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The vagus nerve mediates behavioural depression, but not fever, in response to peripheral immune signals; a functional anatomical analysis

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Introduction

Detection of bacterial lipopolysaccharide (LPS) by immune cells leads to interleukin-1β (IL-1β) and interleukin-6 (IL-6) release. These cytokines act on the brain to provoke fever and behavioural depression (Rothwell & Hopkins, 1995). Fever is an adaptive response to infection (Kluger, 1991) and behavioural depression contributes to it by reducing energy expenditure (Hart, 1988).

Because of their size and hydrophilic nature, cytokines cannot diffuse across the blood–brain barrier (BBB). Three possible mechanisms of cytokine-to-brain signalling have been proposed. Classically, cytokines are thought to act on circumventricular organs where the BBB is absent (Blatteis, 1992). IL-6, for example, rapidly rises in plasma after LPS administration (Luheshi *et al*., 1996) and induces the cellular activation marker c-fos exclusively in circumventricular organs (Vallières *et al*., 1997). Alternatively, LPS and IL-1β induce cyclooxygenase-2 (COX-2), the limiting enzyme for prostaglandin synthesis, in endothelial cells (Cao *et al*., 1996; Laflamme *et al*., 1999). Lipophilic prostaglandins freely diffuse across the BBB and might thus act on receptors found on neuronal populations (Zhang & Rivest, 1999). Recently, the vagus nerve was proposed as an immune-to-brain pathway (Dantzer, 1994). Although evidence exists for each of these mechanisms, their possible intervention has usually been tested separately and by addressing only one physiological response.

Transection of the vagus nerves inhibits fever only in response to low doses of LPS or IL-1β (Hansen & Krueger, 1997; Romanovsky *et al*., 1997). Clearly, higher amounts of circulating cytokines are more prone to act on circumventricular organs or induce COX-2 around blood vessels. However, vagotomy consistently blocks LPS- and IL-1β-induced behavioural depression, as measured by the social interaction test, even in response to high doses (Bluthé *et al*., 1994, 1996b). Since higher doses of IL-1β are needed to induce behavioural depression than fever (Anforth *et al*., 1998), it is possible that fever and behavioural depression are mediated by distinct pathways of immune-to-brain communication, resulting in activation of different brain structures. In the case of fever, the preoptic hypothalamus is the target structure (Boulant, 1981). The neural structures implicated in behavioural changes are still unknown, but limbic structures are good candidates.

This study was designed to test the hypothesis that fever and behavioural depression are due to different mechanisms of immune-to-brain signalling. In addition to fever and behavioural depression, we evaluated LPS-induced Fos expression in rats that underwent subdiaphragmatic vagotomy and compared it with COX-2 induction. Brain targets of IL-6 were investigated by studying expression of Stat3 that translocates into the nucleus after activation by the signal

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transducer gp130 common to the IL-6 receptor family (Hirano *et al*., 1994; Heinrich *et al*., 1998).

The mechanisms behind the effects of vagotomy are still unclear. Vagotomy may interrupt transmission of a peripheral IL-1β signal to the nucleus of the solitary tract (NTS) where vagal fibres terminate. Alternatively, attenuated IL-1β induction in the brain of vagotomized animals has been proposed to underlie its attenuating effects (Layé *et al*., 1995). Therefore, both LPS-induced Fos expression in the NTS and IL-1β immunoreactivity in the brain were assessed.

Materials and methods

Animals

All experiments were performed on adult male Wistar rats (Charles River, Saint Aubin les Elbeuf, France) of 225–250 g body weight. The animals were housed in a temperature-controlled room (21 ± 2 °C) artificially lit between 08.00 h and 20.00 h, and were provided with food (Extralabo, Provins, France) and water *ad libitum*. Juvenile male rats (21–35 days of age) of the same strain served as stimulus animals for the behavioural studies. All animal experiments were conducted in accordance with French legislation.

Surgery

One hundred and sixteen animals were food deprived for 24 h prior to surgery and randomly assigned to vagotomy or sham operations, as previously described (Bluthé *et al*., 1996a). Briefly, animals were anaesthetized with a mixture of ketamine and xylazine (61 and 9 mg/kg, respectively; Rhône Mérieux, France and Bayer Pharma, Sens, France), and the main ventral and dorsal trunks of the vagus nerve were transected immediately above the stomach. All connective tissue 2–3 cm between the gastric artery and the oesophagus was removed to ensure the transection of accessory vagal branches. Sham animals underwent the same surgical procedure excluding nerve transection. All rats received a single intramuscular injection of amoxilin (75 mg/kg) and were allowed to recover for 3 weeks. During the first week of recovery, animals were offered highly palatable food, in addition to normal chow. Palatable food has previously been shown to reduce body weight loss (Kraly *et al*., 1986) and differences in basal body temperature in vagotomized animals (Romanovsky *et al*., 1997). Thirty-five animals which continued to lose weight or which showed apparent signs of sickness, such as piloerection, were euthanized.

In the 34 animals assigned to fever and behavioural studies, vagotomy was verified by blockade of the anorexic effect of intraperitoneal (i.p.) injection of cholecystokinin (4 μg/kg; Sigma, St Fallavier, France) (Smith *et al*., 1981) 2 weeks after the end of the studies. The rats were deprived of food for 18 h, then injected with cholecystokinin or saline and presented with food 5 min later. Food intake was measured over the following 30 min. In the 38 animals assigned to immunocytochemical studies, vagotomy was verified by incubating NTS sections with isolectin I-B4 (1 μg/mL phosphate-buffered saline; L5391, Sigma, St Fallavier, France) which was previously shown to bind to vagal visceral afferents in the NTS (Li *et al*., 1997).

Experimental protocol

Injections were administered i.p. (1.0 mL/kg) to hand-held, lightly restrained rats. Rats were injected with either 25 μg/kg recombinant rat IL-1β (rrIL-1β; specific activity 317 IU/mg; Dr S. Poole, NIBSC, Potters Bar, UK) dissolved in pyrogen-free saline containing 0.1% endotoxin-free bovine serum albumin (BSA; A-8806, Sigma) or 250 μg/kg of LPS (*Escherichia coli* O127:B8, Sigma) dissolved in pyrogen-free saline. Control injections consisted of pyrogen-free saline alone. These doses of rrIL-1β and LPS were previously shown to reliably induce behavioural depression and robust fever responses from which animals recover within 24 h (Luheshi, unpublished observations) (Bluthé *et al*., 1992; Anforth *et al*., 1998; Konsman *et al*., 1999).

