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Ascending caudal medullary catecholamine pathways drive sickness-induced deficits in exploratory behavior: brain substrates for fatigue?

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Abstract

Immune challenges can lead to marked behavioral changes, including fatigue, reduced social interest, anorexia, and somnolence, but the precise neuronal mechanisms that underlie sickness behavior remain elusive. Part of the neurocircuitry influencing behavior associated with illness likely includes viscerosensory nuclei located in the caudal brainstem, based on findings that inactivation of the dorsal vagal complex (DVC) can prevent social withdrawal. These brainstem nuclei contribute multiple neuronal projections that target different components of autonomic and stress-related neurocircuitry. In particular, catecholaminergic neurons in the ventrolateral medulla (VLM) and DVC target the hypothalamus and drive neuroendocrine responses to immune challenge, but their particular role in sickness behavior is not known. To test whether this catecholamine pathway also mediates sickness behavior, we compared effects of DVC inactivation with targeted lesion of the catecholamine pathway on exploratory behavior, which provides an index of motivation and fatigue, and associated patterns of brain activation assessed by immunohistochemical detection of c-Fos protein. LPS treatment dramatically reduced exploratory behavior, and produced a pattern of increased c-Fos expression in brain regions associated with stress and autonomic adjustments paraventricular hypothalamus (PVN), bed nucleus of the stria terminalis (BST), central amygdala (CEA), whereas activation was reduced in regions involved in exploratory behavior (hippocampus, dorsal striatum, ventral tuberomammillary nucleus, and ventral tegmental area). Both DVC inactivation and catecholamine lesion prevented reductions in exploratory behavior and completely blocked the inhibitory LPS effects on c-Fos expression in the behavior-associated regions. In contrast, LPS-induced activation in the CEA and BST was inhibited by DVC inactivation but not by catecholamine lesion. The findings support the idea that parallel pathways from immune-sensory caudal brainstem sources target distinct populations of forebrain neurons that likely mediate different aspects of sickness. The caudal medullary catecholaminergic projections to the hypothalamus may significantly contribute to brain mechanisms that induce behavioral “fatigue” in the context of physiological stressors.

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Keywords

dopamine beta-hydroxylase; saporin toxin; dorsal vagal complex; ventrolateral medulla; hypothalamus; noradrenergic; lipopolysaccharide; behavioral arousal

1. Introduction

Physiological and psychological challenges, including inflammation and stress, can induce a constellation of symptoms referred to as “sickness behavior”. This behavioral pattern involves reductions in motivated behavior including food and water intake, social and sexual behavior, exploratory behavior, and motor activity, and an increase in stress hormones. Sickness behavior supports host defense and recuperation by conserving energy, and is considered to be a motivated and adaptive response to physiological challenges (Dantzer and Kelley, 2007; Miller, 2009). However, in the context of chronic disease, sickness symptoms can lead to prolonged fatigue or symptoms of behavioral depression.

Although the precise neural substrates responsible for the behavioral manifestations of sickness are still emerging, studies using the activation marker c-Fos have indicated that activity in brain regions that support “positive motivation”, including the nucleus accumbens, and several cortical areas including secondary motor, cingulate and piriform, is reduced in animals treated with the immune stimulant lipopolysaccharide (LPS; Stone et al., 2006). Conversely, this inhibition of neuronal activity marker expression occurs concomitant with activation in stress-sensitive, viscerosensory and autonomic control regions including the caudal brainstem, hypothalamus, and extended amygdala (Wan et al., 1994; Stone et al., 2006; Gaykema et al., 2007; Gaykema et al., 2008; Elmquist and Saper, 1996; Elmquist et al., 1996; Frenois et al., 2007). This pattern of inhibition of “positive motivation”-related brain regions and activation of stress-related regions likely contributes to behavioral and mood-related sequelae of infection or inflammation.

The mechanisms by which peripherally generated immune signals are able to influence forebrain neurocircuitry involved in sickness behavior has not been firmly established. Because sickness behavior has been shown to be dependent upon the interaction of cytokines with the brain, constituents of the network of brain regions that subserve sickness behavior must interface with brain regions that can detect central or peripheral cytokines, such as circumventricular organs, vascular endothelium, or viscerosensory relay nuclei.

Of the brain regions that consistently show evidence of activation following immune challenge, the sensory dorsal vagal complex (DVC) located in the dorsomedial caudal medulla [consisting of the nucleus of the solitary tract (NTS) and area postrema], serves as one interface by which peripheral immune-related information influences the brain. The DVC receives immune-sensitive inputs from neural pathways (vagal and spinal) and circulating immune signals via the weak blood barrier in the area postrema (a circumventricular organ). Immune cells within the area postrema that produce interleukin-1 following immune challenge make direct contact with neurons, providing a potential pathway mediating cytokine-neural interface (Goehler et al., 2006). Additionally, the DVC is interconnected with the caudal ventrolateral medulla (VLM), which relays immune sensory information via activation of vascular endothelial cells within the nucleus predominantly by its catecholaminergic projections to the forebrain (Ericsson et al., 1997). Together with the VLM, the DVC projects to forebrain regions that mediate stress responses, autonomic control and arousal. Thus, the caudal brainstem is uniquely situated to transduce and propagate immune-related signals to brain regions likely involved in initiation of sickness behavior.

Functional evidence that the DVC mediates components of sickness behavior derives from findings that inactivation of the DVC with a local anesthetic prior to administration of LPS prevented both social withdrawal behavior and the characteristic brain activation pattern typically that occurs in association with immune activation in resting animals (Marvel et al., 2004). Further, DVC inactivation prevented the suppression of histamine neurons that are normally activated during an exploratory behavior task (Gaykema et al., 2008). These findings support the idea that activation of the DVC and VLM contribute to brain and behavioral responses to immune challenge. However, the specific links (neuronal pathways) between immune-related activation of brainstem viscerosensory regions and the forebrain nuclei that mediate sickness behavior have not been established.

Neuronal projections from the DVC and VLM to the forebrain follow multiple trajectories of catecholaminergic and non-catecholaminergic neurons (Gaykema et al., 2007). The catecholamine pathway arises in both the VLM and the DVC. It projects heavily to the hypothalamus and is well established to activate neuroendocrine responses in context of physiological challenges (e.g. Cunningham et al., 1990; Ericsson et al., 1994; Bienkowski and Rinaman, 2008;) including immune-related stimuli (Ericsson et al., 1997; Elmquist and Saper, 1996). Its contribution to other brain mediated sickness responses is less clear. In contrast, the non-catecholamine projections -which mainly arise from the DVC- preferentially target the external lateral parabrachial nucleus (PBeI) in the pons, which then drives immune-related responses of the amygdala and bed nucleus of the stria terminalis (Tkacs and Li, 1999; Richard et al., 2005). This arrangement suggests that separate neurocircuitries, or parallel pathways, could mediate different aspects of sickness behavior.

As noted previously, immune challenge with LPS can lead to marked reductions in motor activity and motivated behavior that could relate to the psychological experience of “fatigue”. Fatigue is common in acute and chronic illness, but the neurological substrates involved have yet to be established. Our previous studies have indicated that suppression of hypothalamic arousal systems may contribute to reductions in behavior associated with immune challenge (Gaykema et al., 2008; Gaykema and Goehler, 2009; Park et al., 2008). For the experiments reported here, we assessed exploratory motor behavior to provide an index of motivation and fatigue (Stone et al., 2006). To determine functional relationships between the caudal brainstem and the forebrain structures involved in motivation and movement that mediate sickness-induced reductions in exploratory behavior, we addressed three questions. First, can a targeted lesion of the immune-responsive catecholaminergic pathway originating in the caudal medulla prevent LPS-induced deficits in exploratory behavior, as does DVC inactivation? The answer to this question clarifies which of the ascending projections are most relevant for the effects of LPS on behavior. Second, how does LPS change the brain activation patterns normally induced during exploratory behavior, and how does catecholamine lesion or DVC inactivation modify the pattern? The answer to this question establishes potential target brain regions relevant to behavioral deficits during sickness. Finally, if catecholamine lesion is sufficient to ameliorate deficits in exploratory behavior, what are the possible targets of these neurons (i.e. brain regions depleted from catecholaminergic input)? Those brain regions could provide the substrates that link immune challenge and behavior.

