c-Fos generation in the dorsal vagal complex after systemic endotoxin is not dependent on the vagus nerve

G. E. HERMANN, G. S. EMCH, C. A. TOVAR, AND R. C. ROGERS
Department of Neuroscience, Ohio State University, Columbus, Ohio 43210

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Hermann, G. E., G. S. Emch, C. A. Tovar, and R. C. Rogers. c-Fos generation in the dorsal vagal complex after systemic endotoxin is not dependent on the vagus nerve. Am J Physiol Regulatory Integrative Comp Physiol 280: R289–R299, 2001.—The present study used activation of the c-Fos oncogene protein within neurons in the dorsal vagal complex (DVC) as a marker of neuronal excitation in response to systemic endotoxin challenge [i.e., lipopolysaccharide (LPS)]. Specifically, we investigated whether vagal connections with the brain stem are necessary for LPS cytokine-induced activation of DVC neurons. Systemic exposure to LPS elicited a significant activation of c-Fos in neurons in the nucleus of the solitary tract (NST) and area postrema of all thiobutabarbital-anesthetized rats examined, regardless of the integrity of their vagal nerves. That is, rats with both vagi cervically transected were still able to respond with c-Fos activation of neurons in the DVC. Unilateral cervical vagotomy produced a consistent but small reduction in c-Fos activation in the ipsilateral NST of all animals within this experimental group. Given that afferent input to the NST is exclusively excitatory, it is not surprising that unilateral elimination of all vagal afferents would diminish NST responsiveness (on the vagotomized side). These data lead us to conclude that the NST itself is a primary central nervous system detector of cytokines.

lipopolysaccharide; brain stem; nucleus of the solitary tract; vagotomy

CYTOKINES ARE RELEASED by activated macrophages and lymphocytes as part of the immune response to antigenic challenge, injury, or irradiation. Elevation of the early proinflammatory cytokine tumor necrosis factor (TNF)-α in the systemic circulation has been correlated with anorexia, nausea, vomiting, and gastrointestinal stasis (7, 22, 23). This correlation between elevated plasma cytokine levels and changes in physiological state associated with illness implies a communication between the immune and nervous systems. Recent evidence suggests that the dorsal vagal complex (DVC) in the medulla oblongata may be one locus for TNF-α action to control gastrointestinal function (13, 19, 20).

The DVC consists of the sensory nuclei of the solitary tract (NST), the area postrema (AP), and the dorsal motor nucleus of the vagus (DMN). These nuclei comprise the final common pathway of the vago-vagal reflex circuits that control gastric motility (37). This medullary brain stem area has been identified as possessing the characteristics of a circumventricular organ and is essentially devoid of a blood-brain barrier (4, 18, 48). In addition, previous anatomic work (35, 36, 43) demonstrated that dendritic endings of neurons in both the NST and the DMN penetrate the AP and the floor of the fourth ventricle. Together, these anatomic characteristics place the DVC in a position to monitor blood-borne and cerebrospinal fluid-borne factors and to change vagally mediated autonomic functions accordingly (9, 27, 37). The brain stem has a high density of TNF-α binding sites (24) and is in a position to monitor blood-borne peptides. Therefore, it was hypothesized that the DVC may be a site at which circulating TNF-α acts to provoke gastric stasis and the other prodromata of illness such as nausea and emesis.

Endogenous production of TNF-α can be readily elicited by systemic administration of the bacterial cell coat component, lipopolysaccharide (LPS) (47). Our previous studies (20) demonstrated that TNF-α production in response to systemic (i.e., intravenous) LPS is sufficient to suppress centrally stimulated increases in gastric motility. Our earlier study demonstrated that TNF-α injected unilaterally into the DVC abolished a centrally stimulated and vagally dependent increase in gastric motility in a dose-dependent manner (19). The rapidity of the centrally injected TNF-α effect on gastric motility, i.e., within 30 s of application to the DVC, suggested that TNF-α could directly and rapidly affect the firing rate of neurons in the DVC. Electrophysiological studies by Emch et al. (13) showed that neurons of the NST that form the sensory limb of a vago-vagal gastroinhibitory reflex (35, 42) are strongly activated by doses of TNF-α previously shown to affect gastroinhibition (19).