Fever

Fever studies were performed at an ambient temperature of 21 ± 2 °C, that is below the thermal neutrality for rats (28 °C). Hypothermia can occur at ambient temperatures below thermal neutrality when high doses (1000 μg/kg) of LPS are administrated intravenously (Romanovsky *et al*., 1997), raising the possibility that fever phases are masked. However, hypothermia does not occur at lower doses of LPS (10 μg/kg intravenously) (Romanovsky *et al*., 1998). Since i.p. administration of LPS results in 10–100-fold lower circulating levels compared with intravenous administration (Yasui *et al*., 1995), fever phases are very unlikely to be masked after i.p. injection of 250 μg/kg LPS when monitoring body temperature at 21 °C. Core body temperature was measured continuously in conscious, undisturbed, individually housed animals by remote radio-telemetry, via battery-operated biotelemetry transmitters (Data Sciences, St Paul, Minnesota, USA), previously implanted in the abdominal cavity during vagotomy or sham surgery. The output frequency (Hz) of each transmitter was monitored by an antenna mounted in a receiver board situated beneath the cage of each animal, and channelled to a peripheral processor (Dataquest IV, Data Sciences). Frequencies were sampled at 10-min intervals and converted to degrees Celsius (°C). Since the pyrogenic effects of IL-1β and LPS are most pronounced during the light phase (Opp & Toth, 1998), animals received a single injection of rrIL-1β, LPS or vehicle at 10.00 h and their temperatures were measured every 10 min for 7 h. The mean of three consecutive measurements for each individual animal was used for further analysis.

Behavioural depression

Following a 2-week recovery period from the fever study, social interaction was tested in the same group of animals. In this experiment, rats were injected with LPS, rrIL-1β or vehicle. All animals received an injection of vehicle and the treatment groups were arranged such that rats that had received LPS in the fever study were injected with rrIL-1β, whereas those rats that had received rrIL-1β previously were injected with LPS. This experimental design was adopted to avoid the development of tolerance to LPS. Behavioural depression was induced by injection of rrIL-1β, or LPS assessed by reduction in the duration of social interaction with a conspecific juvenile introduced into the home cage of the test animal for a 4-min observation session, immediately before and 2 h after treatment. This time-point was chosen because it corresponds to the appearance of effects of IL-1β and LPS on behaviour (Bluthé *et al*., 1992). Social interaction was measured by the duration of ano-genital sniffing and active interaction of the adult with the juvenile. Different juveniles were presented on each occasion to avoid habituation. Durations of social interaction measured in animals treated with saline were used as baseline values. Social interaction studies were carried out during the dark phase of the cycle, between 20:00 h and 01:00 h, since rats display increased social behaviour during the dark phase.

Tissue processing

Rats assigned to immunocytochemical experiments were killed 2 h after injection of LPS or saline with an overdose of sodium

pentobarbital administered i.p. Once the hind paw reflex upon plantar pinching had disappeared, brains were fixed by intracardiac perfusion of saline via the ascending aorta followed by 4% paraformaldehyde in 0.1 M borate buffer (pH 9.5 at 10 °C). Brains were post-fixed for 4 h, and then cryoprotected in 30% sucrose in 0.1 M phosphate buffer (pH 7.4) for 48 h. Series of 12 frontal 30 μm cryostat sections through the whole brain were collected in cold cryoproectant (0.05 M phosphate buffer, 30% sucrose, 30% ethylene glycol) and stored at –20 °C until immunocytochemical processing.

Immunocytochemistry

Immunocytochemistry was performed as previously described (Konsman *et al*., 1999). Commercially available antisera to Fos, COX-2 and Stat3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted, respectively, 1 : 2000, 1 : 500 and 1 : 2000 were used. The Fos antiserum (cat. number sc-52, lot. J297) was raised against a synthetic peptide corresponding to amino acids 3–16 at the N-terminal of human and mouse Fos and did not cross-react with Fos-related antigens according to the manufacturer. The COX-2 antiserum (cat. number sc-1747, lot. M19) was raised against a synthetic peptide corresponding to amino acids 586–604 at the carboxy terminus of the COX-2 precursor of rat origin. For preabsorption the corresponding peptide was used following the manufacturer's instructions (sc-1747P, lot. L288). Stat3 antiserum (cat. number sc-482X, lot. K247) was generated against a peptide corresponding to amino acids 750–769 of the carboxy terminus of the mouse and did not cross-react with Stat1, Stat2, Stat4, Stat5 or Stat6, according to the manufacturer. The preabsorption test was performed by incubating the antibody with the synthetic peptide (cat. number sc-485P, lot. B028), as specified by the manufacturer. A sheep antiserum generated to rrIL-1β (NIBSC) was used at a final dilution of 1 : 1000.

After washing off the cryoprotectant solution, immunocytochemical processing was performed on a one in four series of free floating sections using the streptavidin-biotin-immunoperoxidase technique. Briefly, nonspecific binding sites were blocked by a 1-h incubation of sections in Tris-buffered saline (pH 7.4; TBS) containing 0.3% Triton X-100 and 0.2% casein. The first antibody was added for 60 h at 4 °C in the same buffer. After four rinses in TBS, sections were treated for 30 min in 0.3% (v/v) hydrogen peroxide, followed by rinsing in TBS. Sections were incubated for 2 h at room temperature with biotinylated donkey antisheep/goat or biotinylated donkey antirabbit immunoglobulins G (Amersham, Les Ulis, France, 1 : 1000) depending on the first antibody, and stained using the ABC protocol (Vectastain Elite, Vector Laboratories, Burlingame, CA, USA, 1 : 1000) with nickel-enhanced diaminobenzidine as a chromogene.

Microscopy

The stained sections were examined with a microscope (Leica Microsystems, Cambridge, UK) and the images were captured by a high-resolution CCD video camera image and fed into a personal computer. Subsequently, the Quantimet 600 Image Analysis System (Leica Microsystems) generated a digitized signal proportional to the intensity of illumination. Photomicrographs of labelled structures were obtained using this system and saved as TIFF files. Image-editing software (Adobe Photoshop, Adobe Systems, San Jose, CA, USA) was used to adjust contrast and brightness only. Image processing was performed on the grey image by defining, for example, brightness and surface above which labelling has to be taken into account. Once established, these parameters remained unchanged. The image was then converted to a binary image and measurements were taken. The number of Fos-immunoreactive cells was measured in at least four sections through the ventromedial preoptic area, the parvocellular part of the paraventricular nucleus of the hypothalamus, the central nucleus of the amygdala, the dorsolateral part of the bed nucleus of the stria terminalis and the nucleus of the solitary tract. These structures were previously shown to contain Fos-positive cells after i.p. injection of the same dose and serotype of LPS (Konsman *et al*., 1999). In the same study, LPS was shown to induce IL-1β immunoreactivity in circumventricular organs. To test the hypothesis that vagotomy reduces the induction of IL-1β in the forebrain after LPS injection, the relative surface of IL-1β-immunoreactive cells in sections of the subfornical organ relative to the total surface of this organ was used as a measure of IL-1β induction. The subfornical organ was chosen among circumventricular organs for its size and regular shape.

