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Noradrenergic inputs to the paraventricular hypothalamus contribute to HPA axis and central Fos activation in rats after acute systemic endotoxin exposure

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Abstract

Noradrenergic (NA) neurons within the nucleus of the solitary tract (NST) and caudal ventrolateral medulla (VLM) innervate the hypothalamic paraventricular nucleus (PVN) to initiate and modulate HPA axis responses to interoceptive stress. Systemic endotoxin (i.e., bacterial lipopolysaccharide, LPS) activates NA neurons within the NST and VLM that project to the PVN and other brain regions that receive interoceptive signals. The present study examined whether NA neurons with axonal inputs to the PVN are necessary for LPS to activate Fos expression within the PVN and other interoceptive-related brain regions, and to increase plasma corticosterone. Male Sprague-Dawley rats received bilateral stereotaxic microinjections of DSAP (saporin toxin conjugated to an antibody against dopamine- β -hydroxylase, DbH) into the PVN to destroy NA inputs. Control rats were microinjected with vehicle into the PVN or received no PVN injections. Two weeks later, DSAP and control rats were injected i.p. with LPS (200 μ g/kg BW) or saline vehicle, and perfused with fixative 2.5–3 hrs later. Brain tissue sections were processed to reveal nuclear Fos protein and cytoplasmic DbH immunolabeling. DSAP lesions depleted NA terminals in the PVN and bed nucleus of the stria terminalis, reduced the number of NA cell bodies in the NST and VLM, attenuated PVN Fos activation after LPS, and attenuated LPS-induced increases in plasma corticosterone. These findings support the view that NA projections from hindbrain to hypothalamus are necessary for a full HPA axis response to systemic immune challenge.

Keywords

saporin toxin; corticosterone; nucleus of the solitary tract; ventrolateral medulla; lipopolysaccharide; interoceptive stress

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria such as *Escherichia coli*, a bacterium commonly found in the mammalian lower intestine. LPS is an endotoxin that elicits a strong response from mammalian immune systems, including the release of pro-inflammatory cytokines such as IL-1, IL-6, and TNF- α that act on immune-responsive cells (Turnbull and Rivier, 1999). Within the CNS, immune-responsive neurons activated by systemic LPS challenge include noradrenergic (NA) neurons in the A1/C1 and A2/C2 cell groups of the caudal ventrolateral and dorsomedial medulla, respectively.

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Axonal projections from activated NA neurons recruit neural activation in the hypothalamus and limbic forebrain, and these ascending projection pathways contribute importantly to hypothalamic-pituitary-adrenal (HPA) axis activation after immune challenge (Ericsson et al., 1994, Gaykema et al., 2007, Schiltz and Sawchenko, 2007).

In a recent report, an axonally-transported neurotoxin was used to unilaterally eliminate NA inputs to the paraventricular nucleus of the hypothalamus (PVN) in order to determine the role of these inputs in central neural activation after intravenous administration of synthetic IL-1 β (Schiltz and Sawchenko, 2007). The toxin, which we call DSAP, comprises an antibody against the NA synthetic enzyme dopamine beta hydroxylase (DbH) conjugated to saporin, a ribosomal toxin. The toxin conjugate is taken up selectively by NA axon terminals located within sites of DSAP injection, and then transported retrogradely to disrupt protein synthesis and thereby destroy NA cell bodies of origin (Madden et al., 1999, Ritter et al., 2001, Banihashemi and Rinaman, 2006, Dinh et al., 2006). Unilateral microinjections of DSAP reduced PVN neural Fos responses to systemic IL-1 β (Schiltz and Sawchenko, 2007), supporting the view that PVN neural recruitment after this cytokine challenge depends on NA inputs. The authors also noted that unilateral PVN DSAP microinjections reduced the density of DbH-immunoreactive NA terminals within the ipsilateral ventral bed nucleus of the stria terminalis (BNST), likely due to loss of collateralized axonal inputs to the PVN and BNST that arise from the same medullary NA neurons (Woulfe et al., 1988, Banihashemi and Rinaman, 2006). However, the authors did not quantify cytokine-induced Fos responses in the BNST or other non-PVN regions in their unilaterally lesioned DSAP rats.

In the present study, we used bilateral DSAP lesions to destroy the majority of medullary NA neurons with axonal inputs to the PVN, and then exposed rats to a more complex immune challenge, systemic endotoxin (LPS). Central LPS-induced Fos activation and DbH immunolabeling density within the PVN, dorsal and ventral BNST, and other brain regions of interest were compared in DSAP lesioned and sham- or non-surgerized (NS) control rats. In addition, the time course of plasma corticosterone responses to LPS was compared in a separate group of DSAP lesioned and sham control rats to determine whether reduced central Fos responses correspond to reduced peak corticosterone levels and/or a faster recovery to baseline after LPS challenge. These findings have been presented in abstract form (Bienkowski and Rinaman, FASEB 2007).

