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Role of Proinflammatory Cytokines on Lipopolysaccharide-Induced Phase Shifts in Locomotor Activity Circadian Rhythm

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We previously reported that early night peripheral bacterial lipopolysaccharide (LPS) injection produces phase delays in the circadian rhythm of locomotor activity in mice. We now assess the effects of proinflammatory cytokines on circadian physiology, including their role in LPS-induced phase shifts. First, we investigated whether differential systemic induction of classic proinflammatory cytokines could explain the time-specific behavioral effects of peripheral LPS. Induction levels for plasma interleukin (IL)-1α, IL-1β, IL-6, or tumor necrosis factor (TNF)-α did not differ between animals receiving a LPS challenge in the early day or early night. We next tested the in vivo effects of central proinflammatory cytokines on circadian physiology. We found that intracerebroventricular (i.c.v.) delivery of TNF-α or interleukin IL-1β induced phase delays on wheel-running activity rhythms. Furthermore, we analyzed if these cytokines mediate the LPS-induced phase shifts and found that i.c.v. administration of soluble TNF-α receptor (but not an IL-1β antagonistic) prior to LPS stimulation inhibited the phase delays. Our work suggests that the suprachiasmatic nucleus (SCN) responds to central proinflammatory cytokines in vivo, producing phase shifts in locomotor activity rhythms. Moreover, we show that the LPS-induced phase delays are mediated through the action of TNF-α at the central level, and that systemic induction of proinflammatory cytokines might be necessary, but not sufficient, for this behavioral outcome. (Author correspondence: dgolombek@unq.edu.ar)

Keywords: Circadian, IL-1β, Lipopolysaccharide, Phase shift, Proinflammatory cytokines, Suprachiasmatic nucleus, TNF-α

INTRODUCTION

Mammalian circadian timing is controlled by a central pacemaker located within the hypothalamic suprachiasmatic nucleus (SCN). The SCN governs the temporal organization of a great number of physiological and behavioral variables and can be entrained to the light-dark (LD) cycle by means of photic stimuli (Golombek & Rosenstein, 2010). The molecular mechanisms underlying the generation of circadian rhythmicity consist of a transcriptional/translational feedback loop that includes a number of "clock" genes (e.g., *Clock*, *Bmal1*, *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *Rev-erb-α*, *Dec1*, *Dec2*) and drives the expression of several clock-controlled genes (Okamura et al., 2010).

A bidirectional communication has been described between the immune and the circadian system (reviewed in Coogan & Wyse, 2008). Several immune cells and cytokines show daily variation in their plasma levels (Born et al., 1997; Leone et al., 2007), and immune cells and tissues display a functional molecular clock (Arjona & Sarkar, 2005; Davidson et al., 2009; Hayashi et al., 2007; Keller et al., 2009; Wang et al., 2011). The response to an immune challenge is also time dependent, both at the cellular and the systemic level (Halberg et al., 1960; Hrushesky et al., 1994; Keller et al., 2009; Marpegan et al., 2009), and circadian disruption can lead to altered immunological response (Castanon-Cervantes et al., 2010). On the other hand, the immune system is also capable of affecting the circadian clock. Lipopolysaccharide (LPS) challenge has been used as an immune stimuli in several works as a pathologic (Halberg et al., 1960) or physiologic model, depending on dose (Mullington et al., 2000; Thomson & Sutherland, 2005). High LPS doses have lethal (when >10 mg/kg) or sublethal effects in mice, exhibiting a circadian response in mortality and a lower amplitude in activity without significant effects on circadian phase (Marpegan et al., 2009). In this pathological model, core body temperature is profoundly affected in mice, inducing hypothermia for several days. A peripheral challenge with low-dose LPS (25 μg/kg) induces photic-like circadian phase delays on wheel-running activity in mice, but only when delivered at circadian time (CT) 15, and this effect is mediated by Toll-like receptor 4 (TLR-4) and nuclear factor-κB (NF-κB)

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activity (Marpegan et al., 2005; Paladino et al., 2010). In addition, LPS effects are mediated by proinflammatory cytokines in several experimental models. In particular, the effect of proinflammatory cytokines on circadian physiology have been shown for interferon (IFN)-α (Koyanagi & Ohdo, 2002; Ohdo et al., 2001), IFN-γ (Kwak et al., 2008; Lundkvist et al., 2002), interleukin (IL)-6 (Motzkus et al., 2002), IL-1β (Cavadini et al., 2007), and tumor necrosis factor (TNF)-α (Cavadini et al., 2007; Nygard et al., 2009). The aim of the present work was to study if proinflammatory cytokines are involved in the LPS pathway that induces phase shifts in the locomotor activity circadian rhythm. We report that peripheral cytokine and chemokine induction was independent of the time of the immune challenge, suggesting that the peripheral response does not explain the time differences in behavior. Intracerebroventricular (i.c.v.) delivery of both TNF-α and IL-1β in vivo induced phase delays in wheel-running activity at CT 15. Finally, TNF-α was found to be necessary for the circadian behavioral outcome of systemic endotoxin challenge, as blockade of TNF-α (but not IL-1β) action at the central level inhibited phase delays induced by peripheral LPS.

