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Sepsis-induced morbidity in mice: effects on body temperature, body weight, cage activity, social behavior and cytokines in brain

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Abstract

Infection negatively impacts mental health, as evidenced by the lethargy, malaise, and cognitive deficits experienced during illness. These changes in central nervous system processes, collectively termed sickness behavior, have been shown in animal models to be mediated primarily by the actions of cytokines in brain. Most studies of sickness behavior to date have used bolus injection of bacterial lipopolysaccharide (LPS) or selective administration of the proinflammatory cytokines interleukin-1 β (IL-1 β) or IL-6 as the immune challenge. Such models, although useful for determining mechanisms responsible for acute changes in physiology and behavior, do not adequately represent the more complex effects on central nervous system (CNS) processes of a true infection with replicating pathogens. In the present study, we used the cecal ligation and puncture (CLP) model to quantify sepsis-induced alterations in several facets of physiology and behavior of mice. We determined the impact of sepsis on cage activity, body temperature, food and water consumption and body weights of mice. Because cytokines are critical mediators of changes in behavior and temperature regulation during immune challenge, we also quantified sepsis-induced alterations in cytokine mRNA and protein in brain during the acute period of sepsis onset. We now report that cage activity and temperature regulation in mice that

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Conflicts of Interest

The authors declare they have no Conflicts of Interest.

Contributors

JIG: performed the surgical procedures on mice, processed data, and wrote the first draft of the manuscript.

LR: conducted the social exploration experiment, scored the video tapes of the behavioral interactions, and contributed to the writing of the manuscript.

SCD: analyzed brain samples for mRNA and protein content, and contributed to the writing of the manuscript.

RMR: analyzed data, produced figures, and contributed to the writing of the manuscript.

MRO: conceived of the project, analyzed data, contributed to the writing of the manuscript.

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survive are altered for up to 23 days after sepsis induction. Food and water consumption are transiently reduced, and body weight is lost during sepsis. Furthermore, sepsis decreases social interactions for 24 – 48 hours. Finally, mRNA and protein for IL-1 β , IL-6, and tumor necrosis factor- α (TNF α) are upregulated in the hypothalamus, hippocampus, and brain stem during sepsis onset, from 6–72 hour post sepsis induction. Collectively, these data indicate that sepsis not only acutely alters physiology, behavior and cytokine profiles in brain, but that some brain functions are impaired for long periods in animals that survive.

Keywords

behavior; thermoregulation; cytokine; sleep; rodent; LPS; brain; central nervous system

Introduction

Hart and others (Hart, 1988; Dantzer et al. 2008) suggest that changes in physiology and behavior during infection are adaptive. Changes in physiology and behavior during infection include altered temperature regulation, feeding and drinking, locomotor activity, sleep and sexual behavior, among others. The collective constellation of these adaptive behaviors has been referred to as sickness. The adaptive value of the complex alterations in the behavior of sick animals is thought to be part of a motivational state that better enables the host to survive infection (Hart, 1988). Numerous studies demonstrate that these changes in physiology and behavior (sickness) during an immune challenge are mediated, in part by actions in brain of proinflammatory cytokines such as interleukin-1 β (IL-1 β), IL-6, and tumor necrosis factor- α (TNF α), (Opp and Krueger, 1991; Bluthé et al., 1994; Kozak et al., 1998; Dantzer, 2001a; Chida and Iwakura, 2007).

In many studies of the relationship between the immune system and behavior, activation of the immune system has been achieved by using central or peripheral administration of recombinant cytokines such as IL-1 β , TNF α or IL-6. Whereas central or peripheral administration of these cytokines induces an acute response, these responses differ from those elicited during infection, in part because there is no replicating pathogen. Within the context of bacterial infections, lipopolysaccharide (LPS) is frequently used as a stimulus to induce sickness behavior (Konsman et al., 2008) because it is derived from the cell wall of Gram-negative bacteria. However, as with administration of cytokines, LPS administration only mimics some facets of immune responses to infection because there is no replicating pathogen. In this present study we tested the hypothesis that infection with a replicating pathogen would also alter complex behavior and physiology of mice. The infectious process which we use as a model is sepsis.

Sepsis has been the focus of intense investigation because of its clinical impact in terms of lives lost and financial burden on the health care system. However, most clinical and preclinical studies of sepsis have focused on the presumed site of the insult, and relatively few have focused on alterations of brain processes and function during sepsis. Clinical studies demonstrate the brain may be one of the first organs affected by sepsis (Freedman et al., 2001), and septic patients often suffer from cognitive impairment and loss of cognitive function after discharge from the hospital (Iwashyna et al., 2010). We have demonstrated that sepsis alters sleep and EEG parameters in rats (Baracchi et al., 2011). Collectively, these and other data demonstrate an impact of sepsis on central nervous system (CNS) processes. However, the underlying pathogenesis of sepsis-induced alterations in CNS processes is not well understood, though several potential mechanisms, such as alterations in the blood-brain barrier, metabolism, and cerebral circulation have been explored (du Moulin et al., 1985; Miller et al., 1987; Koo et al., 2001; Tsao et al., 2001; Basler et al., 2002).