Experimental Procedures

Animals

Adult male Sprague-Dawley rats (200–250 g BW; Harlan Laboratories; n=31) were housed singly in stainless steel cages in a controlled environment (20–22°C, 12:12 hr light:dark cycle; lights off at 1900 hr) with *ad libitum* access to water and pelleted chow (Purina 5001). Experimental protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

DSAP Injections

DSAP toxin was used to specifically lesion NA neurons with inputs to the PVN. DSAP binds to vesicular DbH when vesicles are exposed to the synaptic cleft during transmitter exocytosis (Wrenn et al., 1996). The DSAP enzyme-antibody-toxin complex is internalized during vesicle endocytosis and is retrogradely transported. Upon reaching the cell body, saporin inactivates ribosomes (Ippoliti et al., 1992) to interrupt protein synthesis and produce NA cell death within 1–2 weeks (Madden et al., 1999, Ritter et al., 2001, Madden et al., 2006). The neurochemical specificity of DSAP as a NA lesioning agent has been demonstrated in several reports (Madden et al., 1999, Ritter et al., 2001, Rinaman, 2003, Ritter et al., 2003, Madden et al., 2006).

For bilateral injections of DSAP or vehicle into the PVN, rats (n=17 DSAP; n=10 sham control) were anesthetized by halothane inhalation (Halocarbon Laboratories; 1–3% in oxygen) and mounted into a stereotaxic frame in the flat-skull position. A 1.0 µl Hamilton syringe was attached to the stereotaxic arm. Injection coordinates targeting the left and right medial PVN (1.9 mm posterior, 0.4 mm lateral, and 9.2 mm ventral to bregma at the skull surface) were selected based on a standard rat brain atlas (Paxinos and Watson, 1997). DSAP (44 ng delivered in 200 nl of 0.15M NaCl vehicle; Advanced Targeting Systems, San Diego, CA) was pressure injected over a 1 min period. Sham control rats were similarly injected with 200 nl of vehicle alone. The syringe was left in place for 5 min after each injection to reduce injectate diffusion up the needle tract. PVN injections were repeated on the opposite side of the brain in the same surgical session. The skin was closed with sutures and rats were returned to their home cages after recovery from anesthesia. DSAP and sham control rats recovered for 2 weeks after surgery before being used in either Experiment 1 or Experiment 2, described below. An additional group of rats with no PVN injections [non-surgerized, (NS) controls, n=4] was added to Experiment 2 (see below) for between-group comparisons of brainstem and forebrain Fos activation after i.p. injection of saline vehicle.

Experiment 1: DSAP lesion effects on plasma corticosterone responses to LPS

A subset of DSAP (n=4) and sham control rats (n=6) were anesthetized with halothane. With the aid of a surgical microscope, the right femoral artery was cannulated with PE 50 tubing (Intramedic Clay Adams Brand, Becton Dickinson) and the cannula tip secured within the artery using silk sutures. PE tubing extending from the artery was tunneled subcutaneously to emerge through a small incision between the scapulae. The cannula tubing was secured at the exit site with a purse-string suture and was protected by a lightweight flexible harness system (Instech Laboratories, Plymouth Meeting, PA). Cannula tubing was extended distally and connected to a liquid swivel tether system (Instech Laboratories) mounted to a counterbalanced arm. The arm was attached to the stainless steel top of a standard shoebox cage in which each cannulated rat was individually housed with corncob bedding, with pelleted rat chow and water available ad libitum. This tether system allowed remote arterial blood sampling in freely moving rats.

Rats were allowed to recover from surgery and acclimate to the tether system and cage for 48 hrs before beginning the experiment. During this time, the arterial cannula line was opened twice each day (at 1000 and 1600 hr) to allow a few drops of blood to flow, followed by infusion of 200 µl of heparinized saline into the line to maintain patency.

On experimental day 1, a baseline blood sample (150 µl; time 0) was collected from each rat into a vial containing 3.75 IU heparin at time 0 (1000 hr). Immediately afterwards, each rat was injected i.p. with 2.0 ml of either 0.15M NaCl vehicle or LPS (0.2 mg/kg BW in 0.15M NaCl). Additional blood samples (150 µl) were collected 30, 60, 90, and 120 min after i.p. injection. The small volumes of blood collected at each time point were not replaced. Rats were left undisturbed after the final 120 min sample, although arterial lines were briefly opened and cleared with heparinized saline on the following day. The blood sampling procedure was repeated 48 hrs after the initial sampling procedure, with each rat receiving the alternate i.p. injection (i.e., LPS or vehicle) after the time 0 baseline sample. In this manner, each rat served as its own control for determining baseline corticosterone levels and the temporal effect of LPS and vehicle injections.

Blood samples were collected on ice and centrifuged within 5 minutes of collection to separate plasma. Plasma samples were stored at –20 °C until being assayed for corticosterone concentrations using an EIA kit (Immunodiagnostic Systems Ltd). Kit assay sensitivity for corticosterone was 0.52 ng/ml, with synthetic corticosterone recovery of 98.8% ± 6.0, and linearity of 99.6% ± 4.1.