Work by Sehic and Blatteis (3, 42) and others (14, 15, 17, 28) describes a potential alternate pathway by which information about immune activation may be transmitted to the central nervous system (CNS). There is evidence suggesting that vagal afferents, especially those in the hepatic branch, contain receptive elements responsive to cytokine or complement levels (3, 44). The mechanism implied is similar to that re-
sponsible for the integrative physiological and behav-
ioral actions of CCK. In this case, CCK, released by
duodenal enterocytes, activates vagal afferent fibers
that, in turn, produce a suppression of gastric motility
as well as food intake (33, 41). The hypothesis had been
made that systemic levels of cytokines are monitored
by vagal afferents in the periphery, and their activa-
tion is responsible for illness behaviors and physi-
ological responses such as fever and gastrointestinal
malaise (14, 15, 17, 28). However, the role for vagal
afferents in the transmission of information about pe-
ripheral cytokine release provoking gastrointestinal
malaise and other illness behaviors has been called
into question recently. That is, some investigators have
shown that elimination of vagal afferent pathways
does not block the malaise-inducing, anorexic, somno-
genic, or febrile effects of cytokines (6, 21, 31, 41).

However, it is possible that both the vagal afferent
and direct NST mechanisms operate parallel to moni-
tor the portal circulation and the general systemic
circulation, respectively. This hypothesis is supported
by some reports that CNS effects (i.e., fever, illness
behavior, etc.) of either low-dose intravenous or intra-
peritoneal LPS may be blunted by vagotomy, whereas
the same CNS effects after high-dose intravenous LPS
administration are not blocked by vagotomy (6, 21, 42).

We decided to test the hypothesis that vagal path-
ways are essential to the CNS signaling of peripheral
cytokine production by using the generation of the
protein product of the proto-oncogene c-Fos as an ana-
tomic identification of functionally activated neurons
(32) in the NST. Previous studies (12, 45) showed that
LPS-induced cytokine generation produces a signifi-
cant increase in c-Fos labeling of neurons in the NST,
i.e., the neurons in the medulla that receive vagal affer-
ent projections. However, this earlier work did not
establish whether the NST neurons were activated
directly by circulating cytokine action or by vagal af-
ferent pathways. It should be noted, however, that
studies by Gaykema et al. (15) showed that subdia-
phragmatic vagotomy abolished c-Fos expression in
vaginal sensory ganglia after intraperitoneal adminis-
tration of LPS but only attenuated c-Fos expression in
these nuclei when LPS was administered intrave-
nously. These results suggest that different or redu-

dant pathways are employed to inform the CNS about
peripheral levels of cytokines.

The majority of the hepatic vagal afferents (i.e., the
most likely peripheral afferent target for cytokines;
Ref. 44), in addition to the usual complement of general
visceral afferents from the thorax and abdomen, ascends
via the left cervical vagal trunk (2, 34). There-

fore, we propose the following hypotheses. 1) If intact
vagal pathways are critical to the transmission of in-
formation to the NST concerning peripheral cytokine
generation, then section of the left cervical vagal trunk
should eliminate c-Fos generation in the NST on the
side of section. 2) If direct action of cytokines at the
NST is primary, then vagal transection should have no
effect on NST c-Fos generation, and the numbers of
nous volumes (0.1 ml/100 g body wt) of either PBS (n = 4) or LPS (1,000 μg/kg body wt; n = 6).

Experiment 2 was designed to determine whether transection of the left cervical vagal trunk (with its predominance of hepatic afferent components) would eliminate c-Fos activation in the NST. These studies were performed in unilateral (i.e., left), cervical vagotomized rats that were exposed to one of four drug conditions: 1) PBS (n = 4), 2) 1,000 μg/kg LPS dose (n = 6), 3) 100 μg/kg LPS dose (n = 6), or 4) 25 μg/kg LPS dose (n = 6).

Experiment 3 was designed to determine if loss of all vagal afferents (and efferents) would prevent c-Fos activation of NST neurons in response to endotoxin challenge via either intravenous or intraperitoneal routes. Thus these studies were performed in bilateral, cervical vagotomized rats. Given that other studies have suggested that lower doses of intravenous or intraperitoneal LPS may be more susceptible to produce fever, elicit c-Fos expression in the central amygdala, shown to effectively elevate plasma TNF-α levels (16), produce a significant gastric stasis under similar anesthetic conditions (20). This dose also produces a modest but consistent hypotension. Our “intermediate” dose (100 μg/kg body wt) has been shown to elicit anorexic effects (31, 41) or fever (28) in awake rats. Finally, our “low” dose (25 μg/kg) has been shown to effectively elevate plasma TNF-α levels (16), produce fever, elicit c-Fos expression in the central amygdala, but does not provoke hypotension (45). The effects (or lack thereof) of LPS on blood pressure at these doses (i.e., 25, 100, or 1,000 μg/kg) were verified under Inactin anesthesia in our preliminary studies (data not shown).