MATERIALS AND METHODS

Animals

Adult (3–5 mo old) C57-BL/6J male mice (*Mus musculus*) were raised in our colony and housed under a 12 h light (L):12 h dark (D) photoperiod (lights-on at 08:00 h), with food and water access ad libitum. All animal manipulations and experimental protocols performed in this work were supervised and approved by the University Institutional Animal Care and Use Committee, in agreement with policies and laws of the Office of Laboratory Animal Welfare, National Institutes of Health, USA, and the ethical standards of the journal (Portaluppi et al., 2010).

Behavioral Analysis

Animals were kept in individual cages equipped with running wheels. Wheel revolutions were recorded by magnetic microswitches, with the accumulated counts collected every 5 min. For LD conditions, time is expressed as zeitgeber time (ZT), with ZT 12 defined as the time of lights-off. For experiments in constant darkness conditions (DD), mice were transferred from LD to a constant dark environment (DD) in single cages at least 10 d prior to the treatments, and reference time was set by wheel-running activity-onset of each animal (CT 12). Dim red light was used for animal manipulation in DD conditions. Phase shifts on wheel-running activity were calculated with *El Temps* software (Antoni Díez Noguera, University of Barcelona), using activity-onset as phase reference point. Total wheel-running activity was measured immediately after each treatment (CT 15–24), and then it was expressed relative to the total activity mean (in the same time interval) of the 5 previous days.

Surgery and Microinjections

Mice were surgically implanted with 26-gauge stainless steel guide cannulae (PlasticsOne, Roanoke, VA, USA) for i.c.v. injections. Animals were deeply anesthetized with a 70 mg/kg ketamine and 10 mg/kg xylazine cocktail by intraperitoneal route (i.p.). Stereotaxic surgery allowed the implantation of a cannula aimed at the bottom of the third ventricle in the SCN region (coordinates from bregma: –.5 mm anteroposterior, –5.0 mm dorsoventral, .0 mm from midline). Once recovered from the anesthesia, animals were kept in LD for 24 h and transferred to DD conditions afterwards. i.c.v. microinjections were performed with a 33-gauge internal injector (PlasticsOne) connected to a microsyringe (Hamilton, Reno, NV, USA), delivering a total volume of 1 μL at .2 μL/min.

Cytokine and Chemokine Measurements

Blood serum samples were collected at 0 and 60 min post injection for each time point and stored at –80°C. Levels of selected cytokines and chemokines—IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemotactic protein 1 (JE/MCP1), IL-1α, macrophage inflammatory protein (MIP) 2, and regulated on activation normal T cell expressed and secreted (RANTES)—were measured in triplicate from each sample using the search light proteome arrays/multiplex assay (Pierce Protein Research Products, Rockford, IL, USA; Toedter et al., 2008).

Treatment With LPS, Cytokines, and Cytokine Antagonists

A brief description of the experimental protocol is presented in Figure 1.

For the cytokine/chemokine plasma-induction experiment, animals (n = 3/group) received 25 μg/kg i.p. LPS from *Escherichia coli* serotype 0111:B4 (Sigma-Aldrich, St. Louis, MO, USA) at two different time points in LD (ZT 3 and ZT 15).

To study the behavioral effects of central proinflammatory cytokines, wheel-running animals (n = 4/group) received i.c.v. microinjections of either IL-1β (1 ng; Sigma-Aldrich), TNF-α (5 ng; Peprotech, Rocky Hill, NJ, USA), or vehicle (saline) at CT 15.

For the cytokine antagonist experiments, wheel-running mice received i.c.v. administration of either saline, TNF-α soluble receptor I (TNFsRI; 50 ng; Sigma-Aldrich), or IL-1 receptor antagonist (IL-1Ra; 200 ng; Valeant Pharmaceuticals, Mississauga, Ontario, Canada) 30 min prior to a 50 μg/kg i.p. LPS challenge at CT 15. Animals were divided into two groups, and each mouse received four different treatments. One group received Saline (i.c.v.)-LPS (i.p.), TNFsRI-Saline, TNFsRI-LPS, and Saline-Saline treatments (n = 10), whereas the other group received Saline-LPS, IL-1Ra-Saline, IL-1Ra-LPS, and Saline-Saline treatments (n = 6). In both groups, treatments were separated by least 15 d. For the control experiment of LPS tolerance, mice (n = 3)

