Circulating cytokines and endotoxin are not necessary for the activation of the sickness or corticosterone response produced by peripheral *E. coli* challenge

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Although it is clear that proinflammatory cytokines signal the brain, the pathways by which this communication occurs is equivocal. The observation that receptors for cytokines exist in the brain (1, 15, 46) has prompted the idea that, after peripheral production at a site of infection, cytokines spill over into the bloodstream and are carried to the brain where they bind to their receptors and initiate a neural response (38). However, cytokines are large peptides that cannot passively cross the blood-brain barrier. Thus it has been postulated that 1) blood-borne cytokines initiate signaling at regions of the brain where the blood-brain barrier is weak or absent and 2) cytokines are actively produced by peripheral immune cells in response to a site of inflammation. This response is a prerequisite for these responses. Male Sprague-Dawley rats were injected subcutaneously with one of three doses (2.5 × 107, 2.5 × 108, 2.5 × 109 colony-forming units) of replicating *E. coli*, a ubiquitous bacterial strain, or vehicle. Core body temperature (Tc) and activity were measured for 3 days after the injection. A second set of groups of animals were killed 3, 6, 12, 18, 24, and 48 h after the injection, and blood samples and brains were collected. Injections dose dependently and consistently increased Tc and decreased activity, with increases in Tc beginning 4 h after the injection. In addition, *E. coli* significantly increased serum interleukin (IL)-1β, IL-6, and tumor necrosis factor-α and brain IL-1β levels beginning at the 6-h time point. Corticosterone and endotoxin were first elevated in the circulation at 3 and 18 h after the injection, respectively. Because fever onset preceded brain cytokine induction, we also examined cytokine levels in the serum, brain, and inflammation site 2 and 4 h after injection. Cytokines were elevated at the inflammation site but were not detectable in the serum or brain at 2 and 4 h. We conclude that subcutaneous injection of replicating *E. coli* induces a consistent and naturalistic infection that includes features of the sickness response as well as increases in circulating, brain, and inflammation site tissue cytokines. In addition, injection of replicating *E. coli* produces a robust fever and corticosterone response at a time when there are no detectable increases in circulating cytokines or endotoxin. These results suggest that elevated levels of circulating cytokines and endotoxin are not necessary for the activation of the sickness or corticosterone response. Therefore, fever, activity reduction, and corticosterone elevation induced by *E. coli* infection may have been evoked by a neural, rather than a humoral, pathway from the periphery to the brain.
transports across the blood-brain barrier (2, or 3) cytokines bind to receptors expressed inside the cerebral vasculature and induce the formation of soluble mediators that pass through the blood-brain barrier and act as second messengers (14, 57). However, sickness responses, such as fever, have been observed in the absence of detectable blood levels of proinflammatory cytokines (33). Consequently, it has been suggested that immune-to-brain communication can also be mediated by activation of specialized sensory nerves that carry immune information to the brain (12, 38, 39). It is not entirely surprising that organisms have evolved multiple immune-to-brain signaling pathways given the importance of the brain in mediating host defense during infection.

A thorough understanding of immune-to-brain communication will provide new insights into the bidirectional regulation of these systems, potentially elucidate how disruptions in communication between these systems contribute to disease, and ultimately might provide new avenues of therapy (16). Therefore, additional studies are required (12). To investigate immune-to-brain communication, most previous research has used intraperitoneal bolus injections of LPS (26, 29–31, 35, 36). Conclusions drawn from these past studies, however, are limited for a number of reasons. First, because an intraperitoneal injection of LPS results in a rapid and exaggerated stimulation of circulating cytokines (30, 31), the use of LPS might be masking the contribution of neural pathways. Second, given that animals and humans typically encounter infectious pathogens that replicate in vivo, and are exposed to lower concentrations of LPS over a more prolonged period of time, the use of bolus injection of nonreplicating pieces of bacteria (i.e., LPS) to examine immune-to-brain communication may not be physiologically relevant. Moreover, intraperitoneal injection of LPS often results in “nonresponders” (i.e., animals that have no elevation in cytokines or sickness response) that have to be excluded from the study (26, 29–31, 35, 36). Finally, significant lot-to-lot differences in commercially available LPS exist, making the replication and interpretation of previously reported studies difficult (Fleshner, unpublished observation).