In this study, we use a model of polymicrobial intra-abdominal infection induced by cecal ligation and puncture (CLP) to determine effects of sepsis on physiological and behavioral processes associated with sickness behavior. Because behavior is regulated by the brain, we use behavioral outcome measures as a readout of some facets of brain function. The CLP model has several characteristics that make it suitable for studies such as this (Nemzek et al., 2008; Rittirsch et al., 2009): CLP is clinically relevant because of the progressive release of proinflammatory cytokines, because it is responsive to antibiotic treatment and fluid resuscitation, and because of the rate (days) at which the disease progresses. Furthermore, the severity of the ensuing infection, and as such morbidity, mortality and clinical outcome, can be modulated by varying the size of needle used to puncture the cecum (Ebong et al., 1999a; 1999b; Rittirsch et al., 2009). The goals of this study were to use a well-defined model of social exploration as an indicator of motivational state (Dantzer et al., 2008) and measures of body temperature, activity patterns, and food and water consumption to assess morbidity and the impact of sepsis on some brain regulatory mechanisms. Because IL-1 β , TNF α , and IL-6 have been implicated in the regulation of these aforementioned CNS processes, we also determined sepsis-induced alterations in mRNA and protein in discrete brain regions that are involved in regulating them. We tested the hypotheses that sepsis in laboratory mice alters body temperature and activity rhythms, reduces their social interactions, and upregulate cytokine expression in brain. We now report that during the acute stages of sepsis, social exploration of mice is reduced. Furthermore, there are acute changes in cytokine mRNA and protein expression in brain that suggest these systems may mediate or play a role in the initial organismal responses to sepsis under the conditions of this study. Finally, in mice that survive, daily rhythms of locomotor activity and body temperature are disrupted for at least 23 days after CLP-induced sepsis. Collectively, results of this study demonstrate short-term effects and long-term consequences of sepsis on physiology and behavior of mice.

Methods

Animals

All procedures were approved by the University of Michigan Committee on the Use and Care of Animals in accordance with the US Department of Agriculture Animal Welfare Act and the National Institutes of Health policy on Humane Care and Use of Laboratory animals. Adult male BALB/c mice (~ 25 g; Harlan, Indianapolis, IN) were used in these studies. All mice were housed individually in a temperature-controlled room at 29 $\pm$ 1 $^{\circ}$ C on a 12:12 h light:dark cycle. Standard rodent chow and water were available ad libitum.

Surgical Procedures

Telemeter Implantation—Core body temperature of mice was recorded using implantable telemeters. Pre-calibrated telemeters (model #TA-F20, Data Sciences International, St. Paul, MN USA) were implanted into the abdominal cavity of mice under deep isoflurane anesthesia. The animals were then placed in a warm incubator (37 $^{\circ}$ C) for 15 minutes, after which 1 ml warmed pyrogen-free saline was given subcutaneously for fluid resuscitation. When ambulatory, the mice were placed into standard mouse shoeboxes. Analgesia was provided by administering Ibuprofen (0.2 mg/mL) into the drinking water beginning 24 h before surgery and continuing for 48 h after surgery (Hayes et al., 2000). A broad-spectrum antibiotic (Imipenem, Merck, West Point, PA; 25 mg / kg in 5% dextrose and lactated Ringer's) was administered before surgery to minimize risk of infection during the implantation of the telemetry device.

Cecal Ligation and Puncture (CLP)—Polymicrobial intra-abdominal infection was induced by CLP as previously described (Ebong et al., 1999a; Remick et al., 2000). Briefly,

mice were anesthetized with isoflurane and the cecum exposed and ligated with 4.0 silk below the ileocecal junction, approximately 1.5 cm from the distal end. The cecum was then twice punctured through-and-through with a sterile 21-gauge needle and compressed to extrude fecal contents. For those mice that served as controls (*Experiments 2, 3, and 4*), a SHAM surgery was performed. During SHAM (control) surgeries, the cecum was exposed in the same manner as during CLP surgeries, but was not ligated or punctured. All mice received a broad spectrum antibiotic (Imipenem) in 1 ml lactated Ringers/D5W subcutaneously 2 h post-surgery and twice daily for five days. Antibiotics are generally administered to rodents used in CLP models of sepsis [see for example (Newcomb et al., 1998)] because antibiotic treatment increases the number of animals that survive. Furthermore, human patients who become septic are treated with antibiotics, and CLP has been used as a model to determine the effectiveness of antibiotics as a therapeutic intervention (Vyas et al., 2005). In this study, we administered antibiotics to reduce mortality so that sufficient numbers of mice would survive for study. To account for the impact of antibiotics *per se*, control (SHAM) mice also received the same antibiotic treatment.

Experimental Protocols

Experiment 1: Impact of sepsis on morbidity and mortality, body temperature and cage activity—

In this study we operationally define morbidity as sepsis-induced disruption to daily activity and temperature rhythms, reductions in food and water consumption, and loss of body weight. Collectively, these parameters may be viewed as clinical measures that reflect the overall health of the animal. We used a within-subjects experimental design to determine the impact of sepsis on these clinical measures. Ten days after the abdominal implantation of the telemeters, mice ($n = 18$) in their own shoeboxes were placed on a receiver plate (DSI RPC-1) of the telemeter system. Body temperature values were collected at 10 – min intervals using the ART Analog-8 system (DSI), with subsequent data processing done using custom software (ICELUS, M. Opp, University of Michigan). Cage activity was determined using infrared sensors [BioBserv, BmgH, Bonn, Germany (Olivadoti and Opp, 2008)]. Data collection began at light onset and continuing for 72 h. After 3 days of un-interrupted data collection, all mice were subjected to CLP surgeries as described. CLP and SHAM surgeries were done early in the light period, on an alternating basis such that pairs of mice (CLP, SHAM) were subjected to surgery at essentially the same time of day. After ambulatory, the mice were returned to the recording apparatus, and post-surgical recordings were initiated at dark onset and continued for 23 days. This approach allowed pre-sepsis / post-sepsis comparisons to be made within the same subject. Body weights were obtained daily, and food and water consumption measured. During the recording period, mice were undisturbed except briefly at the same time each day for weights and measurements and for administration of antibiotics and fluids as detailed.