Endogenous TNF-α production reaches maximal plasma levels within 90 min of systemic administration of LPS (47). Studies by Rinaman et al. (32) demonstrated that maximal nuclear c-Fos immunoreactivity is present ~60–90 min following the presence of the presumptive stimulus. Therefore, comparable to other studies on c-Fos activation within the CNS after systemic exposure to endotoxin (40, 45), survival time before perfusion was selected to be 3 h after systemic injections of either PBS or LPS to maximize c-Fos activation expression. At the end of 3 h, rats were given a 0.2-ml bolus intravenous injection of lidocaine to stop respiration and cardiac function. The chest cavity was opened, and a blood sample by ventricular puncture was taken for subsequent ELISA verification of TNF-α production. Animals were then transcardially perfused with PBS followed by 4% paraformaldehyde in PBS. The brain stems were then removed to a solution of 4% paraformaldehyde and 20% sucrose in PBS to postfix for 16 h.

Animals

Male Long-Evans rats (Charles River Labs, Wilmington, MA) were maintained in a temperature-controlled vivarium with a 12:12-h day-night cycle. Animals had ad libitum access to food and water. All experimental procedures were performed according to guidelines set forth by the National Institutes of Health and were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee.

Surgical Preparations

Rats were anesthetized with Inactin (100 mg/kg body wt ip). All subjects received tracheal cannulas to ensure the maintenance of an open airway for the duration of the experiment. Animals assigned to intravenous studies were equipped with sterile jugular cannulas. Depending on the assigned vagal status, each animal received one of three surgical manipulations: 1) exposure of cervical vagi without sectioning of the vagal trunks, i.e., intact (n = 10), 2) left cervical vagal trunk section (n = 22), or 3) bilateral cervical trunk section (n = 15). In the rats receiving bilateral cervical vagotomies, the left vagus was cut ~20 min before the section of the right vagus. Although the rats developed apneustic breathing after the section of the remaining vagus, all survived without auxiliary ventilation (39).

Histological Processing for c-Fos Protein

Brain stems were sectioned on a freezing microtome at 50-μm thickness; sections were collected in PBS. After being rinsed in PBS, sections were treated with 1% sodium borohydride to reduce the fixative remaining in the tissue. After being rinsed in PBS, tissue sections were incubated for 1 h on a shaker in 10% normal sheep serum plus 0.3% Triton X in PBS to block nonspecific binding of the primary c-Fos antibody. After being rinsed, tissue sections were incubated in primary c-Fos antibody (Oncogene AB-5; rabbit c-Fos, 1:10,000) in 0.3% Triton in PBS for 16 h at room temperature with gentle agitation. Tissue sections were rinsed and incubated in biotinylated goat anti-rabbit IgG (Vector, 1:600) for 1 h. Sections were rinsed and reacted with Vector elite avidin-biotin-peroxidase complex (1:600 in PBS) for 1 h, followed by Vector SG peroxidase detection reagents. Specificity of the c-Fos immunocytochemical reaction was verified by omitting the c-Fos antibody from randomly selected sections. Sections were rinsed, mounted on glass slides, dried, cleared in Hemo-De (Fisher Scientific, Pittsburgh, PA), and placed under a coverslip with Entellan (Electron Microscopy Sciences, Fort Washington, PA).

Counting c-Fos Nuclei in the Dorsal Medulla

c-Fos-labeled nuclei were counted manually with the aid of an MD2 Microscope Digitizer (Minnesota Datamektrics, St. Paul, MN) encoder attached to the stage of a Leitz Dialux Microscope. Inclusion of c-Fos-labeled neurons required that the nuclei be a minimum of 6 μm in diameter and exhibit a nucleolus. These criteria guaranteed that staining artifacts and nuclear fragments would not be included in the count (see Figs. 2, 4, and 6). c-Fos-stained nuclei were counted without knowledge of the experimental condition, and counts were verified by a second observer. The agreement between counts of the two observers was within 5%. c-Fos activation of medullary neurons was analyzed at four specific coronal levels for each animal: 0.5 mm posterior to the calamus scriptorius, the level of the calamus, the level of the area postrema (0.5 mm anterior to calamus), and the level of the anterior NST (1.0 mm anterior to calamus). The cumulated number of activated cells was totaled for the right and left side of each animal’s brain for statistical analysis. The distribution of labeled nuclei from the NST, AP, and DMN regions was analyzed separately.