2. Materials and methods

2.1. Animals

The experiments involved 41 male Sprague-Dawley rats (Taconic Laboratories, Germantown, NY, USA) with initial body weights of 250-270 g. The rats were housed in pairs in polypropylene boxes on a barrier cage rack (Allentown, Caging Smart Bio-Pak, Allentown, NJ, USA) in a temperature and humidity controlled room. The rats were

maintained on a 12-hour light-dark cycle (lights on at 7:00 AM) with free access to Purina Rat Chow #R001 and water. Rats were acclimatized for at least a week before they received surgery (guide cannulae aimed at the DVC or DSAP micro-injection targeted to hypothalamus). Directly after surgery, all rats were singly housed during one week. DSAP injected animals and controls were re-housed with their cage mate thereafter. Animals that received guide cannulae for DVC inactivation remained singly housed, to reduce likelihood the cannulae would be damaged. All procedures were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publications No. 80-23; revised 1996) and were in accordance with protocols approved by the University of Virginia Animal Care and Use Committee. Every attempt was made to minimize the number of animals used in these studies and to limit their distress and potential suffering.

2.2. Surgical procedures

2.2.1. Lesion of the ascending noradrenergic/adrenergic projections

Rationale: To investigate the role of the catecholaminergic component of the immune-responsive brainstem-derived pathway, we applied a targeted lesion approach using an anti-dopamine beta hydroxylase antibody conjugated to saporin (DSAP). When injected into a target region, DSAP is taken up selectively by noradrenergic/adrenergic neurons that innervate the target (Fig. 1A,B). The toxin is retrogradely transported to the soma, which it destroys. DSAP was micro-injected bilaterally into the hypothalamic paraventricular nucleus (PVN) because this structure receives particularly dense innervation from the medullary catecholamine projections, and because pilot experiments showed that DSAP injection into the PVN produced a dramatic reduction in the number of DBH-positive (catecholaminergic) cell bodies in the VLM and NTS.

Anti-DBH saporin (DSAP) injection: Twenty four male Sprague-Dawley rats received DSAP or control injection of unconjugated saporin (SAP) into the hypothalamus. The tips of beveled glass micropipettes were directed bilaterally at the PVN, (coordinates 1.8 mm posterior to bregma, $\pm$ 0.4 mm lateral to the midline, and -7.6 mm below the dura; based on Paxinos and Watson, 1998). The glass micropipets were backfilled with a saline solution containing the immunotoxin anti-DBH saporin (DSAP, IT-03, Advanced Targeting Systems, San Diego, CA) or with the control saline solution containing saporin alone. A total of 40 ng DSAP in 200 nl saline was injected into each injection site using a MPPI-2 pressure injector (Stoelting Co., Wood Dale, IL) over a period of 5 minutes. Control rats received bilateral injections of 9 ng SAP in 200 nl saline. The micropipette was left in place for at least another 3 minutes before withdrawal. Following the injections the rats were sutured, and post-operative care was administered. The rats were returned to their home-cages, where they remained for three weeks post-injection before testing. This time period was necessary to allow the DSAP to be internalized, retrogradely transported in DBH-expressing neurons, and to eventually lesion them.

2.2.2. Dorsal vagal complex (DVC) inactivation

Rationale: We have previously used inactivation of the NTS/dorsal vagal complex (DVC) with infused bupivacaine to demonstrate a significant contribution of the DVC to LPS-induced inhibition of social behaviors and induction of c-Fos protein in LPS-responsive forebrain regions (Marvel et al. 2004) and in the LPS-associated inhibition of exploratory behavior (Gaykema et al., 2008). Bupivacaine blocks sodium channels for at least two hours, and was chosen because it has the longest duration of local anesthetics currently in use. This technique produces limited brain damage and has no effect on social or exploratory behavior by itself (Marvel et al., 2004; Gaykema et al., 2008). The technique is used explicitly to avoid problematic autonomic effects due to structural lesions in critical viscerosensory relay

regions (e.g., Williams and McGaugh, 1993). The goals of this analysis were to reveal how DVC inactivation, which prevents LPS-induced behavioral deficits, modifies LPS-induced changes in patterns of brain c-Fos expression (Fig. 1C).

Cannula implantation: For this experiment, a group of 17 rats received double-barrel stainless steel guide cannulae (26 gauge, 1.5 mm distance, Plastics One, Roanoke VA) as described (Marvel et al., 2004). The stainless steel guide cannulae were implanted bilaterally 1mm above the site of injection (NTS) according to the following coordinates: 13.6 mm caudal from bregma, 0.75 mm lateral from the midline and 6.5 mm below the skull surface (Paxinos and Watson, 1998). Stylets were placed inside the guide cannulae to prevent obstruction. After cannulae implantation, the animals received injections of the analgesic ketoprofen (0.1 mg s.c.) and the antibiotic enrofloxacin (Baytril, Bayer; 2.27%, 0.2 ml s.c.). During the recovery period of 10-14 days, the animals were housed individually and handled daily to habituate them to manual contact and mild restraint.

2.3. LPS or saline injection

LPS (Sigma, serotype 0111 B4) was prepared in sterile saline at a concentration of 0.1 mg/ml. Rats were injected i.p. with either LPS (0.1 mg/kg) or sterile saline. This dose of LPS was chosen because we have determined using dose-effect studies (Marvel et al., 2004) with this serotype that a dose of 0.1 mg/kg results in marked, but not complete, reduction in behavior.

2.4. Behavioral testing

Exploratory behavior is assessed using experimental procedures and equipment that allow the animal to move around unrestricted in a novel environment, such as an open field, holeboard or plus or Y maze. Exploratory behavior is related to a drive in foraging species toward potential rewards such as food or mates (Duzel et al., 2009), and is considered to index motivation based on the preferential stimulus novelty-induced firing of midbrain dopamine neurons. Exploratory behavior also reveals motor capability and is reduced in animals treated with LPS (Stone et al., 2006).

Open field exploration (DSAP lesion study)—The animals were habituated to handling and transport to the adjacent experiment room. The rats were randomly assigned to the treatment groups, using a two-by-two factorial design yielding four groups: DSAP/LPS (n=5), DSAP/saline (n=5), control/LPS (n=8) and control/saline (n= 6). On the day of the experiment the rats were transported in their home cage to the adjacent experiment room one hour prior to receiving the injections of LPS or saline). This time point was chosen to minimize c-Fos induction due to transport to the procedure room. Ninety minutes following LPS or saline injection, each animal was placed in the open field (black box of 80 × 80 cm and 50 cm high walls) and allowed to freely explore the arena for 10 minutes. The movements were recorded with a digital camcorder mounted above the maze. After 10 minutes the rats were removed and returned to their home cage. The recorded movements were analyzed using video tracking software (Ethovision Version 3.1 Noldus, Leesburg, VA). The total distance moved (in centimeters) and average locomotion speed was calculated for each animal.

Elevated plus maze (DVC inactivation study)—During the 4 days prior to testing, the animals were handled and mildly restrained to habituate them to the infusion procedure. The rats were randomly assigned to the treatment groups, using a two-by-two factorial design yielding four groups: DVC bupivacaine/LPS (n=5), DVC bupivacaine/saline (n=4), DVC vehicle/LPS (n=4) and DVC vehicle/saline (n= 4). On the day of the experiment the rats were transported in their home cage to the adjacent experiment room between 30 minutes

and hour before receiving the brain infusions and the injections of LPS or saline. The animals were mildly restrained by hand and beveled injector cannulae (33-gauge, Plastics One) connected to 10 μ l Hamilton syringes via PE20 tubing were inserted that extended 1 mm below tip of the guide cannula to extend bilaterally into the DVC. Thereafter, an automated syringe pump (Kd-Scientific, Holliston, MA) infused the nerve block bupivacaine (0.5%, Marcain, Abbot Laboratories, North Chicago) or sterile saline at a rate of 0.1 μ l/min over a 5 min period (final volume administered: 0.5 μ l). After completion of the infusion, the injectors were left in place for 2 minutes before removal after which the animals received an i.p. injection of either LPS or sterile saline, and were then returned to their home cage, where they were observed for signs of illness or distress. Approximately 90 minutes after i.p. injection, each rat from the test group was placed in the center of the elevated plus maze and allowed to explore the maze for 5 minutes. After the test, the rats were returned to their home cage and sacrificed by perfusion fixation one hour later, for preparation of the brain for functional neuroanatomical analysis. Behavioral data from this experiment, showing that DVC bupivacaine prevents LPS-induced exploratory behavior deficits, has been previously reported (Gaykema et al., 2008). Here we report the c-Fos expression patterns concomitant with the behavior changes.