Experiment 2: Sepsis-induced alterations in social exploration—Rodents exhibit stereotypic behaviors when in the presence of naïve conspecifics with which there has previously not been contact or interaction. These stereotypic behaviors, which include sniffing, anal-genital contact, and fighting, are collectively referred to as social exploration. We adapted the model refined and characterized by Dantzer and colleagues (Bluthé et al., 2000a; 2000b) to determine the impact of sepsis on social exploration. Adult male mice ($n = 16$) were used in this experiment. Baseline behavioral testing consisted of placing a juvenile conspecific (aged 3 – 4 weeks, 11 – 16 g) into the home cage of an adult male for 30 minutes. These testing sessions were conducted between 5 and 8 h after lights on, i.e., during the middle of the light period of the light:dark cycle. Each session was videotaped, and the tapes were subsequently visually scored for three types of social interactions: *simple*

contacts, fights, and incidence of anal-genital sniffing. Simple contacts were those contacts by the nose of the adult mouse directed at any body part of the juvenile, exclusive of the anal-genital region. *Fighting* was scored when the adult initiated aggressive actions and biting toward the juvenile. *Anal-genital sniffing* consisted of direct contact of the nose of the adult with the genitals of the juvenile. The juveniles were removed at the end of the session. In this experiment, a between-subjects approach was used such that 24 h after baseline behavioral testing, the adult male mice were randomized into either CLP or SHAM surgery groups (n = 8 per group). Behavioral test sessions were then repeated at 24-, 48-, and 72-h post-surgery, at the same time each day during which baseline testing was done. The juvenile-adult pairings were structured such that each juvenile was used only once, i.e., there had been no previous contacts between the adult and juvenile during any given behavioral test session.

Experiment 3: Cytokine mRNA and protein expression in brain—Separate groups of mice were used to determine effects of sepsis on cytokine mRNA and protein expression in brain. Cytokine mRNA expression was evaluated in mice subjected to CLP or SHAM surgeries (n = 5 – 6 / group). Mice from these groups were sacrificed at 6-, 12-, 24-, 48-, or 72- h post-surgery. All animals in this experiment were quickly anesthetized with isoflurane, decapitated and the brain quickly removed and placed on an ice-cold surface. The hypothalamus, hippocampus, and brainstem were dissected and total RNA immediately extracted using Tri-Reagent (Molecular Research Center, Cincinnati, OH). cDNA was prepared from total RNA using High-Capacity cDNA Archive Kit (P/N 4322171, Applied Biosystem) and TaqMan gene expression assays were performed for individual cytokines (primer assay ID: IL1 β , Mm00434228m1; IL6, Mm00446190m1; TNF α , Mm00443258m1, Applied Biosystems). Assays used an Applied Biosystems 7300 Real Time PCR System (Foster City, CA). Forty cycles consisted of 15 s denaturation at 95 °C followed by annealing/extension for 1 min at 60 °C. Changes in gene expression were evaluated using REST 2009 (<http://www.qiagen.com/products/rest2009software.aspx>). REST 2009 uses integrated randomization and bootstrapping methods to determine statistical significance on the basis of calculated expression ratios. In our study, expression ratios of cytokine mRNA in samples obtained from animals after CLP were calculated on the basis of cycle thresholds (CT) for an internal housekeeping gene (GAPDH: primer assay ID, Mm99999915g1, Applied Biosystems) and for control animals subjected to SHAM surgeries, i.e., the $\Delta\Delta^{CT}$ method.

Sepsis-induced alterations in cytokine protein in brain were determined from a separate group of mice (n = 24) subjected to CLP or SHAM surgeries. These mice were sacrificed at 6-, 24-, or 48-h post-surgery (n = 4 / group), and brain regions dissected as above. Protein from brain tissue samples was extracted using Bioplex cell lysis buffer kits (catalog numbers 171–304011 and 171–304012, BioRad, Hercules, CA) containing protease inhibitors (PMSF, 500mM, Sigma-Aldrich, St. Louis, MO). The protein content of each sample was determined by the BCA method (Pierce, Rockford, IL). For cytokine protein assays equal amounts of protein were loaded into each well of a 96 well plate and multiplex bead assays were conducted using the Luminex 200 system as previously described (Datta and Opp, 2008). All samples were run in duplicate. Per manufacturer specifications, the lower limit of quantitation for the cytokine targets in this multiplex assay is 0.74 pg/ml for IL-6, 10.36 pg/ml for IL-1 β , and 5.8 pg/ml for TNF α . Our experience with these multiplex cytokine assay kits analyzing samples from brain tissue is that the functional lower limit of quantitation is 2.2 pg/ml for IL-6, and 8.0 pg/ml for IL-1 β based upon assessment of the linear portion of the standard curves (Datta and Opp, 2008).

Statistics

Analyses of variance were used to reveal statistically significant differences between conditions. In *Experiment 1*, we assessed the impact of sepsis on food intake and water consumption, body weights, core body temperature and cage activity using repeated measures ANOVA. For body temperature and cage activity, difference scores were calculated for each animal between the average 12 h values obtained during the dark period and the 12 h average values obtained during the light period (“dark” – “light”). These difference scores from the 3 day pre-surgical baseline recordings (protocol days –3 to 0) were compared the difference scores calculated from the same animals during the last 3 days of the recording protocol (post-CLP days 21 – 23). Although we graphically present data from all animals that began the protocol (see Fig. 1), statistical analyses were limited to survivors. That is, only pre-surgical baseline data for the animals that survived the entire protocol were included in the repeated measures analysis. A P value ≤ 0.05 was accepted as indicating significant differences between manipulations.

Values for social behavior obtained in *Experiment 2* were evaluated using a oneway ANOVA in which *manipulation* (CLP, SHAM) was the main (fixed) effect and *behavior* (simple contacts, fights anal-genital sniffing) was the random effect. A P value ≤ 0.05 was accepted as indicating significant differences between manipulations.

Results of the REST tool were used to demonstrate a statistically significant effect of sepsis on cytokine gene expression as evidenced by $\Delta\Delta^{CT}$ values (*Experiment 3*). To determine the impact of sepsis on cytokine mRNA, each cytokine (IL-1 β , IL-6, TNF) was evaluated individually within each brain region and time point. A P-value of $P < 0.05$ was used as denoting statistically significant changes in mRNA expression across time.

Cytokine protein concentrations obtained in *Experiment 4* were analyzed using a oneway ANOVA with *manipulation* (CLP, SHAM) as the fixed effect and protein (IL-1 β , IL-6, TNF α) as the *random* effect. The Bonferroni correction was used for multiple comparisons to determine changes in cytokine protein across time. However, cytokine values were only analyzed for those time-points at which values were above what we consider to be the lower limit of detection of the Bioplex kits used in this study (see methods). A P value ≤ 0.05 was accepted as indicating statistical significance for all analyses.

