Neuroscience 131 (2005) 235–245

THE SUBDIAPHRAGMATIC VAGUS NERVES MEDIATE ACTIVATION OF LOCUS COERULEUS NEURONS BY PERIPHERALLY ADMINISTERED MICROBIAL SUBSTANCES

M. K. BORSODY* AND J. M. WEISS

Department of Psychiatry and Behavioral Sciences, Emory University Medical School, Emory West Campus, 1256 Briarcliff Road Northeast, Atlanta, GA 30306, USA

Abstract—Our earlier studies demonstrated that representative microbial substances—lipopolysaccharide, peptidoglycan, and poly-inosine: poly-cytosine (poly(I):(C))—increased the spontaneous discharge rates and sensory-evoked responses of isolated locus coeruleus (LC) neurons in a dose- and time-related manner after i.p. injection into rats. We then turned our attention to the mechanism by which microbial substances administered into the peritoneal cavity affect the LC neurons. The involvement of the subdiaphragmatic vagus nerves was examined in this regard since several brain responses to peripherally administered lipopolysaccharide have been found to depend upon the integrity of these nerves. The experiments reported here show that lipopolysaccharide, peptidoglycan, and poly(I):(C) all failed to excite LC neurons after i.p. injection into rats that had previously been subjected to complete transection of the subdiaphragmatic vagus nerves. Furthermore, selective transection of the subdiaphragmatic vagus nerve trunks indicated that the dorsal trunk, and not the ventral trunk, was necessary to excite LC neurons in response to i.p. lipopolysaccharide. The inability of LC neurons to respond to i.p. lipopolysaccharide in vagotomized rats is unlikely to be attributed to a desensitization of the neurons to lipopolysaccharide since i.c.v. injection of lipopolysaccharide excited LC neurons in vagotomized rats as it did in vagus-intact rats. These findings suggest that a variety of microbial substances excited LC neurons after administration into the peritoneal cavity in a manner involving the subdiaphragmatic vagus nerves.

Key words: vagotomy, electrophysiology, lipopolysaccharide, peptidoglycan, poly(I):(C), rat.

A recent publication from our laboratory reported that the sensory-evoked responses and, to a lesser degree, the spontaneous discharge rates of locus coeruleus (LC) neurons in rats are increased by i.p. injection of various microbial substances (Borsody and Weiss, 2004). That series of experiments focused on lipopolysaccharide (LPS), which is a chief component of Gram-negative bacterial membranes and which by itself induces many of the behavioral and physiological changes of sickness. LPS excited LC neurons not only after it was injected i.p. but also after it was microinjected directly into the LC region. Administration of LPS either by i.p. injection or by microinjection into the LC was also similar in that the excitation of LC neurons appeared to depend upon locally acting interleukin-1 (IL-1), i.e. the response of the LC neurons could be blocked by microinfusion of the IL-1 receptor antagonist (IL-1RA) protein into the LC region.

When it is administered directly into the brain, LPS appears to stimulate the local brain parenchyma (Higgins and Olschowka, 1991; Borsody and Weiss, 2004). However, direct stimulation of brain parenchyma by a low dose of i.p. LPS is unlikely because (1) LPS does not appear to penetrate the blood–brain barrier (Musson et al., 1978) and (2) LPS is rapidly sequestered by the liver and spleen after its i.v. or i.p. injection (Freudenberg and Galanos, 1988). Instead, low doses of i.p. LPS may depend upon signal transduction through the subdiaphragmatic vagus nerves in order to affect brain function. Several brain responses to i.p. LPS are thought to depend upon the integrity of the subdiaphragmatic vagus nerves because the responses do not occur in animals that have been subjected to subdiaphragmatic vagotomy (Levy and Blattberg, 1967; Bluthe et al., 1994; Bret-Dibat et al., 1995; Gaykema et al., 1995; Laye et al., 1995; Bluthe et al., 1996b; Konsman et al., 2000). Specifically, one study has shown that the integrity of the subdiaphragmatic vagus nerves is necessary for the induction of brain IL-1 by i.p. LPS (Laye et al., 1995); this particular finding, however, has been contested by others (Hansen et al., 2000). We here examined whether the responses of LC neurons to i.p. LPS and other microbial substances are dependent upon the subdiaphragmatic vagus nerves by determining whether the responses to these substances are changed by subdiaphragmatic vagotomy.

EXPERIMENTAL PROCEDURES

Animals

Female albino Sprague–Dawley rats (virus/antigen-free; Charles River) were used in all experiments. Female rats were used instead of male rats because we initially were interested in detecting any effect of the estrous state upon the response of LC neurons to the microbial substances. However, no relationship between estrous state and the response of LC neurons to i.p. LPS was found (data not shown) and this line of investigation was abandoned. Rats were bred and housed in a colony room at the Emory West Hospital and were provided food and water *ad libitum*. In accordance with the National Institutes of Health Guide for the Care and Use for Laboratory Animals all efforts were made to minimize animal suffering and to minimize the use of animal

*Corresponding author. Tel: +1-313-253-1062.
E-mail address: mborsody@hotmail.com (M. K. Borsody).
Abbreviations: aCSF, artificial cerebrospinal fluid; IL-1, interleukin-1; IL-1RA, IL-1 receptor antagonist; LC, locus coeruleus; LPS, lipopolysaccharide; poly (I):C), poly-inosine:poly-cytosine.

0306-4522/05$30.00+0.00 © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2004.09.061

subjects, and the techniques used herein were approved by the IACUC of Emory University. All surgical procedures and injections were performed under sterile conditions.

Surgical procedure for subdiaphragmatic vagotomy

Two weeks prior to surgery, rats were acclimated to a diet consisting only of sweetened, condensed milk diluted 1:10 with distilled water. Rats that demonstrated stable body weight toward the end of the diet acclimation period were subjected to vagotomy under halothane anesthesia. While anesthetized, the rat was placed supine, its abdominal surface was shaved, and the exposed skin was sterilized with 70% ethanol. A 5 cm longitudinal midline incision in the abdominal wall was then made beginning just below the xiphoid process. The subdiaphragmatic esophagus was isolated from the mesoesophagus by blunt dissection with cotton-tipped swabs, and the dorsal and/or ventral vagus trunks were isolated close to the diaphragm with dental probes. A nerve trunk was considered transected if a section could be cut free with iridectomy scissors and removed. Complete subdiaphragmatic vagotomy consisted of transection of both dorsal and ventral trunks, and partial subdiaphragmatic vagotomy consisted of transection of either the dorsal trunk or ventral trunk. After closure of the muscular and skin incisions, each rat received 1×10^6 units/kg body weight of penicillin s.c. and was returned to its home cage for 3 weeks before experimental use. During the recovery period, all vagotomized rats were maintained on the milk diet. The success of the vagotomy procedure was assessed by post-surgical weight loss, and by microscopic examination of the esophagus and gross hypertrophy of the stomach at autopsy (Simons et al., 1998).

Preparation for the recording of LC neurons

In order to perform extracellular recordings of single LC neurons, rats were anesthetized with halothane (3% in 100% oxygen, 3 l/min flow rate) and placed onto a stereotaxic apparatus (nosebar 10 mm below level) equipped with a gas mask for continuous halothane delivery. This technique for anesthesia has been described and verified elsewhere (Borsody and Weiss, 1996). Adequate depth of anesthesia was defined as the minimum amount necessary to inhibit the withdrawal response to paw compression (described below). Throughout the experiment body temperature was continuously monitored and was maintained within the normal range (37.0–37.5 °C) by feedback from a rectal thermometer to a circulating water heating blanket. Surgical preparation for the recording of LC neurons was then performed as described elsewhere (Borsody and Weiss, 1996).

Electrodes and signal processing, and isolation of single LC neurons

Electrodes were introduced into the brain and advanced to the LC using a technique described and verified in our previous publication (Borsody and Weiss, 1996). LC neurons were identified as such by the criteria described in that publication.