2.5. Perfusion fixation and tissue preparation

The animals were deeply anesthetized with an injection of pentobarbital (60 mg/kg), and then perfused through the aorta with saline (100 ml) followed by fixative (freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer with 15% saturated picric acid, pH 7.4). The brains were subsequently removed and post-fixed in the same fixative overnight, stored in 0.1 M phosphate buffer containing 0.1% sodium azide, blocked into 4 parts and sectioned in the coronal plane on a Vibratome into free-floating sections at a thickness of 50 μ m. Brain sections were collected serially in one-in-six sets in six-well plates where stored in 0.1 M PB containing 0.1% sodium azide at 4 °C. All sets of brain tissue sections selected for immunostaining were subjected to a pretreatment protocol aimed at reducing staining background and improving quality of staining. Sections were first pretreated in 0.1% sodium borohydrate (NaBH_4) in phosphate-buffered saline (PBS, pH = 7.4) for 10 minutes to reduce remaining free aldehydes, followed by immersion in 0.3% hydrogen peroxide with 0.1% sodium azide for 30 minutes to block endogenous peroxidase activity. Lastly, the sections were immersed overnight in Fab' fragment of anti-rat IgG (Jackson ImmunoResearch Labs, Inc., West Grove PA, 1:1000) diluted in PBS containing 0.1% sodium azide and 0.5% Triton X-100 (incubation buffer). Between each step sections were rinsed three times in PBS.

2.6. Immunostaining procedures

Antibody solutions were made up in incubation buffer (PBS + 0.1% sodium azide and 0.5% Triton X-100). Vector ABC Elite kit reagents were made up in PBS with 0.1% Triton X-100. All incubations were followed by rinses in PBS. Staining procedures were completed with 3'-diaminobenzidine (DAB) dissolved in 0.05M Tris-HCl solution (pH = 7.6). The staining was accomplished with a modified glucose oxidase protocol to generate hydrogen peroxide in the incubation solution. Upon completion, all sections were mounted, dehydrated in ethanol and histoclear, and coverslipped.

c-Fos immunohistochemistry—The first and fourth series of serially collected coronal sections through the brain were stained for c-Fos immunoreactivity (-ir) as previously described (Goehler et al., 2005). Sections were incubated in anti-Fos (Ab5, Oncogene, Cambridge, MA, 1:50,000) for 72 hours followed by overnight incubation in biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch, 1:1,000) and avidin-biotin-peroxidase complex (Vector ABC Elite kit, 1:500) for 4 hours. Staining for c-Fos-ir was completed

using nickel-enhanced 3,3'-diaminobenzidine (DAB, 0.02%, nickelous ammonium sulfate 0.15%) in Tris-HCl (0.05M, pH 7.6) yielding a black reaction product.

Dopamine beta-hydroxylase (DBH) immunostaining—To assess the extent of the depletion of noradrenergic innervation in the DSAP-injected animals as compared to the control injected ones, one series of sections through the brainstem, including main catecholaminergic cell groups of the NTS, VLM and locus coeruleus (LC), and forebrain was stained for dopamine β -hydroxylase (DBH) -ir, a marker for noradrenergic and adrenergic neurons. One set of sections was incubated in mouse anti-DBH (Millipore/Chemicon, MAB308, 1:5,000, 48h), followed by biotinylated goat anti-mouse IgG (1:1,000, overnight) and ABC (1:500, overnight). DBH-ir was visualized using nickel-enhanced DAB (as described above) to yield crisp black axonal branches, varicosities, and terminal boutons. This description yields a set of brain regions that could provide a link between immune-neural interface in the caudal medulla and the brain regions involved in motor behavior.

Double-labeling for c-Fos and DBH immunoreactivity—The extent of the DSAP-induced loss of immune-responsive caudal medullary catecholamine neurons (cell bodies) was assessed in one set of sections through the entire rostrocaudal extent of. This analysis provides the source of catecholamine input relevant to the effects of DSAP on sickness behavior. That is: which catecholamine neurons of the several catecholamine groups including those in the VLM, NTS and LC, provides the functional link? In addition, to assess the extent to which DVC inactivation prevented activation of catecholaminergic neurons in the VLM, and thus verify the idea that behavioral effects of DVC inactivation are mediated in part by ascending projections from the VLM, we quantified numbers of c-Fos expressing and total numbers of DBH-ir neurons in animals from the DVC inactivation experiment. Following c-Fos immunostaining (yielding a black nuclear label as described previously), sections were incubated in mouse anti-DBH (1:10,000 overnight), biotinylated goat anti-mouse IgG (1:500), and ABC (Vector, 1:500), and finally reacted with DAB (0.04%) yielding a reddish-brown cytoplasmic staining.

Double staining for c-Fos and histidine decarboxylase (HDC), orexin, or tyrosine hydroxylase (TH) immunoreactivity—Our previous studies had identified histaminergic neurons in the ventral tuberomammillary region (TMV), and orexin neurons in the lateral hypothalamus as potential targets for immune-responsive medullary projections that might contribute to behavioral deficits (Gaykema et al., 2008; Gaykema and Goehler, 2009). A series of sections through the hypothalamus were stained for c-Fos as described above and subsequently split into a set of sections through the portion of the hypothalamus that contains orexin neurons (roughly from 2.4 to 3.6 mm caudal to bregma) and a more caudal set through the posterior hypothalamus that includes the histaminergic neurons in the ventral tuberomammillary nucleus (3.7 to 4.9 mm caudal to bregma), which were then processed for orexin-A (Orx-A) and histidine decarboxylase (HDC) immunoreactivity, respectively. After immersion in 0.3% hydrogen peroxide for 30 min, sections from the first set were incubated in solutions containing rabbit anti-orexin A (PC362, Calbiochem/EMD Biosciences, San Diego, CA, 1:2,000, 48h) and biotinylated goat anti-rabbit IgG (1:1,000, overnight). Sections from the second set were incubated in solutions containing guinea pig anti-HDC (H5102, US Biological, Swampscott, MA, 1:5,000, 48h), and biotinylated goat anti-guinea pig IgG (Jackson, 1:1,000, overnight). Both sets were then incubated in ABC (Vector Labs, 1:500), and finally reacted with regular DAB (0.04%) yielding a reddish-brown cytoplasmic staining. Prior adsorption of the primary antibody solution in excess control peptide (10 μ g/ml C terminal fragment 14-33 of human Orx-A) completely abolished its immunostaining.

Based on the well-established role of dopamine in motor behavior, another set of sections through the midbrain region that harbors the substantia nigra and ventral tegmental area was stained for tyrosine hydroxylase (TH)-ir following first staining for c-Fos-ir, to identify dopaminergic neuronal cell bodies. After completion of the c-Fos immunostaining, sections were incubated in mouse anti-TH (Imunostar, cat. 22941, 1:10,000), followed by biotinylated goat anti-mouse IgG (1:1000), and ABC (Vector, 1:750), and finally reacted with DAB (0.04%) yielding a reddish-brown cytoplasmic staining.

2.7. Microscopy and brain tissue analysis

The sections were examined with an Olympus BX51 microscope, and digital images were captured using 4×, 10×, and 20× objectives and a Magnafire digital camera (Optronics, Goleta, CA) controlled by a G4 Apple Power Mac (Mac OS 9.2). Digital images were collected in either grey scale (single c-Fos staining) or in color (double staining). Images were then resized, adjusted for brightness and contrast, and labeled in Adobe Photoshop 9.0 (CS2, Adobe Systems, Mountain View, CA). Sets of sections stained for c-Fos-ir alone were used for quantitative assessment by counting c-Fos-ir profiles in the selected brain areas using the 10× objective. Sections double-stained for c-Fos and cytoplasmic phenotypic markers (orexin-A and DBH) were used for manual counting using the 20× objective and with the guidance of a 10× ocular containing a 10×10 division rectangular grid.

Quantitative assessments of c-Fos immunoreactivity—In selected brain regions, c-Fos-labeled cell nuclei in selected brain regions were counted using NIH Image (v. 1.61). The selected regions were outlined based on landmark features with the aid of the Rat Brain Atlas (Paxinos and Watson, 1998). Counts were performed bilaterally in evenly spaced sections 150 µm or 300 µm apart. Counts from both hemispheres and each section used were combined to yield a total number for each brain region. The following regions were selected based on strongly increased c-Fos expression associated with the exploratory behavior task: the dorsomedial caudate putamen (CPu, 4 sections 150 µm apart, 0.0-0.6 mm caudal to bregma; counts were done within a 810 by 640 µm rectangle area just deep to the wall of the lateral ventricle), the dorsal hippocampus (1 section, 3.2 mm caudal to bregma; counts from subdivisions [dentate gyrus, hilus, CA3 and CA1] were combined representing the hippocampus proper), the ventral tuberomammillary nucleus (TMV, 6 sections 150 µm apart, 3.7-4.9 mm caudal to bregma), and the rostral portion of the ventral tegmental area (VTA, 5.2-5.5 mm caudal to bregma).

