Title: Sepsis-Induced Alterations in Sleep of Rats

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Abstract:

Sepsis is a systemic immune response to infection that may result in multiple organ failure and death. Polymicrobial infections remain a serious clinical problem, and in the hospital sepsis is the number one non-cardiac killer. Although the central nervous system may be one of the first systems affected, relatively little effort has been made to determine the impact of sepsis on the brain. In this study we used the cecal ligation and puncture (CLP) model to determine the extent to which sepsis alters sleep, the electroencephalogram (EEG), and brain temperature (Tbr) of rats. Sepsis increases the amount of time rats spend in non-rapid eye movement sleep (NREMS) during the dark period, but not during the light period. Rapid eye movements sleep (REMS) of septic rats is suppressed for about 24 h following CLP surgery, after which REMS increases during dark periods for at least three nights. The EEG is dramatically altered shortly after sepsis induction, as evidenced by reductions in slow frequency components. Furthermore, sleep is fragmented, indicating that the quality of sleep is diminished. Effects on sleep, the EEG and Tbr persist for at least 84 hours after sepsis induction, the duration of our recording period.

Immunohistochemical assays focused on brainstem mechanisms responsible for alterations in REMS as little information is available concerning infection-induced suppression of this sleep stage. Our immunohistochemical data suggest that REMS suppression after sepsis onset may be mediated, in part, by the brainstem GABAergic system. This study demonstrates for the first time that sleep and EEG patterns are altered during CLP-induced sepsis. These data suggest that the EEG may serve as a biomarker for sepsis onset. These data also contribute to our knowledge of potential mechanisms whereby infections alter sleep and other CNS functions.

Key words: thermoregulation, behavior, cytokines, circadian rhythms, cecal ligation and puncture
Introduction

Sepsis results from an amplified inflammatory response to an infectious process that leads to organ dysfunction or risk for secondary infection. As a clinical syndrome, sepsis is defined by the presence of infection and a systemic inflammatory response (7, 29). In spite of the ever-increasing sophistication of our antimicrobial armamentarium, sepsis continues to kill millions of people world-wide and the diagnosis of sepsis still confers a 20 - 50% risk of mortality (1, 33). The National Center for Health Statistics now lists sepsis among the top 10 leading causes of death in the United States, and in fact, sepsis / septic shock is the leading cause of death among patients in non-coronary hospital units (34, 48). For those who survive, the impact of severe sepsis does not end with hospital discharge. Mortality from all causes increases from 21% at admission to approximately 75% five years after index admission for severe sepsis (60). Furthermore, survivors of sepsis often suffer from new, persistent and dramatic cognitive impairment (25). These epidemiologic data demonstrate that sepsis must also be viewed as a long-term chronic condition and that severe sepsis remains a tremendous public health burden in terms of lives lost and cost. However, although sepsis is often associated with cognitive impairment and end-stage encephalopathy [e.g., (20)], relatively little research has focused on the impact of sepsis on central nervous system (CNS) processes.

Links between the immune system and the central nervous system (CNS) have been identified and characterized during the last 30 years. Infections impact the CNS, and sick individuals experience cognitive deficits, malaise and lethargy. Infections also induces profound changes in sleep-wake behavior, appetite, social contact and other usual daily activities (12), a constellation of symptoms referred to as sickness behavior. Sickness behavior, including changes in sleep, is viewed as an adaptive response of the host organism to infection that ultimately contributes to survival (22, 24).

To advance our understanding of the impact of traumatic infections on CNS processes, and specifically sleep, in this study we determined the extent to which sleep-wake behavior of rats are altered during CLP-induced sepsis. Because sepsis is a heterogeneous and complex pathology, in vivo models are critical tools and have become the mainstay of sepsis research [reviewed (36)]. Two models frequently used to determine mechanisms and mediators of morbidity and mortality in response to sepsis are intraperitoneal administration of high doses of lipopolysaccharide (LPS) and cecal ligation and puncture (CLP). However, bolus administration of LPS does not constitute an infection as there is no replicating pathogen, whereas CLP mimics clinical conditions with respect to bowel perforation and subsequent peritonitis due to mixed intestinal flora. CLP is considered a clinically-relevant sepsis model, in part, because it reproduces the dynamic changes in cardiovascular function observed in septic patients, recreates the progressive release of proinflammatory mediators (13, 14), and is sensitive to antibiotic treatment and fluid resuscitation (37). The CLP technique is relatively simple and yields reliable outcomes (46).

Based on previous studies of laboratory animals infected with select bacterial pathogens, we hypothesized that sepsis, as a polymicrobial infection, also would alter sleep. Specifically, we hypothesized that non-rapid eye movement sleep (NREMS) would increase during sepsis, whereas rapid eye movement sleep (REMS) would be suppressed. Moreover, to begin to elucidate potential mechanisms whereby this infectious process alters sleep, we used immunohistochemistry to determine the impact of sepsis on the activity of brainstem nuclei known to be involved in the regulation of sleep. We now report that sleep of rats is altered for at
least four days during the course of sepsis, and that activity of brainstem nuclei involved in
the regulation of some facets of sleep is altered in a manner demonstrating a potential role for the
GABAergic system as a mediator of these responses.

Methods
Animals
These experiments were performed on male Sprague-Dawley rats (300-350 g at surgery; Harlan,
Indianapolis, IN). Before and after surgical manipulations, all rats were individually housed in
standard cages and kept in temperature controlled chambers at 21 ± 1 °C. Lights were
maintained on a 12:12 h light–dark cycle and food and water were provided ad libitum. All
procedures involving the use of animals were approved by the University of Michigan
Committee on Care and Use of Animals in accordance with the US Department of Agriculture
Animal Welfare Act, and the Public Health Service policy on Humane Care and Use of
Laboratory Animals.