Examination of the sensory-evoked responses of LC neurons

LC neurons exhibit not only spontaneous discharges ("spontaneous discharge rate") but also respond to sensory stimuli with a burst of discharges ("sensory-evoked response"). To elicit the sensory-evoked responses from LC neurons, the contralateral hindpaw was compressed for a period of 1 s between the ends of a pair of 13 cm surgical forceps as described in our previous publication (Borsody and Weiss, 1996).

Measurement of the spontaneous discharge rates and sensory-evoked responses of LC neurons

After the LC was located, a single dorsal–ventral pass of the electrode through the LC was made and any encountered neurons that satisfied the electrophysiological criteria for an LC neuron were recorded. Recording was limited to LC neurons encountered on the first dorsal–ventral pass of the electrode to minimize the possibility of recording from neurons injured during the descent of the electrode.

Following isolation of a single LC neuron, data collection was begun when the waveform amplitude was stable. The spontaneous discharge rate of the LC neuron was measured as the average firing rate over a period of at least 5 min. After determining the spontaneous discharge rate, sensory-evoked responses were then measured. This was done by delivering five paw compressions spaced 4–5 s apart and computing the average firing rate during the 1 s of the paw compression. Whenever sensory-evoked responses of a LC neuron were measured, 2 min were allowed to elapse after the final paw compression before any further procedure was performed.

I.c.v. injection of LPS

In certain experiments, prior to the introduction of the recording electrode into the brain, an additional burr hole was drilled in the skull at the following coordinates (from bregma suture): AP −1.5 mm, ML 0.8 mm. Into this hole was inserted a ventricular cannula constructed from a 26-gauge hypodermic needle (intradermal bevel; Becton Dickenson) to which 3 cm of silastic tubing was attached at the blunt end. The needle end of the cannula was lowered through the brain with mild suction applied by a syringe inserted into the silastic tubing until cerebrospinal fluid appeared in the cannula, after which a stylus was inserted until the cannula was filled for injection. The cannula was prepared for injection by connecting the silastic tubing to a length of PE-10 tubing (approximately 25 cm) attached to a 10 μl Hamilton microsyringe at the distal end; before making this connection, the microsyringe and attached PE-10 tubing was initially filled with distilled water but then was end-loaded with LPS solution, a small air bubble being placed between the distilled water and the LPS solution. After connection of the microsyringe and PE-10 tubing to the silastic tubing, the LPS solution was advanced through the cannula to within 2 mm of the end of the cannula in preparation for subsequent injection. A stereotaxic arm held the cannula in place throughout the experiment.

Drugs and peptides

Lyophylized LPS (*Eserichia coli* serotype 0127:B8) was purchased from Sigma Biochemicals (St. Louis, MO, USA) and dissolved in artificial cerebrospinal fluid (aCSF) to a stock concentration of 10 μg/ml. The doses of LPS used here represented a range of relatively low doses providing a sublethal challenge to rats, and no rat died or even appeared significantly sick as a result of i.p. LPS treatment in the course of our experiments. The highest dose of LPS used in our experiments (10 μg/kg, which produced marked excitation of LC neurons) was roughly one-thousandth the LD50 of LPS in Wistar rats (Nakano et al., 1987), and the lowest dose of LPS used in our experiments (1 ng/kg, which did not affect the spontaneous discharge rates or sensory-evoked responses of LC neurons) has been reported to activate the rat pituitary–adrenal axis (Schotanus et al., 1994). Peptidoglycan was purchased from Lee Laboratories (Atlanta, GA, USA) at a concentration of 3.5 mg/ml and was diluted with aCSF to a stock concentration of 1 mg/ml. Lyophilized poly-inosine: poly-cytosine (poly(I):(C)) was purchased from Sigma Biochemicals and dissolved in aCSF to a stock concentration of 1 mg/ml. In the current experiments we chose a dose of 1 mg/kg as the highest dose for

peptidoglycan and poly(I):(C) injections, similar to what we used in our previous studies (Borsody and Weiss, 2004). Dilutions of the stock solutions were performed with aCSF as necessary. All i.p. injections were made in volumes of 1 ml/kg body weight.

aCSF was made according to a standard recipe (Davson and Segal, 1987) and consisted of the following constituents (in 1.0 l distilled water): 7.46 g NaCl, 0.19 g $CaCl_2$ (anhydrous), 0.20 g KCl, and 0.20 g $MgCl_2$. aCSF was sterilized by passage through a micropore filter before use.

Statistical analysis

In most experiments, up to five LC neurons were measured in a single animal. For the purpose of statistical analysis each neuron was considered a separate observation.

In part one of the Results section, *t*-tests were used to compare the spontaneous discharge rates and sensory-evoked responses of LC neurons from groups of rats subjected to various types of subdiaphragmatic vagotomies. Unpaired (independent) *t*-tests were used to compare the values for spontaneous discharge rates and sensory-evoked responses measured in complete-, dorsal partial-, or ventral partial-vagotomy rats against those measured in vagus-intact rats. Results from *t*-tests were reported as the *t* value and the confidence level, and are two tailed.

All other statistical analyses involved one- or two-way ANOVA. One-way ANOVA was used in the comparison of multiple treatment conditions (e.g. different doses of LPS) against an untreated "baseline" condition. The baseline conditions are defined in section 1 of the Results for vagus-intact rats and for rats with each type of vagotomy. To account for the effects of vagotomy while comparing the effect of a treatment in vagotomized and vagus-intact rats, the statistical comparison must involve the two separate baseline conditions. Two-way ANOVAs were employed in such situations. Results from ANOVA were reported as the *F* value and the confidence level. *H* values were reported instead of *F* values when nonparametric comparisons were required, i.e. when the data were not normally distributed. Post hoc analysis for all ANOVAs was by the Bonferroni method. Significance is noted at $P<0.05$.

RESULTS

Baseline spontaneous discharge rates and sensory-evoked responses of LC neurons in vagus-intact and vagotomized rats

Rats that were not subjected to any experimental manipulation or to vagotomy were used to measure the baseline spontaneous discharge rates and sensory-evoked responses of LC neurons ("vagus-intact baseline"). The values for the vagus-intact baseline ($n=63$ neurons from 40 rats) are as follows: spontaneous discharge rate$=1.61\pm0.08$ Hz; sensory-evoked response$=3.20\pm0.21$ Hz.

LC neurons from a group of rats with transection of both trunks of the subdiaphragmatic vagus nerves ("complete vagotomy") but without any further experimental manipulation were similarly assessed. In comparison to the vagus-intact baseline, LC neurons in complete vagotomy rats ($n=17$ neurons in four rats) exhibited decreased spontaneous discharge rates (1.03 ± 0.11 Hz; [$t=3.52$, $P<0.001$]) while sensory-evoked responses were not different (3.69 ± 0.50 Hz; [$t=1.02$, $P=0.31$]). These values are referred to as the "complete vagotomy baseline."

Baseline values for rats with selective transection of either the dorsal or ventral trunk of the subdiaphragmatic vagus nerves ("partial vagotomy") were also measured in the absence of any experimental manipulation, and are as follows (*t*-test comparison vs. vagus-intact baseline):

- dorsal partial vagotomy baseline values ($n=14$ neurons in four rats)
- spontaneous discharge rates$=1.76\pm0.25$ Hz, $t=0.73$, $P=0.47$
- sensory-evoked responses$=4.20\pm0.55$ Hz, $t=1.94$, $P=0.06$
- ventral partial vagotomy baseline values ($n=18$ neurons in four rats)
- spontaneous discharge rates$=1.46\pm0.18$ Hz, $t=0.84$, $P=0.40$
- sensory-evoked responses$=3.88\pm0.40$ Hz, $t=1.52$, $P=0.13$.