Results

Experiment 1: Impact of sepsis on morbidity and mortality, body temperature and cage activity

Clinical signs of illness were not present during the three day baseline recording period. Food and water consumption and body weights were stable, as were diurnal rhythms of body temperature and cage activity (Fig. 1). As characteristic of mice entrained to a light:dark cycle, body temperature and cage activity counts were greater during the 12 h dark periods as compared to the 12 h light periods. For all animals entering the protocol ($n=18$), average body temperature during the 3 day pre-surgery baseline recordings was 36.9 ± 0.1 °C during the light periods and 38.0 ± 0.1 °C during the dark periods (Fig. 1). Similarly, the average cage activity count was 8.3 ± 0.6 during the light periods and 15.8 ± 1.3 during the dark periods (Fig. 1).

CLP with a 21 gauge needle resulted in the deaths of 8 out of the 18 mice (Fig. 1). The 44% mortality in this experiment is similar to the 50% mortality previously reported in BALB/c mice after CLP with this same gauge needle (Ebong et al., 1999a). Four of the 8 animals that died did so early (days 1 – 3) after CLP, whereas two animals died late, on day 7 post-CLP.

Relative to pre-CLP values, food and water consumption were reduced for 2 – 3 days post-CLP (Fig. 1). As a consequence, body weights of mice dropped and remained lower than baseline values for 7 days post-sepsis.

Rhythms of body temperature and cage activity were severely disrupted during sepsis (Fig. 1). Altered temperature and cage activity rhythms were apparent during the subsequent dark period immediately after the CLP surgery. No discernible light:dark differences (ie., rhythms) in body temperature or cage activity of surviving mice were detected until approximately 12 days after sepsis induction. Statistical analyses revealed that diurnal temperature and cage activity rhythms of mice that survived had not recovered by the end of the recording period 23 days after sepsis induction. The average values for body temperature and cage activity during the light periods and during the dark periods of the last 3 recording days were significantly reduced as compared to values for the same animals prior to sepsis induction (Fig. 1). For the $n=10$ mice that survived sepsis, average body temperatures prior to sepsis onset were 36.7 ± 0.2 °C during the light periods and 37.8 ± 0.2 °C during the dark periods. During the last 3 recording days of the protocol, average post-sepsis body temperatures were 36.2 ± 0.1 and 37.5 ± 0.2 °C during the light and dark periods, respectively. Similarly, average cage activity counts of mice that ultimately survived were 8.0 ± 0.6 and 15.2 ± 1.0 during the pre-sepsis light and dark periods, respectively. These cage activity counts were reduced in post-sepsis surviving mice to 5.7 ± 0.5 and 10.9 ± 0.8 during the light and dark periods, respectively.

Sepsis altered the magnitude of the diurnal rhythms of body temperature and cage activity, not the timing of these rhythms (Fig. 2). When baseline (pre-sepsis) 24 h profiles of these parameters in mice that survived are compared to those of same mice 23 days after sepsis onset, there are no differences in the timing of changes in body temperature or activity that occur around the transition from the light-to-dark period.

Experiment 2: Sepsis-induced alterations in social behavior

One of the mice in the CLP-sepsis group died on day 2 resulting in a sample size of $n = 7$ for the CLP group at the 48- and 72 h testing times. Each of the quantified measures of social exploration of juvenile male mice by adult conspecifics was reduced during the 72 h period following sepsis induction as compared to SHAM-operated controls (Fig. 3). Relative to control mice subjected to SHAM surgeries, animals that had CLP exhibited fewer contacts at 48- and 72-h post-surgery, and reduced incidence of genital sniffing 24- and 48 h post-surgery (Fig. 3). Overt aggression (fights) was non-existent during the 72 h testing periods after sepsis induction.

Experiment 3: Cytokine mRNA and protein expression in brain during sepsis

Relative to the internal housekeeping gene (GAPDH) and control (SHAM operated) mice, mRNA for IL-1 β , IL-6 and TNF α in septic mice exceeded the 2-fold increase criteria for several time points and brain regions (Fig. 4). mRNA for each of these cytokines was elevated in samples obtained 6 h after surgery in all brain regions assayed and remained up-regulated 12 h post-surgery in the hippocampus. The impact of sepsis on cytokine mRNA was less consistent at 48- and 72-h post-surgery, although there was a late (48 h) increase in IL-1 β and IL-6 message in the brainstem 48 h after sepsis induction.

Cytokine protein in each of the brain regions examined increased during sepsis (Figure 4). IL-1 β , IL-6, and TNF α each increased 6 – 24 h after CLP in each brain region examined, except for the brainstem, in which IL-1 was elevated for only 6 h (Fig. 5). IL-6 and TNF remained elevated in samples from the hippocampus and brainstem for 48 h post-CLP.

Discussion

The impact of peripheral immune challenge on CNS processes has been the focus of intense investigation during the last two decades. The ability of the CNS to detect and respond to activation of the peripheral immune system has been firmly established. The impact of immune challenge on CNS processes is evidenced by changes in physiology and behavior, including but not limited to loss of appetite, withdrawal from social activity, altered sleep, temperature regulation and locomotor activity. There are multiple mechanisms whereby bi-directional communication between CNS and the peripheral immune system occurs [reviewed in (Dantzer, 2001b; Dantzer et al., 2008; Imeri and Opp, 2009)], and cytokines are critical components of systems involved in these communication pathways. Of importance to this study, cytokines are mediators of many of the physiological and behavioral responses to immune challenge, which are collectively referred to as sickness [reviewed in (Dantzer et al. 2008)]. The role of cytokines as mediators of sickness has been determined, in part, by using LPS or selected cytokines as a challenge to activate the immune system (Bluthé et al., 1994; Inui, 2001; Harden et al., 2006). These methods have utility as a means to elicit rapid responses that are of relatively short duration. For example, studies using LPS or cytokine administration have demonstrated alterations in temperature regulation and activity patterns of laboratory rodents (Inui, 2001; Harden et al. 2006). However, responses to LPS or cytokine administration cannot mimic all facets of the complex responses to infection because there is no replicating pathogen.