TNF-α Assay

Plasma TNF-α was determined by an ELISA kit for rat TNF-α (R & D Systems, Minnesota, MN). Fifty-microliter plasma samples, in duplicate, were incubated at room tem-
temperature in microwells precoated with monoclonal anti-rat TNF-α antibody. After a 2-h incubation, each well was aspirated and washed with wash buffer; this process was repeated four times. One-hundred microliters of antibody against rat TNF-α conjugated to horseradish peroxidase was added to each well. After a 2-h incubation, each well was aspirated and washed with wash buffer; this process was repeated four times. One-hundred microliters of a tetramethylbenzidine peroxidase substrate was added to all wells. After a 30-min incubation at room temperature, the reaction was stopped by addition of hydrochloric acid. The optical absorbance of each well was read within 30 min with a microplate reader set to 450 nm. Absorbance values were converted to TNF-α concentrations by comparison with a simultaneously generated standard curve. The limits of detection per well of this assay kit were 12.5–800 pg/ml; the intra- and inter-assay variabilities were 8.8 and 2.1%, respectively (manufacturer’s data).

**Analysis**

Although animals were randomly assigned to one of the three surgical groups and experiments were run simultaneously, the analyses of c-Fos data were segregated according to surgical condition, i.e., 1) intact vagi, 2) left cervical vagotomy, and 3) bilateral cervical vagotomy, for two reasons. Most importantly, these three surgical manipulations result in rats with different physiological states (e.g., anesthetic vs. normal breathing) that may be reflected in basal (i.e., PBS challenge) conditions of c-Fos activation of DVC neurons. Second, these three experiments were designed to address different aspects of the DVC response to systemic endotoxin challenge.

**Experiment 1: intact vagi/Inactin (thiobutabarbital) anesthesia.** Previous studies of CNS c-Fos production after LPS have been performed in unanesthetized rodents (12, 40, 45). Studies have shown that different anesthesia types may affect the immune response to LPS challenge, e.g., the reduction of TNF-α production under urethan anesthesia (20, 26). Therefore, it was necessary to first establish that vagally intact, Inactin-anesthetized rats were capable of inducing c-Fos synthesis in response to intravenous LPS challenge.

Second, recent data suggest that the significant cytokine-sensitive sensory pathway to the CNS arises from hepatic vagal afferents (44) that are represented asymmetrically within the two cervical vagal trunks and the NST. That is, the large majority of hepatic vagal afferents travels in the left cervical vagus and terminates in the left medial NST (2, 34). Therefore, the second aim of this experiment was to determine whether there was any intrinsic left versus right “sidedness” to the distribution of c-Fos in the brain stem of rats with both vagi intact.

The cumulated number of activated cells (i.e., total number of c-Fos-activated neurons from the 4 coronal sections) was totaled for the right and left side of each animal’s brain within either the NST or DMN. The NST and DMN c-Fos count results were independently subjected to Student’s t-tests.

AP c-Fos counts were obtained from the single sample section that contained this midline structure. Given that left versus right “sidedness” was not an issue with this structure, c-Fos-activated cell counts of PBS- versus LPS-challenged rats were analyzed using a Student’s t-test.

**Experiment 2: left cervical vagotomy.** Animals received left cervical vagotomy to eliminate vagal connections with half of the brain stem (i.e., the half that may receive a physiologically significant hepatic afferent projection). These unilaterally vagotomized rats were challenged with one of three doses of intravenous LPS or PBS. The cumulated number of activated cells (i.e., total number of c-Fos-activated neurons from the 4 coronal sections) was totaled for the right and left side of each animal’s brain within either the NST or DMN. The NST and DMN c-Fos count results were independently subjected to a repeated-measures ANOVA (i.e., left and right sides from the same animal; PBS vs. LPS groups; Ref. 29). In the event of a significant P value (i.e., P < 0.05), Dunnett’s posttests were used. Given that the AP is a midline structure (i.e., no right or left side), c-Fos-activated cell counts within the AP of the four conditions (i.e., PBS, 25 μg/kg LPS, 100 μg/kg LPS, or 1,000 μg/kg LPS) were analyzed by one-way ANOVA.