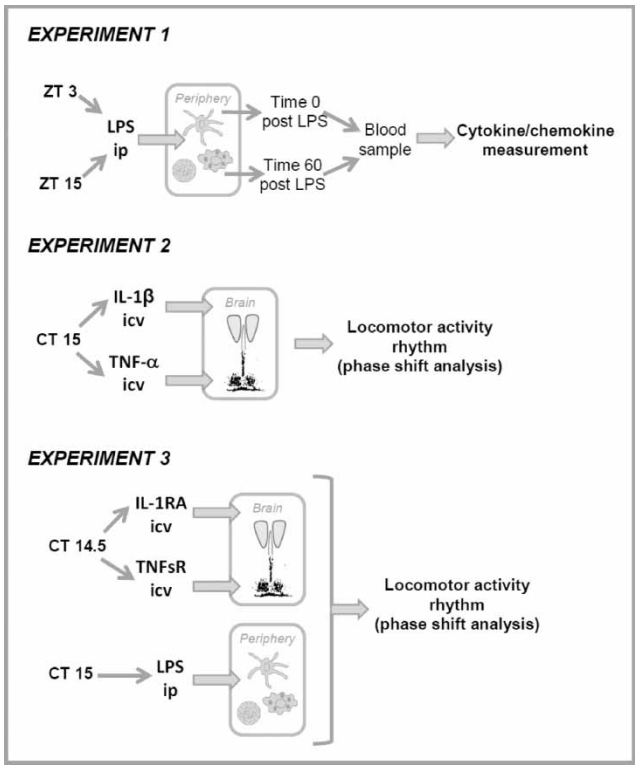

FIGURE 1. Summary of experimental protocol. A set of experiments to assess cytokine effects on circadian physiology was designed, including its role in LPS-induced phase shifts. In Experiment 1, the role of peripheral cytokines/chemokines in the differential effect of LPS on circadian physiology was assessed. Animals were treated with LPS 25 μg/kg ip at two different times of day (ZT3 and ZT15) and then peripheral LPS-induced cytokines/chemokines levels were measured at 0 and 60 min post-challenge. Experiment 2 aimed at testing whether central proinflammatory cytokines could be the mediators of LPS circadian effects. IL-1β or TNF-α were administered icv to assess their ability to induce LPS-like phase delays. Finally, Experiment 3 was designed as to test whether central cytokines are necessary for LPS-induced phase shifts at CT15, by injecting IL-1RA or TNFsR icv 30 min before an LPS 50 μg/kg ip challenge.

received two 50 μg/kg i.p. LPS injections, separated by 29 d, and phase delays were calculated after each challenge.

Statistical Analysis

Differential cytokine/chemokine induction levels were analyzed by assessing 60 min post-LPS challenge values at ZT 3 and ZT 15, subtracting the values at time 0, and compared by Student's *t* test. Variations on basal cytokine/chemokines levels between the two ZTs, when absolute values at time 0 were detectable, were compared by Student's *t* test. The effects of cytokine i.c.v. delivery were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test. For the cytokine antagonism experiments, the effects of the treatments were analyzed by repeated-measures one-way ANOVA followed by Tukey test. Data are presented as mean ± SEM. p values of ≤.05 were considered to be statistically significant.

RESULTS

Peripheral Cytokine Induction After i.p. LPS Administration

Systemic low doses of LPS can alter the circadian system (cause phase delays), only when injected at CT 15 (Marpegan et al., 2005). A possible reason for the time-dependent effects of this challenge on the clock could be a differential response of the immune system to the stimulus, related to a differential increase of humoral factors depending on the time of endotoxin administration. We analyzed the effect of a 25 μg/kg i.p. LPS injection on the plasma levels of selected cytokines and chemokines at two different time points, ZT 15 and ZT 3 (representing the times, respectively, when LPS does or does not induce circadian phase shifts). The basal levels for the molecules analyzed were close to zero, or, in some cases, not detectable, and no difference was found between the two time points analyzed, with the exception of IL-1α, which had higher basal values at ZT 15 (Figure 2). i.p. LPS injection caused strong induction of most of the factors analyzed, at 60 min post administration, but this induction was not dependent on the ZT for the classic proinflammatory cytokines IL-1α, IL-1β, TNF-α, and IL-6 (Figure 2, Table 1). Significant differences were found only for MIP1α and RANTES chemokines, with a stronger induction when LPS was given at ZT 15 than ZT 3 (Table 1).

Circadian Effects of i.c.v. Administration of Proinflammatory Cytokines

Our finding that plasma induction of classic proinflammatory cytokines occurred after an endotoxin challenge given in the early day (a time when no behavioral circadian effects are produced by this treatment), suggests that this peripheral response is not sufficient to explain phase delays upon systemic LPS administration. Therefore, we assessed if this circadian outcome could be mediated by central proinflammatory factors. We first tested if the SCN responds to classic proinflammatory cytokines by investigating the circadian effects of an i.c.v. administration of TNF-α, IL-1β, or vehicle at CT 15. Both TNF-α– and IL-1β – treated animals exhibited a phase delay in their locomotor activity behavior (–51.75 ± 4.44 min and –59 ± 3.03 min, respectively; one-way ANOVA, $F_{(2,9)} = 17.95$, $p < .001$; followed by Dunnett's test, versus Saline, $p < .05$, n = 4; Figure 3). These results confirm the in vivo responsiveness of the mammalian circadian system to central proinflammatory cytokines.