An experimental model to study immune-to-brain signaling has recently been established in which LPS is administered subcutaneously rather than systematically (6, 41, 48). This approach avoids many of the problems associated with intraperitoneal injections (e.g., nonresponders), but it does not address the limitations associated with bolus LPS injections. Therefore, the present studies utilized a more reliable and naturalistic model of infection, namely subcutaneously administered Escherichia coli (5), to investigate immune-to-brain communication. Whereas peripheral administration of endotoxin or cytokines has been reported to result in the induction of fever, suppressed activity, and elevated plasma corticosterone (Cort) (7, 26, 29–31, 35, 36, 47), few data exist regarding the role of endotoxin or cytokines in the induction of sickness during a bacterial infection, per se. Thus the present studies examined whether subcutaneously administered replicating E. coli can activate fever and hypothalamic-pituitary-adrenal (HPA) responses and whether circulating endotoxin and/or proinflammatory cytokines are a prerequisite for these responses. The observation of fever and HPA responses in the absence of elevated peripheral circulating cytokines or endotoxin would suggest that a neural afferent pathway might be playing a role in mediating immune-to-brain communication after subcutaneous E. coli challenge and call into question some of the interpretations and assumptions stemming from previous LPS studies.

**METHODS**

**Animals.** Adult male viral-free Sprague-Dawley rats (300–350 g; Harlan Laboratories) were individually caged in Plexiglas cages (60 × 30 × 24 cm) with food and water available ad libidum. The animal colonies were maintained in a pathogen-free barrier facility at 25°C with a 12:12-h light-dark cycle (lights on 0600–1800). Rats were given at least 1 wk to habituate to the colonies before experimentation. All rats were handled and weighed each day for at least 3 days before each study began (6–7 rats/experimental group). Care and use of the animals were in accordance with protocols approved by the University of Colorado Institutional Animal Care and Use Committee.

**Thermistor implantation.** Precalibrated radio thermistors (Minimitter, Sun River, OR) for measuring core body temperature (Tc) and activity were aseptically implanted in the peritoneal cavity of 28 rats while under isoflurane anesthesia, as described previously (17, 56). Rats were allowed to recover 7 days before the experiments.

**Bacterial culture.** E. coli (ATCC 15746) were purchased from the American Type Culture Collection (Manassas, VA). Vial contents were rehydrated and grown overnight to maximal densities in 30 ml of brain-heart infusion (BHI; Difco Laboratories, Detroit, MI) at 37°C, 95% air–5% CO2. Cultures were then aliquoted into 1.0 ml of BHI supplemented with 10% glycerol and frozen at −70°C. These vials constituted the stock cultures. All experiments utilized bacteria from these stock cultures. One day before experimentation, stock cultures were thawed and incubated overnight (~19 h) in 35 ml of BHI (37°C, 95% air–5% CO2). The number of bacteria in cultures was quantified by extrapolating from previously determined growth curves (5). Cultures were then centrifuged for 15 min at 3,000 rpm, supernatants were discarded, and bacteria were resuspended in sterile PBS.

**Bacterial challenge.** One day before injection, an area on the rat’s dorsal surface measuring 5.0 × 7.0 cm was shaved. On the day of experimentation, injections were given subcutaneously in a volume of 250 μl in the center of the back. Rats were injected with either 2.5 × 108 colony-forming units (CFU) E. coli, 2.5 × 108 CFU E. coli, 2.5 × 107 CFU E. coli, or sterile PBS as vehicle. All injections were done at the same time of day (1500) so that the circadian rhythms of the animals were identical across studies.