Statistical analysis

In order to verify that surgery did not affect behaviour or body temperature of vagotomized animals compared with sham-operated animals, duration of social investigation and body temperature were measured before injection and submitted to a one-way ANOVA. The duration of social investigation 2 h after injection is expressed as a percentage of baseline and submitted to a one-way, repeated-measure ANOVA (surgery as a between-subjects factor and treatment as a within-subject factor). Temperature data from fever experiments are presented every 30 min as means ± SEM and were analysed by a two-way, repeated-measure ANOVA (surgery and treatment as between-subject factors and time within-subject factor, respectively). *Post hoc* comparisons of individual group means were carried out by the Newman–Keuls test. Data from immunocytochemistry experiments are expressed as means ± SEM and were analysed by a two-way ANOVA (surgery and treatment). In all tests a level of $P < 0.05$ was considered as statistically significant.

Results

Verification of vagotomy

In animals assigned to fever and behavioural studies, vagotomy was verified by blockade of the anorexic effect of i.p. injected cholecystokinin. A two-way ANOVA on the amount of food intake over 30 min after 18 h of food deprivation revealed a significant effect of cholecystokinin treatment ($F_{1,17} = 15.1$; $P < 0.01$), surgery ($F_{1,17} = 14.6$; $P < 0.01$) and a significant interaction between cholecystokinin treatment and surgery ($F_{1,17} = 27.56$; $P < 0.001$). Cholecystokinin (4 μg/kg, i.p.) significantly inhibited food intake in sham-operated animals ($P < 0.001$), but had no effect on food consumption of vagotomized animals ($P > 0.10$), as previously shown (Hansen & Krueger, 1997). As abdominal vagotomy blocks the satiety effect of 4 μg/kg of cholecystokinin injected intraperitoneally (Smith *et al*., 1981), this indicates that the animals used in the fever and behavioural studies were successfully vagotomized.

Vagotomy was verified by isolectin I B4 binding in the NTS of those animals used in immunocytochemical studies. Isolectin I B4 binds vagal afferents in the NTS (Li *et al*., 1997) and its distribution in the NTS of sham-operated rats was similar to that reported previously. High isolectin I B4 binding was found in the medial and commissural nuclei, as well as in the tractus solitarius of sham-operated animals, as shown in Fig. 1A. After subdiaphragmatic vagotomy, isolectin I B4 binding in these structures was highly reduced in most animals (Fig. 1C and D). In two animals, VGX 12, shown in Fig. 1B, and VGX 24, however, isolectin I B4 binding was intermediate between sham-operated animals and the

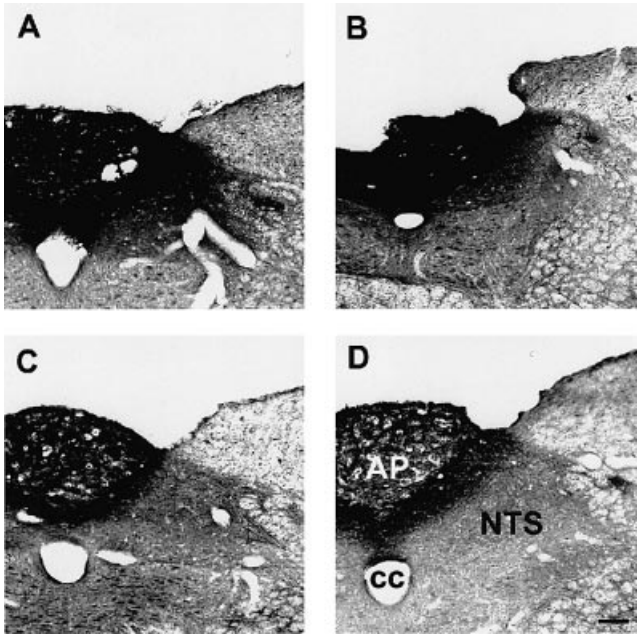

FIG. 1. Isolectin I B4 binding to vagal fibres in the nucleus of the solitary tract (NTS) as a means to verify the extent of subdiaphragmatic vagotomy. (A) Isolectin binding in the commissural and medial NTS of a sham-operated animal (Sham 48). (B) NTS sections of an animal that underwent subdiaphragmatic vagotomy (VGX 12), but still shows considerable isolectin binding compared to the vagotomized animals shown in C and D (VGX 32 and VGX 30, respectively). Animal VGX 12 was therefore judged to be incompletely vagotomized. Note also the high isolectin binding in the area postrema (AP) in A and B compared with C and D (cc, central canal). Scale bar, 100 μm.

remainder of the rats that underwent vagotomy. These animals were considered to be incompletely vagotomized and excluded from further analysis.

Vagotomy blocks i.p. IL-1β- and LPS -induced behavioural depression but not fever

Behavioural depression

A one-way ANOVA on the duration of social investigation measured before injection did not reveal any difference between vagotomized and sham-operated rats ($F_{1,32} = 2.45$; $P > 0.10$). A one-way, repeated measures ANOVA on the duration of social interaction 2 h after injection of rrIL-1β or saline revealed a significant effect of rrIL-1β injection ($F_{1,8} = 16.6$; $P < 0.01$) and a significant interaction between rrIL-1β injection and surgery ($F_{1,8} = 5.71$; $P < 0.05$). rrIL-1β injection significantly reduced social interaction in sham-operated animals ($P < 0.01$), but not in vagotomized animals (Fig. 2A). These animals were immobile, showed piloerection and curled up to minimize heat loss (Hart, 1988). Vagotomy significantly attenuated this effect of rrIL-1β ($P < 0.05$).

A one-way, repeated-measures ANOVA on social interaction 2 h after injection of saline or LPS, revealed a significant effect of surgery ($F_{1,8} = 5.63$; $P < 0.05$), LPS injection ($F_{1,8} = 14.5$; $P < 0.01$) and a significant interaction between surgery and LPS injection ($F_{1,8} = 9.21$; $P < 0.05$). LPS injection significantly reduced social interaction in sham-operated animals ($P < 0.01$), but not in vagotomized animals (Fig. 2B). Vagotomy significantly attenuated this effect of LPS ($P < 0.01$).

Fever

A one-way ANOVA on basal temperatures was measured over the second hour after light onset revealed that body temperature was slightly, but consistently lower in vagotomized animals (mean 36.64 ± 0.28 °C) compared with sham-operated rats (mean 36.89 ± 0.21 °C; $F_{1,68} = 17.0$; $P < 0.001$). This is in accordance with earlier studies conducted at an ambient temperature of 22 °C (Lin & Chern, 1985; Watkins *et al*., 1995; Fleshner *et al*., 1998) and may be due to sectioning of thermosensitive afferent fibres in the hepatic branch of the vagus nerve (Adachi, 1984). However, in contrast to an earlier study (Watkins *et al*., 1995), vagotomy did not prevent the rise in body temperature after handling stress associated with the i.p. injection. This indicates that temperature control mechanisms were largely intact in the present study. This initial rise in temperature normally persisted for 1 h, after which it returned to basal levels.