CLP is considered a clinically relevant model of sepsis because it a) elicits dynamic changes in cardiovascular function that are similar to those observed in septic patients, b) induces the progressive release of inflammatory mediators, and c) is sensitive to antibiotic treatment and fluid resuscitation (Newcomb et al. 1998; Ebong et al. 1999a;1999b). The CLP procedure is relatively simple, yields reliable outcomes, and allows titration of disease severity by varying the gauge of needle used to puncture the cecum (Nemzek et al., 2008; Rittirsch et al., 2009). We used CLP in this study to determine sepsis-induced morbidity (changes in food and water consumption and loss of body weight), and alterations in core body temperature, cage activity and social behavior of mice. Our data are consistent with previous demonstrations of sickness behavior induced by administration of IL-1 β , IL-6 or LPS [e.g., (Bluthé et al., 2000b; Dantzer, 2001b)]. Social withdrawal in this study was evidenced by a reduction in the number of interactions of septic adult mice with healthy juvenile conspecifics, effects that lasted for at least 3 days after sepsis induction. Sepsis-induced reductions in food and water consumption were transient, lasting only 2 – 3 days after sepsis induction. However, this transient reduction in food and water intake resulted in body weights being reduced below pre-sepsis baseline values for about one week.

We extend previous observations of a role for cytokines in responses to sepsis by quantifying changes of cytokine (IL-1 β , IL-6, TNF α) mRNA and protein within the hypothalamus, hippocampus, and brainstem. These brain regions are implicated in the regulation/modulation of many facets of sickness behavior. For example, within the context of sickness the hypothalamus is critical for the regulation of body temperature, locomotor activity, feeding, drinking, and energy metabolism (Klir et al., 1993; Myers et al., 1993; Gayle et al., 1999; Elmquist et al., 2000; Gaykema et al., 2008; Szymusiak and McGinty, 2008; Thaler et al., 2012), as well as the timing of when these physiological and behavioral processes occur (Beynon and Coogan, 2010). The hippocampus is involved in aspects of cognition, learning and memory (Chen et al., 2008) that may be relevant to altered social exploration, and the brainstem is critical for aspects of autonomic responses to this insult (Kafa et al., 2010). These changes in cytokine mRNA and protein occur in a temporal pattern that supports the hypothesis that these cytokines may be mediators of acute responses to sepsis. The extent to which IL-1 β , IL-6 and/or TNF α play a role in the long-

term sepsis-induced alterations of body temperature and activity rhythms remains to be determined because in this study we did not obtain samples at later time points.

Laboratory rodents entrained to a light:dark cycle exhibit diurnal rhythms for many physiological processes and behaviors. These diurnal rhythms include activity patterns, body temperature, and sleep-wake behavior, among others. We have previously demonstrated that sleep, electroencephalogram (EEG) parameters, and brain temperature of rats is profoundly altered for at least 4 days following sepsis induction (Baracchi et al., 2011). One characteristic of responses of rats to sepsis is that the normal diurnal rhythms of sleep, EEG measures and brain temperature are not apparent (Baracchi et al., 2011). In this present study, we did not determine sepsis-induced alterations in sleep, but our recordings reveal that sepsis has a long-term impact on activity and body temperature rhythms of mice. After sepsis induction, there is a complete loss of diurnal rhythmicity of body temperature and activity that lasts about 12 days. The prolonged alterations in diurnal rhythmicity of body temperature and activity after sepsis have been previously reported. Ebong et al., (Ebong et al., 1999a) demonstrate sepsis-induced hypothermia and reduction in cage activity of mice for 8 days after CLP-induced sepsis with the same gauge needle we used in this present study. The acute up-regulation of cytokine gene expression and protein in the CNS during sepsis suggests one mechanism for the loss of diurnal rhythms of body temperature and activity during this 12-day period. IL-1 β and TNF modulate the expression of various clock genes (Cavadini et al., 2007; Beynon and Coogan, 2010), and signaling receptors for IL-1 β and TNF α are found throughout the brain, including the hypothalamus and the suprachiasmatic nucleus [SCN; (Beynon and Coogan, 2010)]. Few studies have determined effects of sepsis on the SCN, but data from our laboratory (unpublished) suggest that the number of c-Fos⁺ cells in the SCN increases during CLP-induced sepsis. In this study, we focused on early changes in cytokine mRNA and protein in brain after sepsis induction (6 – 72 h after CLP). It is presently not known whether cytokines upregulated during acute responses to sepsis play a role in the long-lasting effects on activity and body temperature observed in this study. However, our findings suggest that the CNS is impaired long after the animal is clinically stable and is no longer at risk of death. Patients who survive sepsis often suffer from cognitive impairment long after hospital discharge (Comim et al., 2009; Iwashyna et al., 2010), suggesting that the effects of sepsis on the brain are not limited to the acute period during active infection. Our data obtained from laboratory mice demonstrate persistent alterations in body temperature and activity patterns that are consistent with observations that human patients suffer effects of sepsis after hospital discharge. Ongoing studies in our laboratory aim to elucidate mechanisms responsible for the long-lasting effects of sepsis on the CNS.