Data analysis—A 3-way repeated measures ANOVA was used for statistical analysis of plasma corticosterone levels. Independent variables included lesion group (DSAP vs. sham control), i.p. treatment condition (LPS vs. vehicle) and time (baseline, 30, 60, 90, and 120 min). When *f* values indicated significant main effects and interactions among experimental variables, the ANOVA was followed up with planned *t*-comparisons of interest. Mean area under the curve values also were computed based on corticosterone levels assessed over time within each experimental group. Differences were considered statistically significant when *P* < 0.05.

A few hours after completing the second blood sampling procedure, DSAP and sham control rats were anesthetized and perfused transcardially with fixative as described below for rats used in Experiment 2. Fixed brains were sectioned and processed for immunocytochemical analysis of DbH in order to quantify DSAP lesion extent, as described below.

Experiment 2: DSAP lesion effects on central neural Fos responses to LPS

Remaining DSAP and sham control rats received an i.p. injection of 2.0 ml 0.15M NaCl vehicle (n=4 DSAP) or vehicle containing LPS (0.2 mg/kg BW; n=9 DSAP, n=4 sham control) between 1000 and 1200 hr. An additional group of NS control rats that did not receive any prior PVN surgery (n=4) also were injected with 2.0 ml saline vehicle. Rats were left undisturbed in their home cages for 2.5–3 hr after vehicle or LPS injection, then were deeply anesthetized with sodium pentobarbital (Nembutal, 100 mg/kg BW, i.p.) and transcardially perfused with a brief saline rinse followed by 500 ml of fixative (4% paraformaldehyde in 0.1 M phosphate buffer). Brains were post-fixed in situ overnight at 4°C, then removed from the skull, blocked, and cryoprotected in 20% sucrose solution prior to sectioning.

Histology and immunocytochemistry—Coronal 35 µm-thick tissue sections were cut from the caudal extent of the medullary dorsal vagal complex through the rostral extent of the corpus callosum using a freezing microtome. Sections were collected serially in six adjacent sets and stored at –20°C in a cryopreservant solution (Watson et al., 1986). Sections were removed from storage and rinsed for 1 hr in buffer (0.1 M sodium phosphate, pH 7.4) prior to immunocytochemical procedures.

Antisera were diluted in buffer containing 0.3% Triton-X100 and 1% normal donkey serum. Biotinylated secondary antisera (Jackson Immunochemicals) were used at a dilution of 1:500. Two sets of tissue sections from each rat were processed for immunocytochemical localization of Fos protein using a rabbit polyclonal antiserum (1:50,000; provided by Dr. Philip Larsen, Denmark), biotinylated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch) and Vectastain Elite ABC immunoperoxidase reagents (Vector Laboratories). The specificity of the primary antibody for Fos has been reported (Rinaman et al., 1997). Sections were processed using a nickel sulfate-intensified DAB reaction to generate a blue-black nuclear reaction product identifying Fos-positive cells. One set of Fos-labeled tissue sections was subsequently processed for dual immunoperoxidase localization of NA synthetic enzyme using a monoclonal anti-DbH antibody (1:30,000; Chemicon), biotinylated donkey anti-mouse IgG (1:500), and Vectastain Elite ABC immunoperoxidase reagents. DbH immunolabeling was visualized using a non-intensified DAB reaction to generate a brown cytoplasmic reaction product. A third set of tissue sections from each DSAP and sham control rat was processed for single immunoperoxidase localization of DbH alone in order to document the extent of NA lesions. After immunocytochemical processing, tissue sections were mounted onto Superfrost Plus microscope slides (Fisher Scientific), allowed to dry overnight, dehydrated and defatted in graded ethanols and xylene, and coverslipped using Cytoseal 60 (VWR).

Quantification of Fos activation and NA neuronal lesion extent—Dual

immunoperoxidase-labeled tissue sections from DSAP, sham control, and NS control rats used in Experiment 2 were analyzed with a light microscope to determine the number and proportion of DbH-positive medullary NA neurons activated to express Fos after LPS or vehicle treatment. Criteria for counting a neuron as DbH-positive included the presence of brown cytoplasmic immunoreactivity and a visible nucleus. NA neurons thus identified were considered Fos-positive (i.e., activated) if their nucleus contained blue-black immunolabel, regardless of intensity, and Fos-negative if their nucleus was unlabeled. DbH-positive neurons were counted bilaterally in the NST and VLM using a 40× microscope objective. Counts of DbH-positive NST and VLM neurons were made in tissue sections spaced 210 μm apart, beginning caudal to the area postrema (AP) at the upper cervical spinal cord where DbH-positive neurons first appear in the dorsomedial medulla (i.e., ~ 15.8 mm caudal to bregma) and continuing rostrally to the level at which the dorsal vagal complex moves laterally away from the floor of the fourth ventricle (i.e., ~ 13.2 mm caudal to bregma). In each animal, counts of Fos-positive and Fos-negative DbH neurons were summed within the NST and VLM, and then averaged across the number of sections analyzed to obtain mean cell counts per section. Data were then averaged within the three surgical groups (i.e., DSAP vs. sham control vs. no lesion control).