A two-way, repeated measures ANOVA revealed a significant effect of treatment between subjects ($F_{2,28} = 10.9$; $P < 0.001$) and a significant effect of time within subjects ($F_{13,364} = 12.5$; $P < 0.001$) as well as a significant interaction between treatment and time ($F_{26,364} = 7.21$; $P < 0.001$). Both i.p. injection of rrIL-1β and LPS induced robust fevers, compared with saline injection ($P < 0.001$ and $P < 0.01$, respectively). Body temperature started to rise 2 h after rrIL-1β or LPS injection ($P < 0.01$), and was still elevated at the end of the experiment ($P < 0.001$) (Fig. 3). Apart from the early stress-related transient rise, there were no differences in febrile responses to rrIL-1β and LPS between vagotomized and sham-operated animals ($P > 0.10$).

Effects of vagotomy on i.p. LPS-induced Fos expression in the forebrain

As 25 μg/kg of rrIL-1β and 250 μg/kg of LPS gave rise to similar febrile responses and behavioural depression, immunocytochemical studies were not performed for rrIL-1β-injected rats. In addition, it has previously been shown that IL-1β and LPS induce Fos expression in the same brain structures (Ericsson *et al*., 1994; Elmquist *et al*., 1996).

A two-way ANOVA on the number of Fos-positive cells in the paraventricular nucleus of the hypothalamus (PVN) revealed a significant effect of treatment (saline or LPS) ($F_{1,29} = 12.1$; $P < 0.01$), surgery (sham or vagotomy) ($F_{1,29} = 10.1$; $P < 0.01$) and a significant interaction between treatment and surgery ($F_{1,29} = 8.64$; $P < 0.01$). LPS significantly increased the number of Fos-positive cells in the PVN of sham-operated animals ($P < 0.001$). The PVN of LPS-injected, sham-operated animals showed significantly more Fos-immunoreactive cells ($P < 0.001$) (Fig. 4A) compared with LPS-treated vagotomized animals in which no significant Fos induction was found compared with saline-injected rats ($P > 0.10$). A two-way ANOVA on the number of Fos-immunoreactive cells in the ventromedial preoptic area (VMPO) revealed a significant effect of treatment and a significant interaction between treatment and surgery ($F_{1,29} = 10.7$; $P < 0.01$ and ($F_{1,29} = 4.80$; $P < 0.05$), respectively). The VMPO of LPS-treated sham-operated animals contained significantly greater numbers of Fos-immunoreactive cells compared with saline injection ($P < 0.01$). This was not the case in animals that underwent vagotomy ($P > 0.10$). As a result, Fos expression was significantly higher in sham-operated rats treated with LPS compared with vagotomized animals ($P < 0.01$, Fig. 4B).

A two-way ANOVA on the number of Fos-positive nuclei in the central amygdala (Fig. 4C) and dorsolateral bed nucleus of the stria terminalis (BNST; Fig. 4D) revealed a significant effect of surgery and a significant interaction between treatment and surgery (central

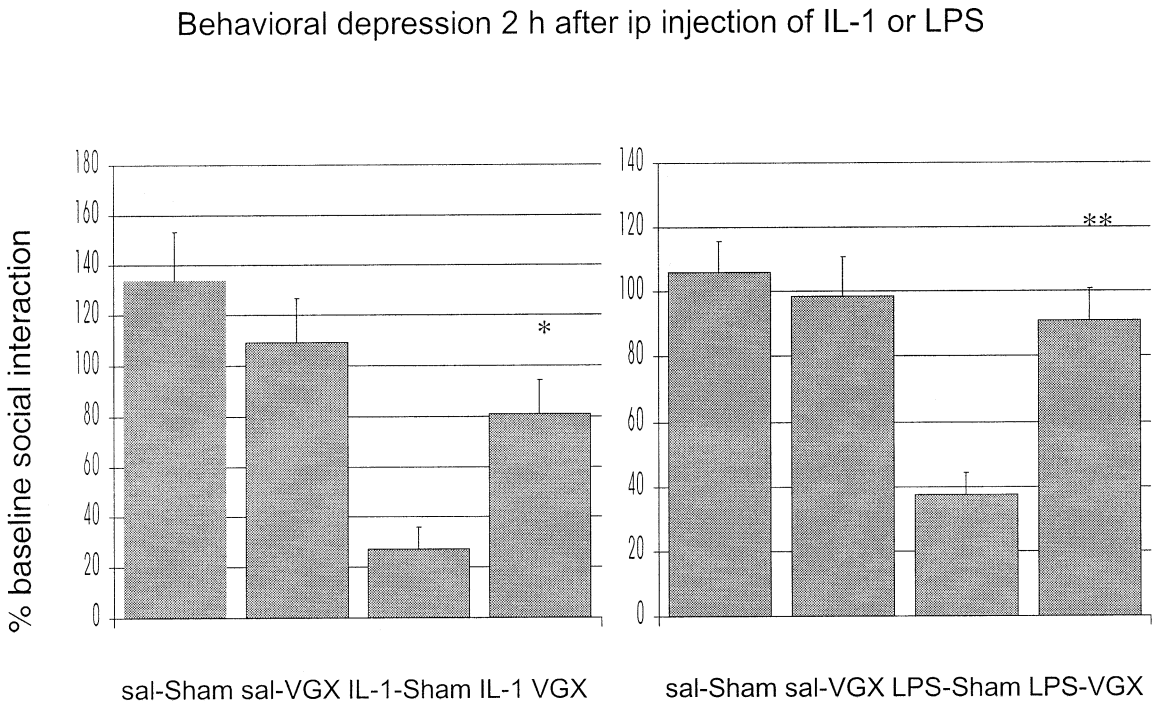

FIG. 2. Behavioural depression in sham-operated and vagotomized rats 2 h after intraperitoneal injection of recombinant rat interleukin-1β (rrIL-1β; 25 μg) or lipopolysaccharide (LPS; 250 μg/kg). Baseline social interaction with a juvenile introduced during 4 min into the cage of the experimental animal was measured just before injection. Social interaction was assessed 2 h later and expressed as percentage of baseline social interaction. Both rrIL-1β and LPS induce a reduction in social interaction in sham-operated animals. This behavioural depression does not occur in vagotomized rats. sal-Sham, sham-operated animals injected with saline; IL-1-Sham, sham-operated animals injected with rrIL-1β; LPS-Sham, sham-operated animals injected with LPS; sal-VGX, vagotomized animals injected with saline; IL-1-VGX, vagotomized animals injected with rrIL-1β; LPS-VGX, vagotomized animals injected with LPS. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; LPS-VGX vs. LPS-Sham. $*P < 0.05$; $**P < 0.01$ IL-1-VGX or LPS-VGX vs. IL-1-Sham or LPS-Sham. Each datapoint is based on $n = 5$.

amygdala; $F_{1,28} = 4.78$; $P < 0.05$ and $F_{1,28} = 5.17$; $P < 0.05$, bed nucleus; $F_{1,29} = 8.05$; $P < 0.01$ and $F_{1,29} = 7.89$; $P < 0.01$). LPS injection significantly increased Fos immunoreactivity in the central amygdala of rats that underwent sham surgery ($P < 0.01$), and this increase was blocked by abdominal vagotomy ($P < 0.01$) (Fig. 4C). In the dorsolateral bed nucleus of the stria terminalis of sham-operated rats, LPS administration significantly increased the number of Fos-positive cells ($P < 0.01$), an effect not occurring in vagotomized animals ($P > 0.10$). Fos expression was significantly higher in the BNST of LPS-injected, sham-operated animals compared with vagotomized rats. ($P < 0.001$) (Fig. 4D).