**Experiment 3: bilateral cervical vagotomy.** It could be argued that any c-Fos label observed in the dorsal medulla ipsilateral to the unilateral vagotomy might be attributed to afferent activity from the remaining intact vagal trunk. Therefore, in this experiment, rats received bilateral cervical vagotomies to totally eliminate vagal connections with the NST. These animals received either intravenous PBS or our lowest LPS dose (25 μg/kg body wt) via either intravenous or intraperitoneal routes. As in the previous experiments, the cumulated number of activated cells (i.e., total number of c-Fos-activated neurons from the 4 coronal sections) was totaled for the right and left side of each animal’s brain.

**Table 1. Summary of c-Fos-activated neurons**

<table>
<thead>
<tr>
<th></th>
<th>Intact</th>
<th>DMN</th>
<th>AP</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Left</td>
<td>Right</td>
<td>Left</td>
</tr>
<tr>
<td>PBS 1,000 μg/kg LPS</td>
<td>64.5 ± 10.9</td>
<td>57.25 ± 13.8</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>CVX 1,000 μg/kg LPS</td>
<td>275.8 ± 13.3</td>
<td>272.0 ± 23.1</td>
<td>10.3 ± 2.2</td>
</tr>
</tbody>
</table>

Number of c-Fos-activated cells (means ± SE) in nucleus of solitary tract (NST), dorsal motor nucleus (DMN), and area postrema (AP) of Inactin-anesthetized, rats under 1 of 3 surgical conditions [i.e., intact vagi, unilateral cervical vagotomy (CVX), or bilateral cervical vagotomy (BVX)] in response to systemic challenges of PBS or lipopolysaccharide (LPS), as designated.
within either the NST or DMN. The NST and DMN c-Fos count results were independently subjected to a repeated-measures ANOVA (i.e., left and right sides from the same animal; PBS vs. LPS groups; Ref. 29). c-Fos-activated cell counts within the AP of the three different conditions (i.e., PBS, LPS [intravenous], or LPS [intraperitoneal]) rats were analyzed by one-way ANOVA.

**Plasma TNF-α Levels**

Plasma TNF-α levels were analyzed according to the systemic challenge (i.e., PBS or different doses of LPS; n = 35) by using the Kruskal-Wallis test for nonparametric samples. Statistical significance was defined as an overall P < 0.05; Dunn’s multiple-comparison posttests were applied.

**RESULTS**

**Experiment 1: c-Fos Labeling in the NST, DMN, and AP in the Vagus-Intact, Inactin-Anesthetized Rat**

Systemic LPS challenge induced a significant rise in NST c-Fos labeling in vagus-intact, Inactin-anesthetized rats (Table 1; Figs. 1A and 2; t = 11.3, df = 8, P < 0.0001). This elevation in NST c-Fos count after intravenous LPS was symmetrical in intact non vagotomized rats; i.e., there was no intrinsic difference in distribution (sidedness) of NST neurons c-Fos activated in response to systemic endotoxin challenge (P = 0.3223). If hepatic afferents (which ascend predominantly within the left cervical vagal trunk to terminate within the left NST) were the principal pathway by which systemic exposure to endotoxin provoked c-Fos activation of brain stem neurons, then one might have expected more NST neurons to be labeled on the left as opposed to the right side.

Systemic LPS challenge produced a statistically significant but small increase in the numbers of DMN neurons containing c-Fos nuclear staining (Table 1; Figs. 1B and 2; t = 3.612, df = 8; P = 0.0069). Again, there was no difference in numbers of c-Fos-labeled nuclei between right and left sides of the brain stem.

C-Fos labeling of neurons in the AP was also significantly increased by LPS challenge in the vagus-intact rat (Table 1; Figs. 1C and 2, t = 4.658; df = 6; P = 0.0035).

**Experiment 2: Effects of LPS on c-Fos Labeling in Rats with Left Cervical Vagotomy**

Animals with left cervical vagotomy demonstrated a significant increase in c-Fos activation of NST neurons, regardless of dose of intravenous LPS or side of brain stem sampled (i.e., ipsilateral or contralateral to vagotomy) (Table 1; Figs. 3A and 4; F = 9.71; df = 3,17; P = 0.006; Dunnett’s posttest, P < 0.05).

The effect of unilateral vagotomy on the distribution pattern of c-Fos-activated neurons (i.e., left vs. right) was not significant at any individual LPS dose. However, when the data were collapsed across all groups, including the PBS controls, the right side (i.e., nonvagotomized) of each brain stem contained somewhat more c-Fos-activated NST cells than the corresponding (or paired) left side. This sidedness was statistically significant as a consequence of the consistency of this observation across all groups as opposed to the actual magnitude of the difference within any particular group (F = 40.909, df = 1,17, P = 0.0001).