I.p. LPS injection

Initially the LC neurons were measured 6 h after i.p. injection of various doses of LPS in vagus-intact rats (Fig. 1a). LPS was administered in doses of 1 ng/kg, 100 ng/kg, and 10 μg/kg body weight in order to identify the most effective dose. One-way ANOVA revealed an effect of LPS dose on the sensory-evoked responses of the LC neurons ($H=13.9$, $P<0.05$), which according to post hoc tests achieved statistical significance after injection of as little as 100 ng/kg LPS. No effect of i.p. LPS on the spontaneous discharge rates of the LC neurons was observed ($F=2.45$, $P=0.06$).

The effect of a single i.p. injection of LPS on the spontaneous discharge rates and sensory-evoked responses of the LC neurons was next examined at various times after injection (Fig. 1b). Rats were injected with 10 μg/kg LPS and were returned to their home cages until measurement was made 12 h to 6 weeks later. When measurement was made at shorter time periods after i.p. injection (i.e. 1 h and 2 h after LPS injection), the rat was first anesthetized and then injected with LPS, allowing 1 h or 2 h to elapse while the rat remained anesthetized before LC neurons were isolated and recorded. One-way ANOVA revealed effects of LPS treatment across the different time points on the spontaneous discharge rates ($H=50.8$, $P<0.001$) and sensory-evoked responses ($H=55.7$, $P<0.001$) of the LC neurons. Post hoc analysis revealed that the earliest time point at which a significant change from baseline was observed was 2 h after LPS injection, at which time the sensory-evoked responses of the LC neurons were increased without any change in their spontaneous discharge rates. Increases over baseline in both spontaneous discharge rates and sensory-evoked responses were first noted 12 h after i.p. LPS injection, and both spontaneous discharge rates and sensory-evoked responses of the LC neurons continued to grow over time until they peaked between 2 days and 7 days after i.p. LPS injection. The increase in sensory-evoked responses after i.p. LPS injection was not present 3 weeks post-injection, but instead at this time spontaneous discharge rates were significantly lower than baseline. Complete return to base-

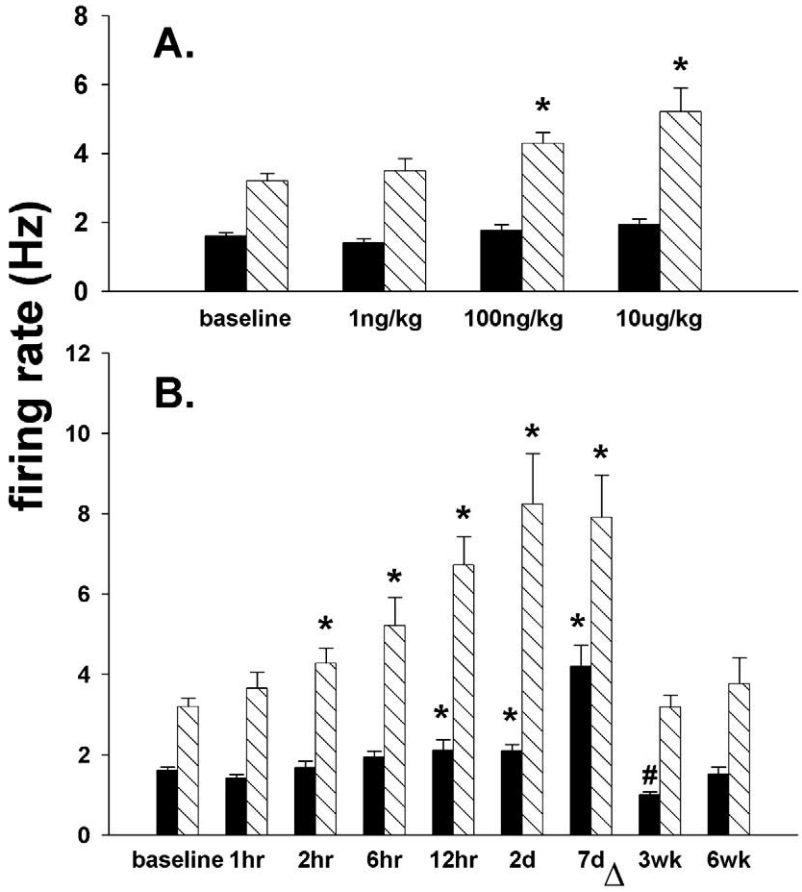

Fig. 1. Effects of i.p. injection of LPS on the spontaneous discharge rates and sensory-evoked responses of LC neurons. (A) Dose-related effects. Six hours after i.p. injection of various doses of LPS, LC neurons were isolated and their spontaneous discharge rates (solid bars) and sensory-evoked responses (hatched bars) were measured. LPS was injected in doses of 1 ng/kg (n=22 neurons in five rats), 100 ng/kg (n=19 neurons in four rats), and 10 μg/kg (n=18 neurons in four rats). (B) Time-related effects. The LC neurons were measured 1 h (n=25 neurons in five rats), 2 h (n=26 neurons in six rats), 12 h (n=23 neurons in five rats), 2 day (n=10 neurons in three rats), 7 days (n=7 neurons in five rats), 3 weeks (n=23 neurons in five rats), and 6 weeks (n=19 neurons in four rats) after i.p. injection. Two of the rats subjected to recording of LC neurons 3 weeks after LPS injection were allowed to recover from the recording procedure and were used a second time after another 3 weeks (i.e. 6 weeks after LPS injection); at the second recording session, LC neurons were recorded from the undamaged side of the brain. Data are displayed as mean±standard error of the mean. * Significantly (P<0.05) increased versus baseline. # Significantly (P<0.05) decreased versus baseline; r, frequently unable to positively identify LC neurons based on electrophysiological criteria (see Borsody and Weiss, 2004).

line activity for both spontaneous discharge rates and sensory-evoked responses was observed only 6 weeks after i.p. LPS injection.

Because of the biphasic response of LC neurons to the 10 μg/kg dose of i.p. LPS, we found it necessary to examine its effects in vagotomized rats at time points where vagus-intact rats exhibited an excitatory response (we chose 12 h post-injection) as well as an inhibitory response (3 weeks post-injection). The right side of Fig. 2 shows the effect of 10 μg/kg LPS injected i.p. 12 h prior to the recording of LC neurons in rats that were previously subjected to complete vagotomy. The effect of this LPS dose on LC neurons in vagus-intact rats is shown at the left of this figure for comparison. In contrast to what was seen in vagus-intact rats, in complete vagotomy rats treated 12 h previously with 10 μg/kg i.p. LPS there was no increase in the sensory-evoked responses of the LC neurons. Two-way ANOVA revealed an interaction between vagotomy and LPS treatment on the sensory-evoked responses of the LC neurons (F=5.60, P<0.05). The same comparison for spontaneous discharge rates revealed no such interaction (F=1.63, P=0.21).

Rats with complete vagotomy were next treated with i.p. LPS 3 weeks prior to the recording of LC neurons in order to determine if the spontaneous discharge rates of LC neurons would still be reduced by LPS as it was in vagus-intact rats (Fig. 3). In this case, two-way ANOVA did not reveal an interaction between vagotomy and LPS treatment for either the spontaneous discharge rates (F=0.82, P=0.37) or sensory-evoked responses (F=0.25, P=0.62) of the LC neurons. Thus, 3 weeks after LPS injection in complete vagotomy rats, the spontaneous discharge rates of LC neurons were decreased as they were in vagus-intact rats.