Pontine and forebrain tissue sections from DSAP, sham control, and no lesion control rats from Experiment 2 were used for quantitative analyses of Fos expression bilaterally within the lateral parabrachial nucleus (PBL), dorsal (d) and ventral (v) BNST, CeA, and PVN, using tissue sections immunolabeled only for Fos protein. Fos immunolabeling was quantified using Simple PCI imaging software (Compix Inc., Cranberry, PA) interfaced with a Zeiss Axioplan light microscope. Regions of interest were digitally outlined on high-resolution video images captured using a 10× microscope objective. The imaging software was calibrated and optimized to recognize and highlight Fos-positive profiles while minimizing the incidence of false-positive and false-negative results. Once the optimal setting criteria were established, they were applied consistently to analyze Fos labeling across all brain regions in all cases. In each rat, the total number of Fos-positive neurons counted within each region was divided by the number of nuclei analyzed through each region. Two tissue sections (spaced by 210 μm) containing the PBL, 2 sections containing the PVN, 1 section containing the dBNST and vBNST, and 3 sections containing the CeA were analyzed quantitatively in each rat. Bilateral count values from each region were combined and then averaged to obtain group mean counts ± SE per tissue section.

Quantification of DbH terminal density—DbH terminal immunolabeling density within the PVN, dVNST, and vBNST was quantified in a subset of DSAP and sham control rats from Experiments 1 and 2 using Simple PCI imaging software. Labeling density was assessed in tissue sections immunoreacted only for DbH. Immunoperoxidase labeling within each region was digitized in high-resolution video images (captured using a 10× microscope objective) at the sampling frequency described above, with optimal setting criteria established and then applied consistently across cases. Immunolabeling density was expressed as an areal fraction value, calculated as the proportion of the digitally outlined region of interest occupied by immunopositive profiles. Areal fraction values were then averaged to obtain group mean ± SE values for each brain region.

Data analysis—One-way ANOVAs (separate for each brain region) were used for statistical comparisons of (1) the numbers of DbH-positive NA neurons within the NST and VLM, (2) the proportions of NST and VLM NA neurons activated to express Fos after LPS or vehicle i.p., (3) the numbers of Fos-positive neurons within the NST, VLM, PBL, CeA, dBNST, vBNST and PVN after LPS or vehicle i.p., and (4) DbH terminal density within the dBNST, vBNST and PVN. The independent variable for each ANOVA was lesion group (i.e., DSAP, sham control, NS control). Differences were considered significant when $P < 0.05$.

Results

DSAP-induced loss of medullary NA neurons projecting to the PVN

Post-mortem analyses of DSAP lesion extent (i.e., loss of medullary NA neurons) were conducted in brain tissue sections from all perfused rats ($n=31$), including those used to assess the effects of LPS on plasma corticosterone levels (Experiment 1) and those used for analysis of central neural Fos expression (Experiment 2). NA cell count data from the two control groups (i.e., sham and no lesion) were combined, because NA cell count values within the NST and VLM did not differ between these two groups. DSAP microinjected bilaterally into the PVN significantly reduced the number of DbH-positive NA neurons within the NST ($P < 0.001$) and VLM ($P < 0.001$) compared to counts of DbH-positive NST and VLM neurons in sham and no lesion control rats (Fig. 1). Visual inspection revealed that DSAP injections virtually eliminated DbH terminal immunolabeling within targeted PVN injection sites (Fig. 2), and also reduced DbH immunolabeling within the dorsal and ventral BNST (Fig. 2). These observations were confirmed by quantitative analysis of DbH-immunopositive terminal density in a subset of rats with the best overall tissue preservation and DbH immunolabeling quality ($n=8$ sham control and $n=6$ DSAP rats from Experiments 1 and 2). Repeated-measures ANOVA of areal fraction data (i.e., DbH labeling density) demonstrated that bilateral PVN microinjections of DSAP produced significant loss of DbH terminal immunolabeling within the PVN ($F=100.09$, $P < 0.001$), dBNST ($F=92.32$, $P < 0.001$), and vBNST ($F=18.42$, $P = 0.001$) (Fig. 3). Further, the density of DbH-positive terminals within the PVN was significantly correlated with the density of DbH terminals within the dBNST (Pearson $r = 0.878$, $P < 0.001$) and vBNST (Pearson $r = 0.731$, $P = 0.003$) in sham and DSAP rats. DbH terminal densities within the dBNST and vBNST also were significantly correlated (Pearson $R = 0.888$, $P < 0.001$).