The time-course of increased cytokine mRNA and protein depends on the brain region assayed. In the hypothalamus, IL-1 β , IL-6 and TNF α proteins were elevated at 6 h post-surgery and returned to baseline values by 48 hours post-surgery. In the hippocampus and brainstem, only IL-1 β protein returned to baseline before the 72 h sample. These findings of an early increase in cytokine protein in mouse brain are consistent with those of a recent study demonstrating increased IL-1 β , IL-6 and TNF in hypothalamus of rats as early as 3 – 6 h post CLP (Figueiredo et al. 2012). The brain heterogeneity with respect to cytokine responses to CLP may have functional relevance due to the generalized roles of the hypothalamus, hippocampus and brainstem in different physiological and behavioral processes. Additional studies with better spatial resolution of cytokine upregulation in brain are necessary before definitive conclusions may be made. It is clear, however, that cytokines are critical mediators of CNS responses to sepsis [e.g., (Gourine et al., 1998; Leon et al., 1998; Figueiredo et al., 2012)]. Previous studies demonstrate that TNF is a key mediator of temperature responses of mice to sepsis, whereas IL-6 is implicated in fever and food intake (Leon et al., 1998). Our data indicate that mRNA expression of TNF in hypothalamus,

hippocampus and brainstem is upregulated for only 6 – 12 h post-CLP, whereas TNF protein in brain remains elevated for 48 h or longer. It seems unlikely that TNF mRNA upregulated 6 – 12 h post-CLP would be responsible for elevated protein some 36 h later. Mechanisms exist by which TNF from the periphery is actively transported across the blood brain barrier and into the CNS (Banks et al., 1995; Pan et al., 1997; Banks et al., 2001). The method of cytokine assay used in this study does not allow us to know the source of the protein. As such, it is possible that the elevated TNF detected 48 h after sepsis induction by CLP does not represent de novo synthesis in the brain, but reflects transport of TNF from the periphery into the brain. Studies underway in our laboratory focus on determining the relative contributions of cytokine synthesis in brain and active transport of cytokines across the blood-brain-barrier to the pathophysiology and CNS responses to sepsis. Nevertheless, our observations that TNF protein is elevated in the hypothalamus, the brain region primarily responsible for temperature regulation, for the duration of our sampling interval suggests central actions of TNF as a potential mediator of the prolonged impact of sepsis on body temperature rhythms.

Collectively, our data suggest behavioral outcome measures may be used as global indicators of CNS impairment during the development of sepsis. We have demonstrated that there are acute effects of sepsis on social behavior, potentially mediated by regulation of cytokine protein and mRNA levels in discrete brain regions although further definitive experiments to demonstrate such a role remain to be conducted. Further, we have identified effects on diurnal rhythms of cage activity and body temperature that last long after body weights, food intake and water consumption have returned to baseline (pre-septic) values, and when the animal is no longer at risk of dying from sepsis. The mechanism(s) and molecules responsible for these long-lasting effects of sepsis on the CNS remain to be elucidated. A growing literature suggests the clinical relevance of these types of investigations because patients who survive critical illness, including sepsis, often manifest neurocognitive deficits long after discharge from intensive care (Iwashyna et al., 2010).

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References

- Banks WA, Kastin AJ, Broadwell RD. Passage of cytokines across the blood-brain barrier. *Neuroimmunomodulation*. 1995; 2:241–248. [PubMed: 8963753]
- Banks WA, Moinuddin A, Morley JE. Regional transport of TNF-alpha across the blood-brain barrier in young ICR and young and aged SAMP8 mice. *Neurobiol Aging*. 2001; 22:671–676. [PubMed: 11445267]
- Baracchi F, Ingiosi AM, Raymond RM Jr, Opp MR. Sepsis-induced alterations in sleep of rats. *Am J Physiol Regul Integr Comp Physiol*. 2011; 301:R1467–R1478. [PubMed: 21900639]
- Basler T, Meier-Hellmann A, Bredle D, Reinhart K. Amino acid imbalance early in septic encephalopathy. *Intensive Care Medicine*. 2002; 28:293–298. [PubMed: 11904658]
- Beynon AL, Coogan AN. Diurnal, age, and immune regulation of interleukin-1beta and interleukin-1 type 1 receptor in the mouse suprachiasmatic nucleus. *Chronobiol Int*. 2010; 27:1546–1563. [PubMed: 20854134]

- Bluthé R-M, Layé S, Michaud B, Combe C, Dantzer R, Parnet P. Role of interleukin-1 β and tumour necrosis factor- α in lipopolysaccharide-induced sickness behaviour: a study with interleukin-1 type I receptor-deficient mice. *Eur J Neurosci*. 2000a; 12:4447–4456. [PubMed: 11122355]
- Bluthé R-M, Michaud B, Poli V, Dantzer R. Role of IL-6 in cytokine-induced sickness behavior: a study with IL-6 deficient mice. *Physiol Behav*. 2000b; 70:367–373. [PubMed: 11006436]
- Bluthé RM, Pawlowski M, Suarez S, Parnet P, Pittman Q, Kelley KW, Dantzer R. Synergy between tumor necrosis factor α and interleukin-1 in the induction of sickness behavior in mice. *Psychoneuroendocrinology*. 1994; 19:197–207. [PubMed: 8190839]
- Cavadini G, Petrzilka S, Kohler P, Jud C, Tobler I, Birchler T, Fontana A. TNF-alpha suppresses the expression of clock genes by interfering with E-box-mediated transcription. *Proc Natl Acad Sci U S A*. 2007; 104:12843–12848. [PubMed: 17646651]
- Chen J, Buchanan JB, Sparkman NL, Godbout JP, Freund GG, Johnson RW. Neuroinflammation and disruption in working memory in aged mice after acute stimulation of the peripheral innate immune system. *Brain Behav Immun*. 2008; 22:301–311. [PubMed: 17951027]
- Chida D, Iwakura Y. Peripheral TNFalpha, but not peripheral IL-1, requires endogenous IL-1 or TNFalpha induction in the brain for the febrile response. *Biochem Biophys Res Commun*. 2007; 364:765–770. [PubMed: 17967442]
- Comim CM, Constantino LC, Barichello T, Streck EL, Quevedo J, Dal-Pizzol F. Cognitive impairment in the septic brain. *Curr Neurovasc Res*. 2009; 6:194–203. [PubMed: 19534717]
- Dantzer R. Cytokine-induced sickness behavior: mechanisms and implications. *Ann N Y Acad Sci*. 2001a; 933:222–234. [PubMed: 12000023]
- Dantzer R. Cytokine-induced sickness behavior: where do we stand? *Brain Behav Immun*. 2001b; 15:7–24. [PubMed: 11259077]
- Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat Rev Neurosci*. 2008; 9:46–56. [PubMed: 18073775]
- Datta SC, Opp MR. Lipopolysaccharide-induced increases in cytokines in discrete mouse brain regions are detectable using Luminex xMAP technology. *J Neurosci Methods*. 2008; 175:119–124. [PubMed: 18771691]
- du Moulin GC, Paterson D, Hedley-Whyte J, Broitman SA. *E. coli* peritonitis and bacteremia cause increased blood-brain barrier permeability. *Brain Res*. 1985; 340:261–268. [PubMed: 2411352]
- Ebong S, Call D, Nemzek J, Bolgos G, Newcomb D, Remick D. Immunopathologic alterations in murine models of sepsis of increasing severity. *Infect Immun*. 1999a; 67:6603–6610. [PubMed: 10569781]
- Ebong SJ, Call DR, Bolgos G, Newcomb DE, Granger JI, O'Reilly M, Remick DG. Immunopathologic responses to non-lethal sepsis. *Shock*. 1999b; 12:118–126. [PubMed: 10446892]
- Elmqvist JK, Ahima RS, Elias CF, Flier JS, Saper CB. Leptin activates distinct projections from the dorsomedial and ventromedial hypothalamic nuclei. *Proc Natl Acad Sci USA*. 2000; 95:741–746. [PubMed: 9435263]
- Figueiredo MJ, Soares DM, Martins JM, de Machado RR, Sorgi CA, Faccioli LH, Melo MC, do Malvar DC, Souza GE. Febrile response induced by cecal ligation and puncture (CLP) in rats: involvement of prostaglandin E(2) and cytokines. *Med Microbiol Immunol*. 2012; 201:219–229. [PubMed: 22203392]
- Freedman NS, Gazendam J, Levan L, Pack AI, Schwab RJ. Abnormal sleep/wake cycles and the effect of environmental noise on sleep disruption in the intensive care unit. *Am J Respir Crit Care Med*. 2001; 163:451–457. [PubMed: 11179121]
- Gaykema RP, Park SM, McKibbin CR, Goehler LE. Lipopolysaccharide suppresses activation of the tuberomammillary histaminergic system concomitant with behavior: A novel target of immune-sensory pathways. *Neuroscience*. 2008; 152:273–287. [PubMed: 18082968]
- Gayle D, Sergey EI, Plata-Salamán CR. Feeding status and bacterial LPS-induced cytokine and neuropeptide gene expression in hypothalamus. *Am J Physiol*. 1999; 277:R1188–R1195. [PubMed: 10516261]