The following brain regions were selected based on their response to LPS challenge with strong increase in c-Fos expression: the oval (dorsolateral) portion of bed nucleus of the stria terminalis (BSTov, 2 sections 150 µm apart, +/-1.2 mm relative to bregma), the lateral portion of the central amygdala (CEA, 4 sections 300 µm apart, 2.0-3.2 mm caudal to bregma), the hypothalamic paraventricular nucleus (PVN, 3 sections 150 µm apart, 1.5-2.0 mm caudal to bregma), and the external lateral subnuclei of the parabrachial nucleus (PBel, 2 sections 150 µm apart, 8.9-9.2 mm caudal to bregma).

Double staining for c-Fos and orexin-A immunoreactivity—Orexin-A-ir cells with and without nuclear Fos labeling were counted manually in sections through the lateral hypothalamus between 3.0 and 3.5 mm caudal to bregma. From each animal in the DSAP study, 2 sections were included in the analysis that were spaced 300 µm apart that contain the bulk of Orx-A-ir cells distributed throughout the lateral and perifornical hypothalamus (LH, PF) with some extending into the adjacent dorsomedial hypothalamus (DMH). The cell cluster was divided into lateral and medial portions by a vertical line through the fornix and counted separately in both hemispheres. This division relates to a possible dichotomy in function and connectivity of orexin neurons (Harris and Aston-Jones, 2006) and the large

difference between medial and lateral orexin cells in their expression of c-Fos (Gaykema and Goehler, 2009). Orx-A-labeled cell bodies that clearly displayed cell nuclei were counted as either c-Fos positive or negative. Total numbers for lateral and medial portions were then determined by summation of the counts from each hemisphere and both sections. The percentage of Orx-A cells double-labeled for c-Fos was then calculated for each animal based on numbers of double-labeled cells and all Orx-A cells.

Double staining for c-Fos and DBH immunoreactivity—DBH-ir neurons in the caudal medulla were manually counted in a series of evenly spaced sections (300 μ m apart) to assess the extent of the loss of noradrenergic cell populations in the NTS and VLM due to hypothalamic microinjection of DSAP. The NTS and VLM were subdivided into 3 rostrocaudal parts to determine more specifically the portions of the NTS and VLM that were depleted of DBH cells the most by the DSAP treatment. DBH-labeled cell bodies with and without nuclear c-Fos staining were counted separately to assess whether double-labeled cells (conceivably LPS-responsive) cells were disproportionally affected. For this purpose, counts were assessed in LPS-treated rats that had received DSAP or control (unconjugated SAP) micro-injections. DBH single-labeled and DBH-c-Fos double-labeled cells were counted bilaterally in every sixth sections (300 μ m apart) throughout the rostrocaudal extent the VLM and NTS. The DBH-labeled cell column in the NTS was further subdivided into rostral (12.5-13.7 mm caudal to bregma, 6 sections rostral to the area postrema), middle (13.7-14.3 mm caudal to bregma, 3 sections at the level of and immediately caudal to the AP) and caudal-most portions (14.3-15.5 mm caudal to bregma, 5 sections). Similarly, the VLM was subdivided into a rostral third (12.0-13.2 mm caudal to bregma, a middle portion (13.2-14.5 mm caudal to bregma), and the portion caudal to the pyramidal decussation (14.6-15.8 mm caudal to bregma). The number of DBH neurons with and without nuclear c-Fos staining were counted bilaterally throughout the VLM in the animals from the DVC inactivation experiment to determine the effects on the induction of c-Fos in these cells by LPS challenge. Every sixth section (between 12.5-15.5 mm caudal to bregma) was included in the summation of the counts to yield total numbers in each rat.

2.8. Statistics

The behavioral data (open field distance travelled and average speed) as well as the c-Fos-labeled cell counts in each selected brain region were analyzed by 2-way analyses of variance (ANOVA) for the between-subject variables (i.p. challenge and DSAP lesion or DVC infusion). In the caudal brainstem, DBH-labeled and DBH-c-Fos double cell counts in LPS-treated rats were analyzed using ANOVA with DSAP lesion status as the independent variable. Data are expressed as mean $\pm$ SEM for each brain region and pair-wise post hoc comparisons were done using Fisher's protected least significant difference. If the ANOVA revealed statistically significant main effects and/or interaction. Significant differences were determined by a probability of 0.05 or less.

3. Results

3.1. Hypothalamic DSAP injection inhibits LPS-induced exploratory behavior deficits

Whereas LPS challenge drastically reduced locomotor behavior in the open field (less distance traveled and lower movement velocity) as compared to saline-treated animals, such behavioral deficits were dramatically diminished in LPS-treated rats that had previously received DSAP microinjection into the PVN (Fig. 2). Analysis of variance revealed statistically significant interaction terms for both distance traveled [$F(1,20) = 8.0, p < 0.01$] and mean velocity [$F(1,20) = 9.2, p < 0.01$] indicating a much reduced effect of LPS on behavioral activity in rats that previously received DSAP micro-injection aimed at the PVN resulting in the depletion of hypothalamic noradrenergic input (see below).

3.2. LPS challenge suppresses exploratory behavior-related c-Fos expression in brain regions involved in motor function, arousal and motivation: prevention by both DSAP lesion and DVC inactivation

In saline-treated animals, exploratory behavioral activity (e.g., locomotion, rearing) on both the elevated plus maze (EPM) and open field (OF) was associated with the induction of neuronal nuclear c-Fos protein within many brain regions associated with behavioral arousal, exploration-associated sensory-motor activity, and “positive motivation”. These regions included large areas of the cerebral cortex and thalamus, accumbens nucleus, dorsal striatum, medial septum, anterior, lateral, dorsomedial and supramammillary regions of the hypothalamus, and the periaqueductal grey. The patterns of c-Fos expression were essentially identical in both behavioral experimental conditions. In LPS-treated rats from the control groups (in both DSAP lesion and DVC inactivation experiments), c-Fos expression was generally reduced in these brain regions. In the open field-tested rats that received SAP control microinjection, comparisons of c-Fos counts revealed significant LPS-related reduction in numbers of c-Fos-positive neuronal nuclei in selected brain regions (as illustrated in Figs 3, 4), which are the dorsomedial caudate putamen (CPu dm), dorsal hippocampus (HPCd), rostral ventral tegmental area (VTAr, including the interfascicular nucleus), the ventral tuberomammillary nucleus (TMV, containing the compact histaminergic cell groups), and the orexin cell group in the peduncular lateral and perifornical hypothalamus (LH/PF) (Figs. 3A-G', 5A). In a similar fashion, LPS challenge reduced the numbers of c-Fos-ir cells in the dorsal CPu, dorsal HPC, and VTA of EPM-tested rats when they received DVC infusion of saline (Figs 4A-D', 5B). We previously reported the suppressive effects of LPS on c-Fos expression in the histaminergic cell groups of these EPM-tested rats (Gaykema et al., 2008).

In the DSAP-lesioned animals that were tested in the open field, LPS challenge did not alter the behavior-associated c-Fos induction, as the expression levels of c-Fos remained strong in the dorsal striatum, dorsal hippocampus, rostral VTA, the histaminergic TMV, and the orexin cell groups in the LH and PF, and indistinguishable from the saline-injected DSAP rats (Fig. 3A"-G", 5A). Similarly, DVC infusion of bupivacaine prior to LPS challenge prevented reduction in EPM exploration-associated c-Fos expression in the dorsal striatum, hippocampus, and rostral VTA, as levels of c-Fos were nearly identical to that in the saline-injected counterparts (Figs. 4A"-D", 5B). The prevention by DVC inactivation of LPS-induced suppression of behavior-related c-Fos parallels the same effects reported on the histaminergic neurons of the TMV (Gaykema et al., 2008). Thus, both interventions (DSAP lesion and DVC inactivation) prevented LPS-induced decline of exploratory behavior-associated c-Fos expression that was seen in the LPS-treated groups without intervention, as demonstrated by significant treatment and intervention (DSAP lesion or DVC inactivation) interactions in 2-way ANOVA analyses (summarized in tables 1, 2).