Although the number of c-Fos-labeled cells in the DMN was increased with LPS challenge (Table 1, Figs. 3B and 4), because of the overall small number of DMN cells activated, this increase was not statistically significant (F = 2.206, df = 3,17, P = 0.1298).

There was a significant increase in the number of c-Fos-labeled neurons in the AP in response to all doses...
of LPS challenge (Table 1; Figs. 5C and 6; \( F = 5.108, \) df = 2,10, \( P = 0.0296 \)). Dunnett’s posttest revealed that only the LPS (intravenous) group was statistically significant relative to the PBS group.

**Plasma TNF-\( \alpha \) Levels**

Blood samples were obtained at \( \sim 180 \) min postinjection of either PBS or one of the different doses of LPS, i.e., immediately before transcardial perfusion for histological processing. All three doses of LPS (25, 100, or 1,000 mg/kg body wt) showed a significant elevation in plasma TNF-\( \alpha \) levels compared to the PBS group (PBS, \( P = 0.7011 \); 25 mg/kg, \( P = 0.0011 \); 100 mg/kg, \( P = 0.0004 \); 1,000 mg/kg, \( P = 0.0002 \)).

**Experiment 3: Effects of LPS on c-Fos Labeling in Rats with Bilateral Cervical Vagotomy**

Animals with bilateral cervical vagotomy (i.e., devoid of vagal connections with the CNS) still demonstrated a highly significant elevation in the numbers of c-Fos-labeled neurons in the NST. This response to our low dose (25 mg/kg body wt) of LPS was evident whether delivered via either the intravenous or intraperitoneal route (Table 1; Figs. 5A and 6; \( F = 15.24, \) df = 2,12, \( P = 0.0005 \); Dunnett’s posttest \( P < 0.05 \)).

The number of DMN neurons showing c-Fos activation following endotoxin challenge (via either intravenous or intraperitoneal routes) in these bilaterally vagotomized animals was comparable to the numbers seen in either the unilaterally vagotomized or vagally intact groups. However, this c-Fos response is not different from that elicited by PBS under these physiological conditions (Table 1; Figs. 5B and 6; \( P = 0.7876 \)).

Finally, the number of c-Fos-activated cells in the AP following endotoxin challenge was significantly increased in the bilateral, cervical vagotomized groups (Table 1; Figs. 5C and 6; \( F = 5.108, \) df = 2,10, \( P = 0.0296 \)). Dunnett’s posttest revealed that only the LPS (intravenous) group was statistically significant relative to the PBS group.

**Fig. 2.** Micrographs of original coronal histological sections through the NST at the level of the AP. c-Fos production in response to systemic (intravenous) challenge of either PBS (A) or 1,000 mg/kg LPS (B) is characterized by the dark staining cells measuring at least 6 \( \mu \)m and demonstrating distinct nucleoli. Systemic LPS evoked a substantial, bilaterally symmetrical increase in c-Fos production in the AP, NST, and DMN in the Inactin-anesthetized rat. Scale bar = 0.5 mm. cc, central canal; mNST, medial portion of the NST; st, solitary tract; 12, hypoglossal nucleus.

**Fig. 3.** Experiment 2: graphic representation of cell counts (means \( \pm \)SE) of c-Fos-activated neurons in the brain stem of left cervical-vagotomized rats that received either intravenous PBS or LPS while under Inactin anesthesia. A: intravenous LPS challenges demonstrated a significant increase in the number of c-Fos-labeled nuclei in the NST. This increase in c-Fos-activated cells was seen on either side of the brain stem, regardless of dose of LPS administered (Table 1; \( F = 9.71, \) df = 3,17, \( P = 0.0006 \); Dunnett’s post hoc \(*P < 0.05 \)). The effect of unilateral vagotomy on the distribution pattern of c-Fos-activated neurons (i.e., left vs. right) was not significant at any individual LPS dose. B: LPS exposure also resulted in increases in the number of c-Fos-positive neurons in the DMN, but the absolute numbers are, comparatively, very small and not statistically significant (\( F = 2.206; \) df = 3,17; \( P = 0.1298 \)). C: all doses of LPS significantly increased c-Fos labeling in the AP compared with PBS injections (\( F = 4.634; \) df = 3,16; \( P = 0.0162 \); Dunnett’s posttest \(*P < 0.05 \)).
1,000 μg/kg body wt) elicited significant production of circulating TNF-α in Inactin-anesthetized rats regardless of route of administration (i.e., intravenous or intraperitoneal) or integrity of the vagal nerve trunks (i.e., intact, unilateral, or bilateral vagotomy) (Fig. 7; n = 35; Kruskal-Wallis test P = 0.0001; Dunn’s post-test P < 0.05).