Photomicrographs of Fos expression in VMPO, PVN and central amygdala of sham-operated and vagotomized animals are shown in Fig. 5. The pattern of LPS-induced cellular activation in these structures of sham-operated animals (Fig. 5A, E and I) was blocked in animals that underwent vagotomy (Fig. 5C, G and K). However, only complete subdiaphragmatic vagotomy resulted in blockage of Fos expression, since animals in which isolectin-positive fibres were still clearly present at the level of the medial and commissural NTS (Fig. 1B), showed robust Fos induction after LPS (Fig. 5B, F and J). Despite a dramatic decrease in LPS-induced Fos expression in the PVN, some Fos-positive cells were still observed in the dorsomedial and ventromedial parvocellular part (Fig. 5G). LPS-induced Fos expression in the organum vasculosum of the laminae terminalis (OVLT) was not affected by vagotomy (Fig. 6).

LPS-induced COX-2 immunoreactivity

Western blot analysis was performed to examine the specificity of the COX-2 antibody. In samples of the hippocampus that is known to express COX-2 constitutively (Breder *et al.*, 1995), the COX-2 antibody recognized two bands of ≈70 kDa (results not shown), which is consistent with the molecular weights of the two COX-2 subunits (71 and 73 kDa) described previously (Pomerantz *et al.*, 1993). After LPS injection COX-2-immunoreactive cells were found around blood vessels throughout the brain, as described in earlier studies (Matsumura *et al.*, 1998). This labelling pattern was not influenced by vagotomy: Fig. 7 shows COX-2-immunoreactive cells in the meningal strand penetrating into the OVLT (A) and associated with blood vessels of the VMPO (B) 2 h after LPS injection into a vagotomized animal. (C) Saline injection did not induce COX-2-immunoreactive cells around blood vessels of vagotomized animals. The staining of neuronal processes in the preoptic area (Fig. 7B, upper right corner, and Fig. 7C) is in accordance with earlier studies (Breder *et al.*, 1995; Elmquist *et al.*, 1997a). COX-2 immunoreactivity was abolished when staining sections that were incubated with the preabsorbed antibody (D).

LPS-induced changes in Stat3 expression

A previous study showed that the Stat3 antibody used in this study recognizes one band in brain homogenates (Strömberg *et al.*, 2000). After LPS injection, many Stat3-immunoreactive nuclei, indicating translocation, were found in the OVLT of both sham-operated (Fig. 8A) and vagotomized animals (Fig. 8B). No nuclear Stat3 labelling was seen in the OVLT of sham-operated animals after saline injection (Fig. 8C). Only a few Stat3-positive nuclei were seen in the OVLT of vagotomized animals injected with saline (Fig. 8D). LPS-induced nuclear Stat3 immunoreactivity was

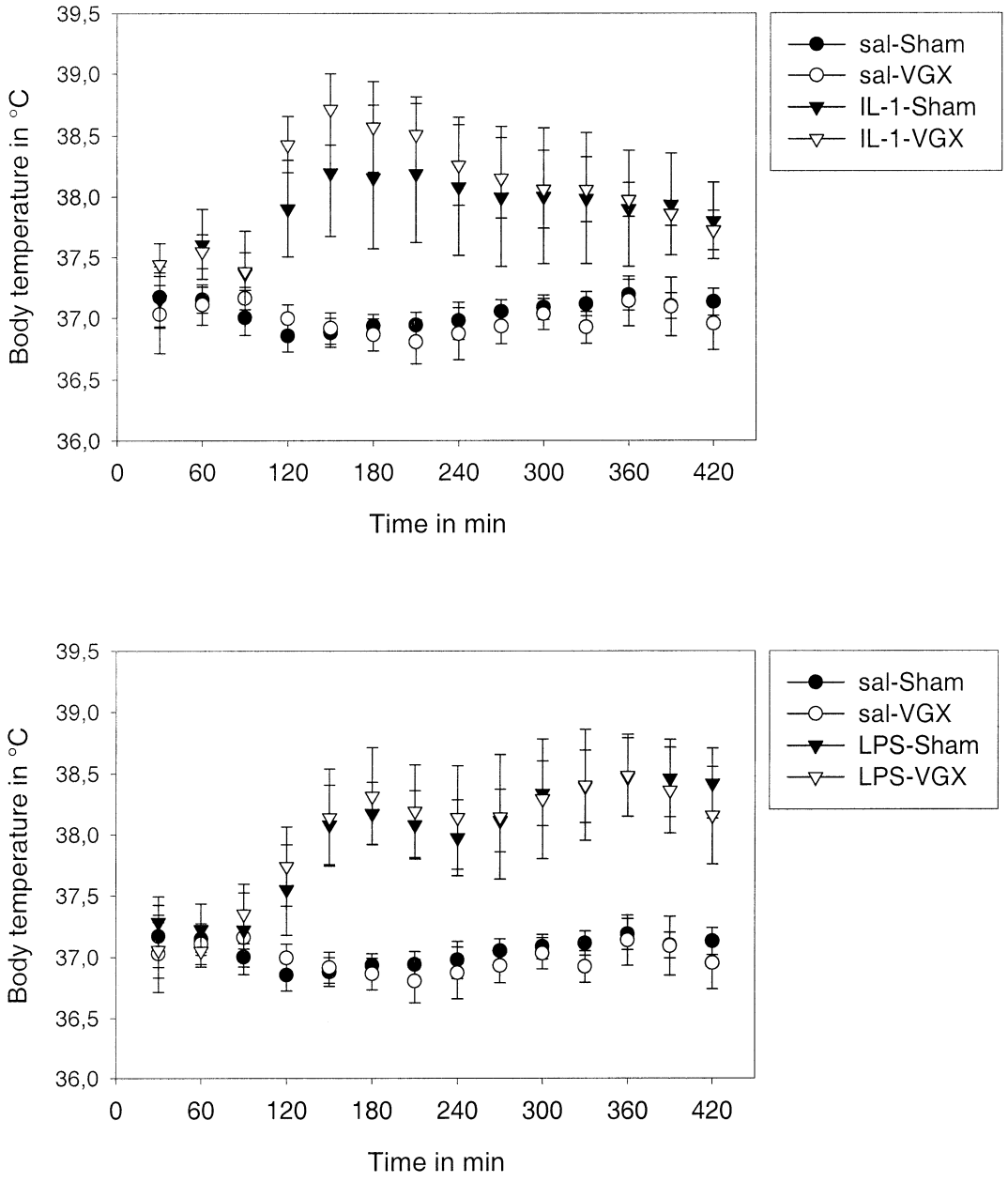

FIG. 3. Changes in abdominal temperature in sham-operated and vagotomized animals after intraperitoneal injection of recombinant rat interleukin-1β (rrIL-1β; 25 μg; upper graph) or lipopolysaccharide (LPS; 250 μg/kg; lower graph). Temperature starts to rise 2 h after injection of rrIL-1β or LPS and remains elevated until the end of the experiment 5 h later. Temperature responses to rrIL-1β or LPS do not differ between sham-operated and vagotomized animals at any time-point. Abbreviations see Fig. 2. Each data-point is based on $n = 5–6$.