Surgical procedures
Two groups of rats were used in this study. Rats in group 1 (n = 37) were used to determine the
impact of sepsis on sleep-wake behavior. These rats were subjected to two surgical procedures.
The first, a cranial surgery, was used to implant instrumentation that allows subsequent
determination of sleep-wake behavior. The second, an abdominal surgery, was to perform the
CLP procedure as a means of inducing sepsis. During the cranial surgery [procedures previously
described in (23)], three stainless steel screws to serve as electroencephalographic (EEG)
recording electrodes were implanted into the skull over frontal and parietal brain cortices. A
calibrated 30-kOhm thermistor (Omega Engineering, Stamford, CT) was implanted between the
dura mater and the skull over a parietal cortex to measure cortical brain temperature (Tbr). At
the end of the surgery the wound was treated with topical analgesic (L.M.X.4, Lidocaine 4%,
Ferndale IP, Inc., MI) and a triple antibiotic ointment (Neomycin and Polymixin B sulfate and
Bacitracin Zinc, E. Fougera & Co., NY). The animals were allowed 10 days of recovery after the
surgical procedures. On the fifth postsurgical day, the rats were connected to the recording
apparatus via a flexible tether for habitation to the recording environment.

On days 10 – 12 after the cranial surgery a 48 h recording period was used to obtain baseline
data for each animal (see later). After this baseline recording period, the abdominal surgery was
performed (46). Some of the rats underwent CLP surgery whereas others had SHAM surgery.
Animals were deeply anesthetized, a 1-cm midline incision made on the abdomen, and the cecum
exposed. The distal 1 cm of the cecum was ligated with a 4–0 Prolenesuture (Ethicon, Johnson &
Johnson, Langhorne, PA) and perforated twice through-and-through with a 21-gauge needle. A
small amount of stool was extruded to ensure that the wound was patent. The cecum was placed
back into the abdominal cavity and the abdomen was closed using silk sutures (4-0, Ethicon
Johnson & Johnson, Langhorne, PA). Animals subjected to SHAM surgery underwent an
identical procedure; however the cecum was exposed but not ligated or punctured and then
replaced. Two hours and 8 hours after surgery (and twice daily for the rest of the protocol), each
animal (SHAM, CLP) received a subcutaneous injection of a broad spectrum antibiotic
(Imipenem, 0.5 mg/rat) in 1 ml of lactated Ringers/D5W. As such, each rat received 10 ml of
fluids during the post-surgical period.

Rats in group 2 (n = 38) were used to determine the impact of sepsis on activity of the
cholinergic and GABAergic systems in selected brainstem nuclei. These animals did not receive
a cranial implant, but were subjected to either CLP or SHAM surgeries, as described.
Experimental protocol

Two separate experiments were conducted in this study. Animals in Experiment 1 were used to determine the impact of sepsis on sleep-wake activity of rats. After recovery from the cranial surgery and adaptation to the recording apparatus, two 24 h undisturbed baseline recordings were obtained from these animals. After this 48 h baseline recording period rats underwent abdominal surgery (CLP, n = 20; SHAM, n = 17) during the first 6 h of the light period. Recordings began at the beginning of the subsequent dark period and continued for as long as 96 h. The rats were undisturbed during these recordings, with the exception of administration of antibiotics twice daily.

Experiment 2 was a “post hoc” experiment, the design of which was guided by results of Experiment 1. Rats in this experiment (SHAM, n = 12; CLP, n = 26) were used to obtain brains for immunohistochemical assessment of the impact of sepsis on selected neurotransmitter systems (see later). After determining the impact of sepsis on sleep of rats (Experiment 1), experimental time points that corresponded to periods during which differences in sleep-wake behavior were revealed were selected. The experimental time points selected for sacrifice were the mid-points of the light periods 30-, 54- and 78 h post-surgery.

Recording apparatus and determination of sleep-wake behavior

Signal conditioning and determination of sleep-wake behavior from rats have been previously described (23). Briefly, signals from the EEG electrodes and thermistors were fed into amplifiers (Colbourn Instruments, Lehigh Valley, PA; models S75–01 and S71–20, respectively). Motor activity was detected using an infrared sensor housed in an observation unit (BioBserve, GmbH, Bonn, Germany). Movements detected by the infrared sensor were converted to a voltage output, the magnitude of which was directly related to the magnitude of movements detected. All the signals were subjected to analog-to-digital conversion (A/D board: PCI.3033E, National Instruments, TX) with 16-bit precision at a sampling rate of 128 Hz for EEG, 1 Hz for Tbr and 8 Hz for motor activity. The digitized EEG waveform, Tbr samples and integrated values for motor activity were stored as binary files until further processing. Arousal states were assigned with a resolution of 12-s by visual scoring of EEG, body movements and Tbr using custom software (ICELUS, M. Opp, University of Michigan). The EEG signal was subjected to fast Fourier transformation (FFT), yielding power spectra between 0.5 and 30 Hz in 0.5-Hz frequency bins. Arousal state was classified as previously described as NREMS, REMS, or wakefulness on the basis of state-dependent changes in multiple parameters, including the EEG, offline FFT values, body movements and Tbr (23). Any 12-s epoch containing movement artifacts or electrical noise was tagged and excluded from subsequent spectral analyses.