The ability of i.p. LPS to excite LC neurons in rats with partial vagotomy was also examined. To do this, a dose of 10 μg/kg LPS was injected 12 h before recording LC neurons into rats that had selective transection of the

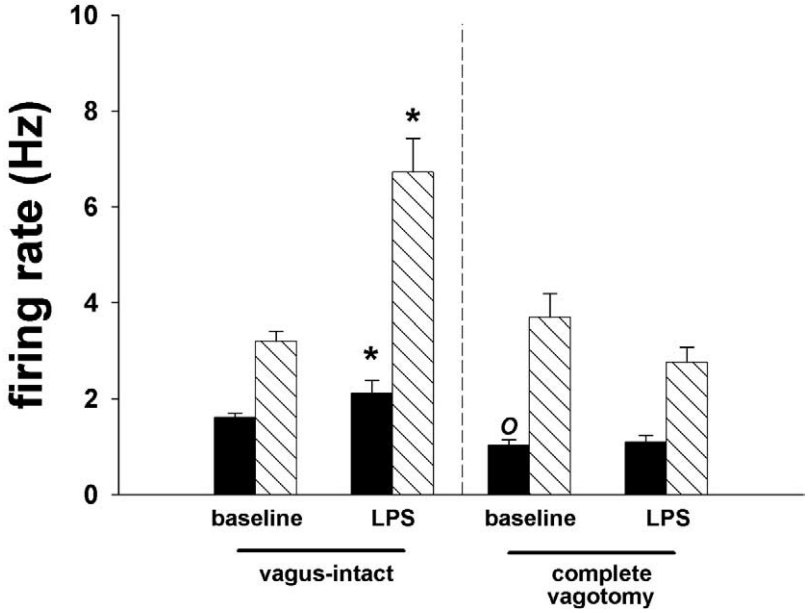

Fig. 2. The effect of i.p. LPS 12 h after injection in rats with complete vagotomy. Twelve hours after i.p. injection of 10 μg/kg LPS into rats previously subjected to complete vagotomy, LC neurons (n=15 neurons in three rats) were isolated and their spontaneous discharge rates (solid bars) and sensory-evoked responses (hatched bars) were measured. The effect of this LPS treatment in vagus-intact rats is shown at the left of the figure for comparison. Data are displayed as mean±standard error of the mean. * Significantly (P<0.05) increased versus vagus-intact baseline. O, significantly (P<0.05) decreased versus vagus-intact baseline.

dorsal trunk or the ventral trunk of the subdiaphragmatic vagus nerves (Fig. 4). Two-way ANOVA revealed an interaction between the type of partial vagotomy and LPS treatment for the sensory-evoked responses of the LC neurons (F=4.92, P<0.05); however, the same analysis of spontaneous discharge rates did not reveal an interaction (F=3.01, P=0.14). Post hoc analysis revealed that dorsal partial vagotomy prevented i.p. LPS from increasing the sensory-evoked responses of the LC neurons whereas ventral partial vagotomy was ineffective in this regard,

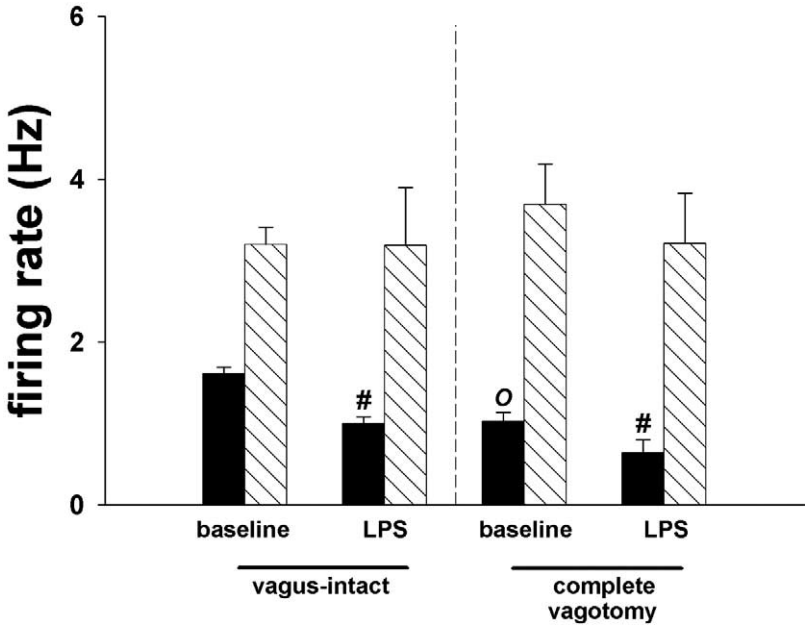

Fig. 3. The effect of i.p. LPS 3 weeks after injection in rats with complete vagotomy. Three weeks after i.p. injection of 10 μg/kg LPS into rats previously subjected to complete vagotomy, LC neurons (n=14 neurons in four rats) were isolated and their spontaneous discharge rates (solid bars) and sensory-evoked responses (hatched bars) were measured. The effect of this LPS treatment in vagus-intact rats is shown at the left of the figure for comparison. Data are displayed as mean±standard error of the mean. # Significantly (P<0.05) decreased versus the corresponding baseline. O, significantly (P<0.05) decreased versus vagus-intact baseline.

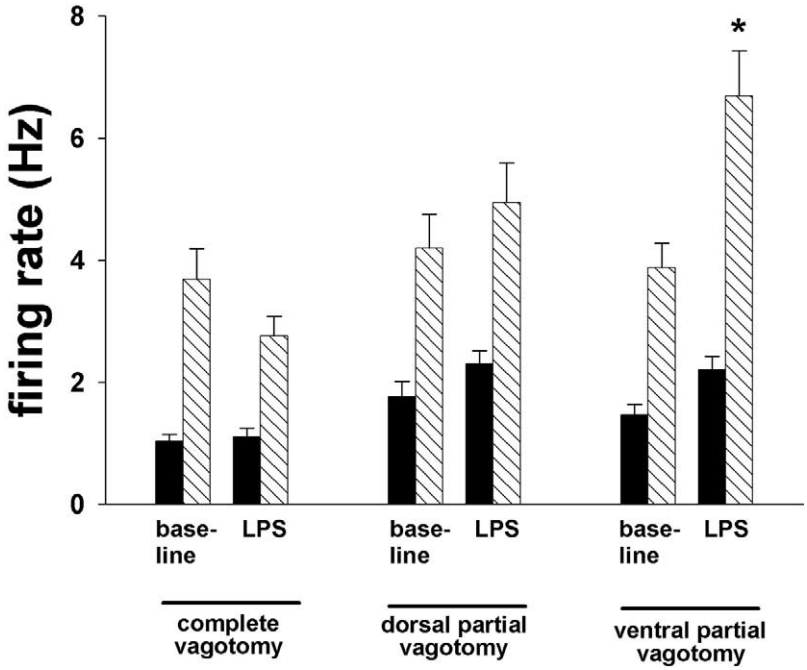

Fig. 4. The effect of i.p. LPS 12 h after injection in rats with partial vagotomies. Twelve hours after i.p. injection of 10 μg/kg LPS into rats previously subjected to dorsal partial (n=19 neurons in four rats) or ventral partial vagotomy (n=15 neurons in three rats), LC neurons were isolated and their spontaneous discharge rates (solid bars) and sensory-evoked responses (hatched bars) were measured. The effect of this LPS treatment in complete vagotomy rats is shown at the left of the figure for comparison. Data are displayed as mean±standard error of the mean. * Significantly (P<0.05) increased versus ventral partial vagotomy baseline.

suggesting that the activation of the LC by i.p. LPS depends only the dorsal trunk of the subdiaphragmatic vagus nerves.

I.c.v. LPS injection

The effect of i.c.v. LPS on the LC neurons was examined to determine if vagotomy indirectly blocked the response of LC neurons to i.p. LPS by making the neurons insensitive to LPS. LPS was injected i.c.v. in vagus-intact rats at doses of 1 ng and 100 ng (in 10 μl volume) and 2 h later LC neurons were isolated and their spontaneous discharge rates and sensory-evoked responses were measured (left side of Fig. 5). One-way ANOVA found that i.c.v. LPS increased the sensory-evoked responses of LC neurons (H=30.2, P<0.001) at the 100 ng dose. No effect of i.c.v. LPS on the spontaneous discharge rates of LC neurons was found (H=5.42, P=0.14).