Experiment 1: DSAP lesion effects on plasma corticosterone responses to LPS

As expected, LPS challenge in sham control rats ($n=6$) provoked significantly increased plasma corticosterone levels compared to levels in the same rats after i.p. vehicle injection (Fig. 4). Repeated-measures ANOVA revealed significant main effects of surgery (DSAP vs. sham control; $F=18.29$; $P = 0.003$), i.p. treatment (LPS vs. saline vehicle; $F=74.0$; $P < 0.001$), and time (0, 30, 60, 90, 120 min; $F=48.3$; $P < 0.001$) on plasma corticosterone levels. There also were significant interactions between surgery and i.p. treatment ($F=10.9$; $P = 0.011$), surgery and time ($F=4.2$; $P = 0.008$), and i.p. treatment and time ($F=17.3$; $P < 0.001$), as well as a 3-way interaction among surgery, i.p. treatment, and time ($F=3.0$; $P = 0.033$). In sham control rats (open symbols in Fig. 4), plasma corticosterone levels reached a peak between 60 and 90 min post-LPS, and remained significantly elevated at the final 120 min time point. Conversely, DSAP rats ($n=4$; closed symbols in Fig. 4) had significantly attenuated plasma corticosterone responses to LPS at the 60 min ($P = 0.01$), 90 min ($P < 0.001$), and 120 min ($P = 0.02$) post-LPS time points compared to sham control rats, including a reduced peak response and an earlier decline. Interestingly, repeated measures ANOVA showed no significant differences between sham control and DSAP rats in their plasma corticosterone responses to i.p. saline injection over all time points, although a trend towards a reduced response by DSAP rats at the 30 min post-injection time point was evident (Fig. 4). The total “area under the curve” (AUC) values for plasma corticosterone after i.p. saline or LPS in both surgical groups are included on the right side of the plot (Fig. 4). AUC values were significantly different between sham control and DSAP rats after LPS treatment ($P = 0.024$; top two AUC values), and approached but did not reach a significant difference between surgical groups after saline treatment ($P = 0.06$; bottom two AUC values). Overall, these results indicate that bilateral NA denervation of the PVN markedly attenuates plasma corticosterone responses to i.p. LPS challenge, without significantly affecting the smaller and more transient corticosterone response to handling and i.p. injection of saline alone.

Rats used in Experiment 1 were perfused with fixative and their brains processed for quantification of DSAP lesion extent (i.e., counts of DbH-positive NA neurons within the NST and VLM). This analysis revealed some variability in lesion extent among the four DSAP rats used for analysis of plasma corticosterone. Compared to the average number of DbH positive NST and VLM neurons counted in sham and no lesion control rats (see Fig. 1), the four DSAP rats in Experiment 1 displayed losses of NA NST neurons ranging from approximately 20% to 75%, and losses of NA VLM neurons ranging from approximately 55% to 95%. Peak plasma corticosterone levels achieved at the 90 min post-LPS time point in the four DSAP rats were highly and significantly correlated with the number of remaining DbH neurons counted within the NST and VLM (Fig. 5). The fewer medullary DbH neurons remaining after DSAP lesion, the more attenuated was the rat's plasma corticosterone response to LPS challenge.

Experiment 2: DSAP lesion effects on central neural Fos responses to LPS

DSAP (n=4) and non-surgerized (NS) control rats (n=4) that were injected i.p. with saline vehicle before perfusion displayed relatively little Fos immunolabeling within the NST, VLM, PBL, PVN, CeA, dBNST, or vBNST compared to Fos labeling in sham and DSAP rats injected with LPS (Fig. 6 and Fig 7). This outcome is similar to previously published results from our laboratory in NS rats after i.p. saline vehicle (Rinaman, 1999; Myers et al., 2005).

Separate multiple-comparison ANOVAs within each brain region revealed significant effects of surgical and i.p. treatment group (i.e., sham, DSAP, or NS after i.p. LPS or saline vehicle) on Fos activation within the PBL [$F(3,17) = 6.07, P = 0.005$], CeA [$F(3,17) = 8.51, P = 0.001$], PVN [$F(3,17) = 21.97, P < 0.001$], and dBNST [$F(3,17) = 3.89, P = 0.028$]. Group differences in vBNST Fos expression approached but not did reach statistical significance [$F(3,17) = 2.89, P = 0.066$], although a trend for reduced vBNST activation in rats after LPS compared to saline vehicle was apparent (Fig. 6), consistent with evidence that the dBNST (which was activated significantly more after i.p. LPS than after i.p. vehicle) provides inhibitory input to the vBNST (Dong et al., 2001). As expected, Fos expression in each region was similar in vehicle-treated NS and DSAP rats (Fig. 6). Except for the vBNST (see below), significantly greater Fos expression was observed in each brain region in sham and DSAP rats after LPS treatment compared to activation in NS and DSAP rats after i.p. vehicle treatment (Fig. 6). The most notable finding was that LPS-induced Fos expression within the PVN was significantly (but not completely) attenuated in DSAP rats compared to sham controls (Fig. 6). Conversely, LPS-induced Fos activation in the PBL, CeA, dBNST, and vBNST did not differ between sham and DSAP rats (Fig. 6).