Role of Central Proinflammatory Cytokines in the Circadian Response to LPS

Taking into account the phase-shifting effects of both centrally administered TNF-α and IL-1β, these molecules appeared to be good candidates for mediating LPS-induced phase delays. To further test this hypothesis, we administered IL-1Ra or TNFsRI i.c.v. 30 min prior to an i.p. LPS 50 μg/kg challenge. We found significant phase delays for mice treated with Saline-LPS (–29.52 ± 6.89

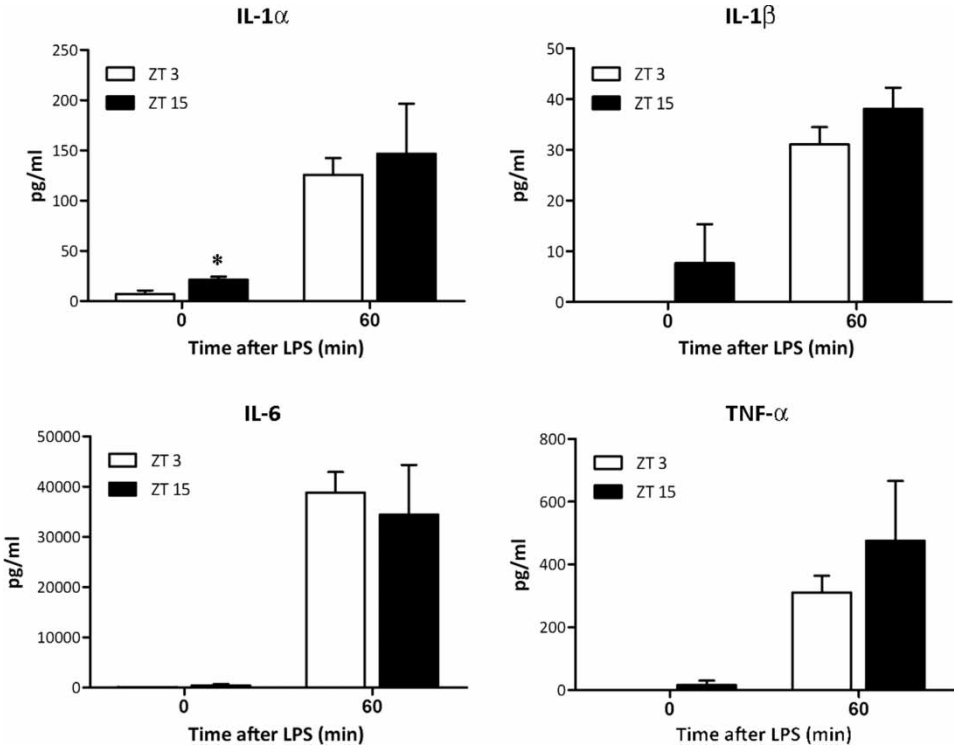

FIGURE 2. Effect of time of LPS challenge on the induction of classic proinflammatory cytokines. Shown are the plasma levels of IL-1α, IL-1β, IL-6, and TNF-α, 0 and 60 min after a 25 μg/kg i.p. LPS challenge given either at ZT 3 or ZT 15. Basal levels differed between ZT 3 and ZT 15 only for IL-1α (two-tailed *t* test, $t(4) = 3.012$, $^*p < .05$, n = 3). A strong induction was observed for the four cytokines, 60 min after LPS injection. No significant differences were found between the two zeitgeber times (ZTs) in the increase of plasma levels in any of the four cytokines ($p > .05$, one-tailed Student's *t* test; when detectable, values at time 0 were subtracted from the values at 60 min post LPS; n = 3/group).

TABLE 1 Cytokine and chemokine plasma levels measured 60 min after a 25 μg/mL i.p. LPS injection

	ZT 3	ZT 15	Statistics
IL-1β	31.08 ± 3.4	30.44 ± 4.22	$t(4) = .118$, $p = .456$
IL-2	23.91 ± 4.21	22 ± 21.06	$t(4) = .089$, $p = .467$
IL-4	23.82 ± 3.97	38.06 ± 13.09	$t(4) = .985$, $p = .190$
IL-6	38 780.73 ± 4064. 72	34 039.1 ± 9840	$t(4) = .445$, $p = .340$
IL-10	475.95 ± 182.64	302.66 ± 133.18	$t(4) = .767$, $p = .243$
TNF-α	310.85 ± 53.27	459.6 ± 190.39	$t(4) = .752$, $p = .247$
GM-CSF	86.99 ± 4.85	110.92 ± 10.44	$t(4) = 2.079$, $p = .053$
JE/MCP1	19 372.9 ± 2947.04	42 392.57 ± 12 864.38	$t(4) = 1.744$, $p = .078$
MIP1α	209.8 ± 44.78	341.25 ± 17.97	**$t(4) = 2.724$, $p = .026$**
IL-1α	118.77 ± 16.66	125.43 ± 49.9	$t(4) = .127$, $p = .453$
MIP2	3130.24 ± 1435.87	3272.23 ± 1907.06	$t(4) = .059$, $p = .478$
RANTES	116.84 ± 28.67	448.4 ± 75.51	**$t(4) = 3.352$, $p = .014$**