The response of LC neurons to 100 ng i.c.v. LPS was then examined in rats with complete vagotomy (right side of Fig. 5). This experiment was otherwise performed as described above. Two-way ANOVA did not find an interaction between LPS treatment and vagotomy for either the spontaneous discharge rates (F=1.85, P=0.38) or sensory-evoked responses (F=0.87, P=0.45) of the LC neurons. These results show that complete vagotomy did not influence the response of LC neurons to i.c.v. LPS, and suggests that in our previous experiments subdiaphragmatic vagotomy did not block the response of LC neurons to i.p. LPS by making the LC neurons unresponsive to LPS.

I.p. peptidoglycan injection

The effects of peptidoglycan at various doses and times after i.p. injection were measured in vagus-intact rats. The left side of Fig. 6 shows the response of LC neurons to 100 ng/kg, 10 μg/kg, or 1 mg/kg peptidoglycan administered 6 h beforehand by i.p. injection. One-way ANOVA found an effect of peptidoglycan on the sensory-evoked responses (H=17.7, P<0.005) but no effect on the spontaneous discharge rates (H=0.47, P=0.93) of the LC neurons. Post hoc analysis showed that sensory-evoked responses of the LC neurons were increased after injection of 10 μg/kg or 1 mg/kg peptidoglycan. The middle of Fig. 6 shows the spontaneous discharge rates and sensory-evoked responses of LC neurons measured 2 days after a single i.p. injection of 1 mg/kg peptidoglycan into vagus-intact rats. One-way ANOVA involving the 6 h and 2 day time points identified an effect of peptidoglycan treatment on the sensory-evoked responses of LC neurons (H=15.9, P<0.005); post hoc analysis indicated that this was attributable to the increase that occurred 6 h after peptidoglycan injection. The spontaneous discharge rates of the LC neurons were also unaffected 2 days after peptidoglycan treatment (H=1.64, P=0.65).

The effect of i.p. peptidoglycan in complete vagotomy rats was next tested. These results are shown at the right of Fig. 6. Peptidoglycan in a dose of 1 mg/kg was injected i.p. and 6 h later LC neurons were isolated and measured. Two-way ANOVA found an interaction between vagotomy and peptidoglycan treatment on the sensory-evoked re-

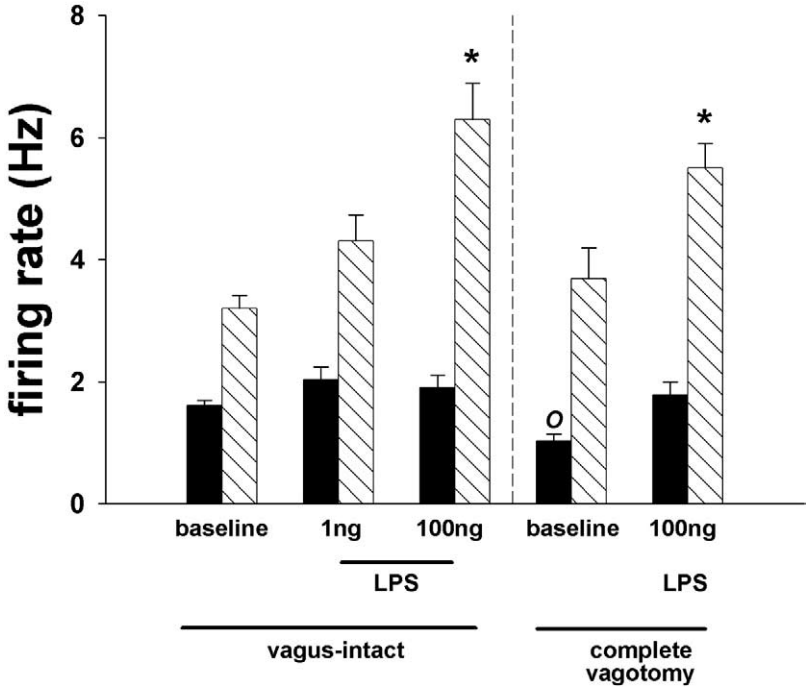

Fig. 5. Effects of i.c.v. injection of LPS on the spontaneous discharge rates and sensory-evoked responses of LC neurons. Dose-related effects of i.c.v. LPS (left side of figure). Various doses of LPS (in 10 μl volume) were injected into the lateral ventricle and after a period of 2 h LC neurons were isolated and their spontaneous discharge rates (solid bars) and sensory-evoked responses (hatched bars) were measured. LPS was injected in doses of 1 ng (n=23 neurons in five rats) or 100 ng (n=19 neurons in five rats). Effect of i.c.v. LPS in complete vagotomy rats (right side of figure). LC neurons were isolated and measured 2 h after i.c.v. injection of 100 ng LPS in rats previously subjected to complete vagotomy (n=18 neurons in four rats). Data are displayed as mean±standard error of the mean. * Significantly (P<0.05) increased versus the corresponding baseline. O, significantly (P<0.05) decreased versus vagus-intact baseline.

sponses of the LC neurons (F=7.04, P<0.01); the same comparison for spontaneous discharge rates did not find an interaction (F=0.79, P=0.43). As with i.p. LPS, then, i.p. peptidoglycan was unable to excite LC neurons in complete vagotomy rats as it did in vagus-intact rats.

I.p. poly(I):(C) injection

The effects of poly(I):(C) at various doses and times after i.p. injection in vagus-intact rats were measured. The left side of Fig. 7 shows the response of LC neurons to 1 ng/kg, 100 ng/kg, 10 μg/kg, or 1 mg/kg of poly(I):(C) administered 6 h beforehand by i.p. injection. One-way ANOVA found an effect of poly(I):(C) treatment on the sensory-evoked responses (H=12.8, P<0.05) but no effect on the spontaneous discharge rates (H=3.63, P=0.46) of the LC neurons. The increase in sensory-evoked responses was observed after injection of 100 ng/kg, 10 μg/kg, and 1 mg/kg poly(I):(C). The middle of Fig. 7 shows the spontaneous discharge rates and sensory-evoked responses of LC neurons measured 2 days after i.p. injection of 1 mg/kg poly(I):(C) into vagus-intact rats. One-way ANOVA involving the 6 h and 2 day time points identified an effect of poly(I):(C) treatment on the sensory-evoked responses of the LC neurons (H=8.27, P<0.05); post hoc analysis indicated that this was attributable to the increase that occurred 6 h after poly(I):(C) injection. The spontaneous discharge rates of the LC neurons were also unaffected 2 days after poly(I):(C) treatment (H=1.93, P=0.15).

The effect of i.p. poly(I):(C) in complete vagotomy rats was then tested. These results are shown at the right of Fig. 7. Poly(I):(C) in a dose of 1 mg/kg was injected i.p. and 6 h later LC neurons were isolated and measured. Two-way ANOVA found an interaction between vagotomy and poly(I):(C) treatment on the sensory-evoked responses of the LC neurons (F=6.38, P<0.01); the same comparison for spontaneous discharge rates did not find an interaction between vagotomy and poly(I):(C) treatment (F=2.49, P=0.16). As with i.p. LPS and peptidoglycan, i.p. poly(I):(C) was unable to increase the sensory-evoked responses in complete vagotomy rats as it did in vagus-intact rats.

DISCUSSION

The role of the subdiaphragmatic vagus nerves in the response of LC neurons to microbial substances

A previous report from this laboratory showed that LPS excited LC neurons when it was injected into the peritoneal cavity (Borsody and Weiss, 2004). The excitation caused by i.p. LPS appeared to involve binding of IL-1 to IL-1 receptors in the LC region because the excitation caused by i.p. LPS could be reversed by microinfusion of IL-1RA into the LC. In that report, LPS was administered not only by i.p. injection but also by direct microinjection into the LC.