3.3. DVC inactivation attenuates LPS increased c-Fos expression in central autonomic network nuclei, but DSAP lesion abrogates LPS-induced activation in the PVN only

Although DSAP lesion and DVC inactivation both blocked the effects of LPS on behavior-related c-Fos expression (described above), these interventions showed rather diverging effects on the pattern of LPS-induced c-Fos expression in central autonomic network nuclei (Figs. 6-8, statistics are summarized in Tables 1, 2). LPS treatment strongly increased c-Fos expression in the PVN, oval BST, CEA, and the external lateral portion of the parabrachial nucleus (PBel), which are brain regions associated with central viscerosensory processing and neuroendocrine and autonomic feedback, consistent with many previous reports (Wan et al., 1994; Stone et al., 2006; Gaykema et al., 2007; Elmquist and Saper, 1996; Elmquist et al., 1996; Frenois et al., 2007). DSAP pretreatment inhibited LPS-induced c-Fos protein only in the PVN, whereas c-Fos induction by LPS treatment in the PBel, CEA and BSTov was

unaffected by DSAP lesion (Figs. 6, 8A). In contrast, DVC inactivation significantly reduced LPS-induced c-Fos expression in all these regions, albeit the blocking effect was strongest in the PVN and partial in the PBel, BSTov, and CEA (Fig. 7, 8B). Thus, targeted loss of noradrenergic input to the PVN and its collateral projections (as described below) interrupted the c-Fos induction due to LPS challenge specifically in the PVN, whereas DVC inactivation led to more widespread attenuation of LPS-related neuronal activation in the PBel, CEA, and BST in addition to the PVN. In addition a strong attenuation after DVC inactivation of c-Fos expression due to LPS treatment occurred in the DBH-labeled neurons of the VLM (which are the predominant target of DSAP injected into the PVN, see below; Fig 8B).

3.4 Catecholamine neurons relevant for sickness-induced inhibition of exploratory behavior primarily reside in the VLM and the rostral/middle portions of the NTS

To determine which of catecholamine neurons were lost after bilateral hypothalamic DSAP injection, and thus potentially providing a major contribution to the effects of LPS on exploratory behavior and associated c-Fos expression, we assessed the DSAP-induced decline in the population of DBH-positive neurons in the NTS and the VLM that are potentially involved in the propagation of the LPS response in forebrain targets (Figs. 9, 10). In comparison with LPS-treated SAP control rats, the numbers of double-labeled (DBH- and c-Fos-positive) neurons in DSAP animals after LPS challenge were drastically reduced throughout the VLM (Fig. 9B, 10A), but the reduction of DBH-c-Fos double-labeled cells within the NTS was restricted to its rostral and middle portions (at the level of the obex and area postrema, Fig. 9A, 10B). In the caudal commissural NTS, DSAP lesion had no significant effect on numbers of double-labeled neurons. However, double-labeled cells in this caudal portion of the NTS neither depended on LPS treatment *per se* as similar numbers occurred in both LPS and saline-injected animals (data not shown). Interestingly, the difference between DSAP and control animals in numbers of DBH-positive, but c-Fos-negative neurons (i.e., noradrenergic/adrenergic but *unresponsive* to LPS challenge) did not reach significance in any part of the VLM or the NTS (Fig. 10C, D), although there was a tendency towards depletion of this subpopulation within the VLM. These findings indicate that the medullary catecholaminergic neurons that target the PVN (and are destroyed by DSAP) are disproportionately responsive to LPS challenge, and the majority are distributed throughout much of the VLM (except for the most rostral portion) whereas a smaller portion reside within the rostral and middle portions of the NTS.

In contrast to the marked reduction in DBH-c-Fos double-labeled neurons in the caudal medulla (Fig. 9A-B'), DSAP injection targeted at the PVN had little or no effect on DBH-positive neurons in the LC (Fig. 9C,C'), which is consistent with the observation that little or no loss of DBH axonal and terminal labeling occurred in forebrain regions that receive exclusive noradrenergic input from the LC, including the cerebral cortex, hippocampus, and anteroventral and lateral thalamic regions (data not shown). Other noradrenergic groups, such as the C5 and C7 groups in the pons and rostral ventrolateral medulla, respectively, were not affected by DSAP either, consistent with the fact that these are known to target the spinal cord rather than the PVN (Tucker et al., 1987; Minson et al., 1990). Thus, the majority of DBH-positive neurons targeting the medial hypothalamus derive from neuronal populations in the caudal ventrolateral and dorsomedial medulla, i.e., the adrenergic C1 and noradrenergic A1 cell groups of the VLM and C2 and A2 cell groups of the NTS, consistent with previous studies utilizing DSAP lesion (Bienkowski and Rinaman, 2008; Ritter et al., 2003) and tract-tracing methods (Gaykema et al., 2007; Ericsson et al., 1994; Palkovits et al., 1992).

3.5. Possible targets of DSAP-lesioned catecholaminergic projections that provide a link between immune challenge and behavior

Although the PVN was targeted with bilateral microinjection of DSAP, depletion of DBH-labeled input was not restricted to this nucleus, but extended to a larger range of brain structures within the ventral and medial parts of the fore- and midbrain. These regions receive collateral input from the same neurons that innervate the PVN. Hypothalamic DSAP injection dramatically reduced DBH axonal and terminal labeling throughout most of the hypothalamus, including the lateral, dorsomedial, perifornical, posterior, tuberomammillary and supramammillary regions (Fig. 11A-E, A'-E'). Outside the hypothalamus, a noticeable decrease in density of DBH-positive fibers was evident in the paraventricular nucleus of the thalamus (PVT; Fig. 11 F, F'), and also in dopaminergic cell-rich regions of the ventral midbrain: the rostral VTA, medial substantia nigra (Fig. 11G, G'), and retrorubial field (Fig. 11I, I'). Other regions of the midbrain showing partially reduced density of DBH fiber and terminal labeling include the lateral and ventral portions of the periaquiductal grey and dorsal raphe nucleus (Fig. 11G, G', H, H'), but this depletion was noticeable only in the rostral part of the PAG. The areas depleted of noradrenergic input after DSAP microinjection into the PVN extended well beyond the PVN, and have in common functional roles in modifying motivational behaviors and behavioral arousal. Thus, any of these regions could constitute relevant targets where LPS-responsive neurons exert depressive effects on motor behaviors and/or arousal states.

4. Discussion

To test the contribution of caudal brainstem-derived pathways to motor/exploratory behavior components of sickness, we compared effects of DVC inactivation and targeted lesion of the catecholaminergic medulla-hypothalamic pathway on exploratory behavior (which provides an index of motivation and “fatigue”) and associated brain activation patterns assessed by induction of c-Fos protein. LPS treatment dramatically reduced exploratory behavior, and produced a pattern of c-Fos induction whereby brain regions associated with endocrine and autonomic adjustments (PVN, BST, CEA) evinced increased evidence of activation, whereas activation in forebrain regions involved in locomotion/exploratory behavior was reduced. Both DVC inactivation and targeted lesion of catecholaminergic projections with DSAP prevented reductions in exploratory behavior, and completely prevented the suppressive LPS effects on c-Fos expression in the hippocampus, VTA, and dorsal striatum. Both interventions strongly inhibit LPS-induced c-Fos expression in the PVN. However, in the PBel, CEA and BSTov the interventions diverged on their effects on the response to LPS challenge. DVC inactivation significantly blunted LPS-induced c-Fos expression in these areas (reduction by approximately 50%), but DSAP lesion had no effect. Thus, the PBel-CEA-BSTov pathway likely does not contribute to behavioral (locomotor) effects of LPS treatment, providing evidence for the idea that separate neural circuits mediate specific sickness responses. The findings support the idea that functional inhibition of neuronal populations associated with exploratory behavior, including the VTA, the hypothalamus (e.g., TMV and LH/PF), dorsal striatum and hippocampus could contribute to the behavioral deficits occurring during illness due to inflammatory insult, and that caudal medullary catecholamine projections originating in the VLM and NTS provide the link between immune-responsive brainstem regions and forebrain areas that control behavior.

4.1. Methodological considerations

Higher levels of exploratory behavior was associated with significantly increased expression of c-Fos protein in brain regions previously implicated in arousal and motor behavior (e.g. the VTA, TMV, dorsal striatum, and hippocampus). This finding supports the idea that c-Fos expression in these regions serves as a reasonable neurobiological correlate with

measures of behavioral activity. However, the functional significance for this association cannot be conclusively ascertained from these studies. Increased functional activity within these selected brain regions could reflect their mediating role in the behavioral response to exposure to the novel maze environment, but could also reflect an increased sensory processing demand in response to increased behavioral activity. Similarly, whereas the “rescue” from LPS effects on behavior and c-Fos expression patterns by prior DSAP lesion or DVC inactivation treatment supports the idea that caudal medullary noradrenergic and adrenergic neurons contribute importantly to the process of behavioral suppression, how this relates to changes in brain activation patterns is unknown. That is: the reduction of neuronal activity in e.g. the hippocampus and striatum may constitute a mechanism for the reduced motor behavior, or it could represent the correlates of reduced motor behavior induced by a different mechanism. Nonetheless, the reduced psychomotor deficits observed in LPS-treated animals in whom the medullary catecholamine input to the midbrain and hypothalamus was lesioned provides strong evidence that medullary catecholaminergic pathways influence motor behavior in the context of systemic inflammation.