When basal values were different form zero, they were subtracted from the values at 60 min. Values are expressed as pg/mL (mean ± SEM, *p* value for one-tailed *t* test). IFN-γ values are not included since measurements at 60 min post LPS were undetectable.

min), but no circadian changes in animals treated with TNFsRI-Saline (–5.89 ± 3.05 min) or IL-1Ra-Saline (–6.02 ± 6.71 min), indicating these drugs do not affect the locomotor activity rhythm per se (Figure 4; repeated-measures one-way ANOVA followed by Tukey post hoc test, $p < .05$ for Saline-LPS versus Saline-Saline and $p > .05$ for IL-1Ra-Saline or TNFsRI-Saline versus Saline-Saline). LPS-induced phase shifts of wheel-running activity were strongly inhibited in mice that received TNFsRI prior to peripheral LPS challenge. This indicates an essential role for central TNF-α in the behavioral effects of a peripheral immune stimulus (repeated-measures one-way ANOVA, $F_{(3,27)} = 12.824$, $p < .0001$; followed by Tukey post hoc test, $p < .001$ compared with the Saline-LPS treatment; Figure 4C and D). On the other hand, no significant difference was found between the effects of Saline-LPS and IL-1Ra-LPS groups (repeated-measures one-way ANOVA, $F_{(3,15)} = 4.984$, $p < .05$; followed by Tukey post hoc test, $p > .05$; Figure 4A and B), which suggests that central IL-1β is not involved in peripheral LPS-triggered phase shifts.

In order to rule out tolerance to two consecutive LPS challenges, we also performed a control experiment in

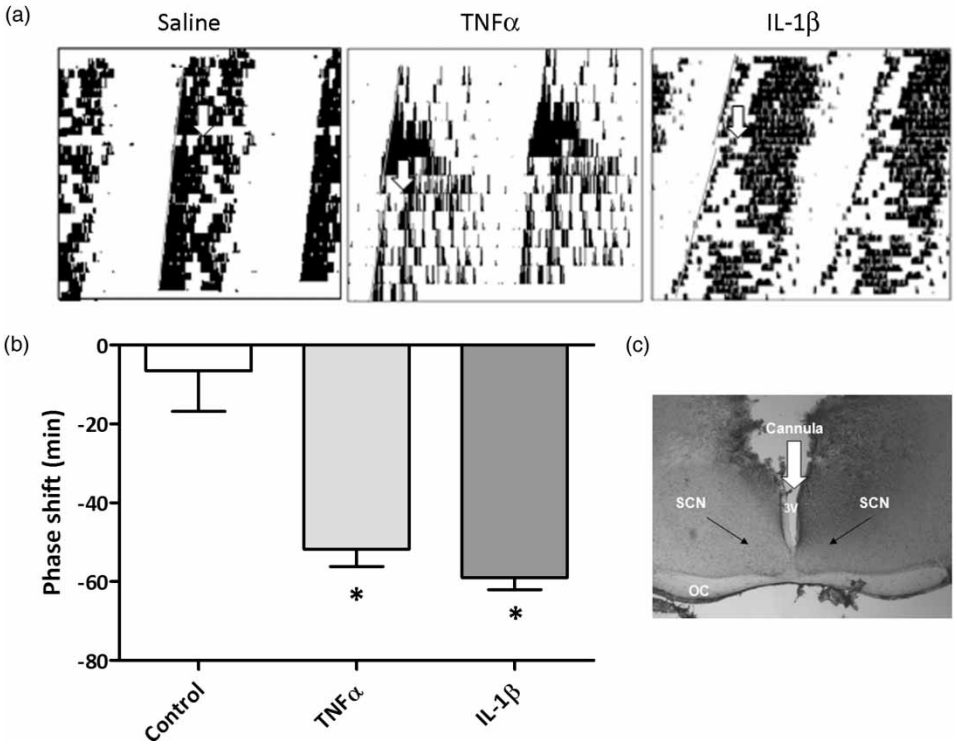

FIGURE 3. i.c.v. cytokine delivery induces phase delays of wheel-running activity. Central (i.c.v.) TNFa or IL-1β administration induces phase shifts in locomotor activity rhythm in mice at CT 15. (A) Representative actograms of animals treated with saline (control), TNF-α (5 ng), or IL-1β (1 ng) i.c.v. (total delivered volume was 1 μL in each case). Arrows indicates day and time of treatment. (B) Cytokine-induced phase shifts at CT 15. Both TNF-α and IL-1β produced significant phase delays, whereas no effect was found for vehicle administration (one-way ANOVA, $F_{(2,9)} = 17.95$, $p < .001$; followed by Dunnett's test, versus Saline, $^*p < .05$; data are expressed as mean ± SEM, n = 4/group). (C) Representative histological mouse brain section showing the cannula trajectory. The image shows a coronal slice containing the SCN. Arrows point towards the SCN beneath the third ventricle (3V) and above the optic chiasm (OC).