- Gourine AV, Rudolph K, Tesfaigzi J, Kluger MJ. Role of hypothalamic interleukin-1 β in fever induced by cecal ligation and puncture in rats. *Am J Physiol.* 1998; 275:R754–R761. [PubMed: 9728072]
- Harden LM, du Plessis I, Poole S, Laburn HP. Interleukin-6 and leptin mediate lipopolysaccharide-induced fever and sickness behavior. *Physiol Behav.* 2006; 89:146–155. [PubMed: 16842830]
- Hart BL. Biological basis of the behavior of sick animals. *Neurosci Biobehav Rev.* 1988; 12:123–137. [PubMed: 3050629]
- Hayes KE, Raucci JA Jr, Gades NM, Toth LA. An evaluation of analgesic regimens for abdominal surgery in mice. *Contemp Top Lab Anim Sci.* 2000; 39:18–23. [PubMed: 11487247]
- Imeri L, Opp MR. How (and why) the immune system makes us sleep. *Nat Rev Neurosci.* 2009; 10:199–210. [PubMed: 19209176]
- Inui A. Cytokines and sickness behavior: implications from knockout animal models. *Trends in Immunology.* 2001; 22:469–473. [PubMed: 11525927]
- Iwashyna TJ, Ely EW, Smith DM, Langa KM. Long-term cognitive impairment and functional disability among survivors of severe sepsis. *JAMA.* 2010; 304:1787–1794. [PubMed: 20978258]
- Kafa IM, Uysal M, Bakirci S, Ayberk KM. Sepsis induces apoptotic cell death in different regions of the brain in a rat model of sepsis. *Acta Neurobiol Exp (Wars).* 2010; 70:246–260. [PubMed: 20871644]
- Klir JJ, Roth J, Szelenyi Z, McClellan JL, Kluger MJ. Role of hypothalamic interleukin-6 and tumor necrosis factor- α in LPS fever in rat. *Am J Physiol.* 1993; 265:R512–R517. [PubMed: 8214140]
- Konsman JP, Veeneman J, Combe C, Poole S, Luheshi GN, Dantzer R. Central nervous action of interleukin-1 mediates activation of limbic structures and behavioural depression in response to peripheral administration of bacterial lipopolysaccharide. *Eur J Neurosci.* 2008; 28:2499–2510. [PubMed: 19087175]
- Koo DJ, Jackman D, Chaudry IH, Wang P. Adrenal insufficiency during the late stage of polymicrobial sepsis. *Critical Care Medicine.* 2001; 29:618–622. [PubMed: 11373430]
- Kozak W, Kluger MJ, Soszynski D, Conn CA, Rudolph K, Leon LR, Zheng H. IL-6 and IL-1 β in fever. Studies using cytokine-deficient (knockout) mice. *Ann N Y Acad Sci.* 1998; 856:33–47. [PubMed: 9917862]
- Leon LR, White AA, Kluger MJ. Role of IL-6 and TNF in thermoregulation and survival during sepsis in mice. *Am J Physiol.* 1998; 275:R269–R277. [PubMed: 9688988]
- Miller CF, Breslow MJ, Shapiro RM, Traystman RJ. Role of hypotension in decreasing cerebral blood flow in porcine endotoxemia. *Am J Physiol.* 1987; 253:H956–H964. [PubMed: 3661743]
- Myers RD, Paez X, Roscoe AK, Sherry B, Cerami A. Fever and feeding: differential actions of macrophage inflammatory protein-1 (MIP-1), MIP-1 α and MIP-1 β on rat hypothalamus. *Neurochem Res.* 1993; 18:667–673. [PubMed: 8510794]
- Nemzek JA, Hugunin KM, Opp MR. Modeling sepsis in the laboratory: merging sound science with animal well-being. *Comp Med.* 2008; 58:120–128. [PubMed: 18524169]
- Newcomb D, Bolgos G, Green L, Remick DG. Antibiotic treatment influences outcome in murine sepsis: mediators of increased morbidity. *Shock.* 1998; 10:110–117. [PubMed: 9721977]
- Olivadoti MD, Opp MR. Effects of i.c.v. administration of interleukin-1 on sleep and body temperature of interleukin-6-deficient mice. *Neuroscience.* 2008; 153:338–348. [PubMed: 18367337]
- Opp MR, Krueger JM. Interleukin 1-receptor antagonist blocks interleukin 1-induced sleep and fever. *Am J Physiol.* 1991; 260:R453–R457. [PubMed: 1825458]
- Pan W, Banks WA, Kastin AJ. Blood-brain barrier permeability to ebitaride and TNF in acute spinal cord injury. *Exp Neurol.* 1997; 146:367–373. [PubMed: 9270046]
- Remick DG, Newcomb DE, Bolgos GL, Call DR. Comparison of the mortality and inflammatory response of two models of sepsis: lipopolysaccharide vs. cecal ligation and puncture. *Shock.* 2000; 13:110–116. [PubMed: 10670840]
- Rittirsch D, Huber-Lang MS, Flierl MA, Ward PA. Immunodesign of experimental sepsis by cecal ligation and puncture. *Nat Protoc.* 2009; 4:31–36. [PubMed: 19131954]