The DSAP injections into the PVN led to both near total depletion of catecholamine fibers and “rescue” of locomotor behavior deficits following LPS treatment. Although taken together these findings implicate the PVN a key nodal point in the circuitry mediating sickness behavior, such a role cannot be established from this data, based on the fact that other brain regions, which were depleted of catecholaminergic input through collateral projections, could also contribute importantly to behavioral effects of LPS.

4.2. Parallel pathways for sickness symptoms

As noted, our previous findings (Marvel et al., 2004) had provided evidence that the immune-responsive DVC in the caudal brainstem contributes importantly to brain and behavioral responses to immune challenge. However, the DVC drives multiple immune-responsive ascending pathways, including a pathway through the VLM, that target pedunculo-pontine, hypothalamic and “limbic” regions including the PBel, BSTov, CEA, and PVN (Gaykema et al., 2007). From that work it could not be ascertained which of the ascending pathways (e.g. catecholaminergic vs. non-catecholaminergic) could be important for inhibition of behavioral activity. Both DSAP-induced noradrenergic lesion and DVC inactivation serve to reduce motor deficits associated with LPS challenge, providing further evidence that ascending pathways emanating from the caudal medulla contribute to behavioral symptoms of sickness. Because the targeted lesion of caudal medullary catecholaminergic neurons that innervate the hypothalamus was sufficient to prevent LPS-induced deficits in exploratory behavior, these catecholamine neurons involved are likely to provide the principal link between viscerosensory relay neurons that interface with immune-related signals and the fore- and midbrain neuronal ensembles that drive behavioral responses in the context of environmental and homeostatic demands.

The present findings further indicate that the parallel non-catecholaminergic pathway involving the PBel, CEA, and BSTov plays a lesser role in the behavioral aspects of sickness. Despite the suppression of c-Fos response to LPS in these structures after inactivation of the DVC, their response to LPS was unaffected by the DSAP lesion, revealing a dissociation of LPS-associated neuronal activation and behavioral response. Put another way: because LPS-treated animals that engaged in exploratory behavior still showed activation of the PBel-CEA-BST pathway, these findings imply that this pathway does not contribute to suppression of behavioral activity. This pathway may contribute to the other aspects of sickness however (Konsman et al., 2008), perhaps related to the negative mood associated with the sickness experience, based on the established links between the function of the extended amygdala (including the BST), psychological stress, and anxiety (Davis et al., 2010). Additionally, or alternatively, because the CEA is part of the network that ensures

appropriate physiological (autonomic) adjustments to stressors, activation in this pathway may not be related to mood or behavior. Taken together, the current findings provide evidence of functional specificity of immune-responsive neural circuits in the brain, consistent with the assertions of Stone et al. (2006) and others (e.g. Konsman et al., 2008; Belevych et al., 2010).

Brain-mediated consequences of challenge with LPS involve autonomic and neuroendocrine responses related to support for host defense, and a set of behavioral responses that serve to promote recuperation (e.g. reduced activity) limit exposure to toxic substances (reduction in food and water intake) and mood disturbances. The neurobiological substrates for these functions overlap (or are redundant) but at the same time include unique brain regions specific to each behavior or function. For instance, the DVC is broadly responsive to immune challenges, and gives rise to multiple ascending neural information pathways, that overlap in some regions, such as the PVN, but overall exhibit unique projection patterns. This “uniquely redundant” arrangement can be considered an organizing feature of physiological regulatory systems, and allows for the coordination of multiple physiological and behavioral adjustments, allowing flexibility in brain-mediated responses supporting host defense.

4.3. The caudal medullary catecholaminergic “danger pathway” drives behavioral aspects of sickness

Unlike the DVC, which responds to a wide variety of viscerosensory stimuli, (first order relay /homeostatic control; e.g. satiety) catecholaminergic neurons of the VLM and the rostral NTS seem to function more specifically as a “danger pathway”, in that these neurons respond exclusively to stimuli that signal stress or physiological danger related to deviations from the homeostatic equilibrium. The VLM catecholaminergic (referred to as adrenergic C1 and noradrenergic A1) neurons respond to virtually all dangerous physiological conditions evaluated, including cancer (Mravec et al. 2009), pathogenic bacteria in the gut (Gaykema et al., 2004; Goehler et al., 2005), hypovolemia and hypotension (Dun et al., 1993, Murphy et al., 1994), hypoxia (Smith et al., 1995), glucoprivic challenge (Ritter & Dinh, 1994; Ritter et al., 2006), intragastric gavage of poison-like bitter taste receptor ligands, (Yamamoto & Sawa, 2000), anxiogenic doses of yohimbine (Myers et al., 2005), joint inflammation (Pinto et al. 2007), opiate withdrawal (Laorden et al., 2002), seizures (Kantor et al., 1996, Silveira et al., 2000), pharmacologic levels of CCK (Schreihöfer et al., 1997), and inescapable stress (Goebel et al., 2009). However, these VLM and rostral NTS catecholaminergic neurons do not respond to non-dangerous viscerosensory stimuli, such as satiating ingestion of sweetened milk (Gaykema et al., 2009) or a meal (Rinaman et al., 1998), even when they produce substantial stomach distension. Taken together the findings support the idea that many caudal medullary catecholamine neurons that give rise to ascending projections to the PVN, and collateral targets in fore- and midbrain, function to signal adverse or dangerous physiological conditions, and allow these targets to initiate a set of coordinated compensatory responses not only in the neuroendocrine and autonomic modalities (in which the PVN plays a central role), but also in the realm of behavioral adaptation.

4.4. How the caudal medullary “danger pathway” integrates with neural circuits mediating behavior

The contribution of VLM and NTS catecholaminergic neurons to the neuroendocrine (i.e., hypothalamo-pituitary-adrenal [HPA] axis) response to systemic LPS or interleukin-1 exposure has previously been advanced using tract tracing, knife cuts, and selective noradrenergic lesion (Bienkowski and Rinaman, 2008; Ericsson et al., 1994; Gaykema et al., 2007; Nance and MacNeil, 2001; Schiltz and Sawchenko, 2007), but a more general role of

these cell groups in *behavioral* responses to immune challenges has been less appreciated. Our findings show that the LPS-responsive caudal medullary catecholaminergic neurons that target the PVN, and hence were eliminated by DSAP, also give rise to extensive collaterals to other parts of the hypothalamus (e.g., LH, PF, DMH, TMV), the midline thalamus including the PVT and the midbrain PAG. Notably, we observed reduced noradrenergic (DBH-labeled) innervation of the dopamine neuron-rich VTA and RRF, consistent with a recent tract-tracing study reporting such innervation (Mejias-Aponte et al., 2009). Beyond the PVN, the regions that show pronounced depletion of collateral noradrenergic input constitute prime candidates for the control and mediation of behavior, and thus represent potential substrates through which branching catecholaminergic projections that arise from the caudal medulla can induce changes in behavior in the event of an inflammatory insult.

4.5. Targets of caudal brainstem immune-responsive projections: arousal networks could mediate sickness behavior

The next step(s) in the circuit linking immune related activation of the medullary viscerosensory regions with forebrain regions that mediate specific behaviors play crucial roles in the control of brain responses to inflammatory challenges. Because the catecholamine neurons in the caudal medulla do not project to the hippocampus and dorsal striatum directly, their influence on these structures must be indirect. The many targets of the medullary catecholaminergic neurons (i.e. those areas showing depletion after DSAP injections) include many regions important in arousal and regulation of behavior, including the parvocellular PVN, which coordinates neuroendocrine and autonomic responses to stress, the histaminergic neurons of the TMV important for behavioral arousal (Gaykema et al. 2008; Valdes et al. 2005), and the orexin/hypocretin neurons of the LH and PF involved in motivation, reward-related behaviors, and arousal (Harris and Aston-Jones, 2006). In turn, these systems provide extensive projections to, e.g., the striatum, hippocampus and cerebral cortex. Depletion of DBH staining also occurred in areas in the midbrain, including the VTA, where the dopamine neurons are integrally involved in motor planning motivation and reward properties (Duzel et al. 2009), and the periaqueductal grey. These cell groups have in common widespread projections throughout the forebrain, and they contribute to different aspects of behavioral arousal. Because sickness behavior involves a generalized reduction in behavioral activities (e.g., ingestive, social, and exploratory behaviors), increases in sleep, and impairments in cognitive function, neurons involved in behavioral arousal make attractive candidates as nodes that integrate information about peripheral inflammation relayed by the medullary viscerosensory circuitry into the various neural networks that mediate behavior.