**Tc and activity measurement.** Body temperatures and activity were measured remotely at minute intervals continuously from the day before bacterial challenge (baseline) to 3 days after challenge with the use of standard biotelemetry procedures (Minimitter). Raw temperature and activity counts were converted to hourly averages to ease the interpretation of data. Animals remained in their home cages during the experiment, while the frequencies emitted by the thermistors were monitored remotely. These frequencies were different.
were then converted to temperature (°C) and activity (number of times rat crossed grid in home cage) values based on each transmitter's calibration data.

Brain and serum cytokines, endotoxin, and corticosterone measurement. Seventy-two additional rats were used in a time course experiment. Rats received either an injection of 2.5 × 10⁶ CFU E. coli or vehicle (as described in Bacterial challenge) and were then killed by decapitation 8, 6, 12, 18, 24, and 48 h after challenge (12 rats/time point; 6 E. coli + 6 vehicle). Brain structures, which included hypothalamus, hippocampus, prefrontal cortex, and dorsal vagal complex, along with the pituitary were quickly removed after decapitation. Care was taken at the macroscopic level to avoid collecting circumventricular organs, dura matter, large blood vessels, or visible choroid plexus during the microdissection procedure. Dissecting scopes were not used, and thus small amounts of pia mater and/or choroid plexus might have been contained in the tissues. Tissues were processed for endogenous rat cytokine measurement as previously described (43).

Briefly, after rapid dissection, all tissues were snap frozen in liquid nitrogen and stored at −80°C until processed. Each tissue was homogenized in 0.25 vol 0.5 mol/l NaCl, 50 mmol/l Tris-HCl (pH 7.5) sonication buffer at 10,000 rpm, 10 min, 4°C, and supernatants were removed and stored at −20°C until assayed. These regions were selected for study because our laboratory has previously reported that LPS administration results in dose-dependent elevations in IL-1β in these regions (26), they are believed to play an integral role in transducing immune-to-brain signals (i.e., dorsal vagal complex (38, 39), and they contribute to the control of sickness responses (i.e., hypothalamus (57)). Bradford protein assays were performed to determine total protein concentrations as described previously (18). Pituitary and brain tissue IL-1β levels were measured as previously described (26, 43). Trunk blood was collected in 50-ml conical tubes for later measurement of serum cytokines, endotoxin, and Cort. Tubes were stored on ice and immediately spun in a refrigerated centrifuge. Serum was aliquoted and stored at −20°C until time of assay. Serum TNF-α, IL-1β (R & D Systems) and IL-6 (Biosource) were measured by using commercially available ELISA kits as previously described for IL-1β (26, 43). Serum endotoxin levels were measured by using a chromogenic Limulus amebocyte lysate assay (BioWhittaker), and serum Cort was measured by using a commercially available radioimmunoassay kit (ICN Pharmaceuticals). All samples were assayed at optimal concentrations and according to the manufacturer’s instructions (26, 43).

Inflammation site culture procedures. An additional study was conducted to examine cytokine levels at the inflammation site, as well as in the serum and brain. All procedures were performed as described above except that the inflammation site was harvested as described previously (5). Briefly, rats received either an injection of 2.5 × 10⁶ CFU E. coli or vehicle (as described in Bacterial challenge) and were then killed by decapitation 2 and 4 h after challenge (12 rats/time point; 6 E. coli + 6 vehicle). Inflammation sites were removed, cut into smaller equal sections, and placed into 2 ml of Hanks’ culture media (GIBCO BRL Life Technologies, Frederick, MD) at 37°C and 95% air-5% CO₂. Brains were microdissected and serum was collected for cytokine measurement as described in Brain and serum cytokines, endotoxin, and corticosterone measurement. Cytokine levels were taken from the inflammation site culture at optimal time points (5) for cytokine measurement (5 h of culture).

Data analyses. The effects of various doses of E. coli on temperature and activity levels were analyzed by two-way repeated-measures ANOVA utilizing a within-subjects/baseline control. All other dependent variables assessed in this study were analyzed by two-way ANOVA. Where appropriate, Fisher’s protected least significant difference post hoc analyses were conducted. Alpha was set at 0.05. In all of the figures, the values shown are as group means ± SE.