which animals received two LPS 50 μg/kg challenges with 29 d between both treatments (i.e., the same dose and timing between LPS challenges as the ones used in the previous experiment); no differences were found in the magnitude of the circadian phase delays (two-tailed *t* test, $t = .97$, $p > .05$; Figure 5).

As predicted, LPS i.p. treatment decreased locomotor activity (Figure 6) immediately after injections at CT 15; this effect was transient, lasting a few hours. However, i.c.v. TNFsRI did not block this inhibition of locomotion. In addition, we found a paradoxical reduction in locomotor activity in the TNFsRI-Saline group (as compared with the Saline-Saline group). These results indicate that the LPS effect on behavior is complex and acts through different pathways when eliciting circadian phase shifts (acting through TNF-α) or when inhibiting locomotion.

DISCUSSION

In the present work, we studied the effects of immune factors on the biological clock, focusing on the role of proinflammatory cytokines in the circadian effects of a peripheral immune challenge. Although systemic LPS caused an increase in the levels of all of the circulating proinflammatory factors assessed, there was no differential induction (with the exception of MIP1α and RANTES) between the time of the day when the endotoxin challenge does and when it does not produce phase delays. Next, we hypothesized that the circadian outcome of the immune challenge could be mediated by proinflammatory cytokines acting in the central nervous system. We first assessed the in vivo effects of central IL-1β and TNF-α (with cytokines delivered by a cannula aimed at the bottom of the third ventricle), and found that i.c.v. delivery of these cytokines at CT 15 elicited phase delays of wheel-running activity. Finally, we tested the role of these cytokines on the behavioral effects of an i.p. LPS challenge, finding that blockade of TNF-α (but not of IL-1β) inhibits the phase delays in wheel-running activity elicited by peripheral endotoxin administration. These findings show the effects of a systemic immune challenge on circadian physiology are mediated through the action of TNF-α at the hypothalamic level.

The lack of temporal variation in peripheral induction of proinflammatory cytokines after an LPS challenge is in accordance with previous findings where no time-of-day effect was found in the plasma levels of TNF-α or IL-6 in humans treated with low-dose endotoxin (Mathias et al., 2000). On the other hand, we have previously described that lethal (20 mg/kg) LPS doses caused a higher increase in plasma IL-1β and IL-6 when the challenge was given at ZT 11 than at ZT 19, in correlation with the time of day when mortality is higher (Marpegan et al., 2009).