Within the caudal medulla, LPS-induced Fos expression was assessed in DbH-positive neurons within the NST and VLM in sham and NS control rats, and in the remaining (i.e., non-lesioned) population of DbH-positive NST and VLM neurons in DSAP rats. Because DSAP lesions significantly reduced the number of DbH-positive NST and VLM neurons (Fig. 1), it is not surprising that the number of DbH-positive NST and VLM neurons that were double labeled for Fos also was significantly reduced in DSAP rats after LPS treatment (Fig. 7; $*P < 0.01$ for each region). The NA NST and VLM neurons that expressed Fos after LPS treatment represented subsets of the total number of DbH-positive NA neurons within each medullary region, as indicated by the % values above each bar in Figure 7. LPS treatment activated ~19% of NA neurons in the NST in sham control rats and ~13.5% of remaining NST DbH neurons in DSAP rats; these proportions are not significantly different ($P = 0.28$). Within the VLM, LPS activated ~57% of DbH neurons in sham control rats and ~33% of remaining DbH neurons in DSAP rats; these proportions are significantly different ($P = 0.02$).

The numbers of double-labeled (i.e., Fos-positive) NA neurons counted within the NST and VLM in individual DSAP and sham control rats after LPS treatment were strongly and significantly correlated with the number of Fos-positive neurons counted within the PVN in

the same rats (Pearson $R = 0.89$ and $P < 0.01$ for each correlation; Fig. 8), supporting the view that LPS-induced activation of NA neurons in both medullary regions participates in driving PVN neural activation. Color photomicrographs of DbH + Fos double labeling within the NST and VLM in representative sham control and DSAP rats after LPS treatment are shown in Figure 9.

Discussion

Systemic administration of LPS is a widely used experimental model to study how the CNS organizes adaptive endocrine, behavioral, autonomic, and emotional responses to circulating proinflammatory cytokines (Elmqvist and Saper, 1996, Turnbull and Rivier, 1999, Zhang et al., 2000, Castanon et al., 2003, Marvel et al., 2004, Gaykema et al., 2007, Schiltz and Sawchenko, 2007). Our new results confirm and extend previous lines of evidence that the ability of systemic immune challenge to activate the stress-responsive HPA axis depends largely on the recruitment of NA inputs from the caudal medulla to the medial hypothalamus.

In the present study, bilateral microinjections of DSAP virtually eliminated DbH terminal immunolabeling within PVN microinjection sites, reduced DbH immunolabeling within the dorsal and ventral BNST, and reduced the number of DbH-positive NA neurons counted within the NST and VLM. DbH-positive terminal densities within the PVN, dBNST, and vBNST were positively correlated across sham control and DSAP rats, supporting the conclusion that significant proportions of these NA terminal fields arise from the same subpopulation of NA neurons within the NST and VLM. These observations extend our previous report that NA inputs to the medial parvocellular PVN (but not the lateral magnocellular PVN) derive from medullary NA neurons that also target the lateral BNST (Banihashemi and Rinaman, 2006). The results also are consistent with a recent qualitative report of reduced DbH immunolabeling within the ipsilateral BNST after unilateral PVN microinjections of DSAP (Schiltz and Sawchenko, 2007). New quantitative results from the present study indicate that the dorsal and ventral BNST receive approximately 75% and 50%, respectively, of their NA innervation from a population of NA neurons whose axon collaterals also innervate the PVN DSAP injection site (values derived from data shown in Figure 3). Our previous findings in rats injected with DSAP into the BNST demonstrated that NA neurons with inputs to the BNST provide virtually all of the NA input to the medial parvocellular PVN (Banihashemi and Rinaman, 2006). Considered together, these findings indicate that the medial parvocellular PVN receives nearly all of its NA input from neurons with axon collaterals that also innervate the BNST, whereas the BNST receives approximately 50–75% of its NA input from neurons that also target the PVN.

Bilateral NA denervation of the PVN markedly attenuated plasma corticosterone responses to acute systemic endotoxin challenge. The fewer medullary DbH neurons remaining in each DSAP rat, the more attenuated was their plasma corticosterone response to LPS. The smaller and more transient plasma corticosterone response to i.p. saline injection also was attenuated in DSAP rats vs. sham controls, although the difference between surgical groups did not reach statistical significance. This result is consistent with prior evidence that NA inputs to the PVN are unnecessary for the ability of “exteroceptive” or “neurogenic” stressors, such as footshock or restraint, to activate CRF-positive neurons at the apex of the HPA axis (Li et al., 1996, Li and Sawchenko, 1998, Schiltz and Sawchenko, 2007). In the present study, the relatively mild stress of handling and i.p. injection likely includes “neurogenic” components that are not affected by loss of NA inputs to the PVN. Conversely, our data support the view that significant portions of the “interoceptive” or “homeostatic” stress evoked by LPS-induced immune challenge depend on NA inputs to the PVN that recruit HPA axis responses, consistent with previous reports (Ericsson et al., 1994, Li et al., 1996, Schiltz and Sawchenko, 2007).