RESULTS

Effects of E. coli on fever and activity. Subcutaneous injection of replicating E. coli produced dose-dependent fever in rats. Repeated-measures ANOVA indicated a main effect of group (preinjection Tc vs. postinjection Tc) and a group × time interaction for all doses of bacteria. Analysis revealed significant differences in Tc between rats that received 2.5 × 10⁶ CFU E. coli [main effect of group: F(3,18) = 89.414, P < 0.0001; and group × time interaction: F(66,396) = 4.777, P < 0.0001], 2.5 × 10⁸ CFU E. coli [main effect of group: F(2,12) = 32.543, P < 0.0001; and group × time interaction: F(44,264) = 3.74, P < 0.0001], 2.5 × 10⁹ CFU E. coli [main effect of group: F(2,12) = 12.594, P = 0.0011; and group × time interaction: F(44,264) = 2.585, P < 0.0001] but not vehicle (P > 0.05) compared with baseline values. The highest dose of bacteria (2.5 × 10⁹ CFU) produced the greatest fever, and post hoc analyses revealed that the fever persisted for 2 days after the injection (P < 0.05) (Fig. 1A). Further analysis indicated that the Tc was elevated compared with baseline values starting at the 4-h time point and returning to baseline values by hour 48 (P < 0.05). The middle dose of bacteria (2.5 × 10⁸ CFU) and the low dose (2.5 × 10⁷ CFU) of bacteria produced elevated Tc that remained significantly elevated over baseline for 1 day after injection (P < 0.05) (Fig. 1, B and C). Further analysis indicated that the Tc was elevated compared with baseline values after injection of 2.5 × 10⁸ CFU starting at the 4-h time point and returning to baseline values by hour 23 (P < 0.05), whereas injection of 2.5 × 10⁷ CFU elevated Tc starting at the 8-h time point and returning to baseline values by hour 12 (P < 0.05). Elevations in Tc after 2.5 × 10⁸ CFU E. coli and 2.5 × 10⁹ CFU E. coli persisted through both the dark (active) and light (inactive) phase of the animals, whereas 2.5 × 10⁷ CFU E. coli resulted in elevated Tc only during the dark phase. Injection of vehicle had no effect on Tc (Fig. 1D). Data collected in the 1 h after injection were dropped from the analysis because of disturbances in Tc due to handling alone.

The pattern of data was similar for activity. Replicating E. coli injection produced dose-dependent decreases in activity. Repeated-measures ANOVA indicated a main effect of group (preinjection activity vs. postinjection activity) and a group × time interaction for all doses of bacteria. Analysis revealed significant differences in activity between rats that received 2.5 × 10⁶ CFU E. coli [main effect of group: F(3,18) = 45.327, P = 0.0001; and group × time interaction: F(66,396) = 2.616, P < 0.0001], 2.5 × 10⁸ CFU E. coli [main effect of group: F(2,12) = 14.357, P = 0.0007; and group × time interaction: F(44,264) = 2.977, P < 0.0001], 2.5 × 10⁹ CFU E. coli [main effect of group: F(2,12) = 3.753,
P = 0.05; and group × time interaction: F(44,264) = 2.585, P < 0.03] but not vehicle (P > 0.05) compared with baseline values. The highest dose of bacteria (2.5 × 10⁹ CFU) produced the greatest decrement in activity, and post hoc analyses revealed that the reduction in activity persisted for 2 days after the injection (P < 0.05) (Fig. 2A). The middle dose of bacteria (2.5 × 10⁸ CFU) and the low dose (2.5 × 10⁷ CFU) of bacteria

Fig. 1. Mean core body temperature for subjects injected at time 0 with 2.5 × 10⁹ colony-forming units (CFU) Escherichia coli (A), 2.5 × 10⁸ CFU E. coli (B), 2.5 × 10⁷ CFU E. coli (C), or vehicle (D). Body temperature was recorded for either 3 (A) or 2 (B–D) days after injection. Solid bar on x-axis, dark phase (active phase) of the light-dark cycle. Values are means ± SE.