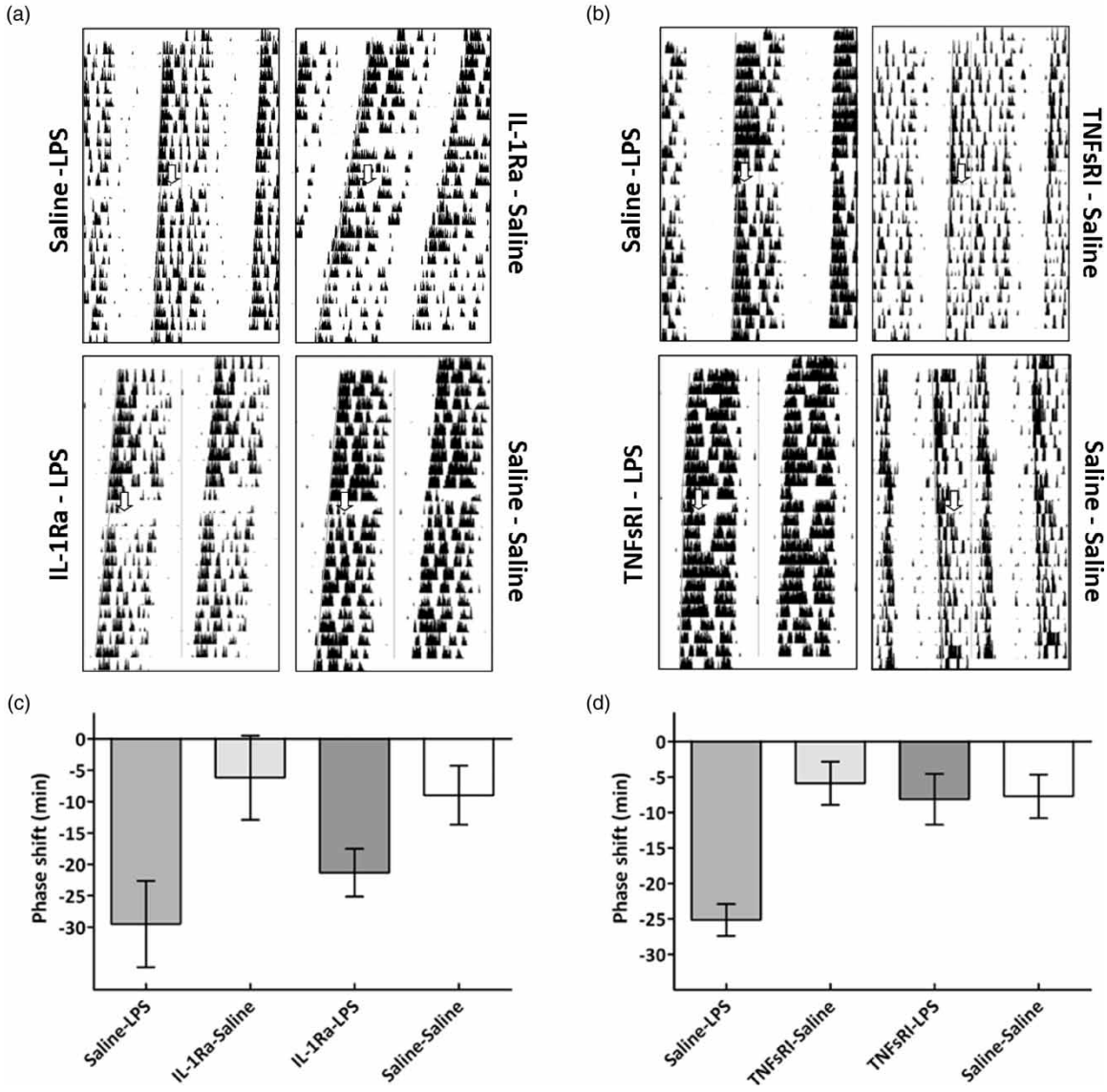

FIGURE 4. Effects of TNF-α and IL-1β i.c.v. blockade on LPS-induced phase shifts. Representative actograms of mice from the group receiving Saline-LPS, IL1-Ra-Saline, IL1-Ra-LPS, and Saline-Saline treatments (A) or from the group receiving Saline-LPS, TNFsRI-Saline, TNFsRI-LPS, and Saline-Saline treatments (C). The route of administration was i.c.v.-i.p., for each pair of treatments. i.c.v. injections were given 30 min prior to the i.p. challenges (CT 14.5 and 15, respectively). Arrows indicate day and time of i.p. treatments. (B and D) Mean ± SEM of phase delay in minutes for each group and treatments. Animals receiving Saline-LPS showed significant phase delays, in both groups. i.c.v. delivery of TNFsRI, but not of IL-1Ra, prior to the endotoxin challenge produced inhibition of LPS-induced phase shifts. Repeated-measures one-way ANOVA, $F_{(3,27)} = 12.824$, $p < .0001$, followed by Tukey post hoc test, $p < .001$ for Saline-LPS versus all other treatments for the TNFsRI group. Repeated-measures one-way ANOVA, $F_{(3,15)} = 4.984$, $p < .05$, followed by Tukey post hoc test, $p < .05$ for Saline-LPS versus IL-1Ra-Saline and Saline-Saline; n = 6 for the IL-1Ra group, and n = 10 for the TNFsRI group.

However, this extremely severe pathological model differs greatly in the general outcomes (high mortality index, no effects on phase or period of the locomotor activity and temperature rhythms) with the ones obtained with lower doses (circadian phase delays with no effects on survival), which might explain the different results regarding proinflammatory cytokine induction.

We also show that central TNF-α is necessary for LPS-induced phase shifts. Peripheral endotoxin challenges may reach the brain through direct transport across the blood-brain barrier (BBB), with a direct effect of LPS on the SCN or paraventricular nucleus (PVN) in the hypothalamus. Indeed, TLR-4 expression (the most important LPS-recognizing receptor) has been described in the hypothalamic PVN and circumventricular organs (Laflamme & Rivest, 1999), and LPS increases arginine-vasopressine secretion in the rat SCN, suggesting that cells in this tissue can recognize and respond to the endotoxin (Nava et al., 2000). However, the amount of LPS reaching the brain after a low-dose administration is unlikely to exert a neuroimmune response, and it is generally accepted that, in models of mild peripheral inflammation, the central effects of LPS are not mediated by the passage of this molecule into the brain (Blatteis,