Although the precise neural substrates of specific behaviors seem to vary, the dorsal striatum contributes to motor aspects of motivated behavior (Balleine and O'Doherty, 2010), and is a critical regulator for motor aspects of behavior (Kravitz et al., 2010). The hippocampus is well recognized to contribute to spatial navigation and exploratory behavior, as well as aspects of cognitive function (Redish, 2001). The finding that DSAP pretreatment largely “rescued” (prevented LPS from suppressing) c-Fos expression in the dorsal striatum and hippocampus concomitant with exploratory behavior supports a role for the “danger pathway” in behavioral, and possibly cognitive, responses to physiological challenges. Thus, the findings reported here indicate that caudal medullary catecholamine neurons exert a broader influence on brain responses to physiological challenges (such as infection) than previously recognized, and imply that reductions in behavioral activity may be an important response to adverse physiological challenges.

4.6. Perspectives

Reduction of behavior is hallmark of recuperation seen in response to stress or immune challenge (Dantzer and Kelley, 2007; Miller, 2009). Indeed, reduced behavior is viewed as necessary for conserving energy need for effective wound healing or host defense. Interference with the brainstem “danger pathway” which responds to most, if not all, dangerous physiological conditions, largely prevented reduction in motor behavior associated with immune challenge (i.e. recuperative behavior). This finding supports the idea that recuperative behavior is a generalized response to physiological stress or challenge, and indicates that these findings could be applicable to other experimental paradigms investigating brain responses to physiological or environmental challenges.

The reductions in exploratory behavior concomitant with motor slowing are consistent with the psychological experience of “fatigue”. Although it is a common companion of chronic illness, reliably effective interventions for fatigue are lacking. For instance, in patients treated with immunotherapy, selective serotonin reuptake inhibitors can ameliorate cognitive-affective symptoms of depression, but have no effect on neurovegetative symptoms including fatigue (Morrow et al., 2003; Musselman et al., 2006). Psychostimulants, including methylphenidate and modafinil, can reduce fatigue, assessed using symptom inventory scores (Minton et al., 2008). Psychostimulants boost dopaminergic and possibly orexin and histaminergic function (Young & Geyer, 2010), supporting the role of reduced or suppressed dopamine and histamine function as biobehavioral substrates for fatigue. Indeed, the findings from the study reported here showing reduced activation of neurons in the dopaminergic VTA and histaminergic TMV, and orexin neurons in the LH concomitant with reduced activity in the dorsal striatum, provides further evidence consistent for a role of reduced function of these systems in “fatigue”. The findings that these fatigue-like symptoms can be ameliorated by interference with caudal brainstem projections of catecholamine-containing neurons suggests that effective interventions for fatigue could target function of these neurons.

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Figure 1.

A,B. The destruction of caudal brainstem catecholaminergic projection neurons by micro-injection of the toxin saporin conjugated to anti-DBH into the PVN, which is densely innervated by VLM and NTS noradrenergic and adrenergic neurons (red filled dots and arrows in A), resulting in a loss of such neurons and their axonal projections to the PVN (indicated as open dots in B), with concomitant loss of collateral innervation of additional regions in fore- and midbrain. C. Functional inactivation of the dorsal vagal complex (DVC, including the NTS) which gives rise to non-catecholaminergic projections (in blue) via the PBel to the BST and the CEA, and direct catecholaminergic projections to the PVN (in red).

Figure 2.

A. LPS challenge-induced inhibition of open field exploration is largely reversed by DSAP lesion of noradrenergic projections from the caudal brainstem to the hypothalamus. These effects are reflected in the total distance moved (A) and average velocity (B). Pair-wise comparisons after significant interaction in 2-way ANOVA are as follows: i.p. LPS vs. saline injections: *, $p < 0.05$, *** $p < 0.0001$; DSAP lesion vs. SAP control: ### $p < 0.0005$.

Figure 3.

Prominent exploratory behavior-associated c-Fos expression: DSAP lesion prevents suppression of c-Fos expression by LPS challenge. Representative images from saline-injected rats (left column) show strong c-Fos expression after open field exploration, compared to diminished c-Fos expression in LPS-challenged SAP control rats (middle column), but not in LPS-treated DSAP rats (right column). Regions that show these LPS and DSAP effects include the dorsomedial portion of the caudate putamen (CPu, A-A''), the dorsal hippocampus (B-B'') with a close-up of the the pyramidal cell layer in cornu ammonis in (CA1, C-C''), the lateral and perifornical parts of the hypothalamus (LH, PF, D-D'') where many orexin-A (Orx-A)-positive neurons express c-Fos (inserted micrographs), the ventral tuberomammillary nucleus (VTM, E-E'' and F-F'') harboring histaminergic neurons (E2 and E3 cell groups; inserts show the c-Fos expression within the HDC-labeled somata), and the rostral part of the ventral tegmental area (VTA) and its interfascicular portion (IF), where c-Fos expression is also present within TH-labeled dopaminergic neurons (inserts). The color photomicrograph images depicted in the inserts (D-G'') were taken from series of double-stained sections adjacent to those in the main panels stained for c-Fos IR only. Scale bars are in micrometers. Other abbreviations: DG, dentate gyrus; ec, external capsule; Hil, hilus.

Figure 4.

Exploratory behavior-associated c-Fos expression (left panels A-D, i.p. saline controls) is suppressed by i.p. LPS challenge in rats when infused intra-DVC with saline (middle column A'-D'), but not in rats that received prior intra-DVC bupivacaine (right column A''-D''). Brain regions that show strong exploration-related c-Fos expression include the dorsomedial portion of the caudate putamen (CPu, A-A''), the hippocampus (B-B'') with in particular an effect the pyramidal cell layer in the CA1 (C-C''), and the rostral VTA and IF in D-D''. Scale bar in micrometers. Abbreviations are as in Fig. 3.

Figure 5.

Effects of LPS challenge and the impact of prior DSAP lesion (A) and bupivacaine infusion into the DVC (B, bottom row) on brain c-Fos expression in rats exposed to a novel environment (open field in A, EPM in B). Bar graphs depict mean $\pm$ SEM of total counts of c-Fos-ir profiles in selected brain regions that show prominent c-Fos induction associated with behavioral activity, including: the dorsomedial CPu, the dorsal hippocampus (HPC d), the rostral VTA, the TMV, and the orexin cells extending throughout the LH and PF and into the adjoining dorsomedial hypothalamus (DMH) divided in lateral and medial portions (the latter expressed as percentage of the total numbers of orexin cells; the latter were not different between groups). Pair-wise comparisons after significant interaction in 2-way ANOVA are as follows: i.p. LPS vs. saline injections: *, $p < 0.05$, **, $p < 0.005$, *** $p < 0.0001$; DSAP lesion vs. SAP control (in A) or DVC bupivacaine vs. saline (in B): #, $p < 0.05$, ##, $p < 0.005$.

Figure 6.

LPS-induced c-Fos expression in autonomic and interoceptive stress-related regions is selectively inhibited in the hypothalamic paraventricular nucleus (PVN) by DSAP lesion. Compared to saline injection, LPS challenge in SAP controls induced marked increases in c-Fos expression in the parvocellular part (pv) of the PVN (A,A'), the lateral central amygdala (CEA; B,B'), the oval bed nucleus of the stria terminalis (BST ov; C,C'), and the external lateral portion of the parabrachial nucleus (PBcl; D,D'). DSAP lesion greatly blunted c-Fos induction in the PVN (A'') as compared to the SAP control (A'), but had no effect on LPS-induced c-Fos induction within the other regions (B''-D''). The left panels depict the much lower levels of c-Fos expression in these regions related to the behavioral exploration task (A-D, left panels). Scale bar in A is in micrometers (applies to all panels). Other abbreviations: 3V, third ventricle; LV, lateral ventricle, mg, magnocellular division; PBcl, central lateral division of the parabrachial nucleus; scp, superior cerebellar peduncle (brachium conjunctivum).

Figure 7.