Fig. 2. Mean activity scores for subjects injected at time 0 with 2.5 × 10⁹ CFU E. coli (A), 2.5 × 10⁸ CFU E. coli (B), 2.5 × 10⁷ CFU E. coli (C), or vehicle (D). Activity was recorded for either 3 (A) or 2 (B–D) days after injection. Solid bar on x-axis, dark phase (active phase) of the light-dark cycle. Values are means ± SE.
produced suppressed activity that persisted for 1 day after injection (P < 0.05) (Fig. 2, B and C). Decreases in activity after E. coli injection could only be detected during the time of day when the animals were awake [i.e., the dark (active) phase]. Vehicle injection had no effect on activity (Fig. 2D). Data collected in the 1 h after injection were dropped from the analysis because of disturbances in activity due to handling alone.

Effects of E. coli on brain, serum, and inflammation site cytokines. Subcutaneous injection of replicating E. coli (2.5 × 10⁹ CFU) increased IL-1β protein content in all brain structures tested as shown in Fig. 3, A–E. ANOVA indicated a main effect of group (E. coli vs. vehicle) and a group × time interaction. Specifically, IL-1β was elevated in the hypothalamus [main effect of group: F(1,9) = 31.877, P = 0.0003; and group × time interaction: F(5,45) = 2.949, P = 0.02; Fig. 3A], hippocampus [main effect of group: F(1,9) = 11.302, P = 0.008; and group × time interaction: F(5,45) = 4.262, P = 0.002; Fig. 3B], prefrontal cortex [main effect of group: F(1,9) = 28.851, P = 0.0004; and group × time interaction: F(5,45) = 9.759, P < 0.0001; Fig. 3C], dorsal vagal complex [main effect of group: F(1,9) = 113.621, P < 0.0001; and group × time interaction: F(5,45) = 8.96, P < 0.0001; Fig. 3D], and the pituitary [main effect of group: F(1,9) = 450.667, P < 0.0001; and group × time interaction: F(5,45) = 7.383, P < 0.0001; Fig. 3E] of rats injected with bacteria compared with those injected with vehicle. IL-1β levels were not elevated in any brain region at 3 h, but were elevated in all brain regions at 6 h, after injection of bacteria. Post hoc analyses indicated that IL-1β was elevated in the hypothalamus 6, 12, 24, and 48 h; in the hippocampus 6 and 48 h; in the prefrontal cortex 6, 12, and 18 h; and in the dorsal vagal complex and pituitary 6, 12, 18, 24, and 48 h after bacteria injection (P < 0.05 for each respective time point). Rats injected with vehicle did not demonstrate elevated IL-1β in any brain region at any time point.

Subcutaneous injection of replicating E. coli (2.5 × 10⁹ CFU) also increased serum levels of TNF-α, IL-1β, and IL-6 over time as shown in Fig. 4, A–C. ANOVA indicated a main effect of group (E. coli vs. vehicle) for IL-1β and IL-6 and a group × time interaction for all
The results of the present study demonstrate that subcutaneously administered replicating *E. coli* produces a robust, consistent, and dose-dependent sickness response in rats. Replicating bacterial challenge results in a significant fever and a decrease in locomotion, both important features of the sickness response, that persist for 1–2 days after the injection. Furthermore, subcutaneous injection of replicating bacteria also results in elevated blood, inflammation site tissue, and brain proinflammatory cytokine levels and in elevated blood levels of endotoxin and Cort. Interestingly, T<sub>e</sub> and Cort are elevated and activity is suppressed before the presence of detectable levels of endotoxin or elevated levels of cytokines in the blood. The results of the present study demonstrate that sickness responses can occur before the presence of detectable levels of endotoxin or elevated levels of cytokines in the blood after injection of replicating bac-