Animal perfusion and tissue sectioning

All rats in Experiment 2 were transcardially perfused under pentobarbital anesthesia (Nembutal; 1.5 ml i.p. of 50 mg/ml). Approximately 75 ml of room temperature saline was perfused transcardially followed by 1000 ml of a 3% paraformaldehyde solution and 250 ml of a 10% sucrose solution at 4 °C. The brains were post fixed overnight in a 30% sucrose solution at 4 °C. Brains were frozen and stored at -80 °C before sectioning. Coronal brainstem sections (25 μm) were cut serially on a sliding microtome with a freezing stage and stored in a cryoprotectant solution (24% v/v Glycerin, 29% v/v Ethylene Glycol, 0.1 M Sodium Phosphate Dibasic) at -20 °C until assay. In contrast to tissue from SHAM control animals, brain tissue from septic rats was difficult to section, presumably as a result of the infection.
Immunostaining

Immunohistochemistry was performed on series of adjacent free-floating frozen brain sections focusing on the cholinergic laterodorsal tegmental/pedunculopontine nuclei (LDT/PPT) and the GABAergic ventrolateral periaqueductal grey (vlPAG) of rats. All brain sections were processed using a double labeling immunoperoxidase procedure to detect the immediate-early gene product c-Fos and either choline acetyltransferase (ChAT) or glutamic acid decarboxylase-67 (GAD67). All reagents used were obtained from Vector Laboratories unless otherwise stated. Tissue was sequentially incubated in rabbit anti-c-Fos polyclonal primary antibody (1:1500, Abcam), biotinylated goat anti-rabbit IgG secondary antibody (1:300) and ABC reagent (Vectastain® Elite® ABC kit). DAB (3,3'-diaminobenzidine) chromagen substrate (DAB Substrate Kit for Peroxidase) was used to visualize c-Fos+ nuclei. Double labeling for ChAT was performed using successive incubations in sheep anti-ChAT primary antibody (1:18,000, Abcam), biotinylated rabbit anti-sheep IgG secondary antibody (1:200) and ABC reagent. Double labeling for GAD67 was achieved by serial incubations in mouse anti-GAD67 monoclonal primary antibody (1:2000, Chemicon International), rat adsorbed biotinylated horse anti-mouse IgG (1:200) and ABC reagent. Silver gray chromagen (Vector® SG Substrate Kit for Peroxidase) was used to visualize ChAT and GAD67 immunoreactivity in the cytoplasm. Negative controls were included in each assay by processing tissue without the respective primary antibodies. At the conclusion of the immunostaining procedure, sections were transferred to glass microscope slides (Superfrost Plus®, Fisherbrand®, Fisher Scientific), dehydrated through a series of graded alcohols (50%, 70%, 95%, and 100%), incubated in xylene and coverslipped with Permount® mounting medium (Fisher Scientific).

Cell counts in LDT/PPT and vlPAG

Coronal brainstem sections were taken from -6.72 to -9.23 mm relative to Bregma to include LDT, PPT and vlPAG. To assess the impact of sepsis on the cholinergic system, ChAT+, c-Fos+ and c-Fos+/ChAT+ dual-labeled cells were counted from a 1 in 200 μm series (1 in 8 series). GABAergic activation during sepsis was determined by counting GAD67+, c-Fos+, and c-Fos+/GAD67+ cells a 1 in 400 μm series (1 in 16 series). These series of sections were selected on the basis of previous studies by Jones and colleagues (31, 32). Stained sections were visualized using light microscopy. ChAT, GAD67 and c-Fos positive cells were counted using the Optical Fractionator workflow of Stereo Investigator version 7.0 (MicroBrightfield, Willison, VT). Contours were drawn at 2X and 4X magnification and were based on region outlines depicted in the Paxinos and Watson Rat Brain Atlas (44). Individual neurons were counted within contours at 60X magnification by an individual blinded to the experimental manipulation of the animal. Counts were made bilaterally on sections through the LDT/PPT and the vlPAG. Sections between animals were matched as closely as possible according to their position relative to Bregma and the same number of sections per region/nuclei were selected for counting from each animal. Individual sections were counted and numbers summed to produce a single value for that animal based on brain region.

Statistical analysis

Results are presented as mean ± SEM. Tests for statistical significance were performed using SPSS for Windows. Two types of statistical comparisons were made. To determine the impact of manipulations across time, analyses were restricted to within group (SHAM, CLP) comparisons for time spent in each behavioral state, motor activity, Tbr and delta power during NREMS. These within-group comparisons were made by means of a general linear model for repeated measures. The second comparison was used to determine if the responses to CLP
differed from those of control (SHAM) animals, i.e., these were between group comparisons. For these analyses, difference scores were calculated for time spent in each behavioral state, Tbr and delta power during NREMS. These difference scores were obtained by subtracting baseline values for each parameter from values obtained after CLP or SHAM surgery. Between group differences were analyzed with a mixed model for repeated measures, using a first-order regression covariance structure where time was the repeated measure. Within- and between group comparisons were performed on 6 h time blocks.

Within- and between-group analyses of dark/light differences in each behavioral state, motor activity, Tbr and delta power during NREMS were performed by means of a one-way ANOVA, with manipulation (CLP, SHAM) as the independent variable. Post-hoc comparisons, with Bonferroni correction, were performed within-groups to evaluate differences between baseline and each experimental day.

The numbers of c-Fos+, ChAT+, and GAD67+ cells as a function of manipulation (CLP, SHAM) were analyzed by one-way ANOVA. For all analyses, an alpha level of \( p < 0.05 \) was accepted as indicating statistically significant differences in values.

**Results**

Data derived from this study are presented in two ways. First, comparisons within group (SHAM, CLP) demonstrate responses to respective surgical procedures across time. Second, comparisons between groups demonstrate the impact of sepsis on outcome measures as compared to values obtained from control animals.

