this region has direct neuronal connection with the endocrine PVN (Sawchenko and Swanson, 1983). Stimulation of the rat POA increases the firing rates of neurons located in the PVN and raises plasma corticosterone levels (Saphier and Feldman, 1986), whereas microinjection of PGE<sub>2</sub> in the POA induces Fos in parvocellular division of the hypothalamic PVN (Scammell et al., 1996). Systemic injection with bacterial LPS causes PG production in the OVLT/preoptic area (Ueno et al., 1982),

and i.v. administration of IL-1 $\beta$  raises PGE<sub>2</sub> levels in these structures (Komaki et al., 1992). In addition, microinjection of PGE<sub>2</sub> within the OVLT/MPOA increases plasma ACTH release, whereas infusion of indomethacin or a PGE antagonist into this area is able to significantly prevent the stimulatory influence of i.v. IL-1 $\beta$  administration on the HPA axis (Katsuura et al., 1990). Induction of *c-fos* mRNA in the MPOA/OVLT, transcriptional activation of neuroendocrine CRF (Lacroix et al., 1996), and release of ACTH and corticosterone (Rassnick et al., 1995) after i.c.v. PGE<sub>2</sub> injection provide additional evidence that OVLT/MPOA PGs may play an important role in mediating the information received from circulating cytokines to the endocrine hypothalamus.

Expression of *c-fos* mRNA was detected in the VLM after systemic injections of IL-1 (Ericsson et al., 1994) and LPS (Rivest and Laflamme, 1995) and after central treatment with PGE<sub>2</sub> (Lacroix et al., 1996). Here, we demonstrate that a high concentration of these cells are TH-ir neurons and that indomethacin essentially abolished A1/C1 activation in animals that received the middle dose of LPS in this study. These results suggest that PGs participate in the activation of A1/C1 catecholaminergic neurons in response to immunogenic challenges. Because rat brain endothelial cells have been shown to express the type 1 IL-1 receptor (IL-1R1; Ericsson et al., 1995b; Van Dam et al., 1996), it is possible that such circulating cytokine acts on its receptor at the level of the medullary microvasculature to stimulate the synthesis PGs in response to LPS. Injection of LPS (Cao et al., 1995) and IL-1 $\beta$  (Cao et al., 1996), as mentioned previously, induces COX-2 mRNA within the brain microvasculature (most likely in endothelial and/or perivascular microglial-associated cells), a phenomenon that is particularly robust in the VLM (Lacroix and Rivest, unpublished data). The facts that the mRNA encoding the EP3 PGE<sub>2</sub> receptor subtype is expressed over neurons of the VLM (Ericsson et al., 1995a) and that A1/C1 neurons responsive to i.v. IL-1 $\beta$  project to the PVN (Ericsson et al., 1994) indicate the presence of an additional circuitry through which PGs could modulate neuroendocrine functions during immunogenic challenges.

The VLM and the NTS are two interconnected structures, whereas the latter (A2/C2) is known to provide the largest NA input to the PVN (Cunningham and Sawchenko, 1988; Cunningham et al., 1990; Sawchenko and Swanson, 1982; Swanson et al., 1983). Interestingly, disruption of afferent medullary catecholaminergic pathways has been shown to significantly prevent the increase in CRF mRNA levels in the PVN of IL-1 $\beta$ -injected rats (Ericsson et al., 1994). It has also been demonstrated that the NTS displayed the highest density of PGE<sub>2</sub> binding sites (Matsumura et al., 1992), and PG facilitates excitatory synaptic transmission in voltage-clamped neurons in rat NTS slices (Seriya et al., 1995). The role of PGs in the activation of A2/C2 group of cells in response to LPS, however, remains uncertain; in contrast to the VLM, indomethacin did not significantly alter the number of TH-ir cells positive for *c-fos* mRNA in the NTS 3 hours after the i.p. treatment with the middle dose of the endotoxin, suggesting a PG-independent mechanism. Interestingly, the involvement of the vagus nerve in mediating the effect of systemic (i.p.) LPS on Fos-ir in the NTS and the PVN has been demonstrated (Wan et al., 1994). On the other hand, neurons of the NTS receive efferent projections from the AP (Cunningham et al., 1994), a circumventricular organ

that is devoid of the blood-brain barrier. Circulating cytokines (such as IL-1) produced during the acute-phase response, therefore, could reach their receptors (IL-1R1) associated with either neurons and/or perivascular elements of the AP (Ericsson et al., 1995b) and indirectly activate, without the implication of PGs, catecholaminergic neurons of the NTS. In accordance with this hypothesis is the elegant study performed by Lee and Herkenham (1996) that demonstrated the essential role played by the AP in the effects of i.v. IL-1 $\beta$  on the endocrine hypothalamus. More precisely, AP destruction was able to prevent the IL-1-induced activation of the HPA axis and transcription of *c-fos* in the NTS and PVN, but not in any other regions.

In conclusion, systemic i.p. injection of a low dose of endotoxin (2.5  $\mu$ g LPS/100 g b.w.) induced expression of the IEGs quite selectively within the sensorial CVOs, whereas 25  $\mu$ g and 250  $\mu$ g LPS/100 g b.w. caused strong transcription of *c-fos* mRNA in numerous structures throughout the rat brain. Administration of the eicosanoid cyclooxygenase inhibitor indomethacin attenuated IEG induction in the PV, PVN, cVLM (LRN/AMBd), and leptomeninges of rats treated with the middle dose (25  $\mu$ g/100 g b.w.), but not in animals receiving the high dose (250  $\mu$ g/100 g b.w.), of LPS i.p. In a similar manner, pretreatment with indomethacin was effective enough to prevent the transcription of neuroendocrine CRF only in rats treated with the middle dose of LPS. Finally, PG production in response to the middle dose of LPS seems largely responsible for the activation of A1/C1, but not A2/C2, groups of brainstem catecholaminergic neurons. These results indicate that the role of PGs as mediators of systemic immune response is partial and is dependent on various factors, such as the severity of the stressful situation, the brain regions, and the cell groups, as well as the activated target genes.

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