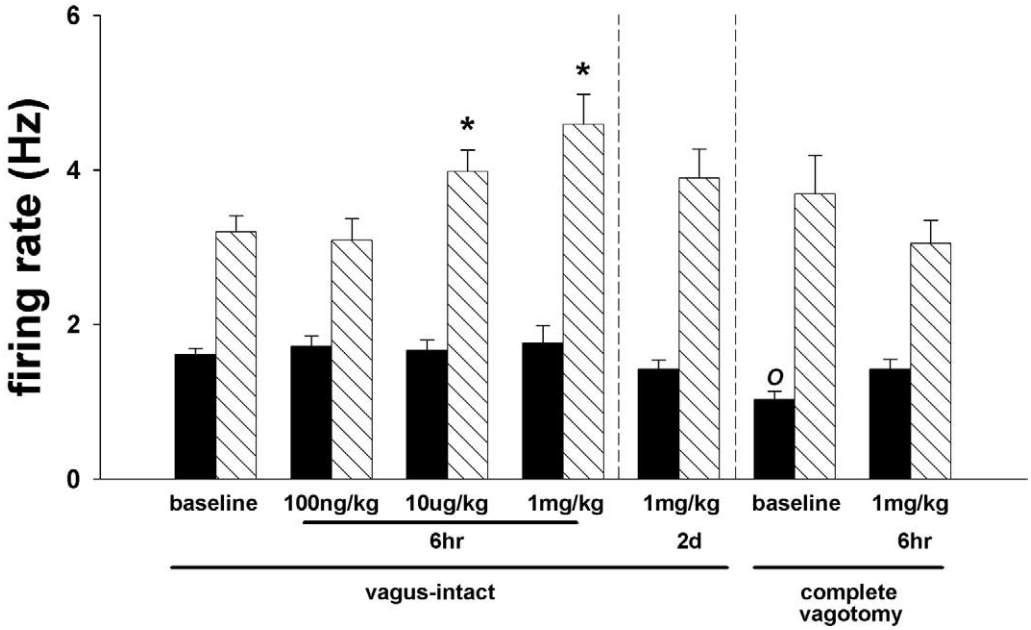

Fig. 6. Effects of i.p. peptidoglycan on the spontaneous discharge rates and sensory-evoked responses of LC neurons. Dose-related effects of i.p. peptidoglycan (left side of figure). Peptidoglycan was injected i.p. in doses of 100 ng/kg ($n=20$ neurons in four rats), 10 μg/kg ($n=19$ neurons in four rats), and 1 mg/kg ($n=17$ neurons in five rats). Six hours after injection, LC neurons were isolated and their spontaneous discharge rates (solid bars) and sensory-evoked responses (hatched bars) were measured. Time-related effects of i.p. peptidoglycan (middle of figure). The LC neurons were measured 2 day after i.p. injection of 1 mg/kg peptidoglycan ($n=18$ neurons in four rats). The effect of vagotomy (right side of figure). Six hours after i.p. injection of 1 mg/kg peptidoglycan into rats previously subjected to complete vagotomy ($n=19$ neurons in four rats), LC neurons were isolated and their spontaneous discharge rates and sensory-evoked responses were measured. Data are displayed as mean±standard error of the mean. * Significantly ($P<0.05$) increased versus the vagus-intact baseline. O, significantly ($P<0.05$) decreased versus vagus-intact baseline.

LPS microinjected directly into the LC region also excited LC neurons in a dose-dependent manner that appears to involve IL-1 bioactivity within the LC because its effect also could be blocked by IL-1RA. In the most simplistic interpretation, the similarity of the responses of LC neurons to LPS administered by i.p. injection and microinjection into the LC would suggest that the i.p. LPS diffused from its injection site into the LC region in order to activate the LC neurons. However, LPS does not enter the brain in appreciable amounts when it is administered peripherally (Musson et al., 1978). Rather, i.p. LPS appears to affect several brain functions by stimulating sensory fibers of the subdiaphragmatic vagus nerves (Levy and Blattberg, 1967; Bluthe et al., 1994; Bret-Dibat et al., 1995; Gaykema et al., 1995; Laye et al., 1995; Bluthe et al., 1996b; Konsman et al., 2000). The present study sought to determine if the subdiaphragmatic vagus nerves were necessary for the activation of LC neurons by i.p. LPS. In these studies, LPS was administered by i.p. injection into rats after complete or partial transection of the subdiaphragmatic vagus nerves and the response of the LC neurons was compared against that observed in vagus-intact rats. We found that complete vagotomy prevented i.p. LPS treatment from exciting LC neurons as did selective transection of the dorsal trunk of the subdiaphragmatic vagus nerves.

The observation that the subdiaphragmatic vagus nerves are necessary for the activation of LC neurons by i.p. LPS also illuminates the mechanism underlying the prolonged response (i.e. lasting at least 3 weeks) of the LC to a single LPS treatment: namely, that this mechanism may in fact relate to the handling and disposal of LPS in the abdominal viscera. Radiolabeled LPS injected i.v. is removed from the blood and is stored in the liver and spleen where the radiolabel remains for upwards of 5 weeks (Freudenberg and Galanos, 1988). Even though assays of LPS antigenicity suggest that the LPS is extensively metabolized within 9 days of injection, the stored LPS still retains its full bioactivity (Freudenberg et al., 1985). Thus, from storage sites in the abdomen, residual LPS may continue to affect the LC neurons over a period of weeks. This may occur by means of direct stimulation of the subdiaphragmatic vagus nerves by LPS, although our experiments did not distinguish between an active or permissive response from these nerves.

As a preliminary attempt to define the abdominal site from which the prolonged excitation of the LC might be maintained, we injected LPS into rats with selective transection of either the ventral or dorsal trunks of the subdiaphragmatic vagus nerves and found that only transection of the dorsal trunk prevented LPS from exciting the LC neurons. The dorsal trunk has some selectivity in innervating the tail of the pancreas, but in the rat does not seem to innervate the spleen (Rinaman and Miselis, 1987; Nance and Burns, 1989). Furthermore, the dorsal trunk may provide a small degree of innervation to the liver via fibers coursing through the sympathetic celiac plexus (Berthoud and Neuhuber, 2000). Ventral trunk vagotomy was unable to prevent LC neurons from responding to i.p. LPS, and