There were no significant differences among any of the biological parameters obtained during the pre-abdominal surgery baseline recording. As such, the data for these two 24 h recording periods were averaged to provide a 24 h baseline profile of control values. The 24 h baseline profile is sequentially plotted in appropriate figures for 96 h (ie., 4 days).

**Sleep-wake behavior**

During undisturbed baseline recordings, there were no differences in sleep-wake behavior between animals that were subsequently divided into the two manipulation groups. As a group, these rats exhibited normal diurnal variation in sleep-wake behavior, spending less time in wakefulness and more time in both NREMS and REMS during the light period than during the dark period of the 24 h light:dark cycle (\% recording time NREMS: 41.7 ± 0.6 light period, 23.3 ± 0.7, dark period; \% recording time REMS: 11.3 ± 0.2 light period, 5.1 ± 0.2 dark period; \% recording time WAKE: 47.0 ± 0.7 light period, 71.6 ± 0.9 dark period).

There was no mortality in animals randomized into the SHAM group (\( n = 17 \)). Of the \( n = 20 \) animals randomized into the CLP group, \( n = 2 \) (10%) died during sepsis. Corresponding baseline values were omitted from analyses and graphic representation for periods after rats died. In this manner, the within-subjects nature of the protocol was maintained as baseline values and post-CLP values always correspond across animals.

The impact of sepsis on NREMS, REMS, and wakefulness are summarized day-by-day across the post-surgical recording period to simplify data presentation.

*Day 1:* Arousal states were altered immediately after abdominal surgery in rats that had
SHAM (control) surgery and in animals that had CLP surgery to induce sepsis (Figs. 1, 2). During the initial post-surgery dark period the amount of time spent in wakefulness was reduced and NREMS increased in both groups with respect to baseline values (Fig. 1A, B). The magnitude of the increase in NREMS was greater in CLP-septic rats than in control rats (Fig. 2B). Delta Power during NREMS was altered only in CLP rats, which showed a marked reduction compared to baseline values (Fig. 1C, Fig. 2C). REMS was differentially affected in the two groups. REMS of control animals (SHAM) increased during the subsequent dark period following surgery (Fig. 1D), whereas REMS was almost absent in CLP-septic rats (Fig. 1D). Compared to baseline, sleep of rats in both groups was fragmented during the 12 h dark period of Day 1, as indicated by an increase in the number of transitions from one behavioral state to another (Fig. 3).

**Day 2**: Relative to baseline recordings, wakefulness was reduced and NREMS was increased in both groups, but this effect was limited to the dark period (Fig. 1A, B) and was of greater magnitude in septic rats (Fig. 2A, B). Delta power during NREMS was suppressed during the entire 24 h on Day 2 in septic animals, but not in the control group (Fig. 1C). The suppression of REMS observed in septic rats during Day 1 persisted during the 12 h of the light period of Day 2, after which REMS of these animals increased during the dark period relative to baseline values (Fig. 1D). Time spent in REMS differed between groups during the light period, but not during the dark period (Fig. 2D). With respect to baseline values, sleep of septic rats (CLP) was fragmented during the dark period of the second postsurgical recording day (Fig. 3).

**Day 3**: Both groups of rats spent less time awake during Day 3, but the pattern differed between groups. The reduction in wakefulness of control rats was limited to the 12 h of the light period, whereas in septic animals wakefulness was reduced only during the dark period (Fig. 1A). Relative to baseline values, the magnitude of differences between the groups differed statistically only during the dark period (Fig. 2A). NREMS of septic rats (CLP) was increased for the entire 24 h period, although the majority of this effect was apparent during the dark period (Fig. 1B). Direct comparison between groups showed that the difference in the NREMS response to sepsis was limited to the 12 h of the dark period (Fig. 2B). Relative to baseline values, delta power during NREMS was reduced in septic animals during both the light and the dark period (Fig. 1C), and differed between groups during the dark period of Day 3 (Fig. 2C). REMS was increased in SHAM group during the 12 h of the light period and in CLP group during the 12 h of the dark period (Fig. 1D). Sleep of control animals and septic rats was fragmented during the dark period of Day 3 (Fig. 3).

**Day 4**: No differences from baseline were observed in wakefulness, NREMS or delta power during NREMS in control rats (Fig. 1 ABC). In contrast, septic rats still exhibited decreased wakefulness and increased NREMS during the light period (Fig. 1 AB). Delta power during NREMS of septic rats remained decreased compared to baseline values across the 24 h period (Fig 1C). REMS was increased with respect to baseline in both groups. However, in the control group the increase in REMS was limited at the light period, whereas in septic rats it was limited to the dark period (Fig 1 D). Direct comparison between groups indicated that the treatment differences were limited to the dark period for wakefulness, NREMS and REMS (Fig. 2 ABD), whereas the difference in delta power during NREMS was observed across the 24 h (Fig 2C). In control animals, the number of transitions between states did not differ from baseline, whereas sleep was still fragmented during the dark period of septic rats (Fig. 3).

*Brain temperature*
Brain temperature of control (SHAM) animals was not altered by the surgical procedure, although there were modest reductions in Tbr during the dark periods of the recording protocol that did not achieve statistical significance (Fig. 4). In contrast, there was a profound hypothermia manifest in CLP-septic rats during the 12 h dark period following surgery (Fig. 4). In these animals, there were trends for altered Tbr during Day 2 that did not achieve statistical significance. During the dark periods of postsurgical days 3 and 4, Tbr of CLP-septic rats was still lower than during comparable baseline periods for these same animals (Fig. 4).