Although there was no significant difference between the amounts of TNF-α elicited by the different doses of LPS across the various groups, there was a highly significant correlation (Spearman r = 0.6908; P < 0.0001) between levels of plasma TNF-α and number of c-Fos-activated neurons in the NST.

DISCUSSION

These studies demonstrated that, even in Inactin-anesthetized rats, neurons in the NST and AP demonstrate a significant increase in c-Fos nuclear protein labeling after systemic challenge of endotoxin (i.e., via either intravenous or intraperitoneal routes) regardless of the integrity of the vagus nerves (i.e., intact, unilateral, or bilateral cervical vagotomy). This increase occurs at both hypotensive and nonhypotensive doses of LPS, suggesting that the c-Fos labeling is a primary effect of LPS-induced cytokine production on the nervous system, as opposed to a primary effect on peripheral vasculature that is signaled by baroreceptive afferents (10).

Additionally, from the unilateral vagotomy experiments, our data indicate that the vagus nerve exerts a subtle effect on the responsiveness of the NST to other afferent inputs. That is, in the unilateral vagotomized groups (experiment 2), c-Fos labeling was modestly but consistently depressed on the vagotomized side of the brain stem (i.e., left side) at all doses of LPS as well as in the control, PBS, group. Only when the results from all doses were combined does the effect of vagotomy on the distribution of c-Fos-activated cells in the NST achieve statistical significance. Although one cannot rule out the possibility that cutting the vagus removes an important pathway regarding information concerning peripheral cytokine levels (3, 15, 44), it is more likely that this uniform reduction in c-Fos labeling in the NST is due to removal of a significant source of general vagal afferent excitation. That is, general visceral afferent input to the NST is glutaminergic and this input is responsible for a majority of the tonic excitation that NST neurons receive (37). Therefore, removal of vagal inputs to the NST, regardless of the afferent modality, will reduce the overall excitability of NST neurons (30). Nevertheless, the effects of systemic exposure to LPS were certainly detectable by neurons within the NST regardless of the connectivity of any reduction in sensitivity caused by the removal of vagal afferents. Second, the number of c-Fos-activated NST neurons was highly correlated with TNF-α production.

Our studies employed unilateral and bilateral cervical vagotomies (i.e., caudal to the nodose ganglia). Although nodose neurons may still detect LPS-related signals and communicate them to the CNS through their intact synaptic inputs to the DVC, studies by Gaykema et al. (15) have already shown that subdiaphragmatic vagotomy (i.e., even more caudal vagotomies) abolished c-Fos expression in vagal sensory ganglia after intraperitoneal administration of LPS and attenuated c-Fos expression in these nuclei when LPS was administered intravenously (15). Furthermore, elimination of vagal afferent pathways rostral to the...
nodose (31, 41) does not block the anorexia produced by peripheral LPS. Taken together, these results suggest that different or redundant pathways (i.e., both neural and humoral) are employed to inform the NST about circulating levels of cytokines.

c-Fos labeling of cells in the AP parallels that of the NST. It is possible that the AP is the principal CNS detector of cytokines elicited by the LPS challenge. That is, NST labeling may only be a consequence of excitatory inputs from the AP (46). However, morphological and physiological studies do not support the concept of the AP acting only as a specialized chemosensor, with the NST acting only as a processor of general visceral afferent information from the vagus. Rather, the NST and AP share several morphological and functional features: 1) both nuclei receive primary vagal afferent inputs (34), 2) both nuclei are vascularized by fenestrated capillaries (18), and 3) dendrites from the NST (and DMN) are intermingled within the AP (43). It is likely that the c-Fos-labeled NST and AP neurons share a common sensitivity to cytokines and that AP neurons modulate NST and DMN excitability (46).