As predicted by the plasma corticosterone data, significantly less LPS-induced PVN Fos activation was present in DSAP rats vs. sham controls. These Fos data are consistent with a recent report that unilateral PVN DSAP lesions significantly attenuate the ability of intravenous IL-1b to increase Fos immunolabeling and CRF mRNA expression within the lesioned PVN (Schiltz and Sawchenko, 2007). However, DSAP lesions targeting the PVN in the present study did not eliminate either DbH-positive NA terminals or LPS-induced Fos activation within the PVN. As evident in Figure 6, PVN Fos activation was still significantly elevated in DSAP rats after LPS treatment compared to PVN activation in DSAP or NS control rats after saline vehicle injection. The bilateral PVN DSAP lesions also did not reduce the ability of LPS to activate Fos expression in the PBL, CeA, or BNST; therefore, other intact neural pathways appear to be sufficient to activate Fos expression in these regions after LPS.

Our observations are partially consistent with findings that knife-cut lesions of the ventral noradrenergic ascending bundle (VNAB) reduce the ability of intravenous LPS to activate Fos expression within the PVN without reducing Fos activation within the dBNST or CeA (Schiltz and Sawchenko, 2007). However, Schiltz and Sawchenko also reported that VNAB knife cuts reduce the ability of LPS to activate Fos expression within the ipsilateral vBNST, whereas in the present study, bilateral PVN DSAP injections did not significantly alter the relatively low levels of LPS-induced Fos expression within the vBNST, even though DbH-immunopositive NA inputs to this region were significantly reduced by the DSAP lesion. The apparent difference in experimental outcomes between our study and theirs may be due to the more complete and/or less chemically specific denervation of ascending inputs to the hypothalamus and limbic forebrain that is achieved by VNAB knife cuts (Schiltz and Sawchenko, 2007).

Bilateral DSAP injections into the PVN significantly reduced the overall number of DbH-positive NA neurons counted within the NST and VLM, including NA neurons that were activated to express Fos after LPS treatment. However, of the remaining NA neurons that were not destroyed, the proportion within the NST that was activated by LPS (~13.5%) was not significantly different from the proportion activated within the NST in sham control rats (~19%). Conversely, within the VLM, LPS activated a significantly smaller proportion of remaining NA neurons in DSAP rats (i.e., ~33%) compared to activation in sham controls (~57%). These results suggest that LPS-induced recruitment of NA neurons within the VLM may depend, in part, on descending inputs from the PVN (Sawchenko and Swanson, 1982, Buijs et al., 1990) or from other brain regions whose activity was affected by the lesion.

In conclusion, findings from this study support the view that NA projections from hindbrain to hypothalamus are necessary for a full HPA axis response to systemic endotoxin. Bilateral DSAP lesions of NA inputs to the PVN did not eliminate the Fos or plasma corticosterone responses to immune challenge, however, indicating that remaining non-lesioned NA or other chemical signaling pathways are sufficient to sustain some degree of central neural and HPA axis sensitivity to LPS. Additional work will be necessary to determine the extent to which NA inputs to the hypothalamus and limbic forebrain are involved in mediating other well-characterized organismic responses to immune challenge, including behavioral, autonomic, and emotional responses.

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Abbreviations

AUC, area under the curve

BNST, bed nucleus of the stria terminalis (d, dorsal; v, ventral)
 CeA, central nucleus of the amygdala
 DbH, dopamine beta hydroxylase
 DSAP, saporin toxin conjugated to an antibody against DbH
 HPA, hypothalamic-pituitary-adrenal
 LPS, lipopolysaccharide
 NA, noradrenergic
 NS, non-surgerized
 NST, nucleus of the solitary tract
 PBL, lateral parabrachial nucleus
 PVN, paraventricular nucleus of the hypothalamus (mp, medial parvocellular)
 VLM, ventrolateral medulla

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Figure 1.

Bar graphs depicting the number of DbH-positive NA neurons counted within the NST and VLM in sham and no lesion control rats (combined, $n=14$, open bars) and in DSAP rats ($n=17$; filled bars) from Experiments 1 and 2. Counts are group mean averages per section ($\pm$ SE). DSAP lesions significantly reduced NA neuron number within both the NST and VLM. * $P < 0.001$, DSAP vs. sham and no lesion controls.

Figure 2. Black and white photomicrographs of DbH-positive terminals and Fos-positive nuclei within the PVN (A,B) and vBNST (C,D) in a representative sham control (left) and in a DSAP rat (right), perfused after LPS treatment. DSAP lesions reduced DbH terminal labeling in both regions (as well as in the dorsal BNST, not shown), and reduced the ability of LPS to activate Fos in the PVN but not within the BNST (see Figure 6). Scale bar in D = 200 μm; applies to all panels. See Figure 3 for quantification of DbH terminal density.