**Light / Dark rhythms**

Visual inspection of data across the post-surgical recording period (Figs. 1, 4) suggested that the normal diurnal rhythms of sleep-wake behavior and Tbr of CLP-septic animals were generally damped or flattened. To further analyze the impact of sepsis on these daily rhythms, the proportion of each behavioral state that was spent in the light or dark phase was calculated relative to values for that state across the entire 24 h period. For example, in healthy rats entrained to a 12-12 h light-dark cycle, a greater proportion of total daily NREMS or REMS occurs during the light phase, whereas a greater proportion of total wakefulness occurs during the dark phase. Similarly, average Tbr is greater during the dark phase than during the light phase of the light-dark cycle.

During baseline recordings, there were no differences in the light-dark distribution of sleep-wake behavior or Tbr among rats subsequently randomized to the SHAM control group and those randomized to the CLP group (Fig. 5A-D, baseline). SHAM control surgeries had little effect on subsequent light-dark distributions of sleep-wake behavior or Tbr (Fig. 5), although there was a modest shift in the distribution of wakefulness of post-surgical Day 4. In contrast, the light-dark distribution of sleep-wake behavior of rats subjected to CLP-sepsis was severely impacted (Fig. 5A-C). On post-surgical days 2 - 4, the distribution of NREMS and REMS of septic rats shifted such that a greater proportion of these sleep phases occurred during the dark period (Fig. 5BC). Concurrently, the proportion of daily wakefulness exhibited by septic rats was greater during the light period as compared to the distribution normally apparent (Fig. 5A). Average Tbr of septic rats across the 12 h dark period was reduced relative to control rats and to pre-surgical baseline values (Fig 5D).

**Activity of brainstem nuclei involved in REMS regulation**

None of the rats subjected to SHAM surgery died prior to scheduled sacrifice times. As such, n = 4 brains were obtained at each of the three experimental time points. In contrast, 6 out of 26 rats subjected to CLP surgery died before their scheduled sacrifice times, resulting in an n = 6 – 7 brains per time point. Because of the time- and labor-intensive nature of immunohistochemical assays and cell counting from multiple brain regions, a subset of n = 4 brains from the CLP-septic rats was randomly selected before tissue processing and included in this study.

c-Fos+ , ChAT+ and GAD67+ cells could be detected in tissue obtained from control (SHAM) and CLP-septic rats. Representative examples of GAD67 stained cells are given in Fig. 6. Although there was a tendency for the number of c-Fos+ cells to increase through the course of sepsis, statistical changes were revealed only in tissues taken from animals sacrificed 30 h after sepsis onset. Thirty hours after surgery, the number of c-Fos+ cells increased statistically in PPT and vlPAG of septic rats compared to control rats (Fig. 7). Although there was variability across animals, most of the c-Fos+ neurons in PPT were GABAergic, being double stained for c-Fos /GAD67. However the number of c-Fos+ / GAD67+ cells did not differ statistically between CLP and control group in this nucleus (Fig. 7). In vlPAG, about 55% of the c-Fos+ neurons were
GABAergic, and in this region there was a statistical increase in the number of c-Fos$^+$/GAD$^+$
double stained cells in septic animals (Fig. 7). No c-Fos$^+$/ChAT$^+$ double labeled neurons were
found in tissue collected from any of the rats irrespective of manipulation (data not shown).

Discussion
Clinical reports suggest the brain may be one of the first organs affected by sepsis. For example,
in a study designed to determine the effects of the ICU environment on sleep of patients,
Freedman and colleagues observed altered EEG patterns that preceded sepsis onset in patients
who became septic during the recording period (18). Our present study demonstrates for the first
time the impact of CLP-induced sepsis on EEG-defined sleep. Our results demonstrate that sleep
and EEG patterns of laboratory rats are altered during sepsis. These sepsis-induced alterations in
sleep include changes in the amount of sleep and in its diurnal distribution. In general, during
the development of sepsis the amount of time rats spend in NREMS increases during the dark
(primarily active) period, and is not dramatically altered during the light (primarily rest) period.
NREMS during sepsis is fragmented, and is associated with reductions in delta power during
NREMS, suggesting that the quality of sleep is diminished. Furthermore, sepsis in rats may alter
mechanisms of thermoregulation as septic animals exhibit damped diurnal rhythms of body
temperature for at least three days after sepsis induction. We also demonstrate with an initial
survey that the brainstem GABAergic system may contribute to the alterations in sleep during
the early stages of this infectious challenge. Collectively, these results contribute to a modest yet
growing literature that details the manner in which infections alter sleep and other CNS
processes.