not found in sections incubated with the preabsorbed antibody (results not shown). Constitutive cytoplasmic Stat3 expression in the brain was found in the cytoplasm of neurons of the PVN, as well as in the arcuate nucleus (results not shown), as previously described (Håkansson & Meister, 1998; Strömberg *et al*., 2000). LPS injection did not induce nuclear labelling in these structures.

Mechanisms underlying the effects of vagotomy

Vagotomy does not affect i.p. LPS-induced IL-1β immunoreactivity in circumventricular organs

Two hours after i.p. injection of LPS, IL-1β-positive cells were observed in all circumventricular organs and in the choroid plexus, as previously described (Konsman *et al*., 1999). Immunoreactive cells were polar, with several cellular processes arising from the soma that often encompass blood vessels. No IL-1β immunoreactivity was seen in saline-treated, sham-operated rats or vagotomized animals. A two-way ANOVA on the relative surface covered by IL-1β-immunoreactive cells in the subfornical organ revealed a significant effect of treatment ($F_{1,27} = 7.18$; $P < 0.05$) (Fig. 9A). No significant differences in IL-1β immunoreactivity occurred between LPS-treated, sham-operated rats and vagotomized rats ($P > 0.10$) (Fig. 9A), indicating that vagotomy did not affect IL-1β induction after LPS.

Vagotomy blocks LPS-induced Fos expression in the NTS

A two-way ANOVA on the number of Fos-positive nuclei in the NTS (Fig. 9B) revealed a significant effect of treatment ($F_{1,28} = 6.83$;

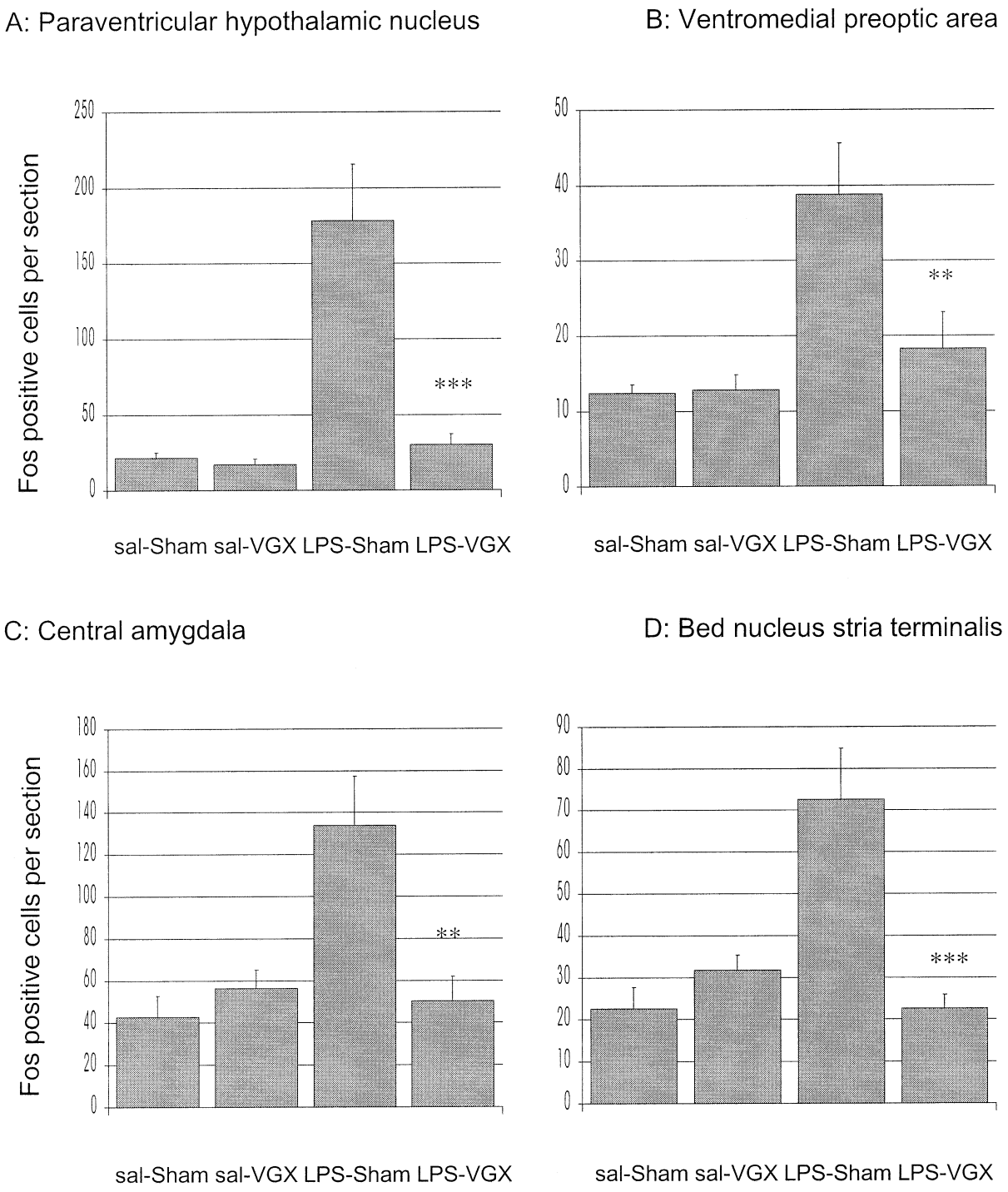

FIG. 4. Subdiaphragmatic vagotomy prevents the increase in the number of Fos-immunoreactive cells in (A) the paraventricular hypothalamus, (B) ventromedial preoptic area, (C) central amygdala and (D) bed nucleus of the stria terminalis 2 h after intraperitoneal injection of 250 μg/kg LPS. For abbreviations see Fig. 1. *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$; LPS-VGX vs. LPS-Sham. sal-Sham: $n = 10$; sal-VGX: $n = 11$; LPS-Sham: $n = 10$; VGX-LPS: $n = 7$.