- Szymusiak R, McGinty D. Hypothalamic regulation of sleep and arousal. *Ann NY Acad Sci.* 2008; 1129:275–286. [PubMed: 18591488]
- Thaler JP, Yi CX, Schur EA, Guyenet SJ, Hwang BH, Dietrich MO, Zhao X, Sarruf DA, Izgur V, Maravilla KR, Nguyen HT, Fischer JD, Matsen ME, Wisse BE, Morton GJ, Horvath TL, Baskin DG, Tschop MH, Schwartz MW. Obesity is associated with hypothalamic injury in rodents and humans. *J Clin Invest.* 2012; 122:153–162. [PubMed: 22201683]
- Tsao N, Hsu HP, Wu CM, Liu CC, Lei HY. Tumour necrosis factor- α causes an increase in blood-brain barrier permeability during sepsis. *J Med Microbiol.* 2001; 50:812–821. [PubMed: 11549183]
- Vyas D, Javadi P, Dipasco PJ, Buchman TG, Hotchkiss RS, Coopersmith CM. Early antibiotic administration but not antibody therapy directed against IL-6 improves survival in septic mice predicted to die on basis of high IL-6 levels. *Am J Physiol Regul Integr Comp Physiol.* 2005; 289:R1048–R1053. [PubMed: 15947070]

Fig. 1. Sepsis induces morbidity and mortality in mice

Baseline measures of body weight, food and water consumption, body temperature and cage activity were obtained for three days from adult male mice ($n = 18$). All animals were then subjected to CLP surgeries with a 21 gauge needle as described (timing indicated by dashed vertical line), and recordings continued for 23 days. Symbols are means $\pm$ SDEV (body weights, food and water consumption) or SEM (body temperature and activity). Measures of body weight, food and water consumption were obtained daily for 8 days post-CLP and then every other day thereafter. Values for body temperature and activity are 12 h averages for the light period (open symbols) and dark period (closed symbols) for each day. Solid horizontal lines depict the average 12 h light period and dark period values obtained during the 3 day baseline recordings. Dotted horizontal lines depict the average 12 h light period and dark period values during the final 3 days of recording. Data for all animals is graphically depicted, but statistical analyses were restricted to survivors ($n = 10$) such that pre-surgical baseline values were statistically compared to those from the same animals during the last 3 days of the recording protocol. * = $p < 0.05$.

Fig. 2. Sepsis alters the 24 h profile of body temperature and activity

Symbols depict the hourly means ($\pm$ SEM) for body temperature and activity of mice that survived sepsis as averaged across the three pre-CLP baseline days (open symbols) and the last three recording days (days 21 – 23) post-CLP (closed symbols). Sepsis reduced the amplitude of these diurnal rhythms, but did not alter their timing. The dark bar on the x-axis depicts the dark period of the light dark cycle. See also Fig. 1.

Fig. 3. Sepsis reduces social exploration

The mean ($\pm$ SDEV) numbers of contacts, overt fights, and incidents of genital sniffing were reduced for 24 – 72 h in mice that were septic (closed bars; CLP surgeries) as compared to control mice (SHAM surgeries). Overt fights were non-existent during the 72 h post-CLP. * = $p < 0.05$ vs. SHAM.

Fig. 4. Sepsis upregulates cytokine mRNA in discrete mouse brain regions

Early responses to sepsis include upregulation of interleukin-1 β (IL-1 β), IL-6, tumor necrosis factor- α (TNF α) mRNA in hypothalamus, hippocampus, and brainstem of mice. Mice were subjected to CLP or SHAM surgeries (n=5 – 6 / group) as described, and sacrificed at 6-, 12-, 24-, 48-, or 72- h post-surgery. The fold increase in cytokine mRNA was calculated using REST 2009 software (see methods) based on the comparative cycle threshold (CT) method relative to both the internal housekeeping gene (GAPDH) and control animals (SHAM surgeries). Asterisks (*) indicate significant changes in gene expression ($\Delta\Delta$ ct) at P = 0.05.

Fig. 5. Sepsis increases cytokine protein in discrete mouse brain regions

Adult mice were subjected to CLP (closed bars) or SHAM (open bars) surgeries and sacrificed at 6-, 24-, and 72-h later (n = 4 / group). Interleukin-1 β , interleukin-6, tumor necrosis factor- α concentrations were determined from hypothalamus, hippocampus and brainstem. Horizontal lines indicate lower limits of detection for the assay. * = p < 0.05 vs. SHAM; # = p < 0.05 vs. 6 h sample.