The pattern of changes in sleep, delta power during NREMS and brain temperature suggests that
CLP-induced sepsis impacts the circadian system. Visual inspection of the data indicates that
these parameters do not exhibit diurnal variation, and essentially represent a “flat line” response
to sepsis. As such, in rats the normal diurnal variation in sleep-wake behavior, EEG parameters
and brain temperature are not manifest during sepsis. These observations are consistent with
other studies that describe similar effects on locomotor activity and body temperature of
laboratory rodents in response to sepsis (13, 19). Sleep and body temperature are regulated by
interactions between circadian timing systems and sleep-regulatory systems (17). The
suprachiasmatic nucleus (SCN) of the hypothalamus is the primary circadian pacemaker that
synchronizes many physiological processes and behaviors. The timekeeping apparatus of the
SCN is a molecular mechanism that involves both transcriptional repressor genes as well as
transcriptional activators, which together create a feedback loop generating circadian oscillations
(17). Cytokines such as IL-1 and TNF down regulate the expression of some of these clock
genes (6, 11). Signaling receptors for IL-1 and TNF are distributed throughout the brain,
including the hypothalamus (2, 5), and IL-1 signaling receptors are present in the SCN (6). As
such, one possibility for the damped diurnal rhythmicity of the parameters reported in this study
is the action of IL-1 and/or TNF, and possibly other inflammatory mediators, on neurons of the
SCN. In addition to direct actions of cytokines on neurons in the SCN, the disruption to the
normal diurnal rhythms for the parameters of interest in this study may also be due to endocrine
responses to sepsis. CLP-induced sepsis alters rhythms of the hypothalamic-pituitary-adrenal
axis (HPA) of rats (10), which is associated with increased immunoreactivity of corticotropin-
releasing hormone in the hypothalamus (9). Cytokine actions in brain are modulated via
negative-feedback effects of corticosterone (56). Therefore, disruption of the normal rhythmicity
of the HPA axis also may contribute to altered rhythms of sleep and brain temperature.
Although few studies have demonstrated effects of sepsis on the SCN, data from our laboratory
(unpublished) indicate that the number of c-Fos positive cells in the SCN increases during CLP-
The impact of sepsis on NREMS of rats is most pronounced during the dark phase, whereas during the light phase the amount of time spent in NREMS does not appreciably differ from either pre-surgical baseline values or from values obtained from control rats subjected to SHAM surgeries. Rats are nocturnal animals, and the dark phase of the light:dark cycle is predominantly their active period. Although these data may be interpreted such that sleep of rats during the light period represents something of a physiological ceiling, it is more likely that the relatively selective increases in NREMS during the dark period are due to the activation of overlapping systems that are involved in sleep regulation and in immune responses to infection. For example, there are diurnal rhythms of IL-1 and TNF mRNA and protein in healthy rat brain, with expression lower during the dark period than during the light period [reviewed (38)]. CLP-sepsis is characterized, in part, by increases in cytokines, notably IL-1, TNF and IL-6 (13, 14, 19). Each of these cytokines is implicated in the regulation / modulation of NREMS. As such, sepsis-induced increases in sleep-regulatory cytokines during periods when their concentrations would normally be at a minimum is expected to increase NREMS. We have not measured cytokine concentrations in septic animals from samples taken during the dark period, and are not aware of others who may have done so. Therefore, although it is likely that sepsis-induced increases in cytokine concentrations during the dark phases are responsible for the increased NREMS observed during these time periods, such findings remain to be definitively demonstrated.

Administration of cytokines, bacterial lipopolysaccharide (LPS), or infection with bacterial, viral or fungal pathogens is consistently reported to suppress REMS of laboratory animals [reviewed (38)]. This study indicates that sepsis also suppresses REMS of rats, but the effects may be more complex than those that occur in response to other types of infections or immune challenges. REMS of septic rats is suppressed for about 24 h following CLP surgery. After this time, REMS increases during the subsequent dark phases for at least three nights. Based upon previous studies of immune challenge on sleep of laboratory animals, these increases in REMS of septic rats are unexpected. Mechanisms underlying the interaction between cytokines and the regulation of REMS are as yet not well understood. Of interest, recent studies of the bi-directional interactions between the CNS and peripheral inflammation have focused on the role of the cholinergic system (55). It is now known that macrophages and other non-neuronal cell types that produce cytokines also express acetylcholine receptors (59). Central activation of acetylcholine receptors, particularly muscarinic receptors, dose-dependently suppresses circulating IL-1 and TNF through mechanisms involving vagal nerve efferents and the $\alpha_7$ subunit of nicotinic receptors on macrophages and other cells (42, 43). Although subdiaphragmatic transection of the vagus nerve does not alter the amount of time rats spend in REMS (21, 40), it does alter the EEG (40), indicating that vagal afferents contribute to properties of the cortical EEG. In addition, vagal nerve stimulation attenuates stimulated cytokine production (47), improves survival of mice subjected to CLP (47), and increases the amount of time cats spend in REMS (57, 58). Although much remains to be learned, these intriguing studies demonstrate that interactions of the central cholinergic network and the vagus nerve not only contribute to the regulation of peripheral cytokine responses, but also to CNS processes, including sleep-wake behavior.

The neural substrates involved in REMS regulation have been extensively investigated for many years [reviewed (26, 35)]. Cholinergic cell populations involved in REMS initiation are found in the laterodorsal tegmental (LDT) and pedunculopontine (PPT) nuclei (31). GABAergic neurons
located in the ventrolateral periaqueductal grey (vlPAG) project to REMS-regulating brainstem nuclei, including LDT/PPT. These vlPAG GABAergic neurons have been suggested to play a role in REMS regulation because lesions of the vlPAG increase REMS (30). As such, activation of these GABAergic neurons is expected to suppress REMS. Our immunohistochemical analyses indicate that the number of c-Fos⁺ / GAD67⁺ neurons in vlPAG increases 30 h after CLP surgeries, but not after control surgeries. These results suggest that the GABAergic system in vlPAG is activated early in the development of sepsis. Additional studies are necessary to determine if activation of central cholinergic inflammatory pathways and circulating cytokines play roles that are complementary to activation of brainstem GABAergic systems in the alterations in REMS of rats observed in these experiments.