$P < 0.05$) and a significant interaction between treatment and surgery ($F_{1,28} = 8.02$; $P < 0.01$). Subdiaphragmatic vagotomy induced Fos expression in the NTS at the level of the area postrema in saline-treated animals, as previously observed (Traub *et al.*, 1996). However, the effect of vagotomy on the number of Fos-immunoreactive cells in the NTS of saline-treated rats was not significantly different from animals that underwent sham surgery ($P > 0.10$). In contrast, LPS induced a significant increase in the number of Fos-positive cells in the NTS of sham-operated animals ($P < 0.01$), and this increase was prevented by abdominal vagotomy ($P < 0.01$) (Fig. 9B).

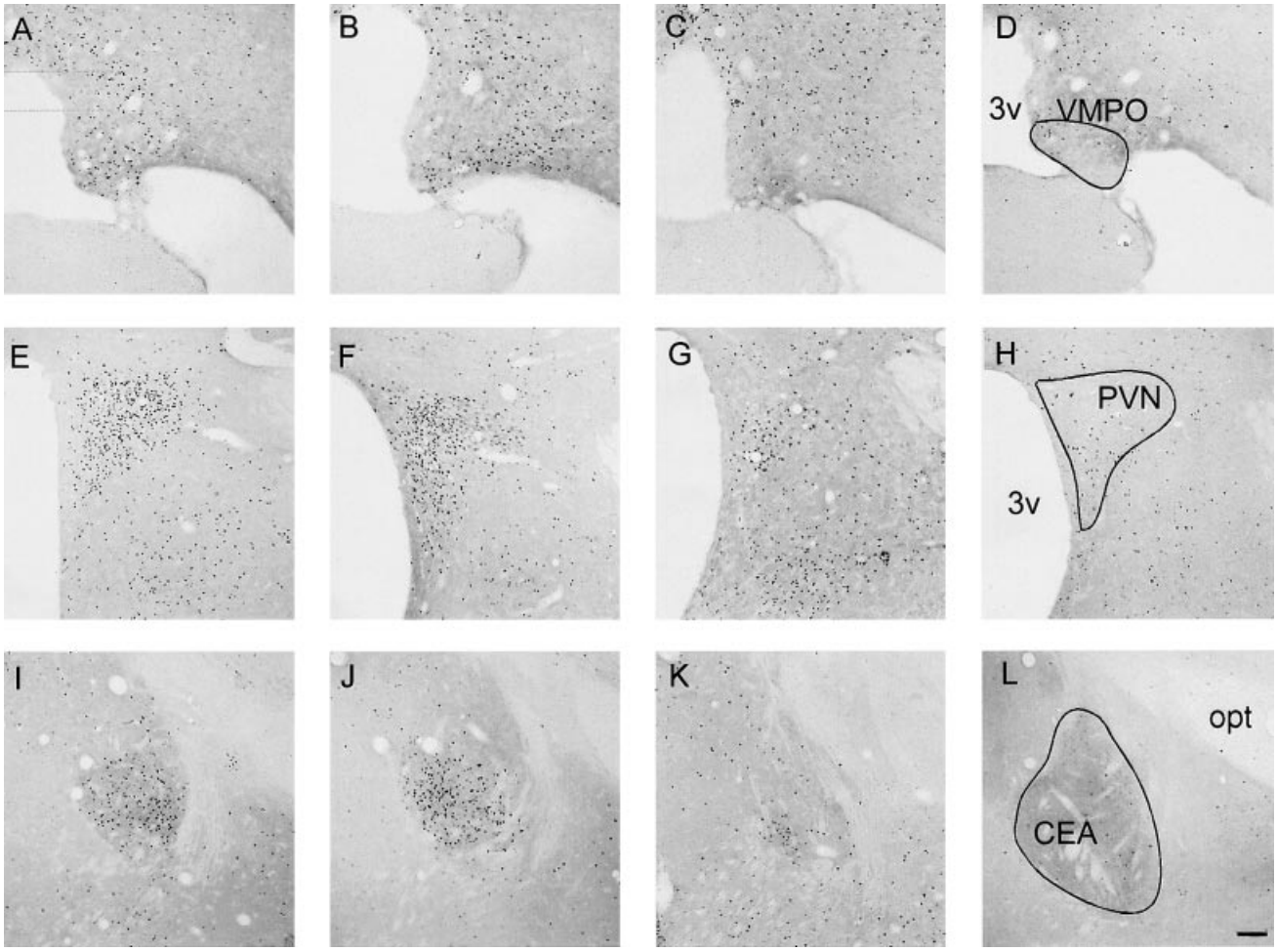

FIG. 5. Fos expression in the ventromedial preoptic area (VMPO; A–D), paraventricular nucleus of the hypothalamus (PVN; E–H) and central amygdala (CEA; I–L) 2 h after intraperitoneal injection of saline or LPS in vagotomized (VGX) and sham-operated rats. The left lane shows sections of a sham-operated animal (Sham 48) injected with 250 μg/kg LPS. The middle left lane displays sections from an animal (VGX 12) injected with LPS, that was judged to be incompletely vagotomized based on the persistence of isolectin binding in nucleus of the solitary tract (Fig. 1B). The pattern of Fos expression after LPS in this animal is similar to that seen in a sham-operated animal (left lane). However, LPS-induced Fos expression is attenuated in a rat that was completely vagotomized (VGX 32) (middle right lane). Despite a dramatic decrease in LPS-induced Fos expression in the PVN, Fos-positive cells were still observed in the ventromedial parvocellular part (G). The right lane shows a completely vagotomized animal injected with saline (VGX 30). Quantified areas are outlined. 3v, third ventricle; opt, optic nerve. Scale bar, 100 μm.

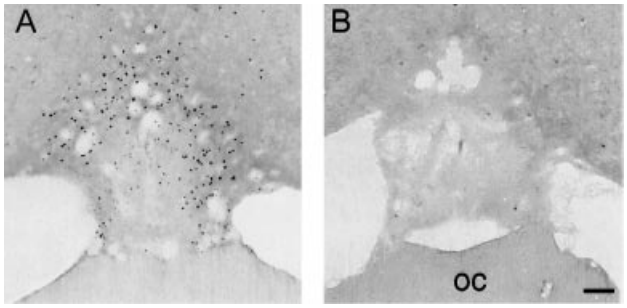

FIG. 6. Vagotomy does not block LPS-induced Fos expression in the organum vasculosum of the laminae terminalis. Intraperitoneal injection of 250 μg/kg LPS results in a robust Fos expression in the organum vasculosum of the laminae terminalis of (A) vagotomized animals 2 h later compared with (B) saline injection. oc, optic chiasm. Scale bar, 100 μm.