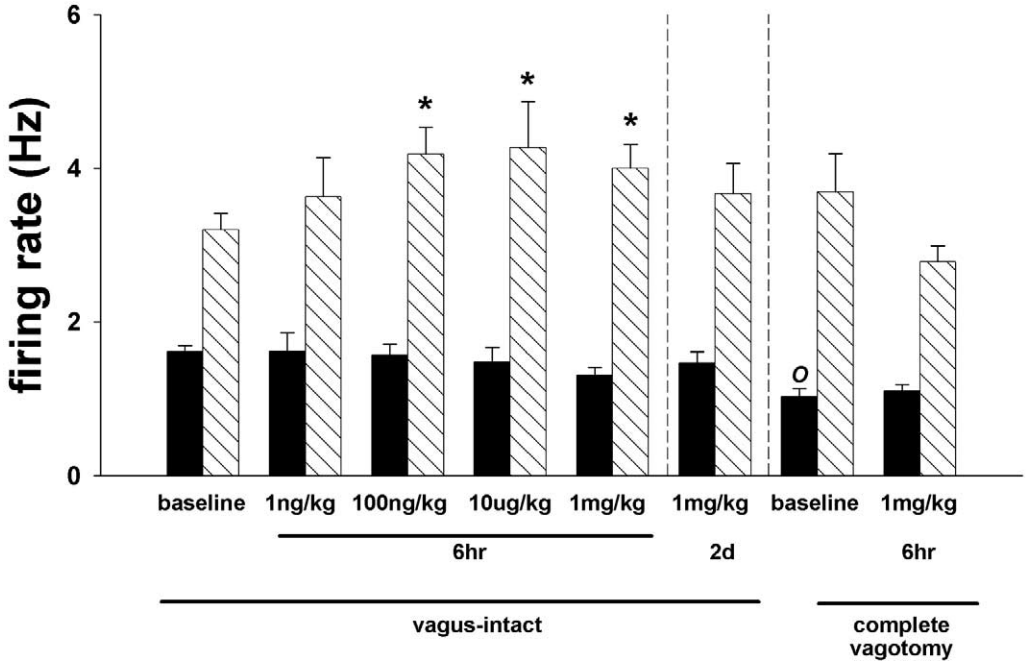

Fig. 7. Effects of i.p. poly(I):(C) on the spontaneous discharge rates and sensory-evoked responses of LC neurons. Dose-related effects of i.p. poly(I):(C) (left of figure). Poly(I):(C) was injected in doses of 1 ng/kg (n=20 neurons in four rats), 100 ng/kg (n=15 neurons in four rats), 10 μg/kg (n=18 neurons in four rats), and 1 mg/kg (n=20 neurons in four rats). Six hours after injection of poly(I):(C), LC neurons were isolated and their spontaneous discharge rates (solid bars) and sensory-evoked responses (hatched bars) measured. Time-related effects of i.p. poly(I):(C) (middle of figure). The LC neurons were measured 2 days after i.p. injection of 1 mg/kg poly(I):(C) (n=17 neurons in four rats). The effect of vagotomy (right of figure). Six hours after i.p. injection of 1 mg/kg poly(I):(C) into rats previously subjected to complete vagotomy (n=20 neurons in four rats), LC neurons were isolated and their spontaneous discharge rates and sensory-evoked responses were measured. Data are displayed as mean±standard error of the mean. * Significantly (P<0.05) increased versus the vagus-intact baseline. O, significantly (P<0.05) decreased versus vagus-intact baseline.

since the ventral trunk of the subdiaphragmatic vagus nerves carries the major hepatic projections (Prechtl and Powley, 1985) it would seem unlikely that LPS stores in the liver are responsible for the prolonged response of the LC neurons. It would not be unexpected that the response of LC neurons to i.p. LPS is dependent upon a specific trunk or branch of the subdiaphragmatic vagus nerves, since specific physiological roles for individual trunks and branches of these nerves have been reported (Fleshner et al., 1995; Watkins et al., 1995).

The aforementioned experiments with vagotomy leave open the possibility that this procedure simply rendered the LC neurons insensitive to LPS wherever it was acting in the body. To control for this possibility, LPS was administered by i.c.v. injection in vagus-intact rats and in rats with complete vagotomy. In vagus-intact rats, i.c.v. LPS increased the sensory-evoked responses of LC neurons in a dose-related manner similar to the effect of microinjecting the LPS directly into the LC (Borsody and Weiss, 2004). Although complete vagotomy prevented LC neurons from responding to i.p. LPS it did not reduce the ability of the LC neurons to respond to i.c.v. LPS. Thus, it is unlikely that the subdiaphragmatic vagus nerves act only to maintain the sensitivity of LC neurons to any source of LPS, as it would appear that there is a specific relation between the LC's response to LPS that is acting within the peritoneal cavity and the integrity of the subdiaphragmatic vagus nerves that is not shared by i.c.v. LPS. A similar role for the subdiaphragmatic vagus nerves has been demonstrated for IL-1-induced suppression of exploratory behavior, which is sensitive to subdiaphragmatic vagotomy when the IL-1 is administered i.p. but not i.c.v. (Bluthe et al., 1996a). These observations do not define the precise nature of the role the subdiaphragmatic vagus nerves play in affecting the LC neurons, and it should be made clear that permissive roles for these nerves cannot be ruled out by our experiments. It is possible, for example, that LPS or LPS-induced cytokines in the circulation act on blood vessels in a manner that only requires vagus nerve integrity; such a mechanism would be unable to stimulate LC neurons in vagotomized rats, which would otherwise remain directly sensitive to LPS.

The mechanism that we have described by which i.p. LPS excites LC neurons via the subdiaphragmatic vagus nerves also appears to be used by other microbial substances. The substances we chose were peptidoglycan (a component of the bacterial cell wall that is used to model infections by Gram-positive bacteria) and poly(I):(C) (a nucleic acid polymer that induces many of the responses of an RNA virus infection). Peptidoglycan and poly(I):(C) injected i.p. were also unable to excite LC neurons in rats that had undergone transection of the subdiaphragmatic vagus nerves, in comparison with the excitatory response they elicit in vagus-intact rats. This is particularly interesting when one considers that the excitation of LC neurons caused by i.p. peptidoglycan and LPS were reversible by

microinfusion of IL-1RA into the LC region but the excitation caused by i.p. poly(I):(C) was not (Borsody and Weiss, 2004). Furthermore, the three microbial substances likely activate different pathogen receptors (i.e. Toll-like receptors) in the peritoneal cavity (Takeda and Akira, 2003), and thereby induce different local cytokine cascades (O'Neill et al., 2003). Thus, the three microbial substances appear to utilize different signaling molecules in the brain to affect LC function and likely also have distinguishable profiles of cytokine expression in the peritoneal cavity. However, all three appear to require the integrity of the subdiaphragmatic vagus nerves in order to affect LC neurons.

Limitations of the current studies

The aforementioned microbial substances were administered by the i.p. route rather than i.v. or intraarterially because (1) the peritoneal cavity is a frequent site of infections due to gut flora leakage from the intestines (Klasen et al., 1994; Panigrahi et al., 1994), (2) the peritoneal cavity contains resident macrophages that readily respond to a variety of microbial substances (Novakovic and Boldogh, 1994), and (3) microbial substances administered i.p. are nevertheless taken up by peritoneal capillaries and distributed by the circulatory system throughout the body (Gilbart and Fox, 1987; Freudenberg and Galanos, 1988). Recent studies have highlighted different mechanisms by which peripherally administered microbial substances may affect brain function depending upon the route of administration. For example, Wan et al. (1994) demonstrated that i.p. and i.v. LPS induce similar patterns of expression of the immediate-early gene product c-Fos; however, only the c-Fos expression caused by i.p. LPS was blocked by vagotomy. Similarly Bluthe et al. (1996b) found that subdiaphragmatic vagotomy effectively prevented the reduction in exploratory behavior that occurred in mice injected i.p. with IL-1 but not when the IL-1 was administered by s.c. or i.v. injection. Clearly, then, our finding that i.p. LPS, peptidoglycan, and poly(I):(C) all excite LC neurons by means of the subdiaphragmatic vagus nerves cannot be readily extrapolated to the administration of such substances by other peripheral routes.

Comparing the potency and efficacy of the three microbial substances is also problematic. Clearly, i.p. LPS produced larger and longer-lasting effects on the LC neurons than did i.p. peptidoglycan or poly(I):(C), and the LPS even increased the spontaneous discharge rates of the LC neurons under certain conditions. We are unable to explain this observation since little is known about the bioactivity of the microbial substances acting in the peritoneal cavity or within the brain parenchyma. Before addressing this issue, key questions must be answered regarding the expression of pathogen receptors within the peritoneal cavity, the accessibility of the microbial substances in the peritoneal cavity to resident leukocytes, and the distribution, metabolism, and excretion of the microbial substances throughout the body. A similar set of questions would have to be answered before any comparison of the potency and efficacy of direct administration of the microbial substances into the brain parenchyma could be undertaken.

Furthermore, our experiments do not directly address the controversy over the role of the subdiaphragmatic vagus nerves in the stimulation of brain IL-1 systems by i.p. LPS. There is considerable evidence that low doses of i.p. LPS require intact subdiaphragmatic vagus nerves to affect several brain functions, including the observation that the expression of IL-1 mRNA in mouse brain after injection of 400 μg/kg i.p. LPS is blockable by subdiaphragmatic vagotomy (Laye et al., 1995). In contrast, Hansen et al. (2000) were unable to demonstrate any reduction of brain IL-1 protein in rats previously subjected to subdiaphragmatic vagotomy that were treated with 10–100 μg/kg LPS. Our experiments examining excitation of the LC caused by i.p. LPS—a phenomenon that is dependent on brain IL-1 bioactivity (Borsody and Weiss, 2004)—would appear to be consistent with the earlier studies and would seem to contradict Hansen et al. (2000), despite the similarity that study has with ours both in terms of the animal model and range of LPS doses. However, our experiments cannot be said to test the dependence of LPS-induced brain IL-1 on the subdiaphragmatic vagus nerves. Although the response of LC neurons to i.p. LPS critically involves IL-1, it does not necessarily follow that this IL-1 is dependent upon the subdiaphragmatic vagus nerves even if the response of the LC neurons to i.p. LPS can be blocked by subdiaphragmatic vagotomy. Instead, for argument's sake, an unknown factor which becomes produced in the brain in response to i.p. LPS treatment might be blocked by vagotomy, and the absence of this factor could prevent the excitation of the LC despite the presence of sufficient IL-1 bioactivity. Thus, the results of our experiments do not directly impact this ongoing debate.