Of the sleep-associated parameters quantified in this study, sepsis had the greatest impact on the EEG. The impact of sepsis on the EEG is evidenced by changes in delta power during NREMS, which was dramatically reduced in septic animals irrespective of whether the amount of time spent in NREMS differed from baseline control values. That is, delta power during NREMS is reduced during periods when NREMS duration is essentially normal and during periods when NREMS duration is increased. Because delta power during NREMS is widely regarded as an indicator of the depth or quality of sleep (8), these data suggest that sleep of septic animals is qualitatively poor as compared to their baseline sleep prior to infection, or relative to the sleep of SHAM-operated control rats. The dissociation between the nocturnal increase in NREMS duration and the prolonged reduction in delta power during NREMS of septic animals could be interpreted as an attempt to compensate for poor quality sleep by spending more time in NREMS. Alternatively, cytokine actions on the EEG could reflect direct neural effects that are independent of the quality or quantity of sleep. The mechanisms through which cytokines influence delta power during NREMS are complex and not fully understood. Early studies (28, 39, 49, 52) demonstrate that cytokines alter EEG parameters, and that these effects depend on the dose, time and route of administration. For example central administration of TNF enhances delta power during NREM sleep (50, 51), whereas intraperitoneal administration increases NREM sleep duration without a concomitant increase of EEG delta power (15, 16, 27, 51). IL-1 increases EEG delta power at doses too low to alter NREMS duration (39, 52). Moreover, both IL-1 and TNF enhance EEG delta power in a dose-dependent manner when locally applied to the surface of the cerebral cortex, but do not alter NREM sleep duration under these conditions (51, 61, 62). Of importance to this present study, some doses of IL-1 administered directly into rat brain reduce EEG delta power even when NREMS duration is enhanced (28, 39). These findings of the impact of IL-1 on EEG power spectra in rats are reminiscent of the suppressed NREMS delta power during periods of enhanced NREMS duration of septic rats in this study.

Collectively, these previous studies demonstrate that the EEG is highly sensitive to cytokine actions. Furthermore, these previously reported data of cytokine actions on the EEG provide a potential mechanistic basis for the hypothesis that the brain is an early responder to septic insult. Data derived from this current study also support the hypothesis that the EEG may serve as a predictor of sepsis onset, an electroencephalographic biomarker. Although we cannot mimic all facets of the manner in which sepsis develops in patients in the ICU, our data do indicate that the EEG is altered within a few hours of the surgical manipulation to induce sepsis. The reduction in delta power during NREMS is not a byproduct of the surgical procedure per se, or anesthesia, because EEG delta power during NREMS of control rats subjected to SHAM surgeries is not altered. Furthermore, the suppression of EEG delta power during NREMS of septic rats does not normalize during the three days following the surgical manipulation, long after effects of anesthesia and the surgical incision are past. We can only conclude that polymicrobial infection
in the periphery has dramatic effects on the cortical EEG of rats during the infection. Whether such changes in the EEG persist for periods longer than the 84 h post-surgery recording duration of this study remains to be determined.

Infection with bacteria, viruses or fungus alters sleep of laboratory animals and humans [reviewed (41)]. For example, previous experimental studies have determined the impact of infection with selected bacterial pathogens (e.g., *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli*) on sleep of laboratory rabbits. NREMS of rabbits inoculated intravenously with *S. aureus* (53), *S. pyogenes*, or *E. coli* (54) increases for periods ranging from 2 – 24 h, whereas REMS is suppressed for 2 – 28 h, depending on the pathogen. In addition to changes in the amount of time spent in NREMS or REMS, the quality of sleep in rabbits infected with these pathogens is diminished, as evidenced by reductions in delta power during NREMS. In these studies, positive blood cultures were evident in most rabbits inoculated with *S. aureus, S. pyogenes* or *E. coli*, indicating septicemia. Therefore, laboratory rabbits made septic by intravenous inoculation of selected bacterial pathogens may exhibit alterations in sleep that are generally of shorter duration than those of rats made septic by CLP. In rats, infection following CLP results from a mixed population of enteric bacteria, with blood cultures positive for multiple bacterial species (36). Whether or not the difference between rabbits and rats in altered sleep during infection is attributable to species differences or to the polymicrobial nature of sepsis in rats induced by CLP is not known.

As with any study in which an animal model is used to mimic the human condition there are limitations to this study. From a technical standpoint, the time and labor intensive nature of immunohistochemical assessment of the impact of sepsis on multiple brain regions necessitated selecting relatively few time points during the development of sepsis at which to sacrifice animals. Ongoing studies of rats and mice aim to further elucidate mechanisms and mediators responsible for the impact of sepsis on the brain. In addition, physical characteristics of brains from septic rats are altered such that the tissue is difficult to section. Importantly from a clinical perspective, because of the heterogeneous nature of sepsis in the ICU, we are unable to mimic all aspects of sepsis onset in patients using this model. Nevertheless, this present study advances our knowledge of how sleep of laboratory animals is altered during infection by focusing on a polymicrobial infection that mimics many of the clinical facets of sepsis in patients.

**Perspectives and Significance**

This study demonstrates that CLP-induced sepsis dramatically affects mechanisms involved in the regulation of sleep, EEG and body temperature. These conclusions are made on the basis of sepsis-induced alterations in the amount of time spent in each behavioral state, the severe fragmentation of the sleep-wake cycle, and the damping of diurnal rhythms of sleep and brain temperature. Limited clinical data suggest that the EEG is altered prior to sepsis onset (18). We cannot mimic the onset of sepsis as might occur in the ICU, but the marked reduction in delta power during NREMS of septic rats supports the notion that sepsis alters the EEG in a manner that may serve as an indicator of pending sepsis onset. Of significance, early intervention is still the most effective management strategy for improving sepsis outcomes. Monitoring in the ICU perhaps could be used as a method to detect alterations in the EEG that may be characteristic of impending sepsis. Such a “warning” that sepsis is eminent could prove to be a valuable tool in sepsis management. Finally, although the protocol used in this study does not allow us to determine the complete duration of sepsis-induced alterations in CNS processes, observations from clinical studies suggest there are long-term consequences of sepsis on cognitive function. For example, patients surviving severe sepsis suffer from new, persistent and dramatic cognitive
impairment or global loss of cognitive function after discharge (25). Pre-clinical data indicate that CLP sepsis alters cognitive function of rats as assessed by an inhibitory avoidance task for at least 30 days (3, 4). Collectively, these clinical observations and data obtained in this present study indicate that additional effort is warranted to elucidate mechanisms underlying the complex relationships between traumatic infection and multiple facets of CNS function.