Fig. 4. Serum levels of tumor necrosis factor-α (TNF-α) (A), IL-1β (B), and IL-6 (C) 2, 3, 4, 6, 12, 18, 24, and 48 h after injection of 2.5 × 10<sup>9</sup> CFU *E. coli* or vehicle. Solid bar on x-axis, dark phase (active phase) of the light-dark cycle. Values are means ± SE. *E. coli* vs. vehicle, *P* < 0.05 (by Fisher’s protected least significant difference test).
This is supported by the observation that rats begin to fever and decrease their activity 4 h after injection of 2.5 \( \times 10^9 \) CFU E. coli but that peripheral blood cytokines and peripheral blood endotoxin are not elevated at this time. These results are contrary to the observation that circulating IL-6 mediates the febrile response to localized inflammation induced by LPS administration into a subcutaneous air pouch, but they are consistent with the observation that LPS administration into a subcutaneous air pouch elicits fever in the absence of detectable endotoxin in the circulation (8). Furthermore, in the present studies, elevations in plasma Cort preceded the appearance of elevated levels of peripheral blood cytokines and endotoxin, which is in contrast to what has been previously reported after injection of LPS (36). Interestingly, sickness responses actually subsided before the disappearance of elevated levels of plasma cytokines and endotoxin after injection of E. coli. These results are consistent with the idea that even when endotoxin or cytokines are detected in the blood, their timing does not always correlate with acute-phase activation after administration of immune stimuli (33). Taken together, the results from the present study suggest that the relevant signal for brain-mediated sickness responses is not dependent on circulating proinflammatory cytokines or endotoxin and illustrate differences between E. coli and previously reported LPS-induced host responses.

Although others have demonstrated that the appearance of endotoxin in the blood is often a prerequisite for the appearance of circulating proinflammatory cytokines...
kines after intraperitoneal injection of LPS (36), the results from the present study indicate that this is not the case after subcutaneous injection of *E. coli*. Blood levels of TNF-α, IL-1β, and IL-6 were elevated at 6 h after subcutaneous injection of 2.5 × 10^8 CFU *E. coli*, but endotoxin did not reach the blood until 18 h after injection. Similarly, it is clear that endotoxin does not need to reach the circulation for proinflammatory cytokines to be expressed in the brain. In fact, expression of cytokines in the brain preceded the appearance of endotoxin in the circulation by at least 12 h. Furthermore, the appearance of proinflammatory cytokines in the blood does not necessarily precede the induction of brain proinflammatory cytokines. This is supported by the observation that both the blood and brain demonstrate elevated cytokine levels at the same time (6 h after injection). However, a more detailed time course characterization is necessary to fully substantiate this observation.

These results demonstrate that elevated circulating cytokines and endotoxin are not necessary in the induction of brain-mediated responses, and they suggest that other, yet to be identified, mediators, as possible mechanisms of immune signaling to the brain during an actual infection. The signal for induction of fever could be due to local cytokine production at the inflammation site as the initiation of inflammation (5), and in the present study, the production of proinflammatory cytokines at the site of infection (Fig. 5, A–C) was observable 2 h after injection. A local inflammatory response at the site of infection might initiate a neural afferent pathway. Evidence for neural pathways serving as conduits for immune-related information is provided by the observations that systemic IL-1β increases electrical activity of vagal afferents (13, 44) and induces c-Fos in vagal primary afferent neurons (13, 19) and that IL-1β binding and immunoreactivity have been localized to abdominal vagal afferents and paraganglia (21, 22). Furthermore, severing the vagus nerve has been found to block or attenuate numerous brain-mediated responses to intraperitoneal challenge (4, 9, 38). However, the pathways used in immune-to-brain communication remain controversial. It is important to elucidate all of the pathways involved in immune-to-brain communication to better understand bidirectional regulation of these systems and because disruptions in communication between these systems contribute to disease (16). Thus insight into underlying mechanisms ultimately might provide new avenues of therapy.