In vagally intact animals, systemic challenge with endotoxin also resulted in a statistically significant...
increase in the number of c-Fos-positive neurons in the DMN, although the increase in absolute cell number per section was relatively small (Table 1). Although this represents a “20-fold increase,” this characterization may convey an overstatement of the data. The ideal index of increased c-Fos activation would be a count of the proportion of activated DMN neurons out of the total number of DMN cells available. Compared with the magnitude of increase in the number of c-Fos-labeled neurons in the NST, the effect on the DMN is relatively minor. Nevertheless, Rinaman et al. (32) observed similar results in the DMN and NST after systemic injections of CCK. CCK produces a significant gastroparesis by acting on vagal afferent mechanoreceptors that, in turn, elicit a withdrawal of cholinergic excitation from the stomach. This withdrawal of excitation in combination with an activation of nonadrenergic-noncholinergic (NANC) vagal efferent neurons would cause a significant gastric relaxation. Rinaman et al. speculated that the few neurons in the DMN that expressed c-Fos in response to CCK may be the activated NANC neurons described above. Cytokines produced in response to LPS challenge may be having a similar effect on gastric vago-vagal control (19, 20, 37). It would be of interest to determine whether the vagal motoneurons that were activated in these studies in response to LPS challenge comprise the subpopulation of DMN neurons that is excited by gastric or intestinal distension, i.e., the NANC vagal efferents.

Data from our plasma TNF-α assay support the conclusion that these brain stem neurons are activated by cytokines elicited by the systemic LPS challenge in that there is a positive correlation between the number of c-Fos-labeled neurons and the amount of TNF-α produced. Furthermore, neurophysiological studies in our laboratory (13) show that NST neurons responsible for coordinating reflex inhibition of gastric function (36, 49) are directly activated by subfemtomole doses of the early cytokine TNF-α. Parallel studies (19) have also shown that subfemtomole doses of TNF-α delivered to the DVC area containing these NST neurons produce a profound reduction in gastric motility. Together, these data support the view that neurons in the DVC, particularly the NST, are intrinsically sensitive to the effects of TNF-α. One physiological result of NST activation would be gastroparesis (37).

TNF-α suppresses gastric motility as part of the constellation of signs and symptoms of the illness behavior of inflammatory disease. These results provide us with a tentative CNS mechanism for this effect. TNF-α released as part of the cytokine cascade probably gains access to the DVC through fenestrated capillaries. It is now well known that the excitability of neurons in this important autonomic integrative zone can be controlled by large circulating peptides, as well as by vagal afferent and descending CNS afferent influences (37, 48). The NST is in position to directly transduce this “hormonal” signal into changes in excitability, although it is extremely likely that NST activity (perhaps, also the DMN) is indirectly modulated by chemosensor neurons in the AP, which, in turn, synapse on NST or DMN neurons (46).

**Perspectives**

These results provide evidence that the NST and AP response to the effects of LPS challenge is mediated locally and does not require the presence of intact vagal afferent innervation. This finding is consistent with previous results showing that these structures directly sense humoral “afferent” signals and control important autonomic and behavioral functions accordingly (27). That is not to say that vagal afferent mechanisms that detect the consequences of immune activation do not exist, but rather that other pathways (i.e., humoral) exist that are sufficient to provide direct CNS activation after immune challenges. Note that these results do not confound previous observations of a primary vagal afferent mechanism that detects the consequences of immune activation do not exist, but rather that other pathways (i.e., humoral) exist that are sufficient to provide direct CNS activation after immune challenges. Note that these results do not confound previous observations of a primary vagal afferent mechanism that detects the consequences of immune activation that may result in a febrile response (3, 44). It is likely that vagal afferents in the hepatic branch of the vagus are involved in detecting activation of the mucosal immune system of the gut. Cytokines and other markers of immune activation produced in the gastrointestinal tract are released into the portal circulation first and could be detected first by hepatic vagal afferents (3, 44). However, the physiological impact of this vagal afferent path must be very brief, because total cervical vagotomy did not block c-Fos production within the NST after intraperitoneal administration of endotoxin (i.e., a model for gastrointestinal immune activation).

It is very likely that the NST and vagal afferent cytokine detection pathways are further examples of serial detection mechanisms that the brain uses to regulate a variety of homeostatic processes. For example, neurons in the NST and general visceral afferent sensors (including the vagus) are sensitive to peptide hormones, CO₂ tension, glucose, sodium, osmolality, and temperature (Refs. 27, 11, 1, 25, and 8, respectively). Removal of the vagal afferent input to the NST may blunt, but not eliminate, the brain’s capacity to regul-
late physiological processes that control these parameters.

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