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Figure legends

Fig. 1. Sepsis alters sleep and the electroencephalogram (EEG) of rats. Depicted is the percentage of recording time spent in wakefulness (panel A), non-rapid eye movements sleep (NREMS) (panel B), EEG delta power (0.5–4.0 Hz) during NREMS (panel C), and rapid eye movements sleep (REMS) (panel D). Data were obtained during pre-surgical baseline recordings (BL, open symbols) and during four days after abdominal surgeries (filled symbols) from control rats (SHAM, circles) and septic rats (CLP, squares). Data are presented as hourly means ± SEM. Values for EEG delta power during NREMS were normalized and expressed as percentage from the 24 h average baseline values, depicted by the “zero” line. Statistical evaluations were performed on consecutive 6 h time blocks. Statistically significant changes relative to baseline values across time blocks are indicated as: *, p < 0.05; **, p < 0.01. The black bars on the X-axis indicate the dark portion of the light–dark cycle.

Fig. 2. The changes in sleep and the electroencephalogram (EEG) of rats are not due to the surgical procedure, but are a direct response to sepsis. Hourly differences between pre-surgical baseline values and postsurgical values were calculated, and are plotted for each group relative to baseline values (depicted by the zero lines). Depicted are group differences in time spent in wakefulness (panel A), non-rapid eye movements sleep (NREMS) (panel B), EEG delta power (0.5–4.0 Hz) during NREMS (panel C), and rapid eye movements sleep (REMS) (panel D) for control rats (SHAM, open circles) and septic rats (CLP, filled circles). Data are presented as means ± SEM. Statistical evaluations were performed on consecutive 6 h time blocks. Statistically significant differences between groups across time blocks are indicated as: *, p < 0.05; **, p < 0.01. The black bars on the X-axis indicate the dark portion of the light–dark cycle.

Fig. 3. Sepsis fragments sleep of rats. The number of transitions from one behavioral state to another were determined hourly from control rats (SHAM, circles) and septic rats (CLP, squares) during pre-surgical baseline recordings (open symbols) and during post-surgical recording days (filled symbols). Data are presented as means ± SEM. Statistical evaluations were performed on consecutive 6 h time blocks. Statistically significant differences between baseline and post-surgical values across time blocks are indicated as: *, p < 0.05; **, p < 0.01. The black bars on the X-axis indicate the dark portion of the light–dark cycle.

Fig. 4. Sepsis alters thermoregulation of rats. Depicted are changes in brain temperature of rats during baseline (thin line) and during four days after abdominal surgery (thick line). Data were obtained from control rats (SHAM) and septic rats (CLP). Data are presented in 10 min intervals as means ± SEM. Statistical evaluations were performed on consecutive 6 h time blocks. Statistical significance across time blocks is indicated as: *, p < 0.05; **, p < 0.01. The black bars on the X-axis indicate the dark portion of the light–dark cycle.

Fig. 5. Sepsis alters the distribution of sleep and reduces the magnitude of diurnal changes in brain temperature. The proportion of time spent in wakefulness (panel A), non-rapid eye movements sleep (NREMS) (panel B), and rapid eye movements sleep (REMS) (panel C) is depicted for pre-surgical baseline values and for post-surgical days 2, 3, and 4. The proportion behavior that occurs during the 12 h dark period and the 12 h light period is shown for control rats (SHAM, open bars) and for septic rats (CLP, filled bars). Brain temperatures (panel D) are 12 h average temperatures during the dark period and the light period, and 36 °C is arbitrarily defined as the “mid-point”. Within- and between-group statistical comparisons are presented: *, p ≤ 0.05 relative to control (SHAM) rats; #, p ≤ 0.05 relative to pre-surgical baseline values.
Fig. 6. Sepsis-induced alterations in activity of the brainstem GABAergic system are apparent in these representative tissue samples taken from a control rat (SHAM surgery; panels A, B) and a septic rat (CLP surgery; panels C,D). Both animals were sacrificed 30h after surgery. Sections contain the ventrolateral peri-aqueductal gray (vlPAG), and are approximately -7.04 mm from Bregma (45). Panels A and C are 10x magnification, whereas the boxed insets (panels B and D) are 60x magnification. Examples of cells stained for glutamic acid decarboxylase-67 (GAD_{67}) are indicated by black arrowheads, whereas examples of cells stained for both GAD_{67} and c-Fos are denoted by black arrows. An example a c-Fos positive cell is indicated in panel B with an asterisk. Scale bars: panel C = 100 um; panel D = 25 um.

Fig. 7. Sepsis alters activity of the brainstem GABAergic system. Presented are numbers of c-Fos^{+} neurons and cFos^{+}/GAD_{67}^{+} neurons in the pedunculopontine tegmental nucleus (PPT), laterodorsal tegmental nucleus (LDT) and the ventrolateral peri-aqueductal grey (vlPAG) of rats after abdominal surgeries. Stereological counts were obtained from tissue in brains of rats sacrificed at 30-, 54-, and 78 h after SHAM surgeries (open bars) or CLP surgeries (filled bars). Values are means ± SEM from n = 4 animals per time point and condition. Statistically significant differences between groups are indicated as: *, p ≤ 0.05; **, p ≤ 0.01.
References:


A. Wakefulness

B. NREM Sleep

C. Delta Power during NREM Sleep

D. REM Sleep

**Fig. 1**
Fig. 3
c-Fos / GAD$_{67}$

30 h SHAM  

30 h CLP

Fig. 6
Fig. 7