Administration of a variety of immune stimuli leads to production of mediators capable of signaling the brain (i.e., local cytokines) (38). Most of what is known about immune-to-brain communication derives from research using administration of LPS. It has recently been reported, however, that T-cell-dependent antigens derived from gram-positive bacteria can activate brain pathways mediating host-defense (20), fever (37), and neuroendocrine responses (37). Compared with T-cell-independent stimuli such as LPS, the onset and magnitude of fever and HPA activation after intraperitoneal administration of T cell-dependent antigen are significantly different (20, 37). The two stimuli also differ in their ability to induce tolerance after repeated injections (24), and gram-positive bacterial fever may involve mediators other than those released after gram-negative stimuli (23, 49).

The results from the present study demonstrate that replicating *E. coli* also induces fever and HPA activation. The onset and magnitude of fever and HPA activation after subcutaneous *E. coli* injection, however, are different from previously observed after LPS or gram-positive bacterial administration. *E. coli* injection resulted in a slower onset of fever, a larger magnitude of fever, a longer lasting fever, and larger degree of neuroendocrine activation than previously reported after LPS or gram-positive bacterial administration (20, 21, 36, 37). These observations are consistent with recent evidence demonstrating that the macrophage response to whole bacteria is of greater magnitude and duration than observed after LPS stimulation (42). However, because both the immune stimuli dose (25–27, 45, 53, 55) and route of administration (36) are critical factors when the induction of brain-mediated symptoms of illness is assessed, it is difficult to directly compare previous studies with the present one.

Although the present study did not directly compare host responses to *E. coli* and LPS, several important differences between previous reports utilizing LPS and the present investigation utilizing replicating *E. coli* are worthy of discussion. First, as previously discussed, in contrast to previous studies utilizing injection of LPS (8, 36), sickness responses can occur before the presence of detectable levels of endotoxin or elevated levels of cytokines in the blood after injection of replicating bacteria. Second, in contrast to previous studies employing LPS (26, 29–31, 35, 36), every animal in the present series of studies responded to bacterial challenge, suggesting there is little, if any, individual variation in host response to replicating *E. coli*. In addition, because the precise quantity of bacteria can be calculated before inoculation, the concentrations of bacteria used in the present studies can easily be replicated in subsequent studies. This is in contrast to the use of LPS where large discrepancies in commercial lot-to-lot LPS exists, requiring significant pilot work to determine appropriate doses. Finally, because organisms typically encounter infectious pathogens that replicate in vivo, injection of replicating *E. coli* might be more physiologically relevant than a bolus injection of nonreplicating LPS.

It is of interest to note the lack of temporal correlation among the experimental variables. For example, as previously mentioned, circulating cytokines and endotoxin did not correlate with the onset or amelioration of sickness responses. Moreover, although central IL-1β has been implicated in regulating sickness responses (57), and a variety of brain regions demonstrated a robust increase in IL-1β expression after *E. coli* infection (Fig. 3), the pattern of IL-1β expression in the brain did not correlate with sickness responses.
Particularly interesting is the IL-1β pattern observed in the hypothalamus. The hypothalamus plays a large role in the regulation of fever (33) and, in concert with the pituitary and adrenal, is responsible for elevations in Cort (51), yet neither the hypothalamus nor the pituitary demonstrated significant elevations in IL-1β when body temperature and Cort were first elevated. Moreover, the hypothalamus demonstrated elevated IL-1β at a time when fever had returned to baseline values. These results suggest that circulating cytokines and central IL-1β expression are likely not necessary for the induction of fever in the present experimental paradigm. Interestingly, recent evidence suggests that prostaglandins are essential components of the manifestation of fever in a subcutaneous model of inflammation (47). However, it remains unknown whether prostaglandins exert their febrile effects directly in the brain and/or on neural afferents and what role they might play during an actual infection.

In conclusion, subcutaneous injection of replicating E. coli induces a dose-dependent sickness response that includes a long-lasting fever, decrease in locomotion, activation of the HPA axis, and an elevation in brain, peripheral circulating, and inflammation site cytokines. Importantly, the results of this study demonstrate that Tc and Cort are elevated and that activity might play during an actual infection.

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