LPS-induced c-Fos expression in autonomic and interoceptive stress-related regions was inhibited by DVC inactivation with bupivacaine. Brain regions that showed marked increase in c-Fos expression after LPS challenge include the PVN (A,A'), the lateral CEA (B,B'), the oval/dorsolateral BST (C,C'), and the PBEL (D,D'). Bupivacaine-infused rats showed greatly blunted c-Fos induction in the PVN (A''), the CEA (B''), BST ov (C''), and PBEL (D''). Scale bar in A is in micrometers (applies to all panels). Abbreviations are as in Fig. 7.

Figure 8.

Effects of LPS challenge and the impact of prior DSAP lesion (A) and bupivacaine infusion into the DVC (B) on brain c-Fos expression in rats exposed to a novel environment (open field in A, EPM in B). Bar graphs depict mean $\pm$ SEM of total counts of c-Fos-ir profiles in selected brain regions that show prominent c-Fos induction in response to LPS challenge, including the PVN, the oval BST, the lateral CEA, and external lateral portions of the PB. DSAP lesion suppresses LPS-induced c-Fos expression only in the PVN (A), whereas DVC inactivation inhibits LPS-related c-Fos induction in each of the brain regions (B) as well as in the DBH-positive neurons of the VLM (expressed as the percentage of the total numbers of DBH cells; total numbers were not different between groups). Pair-wise comparisons

after significant interaction in 2-way ANOVA are as follows: i.p. LPS vs. saline injections: *, $p < 0.05$, **, $p < 0.005$, *** $p < 0.0001$; DSAP lesion vs. SAP control (in A) or DVC bupivacaine vs. saline (in B): #, $p < 0.05$, ##, $p < 0.005$.

Figure 9.

Effects of DSAP microinjection aimed at the PVN on the LPS-responsive DBH-immunoreactive somata in the brainstem. Coronal sections depict both DBH immunolabeling of somata (brown) as well as LPS-induced c-Fos expression (black nuclear stain). In the NTS, loss of DBH somata is modest at the level of the AP but fewer double-labeled cells remain (A). In contrast, most of the double-labeled cells were lost in especially the middle portion of the VLM in DSAP animals (B). In the locus coeruleus (LC) just deep to the fourth ventricle (4V), DBH-ir cells were abundant in both SAP control and DSAP rats (C), indicative of the minimal loss of noradrenergic cells following DSAP injection into the medial hypothalamus. Similarly, no depletion of DBH cells was found in the other noradrenergic cell groups of the rostral medulla and pons (A5 and A7 cell groups, not shown).

Figure 10.

Quantitative analysis of DBH-immunoreactive somata in the VLM and NTS in LPS-treated SAP control and DSAP rats reveal a significant loss of c-Fos-DBH double-labeled neurons (A,B) but not c-Fos-negative DBH cells (C,D). In the VLM, loss of double-labeled neurons occurred at all levels although most prominently in the middle and caudal portions, whereas the rostral-most portion of the VLM showed limited loss of double-labeled cells in DSAP rats. The loss of double-labeled neurons in the NTS was significant in the rostral and mid-portions (at the level of the AP), but not in the caudal (commissural) NTS. DSAP effects: *, $p < 0.05$, **, $p < 0.005$, ***, $p < 0.0001$.

Figure 11.

The extent of the depletion of catecholaminergic innervation as a result of micro-injection of DSAP aimed at the PVN. Photomicrographs arranged in pairs from representative SAP control rats (panels A-I) and DSAP lesioned rats (A'-J') show DBH-ir fiber and terminal labeling and the extent of the depletion due to DSAP lesion in the hypothalamus (A-E) and neighboring fore- and midbrain regions (F-I). Within the hypothalamus, depletion of DBH-ir extended beyond the PVN to the anterior hypothalamic area (AHA), lateral (LH), perifornical (PF), and dorsomedial (DMH) areas (A'-C'), as well as the ventral tuberomammillary nucleus (TMV in D',E'), which latter contains E2 and E3 histaminergic neurons within the dense DBH-ir plexus (shown in more detail in Gaykema et al., 2008). The extra-hypothalamic regions affected by DSAP include the paraventricular thalamus (PVT in F,F'), ventral tegmental area (VTA), lateral and ventrolateral periaqueductal grey (LPAG, VLPAG in G,H), and retrorubal field (RRF in I), all of which show partial depletion. Scale bars are in micrometers. Other abbreviations: 3V, third ventricle; Aq, aqueduct, DR, dorsal raphe; PMD, dorsal premammillary nucleus; PMV, ventral premammillary nucleus.

Figure 12.

Model diagrams outlining potential brain substrates and mechanisms that underlie sickness behavior in response to systemic inflammatory insult (LPS challenge). Panel A depicts the two parallel pathways arising in the caudal brainstem: one non-catecholaminergic that involves the PBel, CEA, and BSTov (in blue), and a catecholaminergic (noradrenergic and adrenergic) pathway aimed at the PVN with collaterals aimed at other regions (in red). The latter involves some of the brain regions that show inhibition of c-Fos expression such as the LH/PF, TMV, and VTA. Inhibition of c-Fos expression (indicated with lighter grey dots) also occurs in the caudate putamen (CPu) and hippocampus (HPC). Suppressed neuronal activity in all these regions may underlie the illness-associated reduced behavioral activity in the exploration task. Panel B shows the effect of DSAP lesion that destroys the ascending catecholaminergic pathway with loss of noradrenergic and adrenergic cell bodies in the NTS and VLM (indicated with red dots). This lesion results in suppression of LPS-induced activation in the PVN (light grey dots in B) and the prevention of suppression of behavioral task-related neuronal activation in LH/PF, TMV, VTA, CPu and HPC (indicated with black dots in B). Unlike after DVC inactivation that blunted the response of the non-catecholaminergic PBel-CEA-BSTov pathway to LPS, DSAP lesion did not affect the response to LPS by this latter pathway, which therefore does not seem to mediate behavioral inhibition.

Table 1
Summary of the statistical analysis of the effects of DSAP lesion and LPS challenge on c-Fos expression in selected brain regions (2-way ANOVA)*

Brain region	main effects DSAP injection		LPS treatment		Interaction LPS × DSAP	
	<i>F</i> (1,20)	<i>p</i>	<i>F</i> (1,20)	<i>p</i>	<i>F</i> (1,20)	<i>p</i>
<i>(behavior related)</i>						
CPu dm	7.18	0.014	5.02	0.037	5.08	0.036
HPC d	11.02	0.0034	2.89	0.1	8.14	0.0098
VTA r	7.99	0.01	6.18	0.022	14.4	0.0011
TMV	9.03	0.0076	37.1	<.0001	14.8	0.0012
LH/PF Orx lat	0.72	0.4	24	0.0001	8.79	0.0077
PF/DMH Orx med	0.25	0.62	10.99	0.0035	4.42	0.048
<i>(LPS-responsive)</i>						
PVN	25.3	<.0001	49	<.0001	12.86	0.0019
BSTov	1.166	0.3	28.35	<.0001	0.24	0.63
CEA	3.07	0.1	103.3	<.0001	0.004	0.95
PBel	0.134	0.72	132.7	<.0001	1.2	0.29

* significant effects ($p < .05$) are bolded. Statistics was performed on the total number of c-Fos-immunoreactive profiles in each brain region, except for the orexin (Orx) cells in the LH/PF/DMH, which involved the percentage of the total number of Orx cells that expressed c-Fos.

Table 2
Summary of the statistical analysis of the effects of DVC inactivation and LPS challenge on c-Fos expression in selected brain regions (2-way ANOVA)*

Brain region	main effects DVC bupivacaine		LPS treatment		Interaction LPS × DVC Bupiv	
	<i>F</i> (1,13)	<i>p</i>	<i>F</i> (1,13)	<i>p</i>	<i>F</i> (1,13)	<i>p</i>
<i>(behavior related)**</i>						
CPu dm	16.3	0.0014	9.18	0.0097	23.86	0.0003
HPC d	7.26	0.018	4.87	0.046	4.79	0.0474
VTA r	13.9	0.0026	1.67	0.22	9.64	0.0084
<i>(LPS-responsive)</i>						
PVN	4.76	0.048	18.41	0.0009	7.52	0.017
BSTov	6.34	0.026	26.44	0.0002	2.03	0.17
CEA	1.06	0.32	39.8	<.0001	5.85	0.031
PB el	4.58	0.052	56.21	<.0001	17.16	0.0012
VLM DBH cells	6.13	0.028	23.92	0.0003	5.74	0.032

* significant effects ($p < .05$) are bolded. Statistics was performed on the total number of c-Fos-immunoreactive profiles in each brain region, except for the DBH-labeled neurons in the VLM, which involved the percentage of the total number of DBH cells that expressed c-Fos.

** c-Fos expression in the TMV: see Gaykema et al., 2008.