Acknowledgments—This work was supported by grants MH50420 and MH56602 to J.M.W. from the National Institute of Mental Health. Some of the data presented here have been summarized in previous publications (Borsody and Weiss, 2002a,b, 2004).

REFERENCES

Berthoud HR, Neuhuber WL (2000) Functional and chemical anatomy of the afferent vagal system. Auton Neurosci 85:1–17.

Bluthe RM, Michaud B, Kelley KW, Dantzer R (1996a) Vagotomy attenuates behavioural effects of interleukin-1 injected peripherally but not centrally. Neuroreport 7:1485–1488.

Bluthe RM, Michaud B, Kelley KW, Dantzer R (1996b) Vagotomy blocks behavioural effects of interleukin-1 injected via the intraperitoneal route but not via other systemic routes. Neuroreport 7:2823–2827.

Bluthe RM, Walter V, Parnet P, Laye S, et al (1994) Lipopolysaccharide induces sickness behaviour in rats by a vagal mediated mechanism. C R Acad Sci III 317:499–503.

Borsody MK, Weiss JM (1996) Influence of corticotropin-releasing hormone on electrophysiological activity of locus coeruleus neurons. Brain Res 724:149–168.

Borsody MK, Weiss JM (2002a) Alteration of locus coeruleus neuronal activity by interleukin-1 and the involvement of endogenous corticotropin-releasing hormone. Neuroimmunomodulation 10:101–121.

Borsody MK, Weiss JM (2002b) Peripheral endotoxin causes long-lasting changes in locus coeruleus activity via IL-1 in the brain. Acta Neuropsychol 14:303–321.

Borsody MK, Weiss JM (2004) The effects of endogenous interleukin-1 bioactivity on locus coeruleus neurons in response to bacterial and viral substances. Brain Res 1007:39–56.

Bret-Dibat JL, Bluthe RM, Kent S, Kelley KW, Dantzer R (1995) Lipopolysaccharide and interleukin-1 depress food-motivated behavior in mice by a vagal-mediated mechanism. Brain Behav Immun 9:242–246.

Davson HKW, Segal MB (1987) The physiology and pathophysiology of cerebrospinal fluid. Edinburgh: Churchill Livingston.

Fleshner M, Goehler LE, Hermann J, Relton JK, Maier SF, Watkins LR (1995) Interleukin-1 beta induced corticosterone elevation and hypothalamic NE depletion is vagally mediated. Brain Res Bull 37:605–610.

Freudenberg MA, Galanos C (1988) The metabolic fate of endotoxins. In: Bacterial endotoxins: pathophysiological effects, clinical significance, and pharmacological control (Levin J, ed), pp 63–75. New York: Alan R. Liss, Inc.

Freudenberg N, Freudenberg MA, Bandara K, Galanos C (1985) Distribution and localization of endotoxin in the reticuloendothelial system (RES) and in the main vessels of the rat during shock. Pathol Res Prac 179:517–527.

Gaykema RP, Dijkstra I, Tilders FJ (1995) Subdiaphragmatic vagotomy suppresses endotoxin-induced activation of hypothalamic corticotropin-releasing hormone neurons and ACTH secretion. Endocrinology 136:4717–4720.

Gilbart J, Fox A (1987) Elimination of group A streptococcal cell walls from mammalian tissues. Infect Immun 55:1526–1528.

Hansen MK, Nguyen KT, Goehler LE, Gaykema RP, et al (2000) Effects of vagotomy on lipopolysaccharide-induced brain interleukin-1beta protein in rats. Auton Neurosci 85:119–126.

Higgins GA, Olschowka JA (1991) Induction of interleukin-1 beta mRNA in adult rat brain. Mol Brain Res 9:143–148.

Klasen IS, Melief MJ, van Halteren AGS, Schouten WR, et al (1994) The presence of peptidoglycan-polysaccharide complexes in the bowel wall and the cellular responses to these complexes in Crohn's disease. Clin Immunol 71:303–308.

Konsman JP, Luheshi GN, Bluthe RM, Dantzer R (2000) The vagus nerve mediates behavioural depression, but not fever, in response to peripheral immune signals: a functional anatomical analysis. Eur J Neurosci 12:4434–4446.

Laye S, Bluthe RM, Kent S, Combe C, et al (1995) Subdiaphragmatic vagotomy blocks induction of IL-1 beta mRNA in mice brain in response to peripheral LPS. Am J Physiol 268:R1327-R1331

Levy MN, Blattberg B (1967) Changes in heart rate induced by bacterial endotoxin. Am J Physiol 213:1485–1492.

Musson RA, Morrison DC, Ulevitch RJ (1978) Distribution of endotoxin (lipopolysaccharide) in the tissues of lipopolysaccharide-responsive and -unresponsive mice. Infect Immun 21:448–457.

Nakano K, Suzuki S, Oh C (1987) Significance of increased secretion of glucocorticoids in mice and rats injected with bacterial endotoxin. Brain Behav Immun 1:159–172.

Nance DM, Burns J (1989) Innervation of the spleen in the rat: evidence for absence of afferent innervation. Brain Behav Immun 3:281–290.

Novakovic S, Boldogh I (1994) In vitro TNF-a production and in vivo alteration of TNF-a RNA in mouse peritoneal macrophages after treatment with different bacterial derived agents. Cancer Lett 81:99–109.

O'Neill LAJ, Fitzgerald KA, Bowier AG (2003) The Toll-IL-1 receptor adaptor family grows to five members. Trends Immunol 24:286–289.

Panigrahi P, Gupta S, Gewolb IH, Morris JG (1994) Occurrence of necrotizing enterocolitis may be dependent on patterns of bacterial adherence and intestinal colonization: studies in Caco-2 tissue culture and weanling rabbit models. Ped Res 36:115–121.

Prechtl JC, Powley TL (1985) Organization and distribution of the rat subdiaphragmatic vagus and associated paraganglia. J Comp Neurol 235:182–195.

Rinaman L, Miselis RR (1987) The organization of vagal innervation of rat pancreas using cholera toxin-horseradish peroxidase conjugate. J Autonom Nervous Syst 21:109–125.

Schotanus K, Makara GB, Tilders FJH, Berkenbosch F (1994) ACTH response to a low dose but not a high dose of bacterial endotoxin in rats is completely mediated by corticotropin-releasing hormone. Neuroimmunomodulation 1:300–307.

Simons CT, Kulchitsky VA, Sugimoto N, Homer LD, Szekely M, Romanovsky AA (1998) Signaling the brain in systemic inflammation: which vagal branch is involved in fever genesis? Am J Physiol 275:R63–R68.

Takeda K, Akira S (2003) Toll receptors and pathogen resistance. Cell Microbiol 5:143–153.

Wan W, Wetmore L, Sorensen CM, Greenberg AH, Nance DM (1994) Neural and biochemical mediators of endotoxin and stress-induced c-fos expression in the rat brain. Brain Res Bull 34:7–14.

Watkins LR, Goehler LE, Relton JK, Tartaglia N, et al (1995) Blockade of interleukin-1 induced hyperthermia by subdiaphragmatic vagotomy: evidence for vagal mediation of immune-brain communication. Neurosci Lett 183:27–31.

(Accepted 17 September 2004)