Figure 3. Bar graphs depicting the density of DbH-positive NA terminals within the PVN, dBNST, and vBNST in a selected subset of sham control (n=8) and DSAP rats (n=6) with optimal tissue preservation and immunolabeling quality. Areal fraction values represent the proportion of each sampled region that contained DbH-positive profiles (group mean $\pm$ SE). DSAP lesions significantly reduced NA terminal density within all three regions. * $P \leq 0.001$, DSAP vs. sham controls.

DSAP Lesions Attenuate LPS-Induced Increases in Plasma Corticosterone

Figure 4. Line graphs depicting plasma corticosterone levels (pg/ml; mean $\pm$ SE) at five time points in sham control (n=6) and DSAP rats (n=4) before and after i.p. injection of saline or LPS. Total area under the curve (AUC) values for each line are provided on the right side of the graph (group mean $\pm$ SE).

Figure 5. Peak plasma corticosterone levels achieved at the 90 min post-LPS time point (y-axis) in four DSAP rats (individual symbols) are significantly correlated with the average number of remaining DbH neurons counted per section within the NST (top panel, x-axis) and VLM (bottom panel) in each rat.

DSAP Lesions Attenuate LPS-Induced Fos Expression within the PVN

Figure 6.

Bar graphs depicting the number of Fos-positive neurons counted via image analysis within the PBL, CeA, PVN, dBNST, and vBNST in sham control rats after LPS (sham-LPS; $n=4$; open bars), in DSAP rats after LPS (DSAP-LPS; $n=10$; solid bars), in non-surgerized (NS) rats after i.p. saline vehicle (NS-vehicle; $n=4$; dark stippled bars), and in DSAP rats after i.p. saline vehicle (DSAP-vehicle; $n=4$; light gray bars). Values are group mean averages per nucleus ($\pm$ SE). Relatively little (and statistically similar) Fos activation was observed in DSAP and NS rats after i.p. vehicle injection. In each brain region, LPS treatment was associated with significantly greater Fos activation compared to activation after vehicle treatment (@, $P < 0.05$). DSAP rats displayed significantly attenuated LPS-induced Fos activation only within the PVN (* $P = 0.001$, DSAP vs. sham controls). Conversely, DSAP lesions did not significantly alter LPS-induced Fos activation within the PBL, CeA, dBNST, or vBNST. Fos labeling within the

vBNST was somewhat higher in vehicle-injected rats compared to activation in rats after LPS treatment, although this difference did not reach statistical significance.

DSAP Lesions Reduce the Number of LPS-Activated NA Neurons within the NST and VLM

Figure 7.

Bar graphs depicting the number of double-labeled (i.e., Fos-positive and DbH-positive) NA neurons counted within the NST and VLM of sham control rats after LPS (n=4), in DSAP rats after LPS (n=9), in “non-surgerized” (NS) control rats after saline vehicle (n=4), and in DSAP rats after saline vehicle (n=4). After LPS treatment, rats with DSAP lesions displayed significantly reduced numbers of activated NA neurons within both the NST and VLM (* $P < 0.01$ compared to sham LPS). Significantly fewer DbH-positive NST and VLM neurons were activated in NS and DSAP rats after saline injection (@ $P < 0.05$ compared to DSAP LPS, and @ $P < 0.001$ compared to sham LPS), with no significant difference between i.p. saline-induced Fos activation of NA neurons in the NS and DSAP groups. The % values above each

bar report the proportion of counted DbH-positive NST and VLM neurons that were activated to express Fos. For example, in sham control rats, LPS treatment activated approximately 12 DbH-positive NST neurons per section and approximately 20 DbH-positive VLM neurons per section (open bars), which represented ~18.6% of all DbH-positive NST neurons and ~57.0% of all DbH-positive VLM neurons counted per section within that group.

Correlation Between Activation of Non-Lesioned NA Neurons and Activation of PVN Neurons in DSAP Rats after LPS Treatment

Figure 8.

The average number of double-labeled (i.e., Fos-positive) NA neurons counted per section within the NST (top panel) and VLM (bottom panel) in DSAP rats (open symbols; $n=9$) and sham control rats (closed symbols; $n=4$) after LPS treatment were strongly and significantly correlated with the number of Fos-positive neurons counted within the PVN in the same rats.

Figure 9. Color photomicrographs of DbH-positive neurons and processes (brown) and Fos-positive nuclei (blue-black) within the NST (A,B) and VLM (C,D) in a representative sham control (left) and DSAP rat (right). Tissue sections are through the mid-level of the area postrema. Arrows point out some of the double-labeled neurons in each panel (DbH + Fos). Scale bar in D = 200 μm; applies to all panels. See Figure 6 for quantification of cell count data.