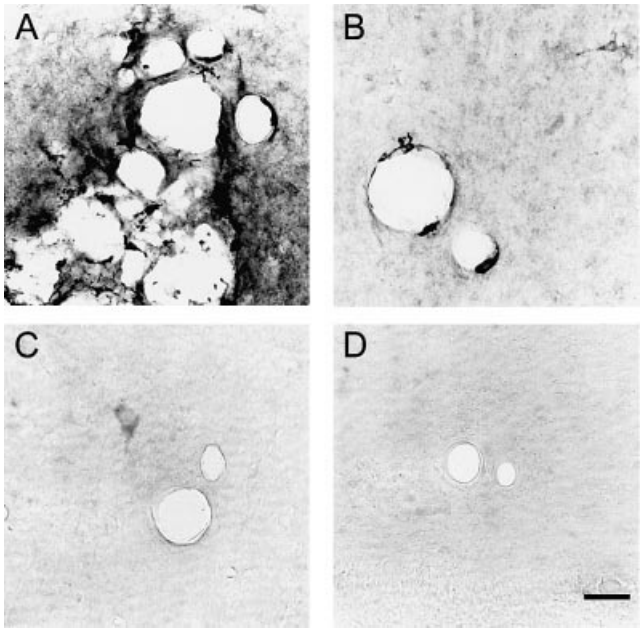

FIG. 7. Cyclooxygenase-2 induction in the preoptic area 2 h after intraperitoneal injection of 250 μg/kg LPS in vagotomized animals. (A) Cyclooxygenase-2 is found in meninges just rostral to the organum vasculosum of the laminae terminalis and (B) around blood vessels in the medial preoptic area after LPS injection. (C) No cyclooxygenase-2 is found around blood vessels after injection of saline. (D) Note that some neuronal processes were also stained in the preoptic area (B, upper right corner, and C). No staining is found after preabsorbing the antibody with its antigen. 3v, third ventricle; oc, optic chiasm. Scale bar, 50 μm.

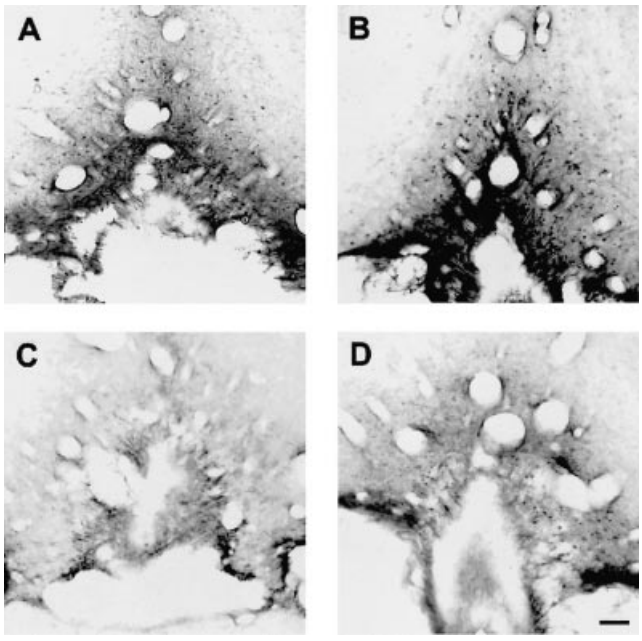

FIG. 8. Nuclear Stat3 expression indicating translocation in the organum vasculosum of the laminae terminalis of (A) a sham-operated and (B) vagotomized rat 2 h after intraperitoneal injection of 250 μg/kg LPS. No or very little nuclear labelling is found after saline injection in (C) sham-operated and (D) vagotomized animals. Scale bar, 100 μm.

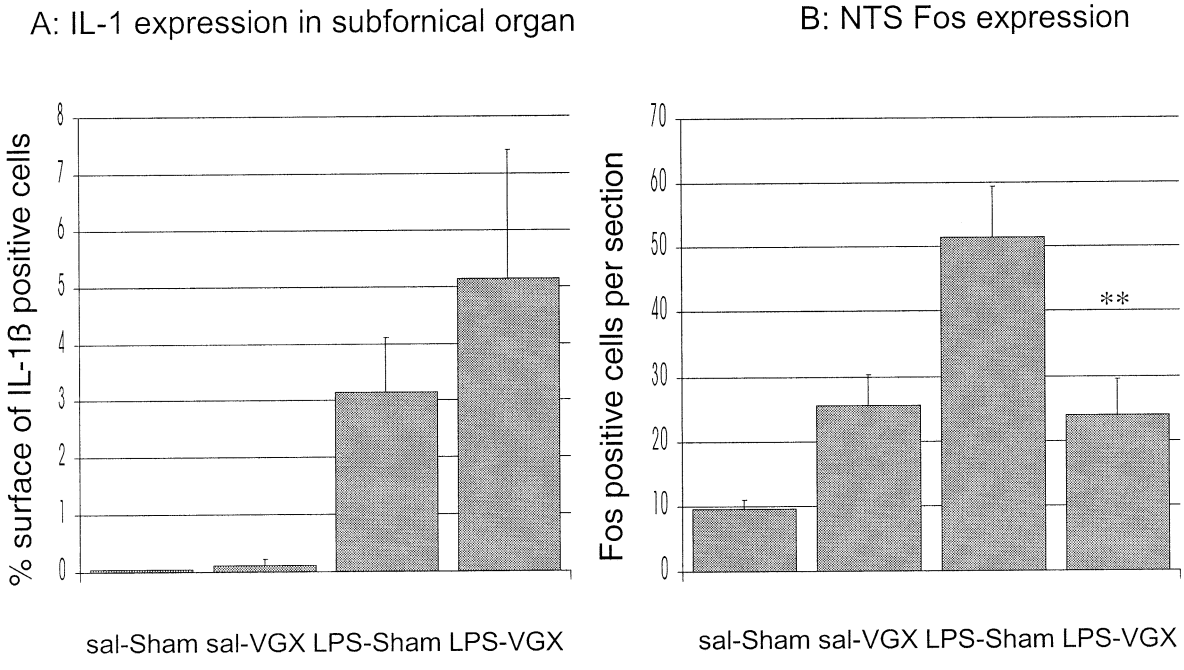

FIG. 9. Mechanisms underlying the effects of vagotomy. (A) The relative surface occupied by interleukin-1β-positive cells in the subfornical organ 2 h after intraperitoneal injection of 250 μg/kg LPS does not differ between sham-operated and vagotomized animals. Abbreviations see Fig. 1. sal-Sham: $n = 9$; sal-VGX: $n = 10$; LPS-Sham: $n = 8$; VGX-LPS: $n = 9$. (B) Subdiaphragmatic vagotomy prevents the induction of Fos in the nucleus of the solitary tract (NTS) 2 h after intraperitoneal injection of 250 μg/kg LPS in vagotomized animals. For abbreviations, see Fig. 1. ** $P < 0.01$, LPS-VGX vs. LPS-Sham. sal-Sham: $n = 9$; sal-VGX: $n = 11$; LPS-Sham: $n = 10$; VGX-LPS: $n = 7$.