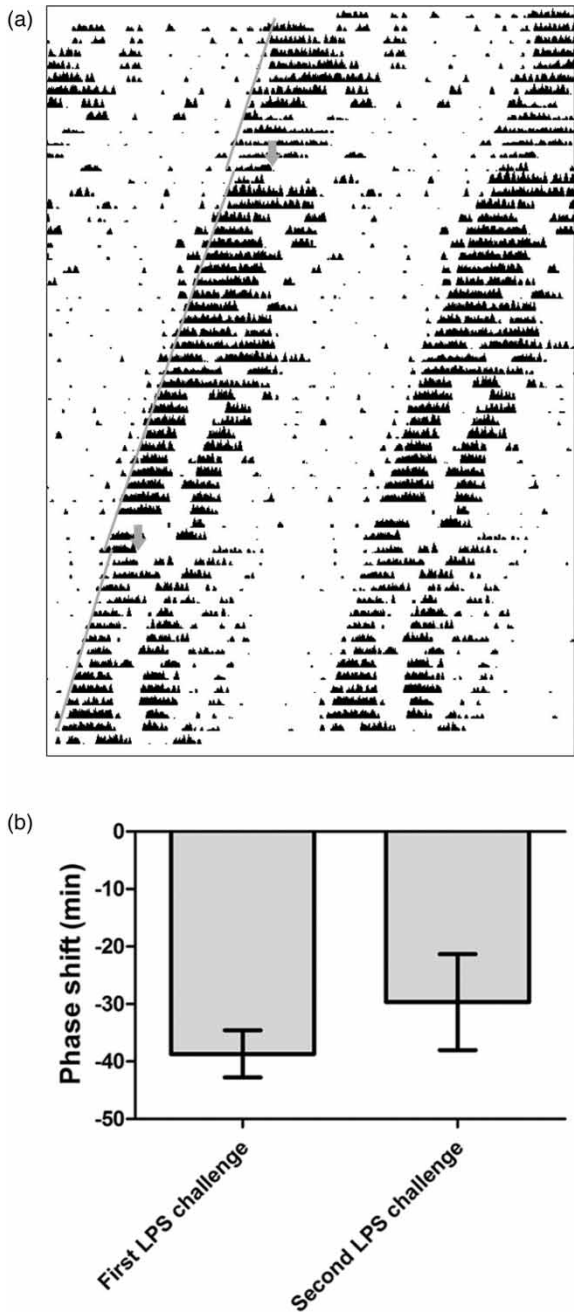

FIGURE 5. Two successive LPS 50 μg/kg challenges (with 29 d between treatments) induce phase shifts of similar magnitude. (A) Wheel-running activity of a mouse treated with LPS 50 μg/kg i.p. at CT 15 and then rechallenged with the same LPS dose 29 d after the first stimulus (arrows indicates time of treatment). (B) Average phase delays induced by LPS challenges (two-tailed Student's t test, $t(4) = .97$, $p > .05$, n = 3).

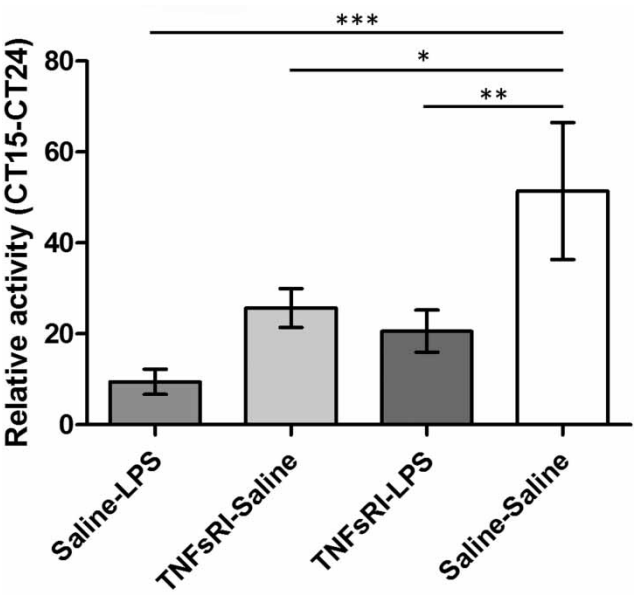

FIGURE 6. An LPS i.p. challenge induces a significant decrease in wheel-running activity after treatment (CT 15–24), which is not affected by pretreatment with TNFsRI i.c.v. ($p < .05$, repeated-measures ANOVA followed by Tukey post hoc test, $^{***}p < .001$, $^{**}p < .01$, $^{*}p < .05$). Data are shown as mean ± SEM.

1992; Pan et al., 1997; Singh & Jiang, 2004). It was reported that a 100 μg/kg LPS dose does not cross the BBB, but is able to bind to endothelial cells and induce an increase in proinflammatory cytokines levels and inducible nitric oxide synthase (iNOS) activity in the rat brain (Singh & Jiang, 2004). Systemic cytokines induced by LPS may also enter the brain through sites outside the BBB, such as the organum vasculosum laminae terminalis (Romanovsky et al., 2003), a possibility that remains to be addressed. Finally, a role for peripheral neural terminals, such as the vagus nerve, cannot be ruled out, since this pathway was shown to participate in behavioral effects of an i.p. immune challenge, such as TNF-α–induced non–rapid eye movement (REM) sleep responses (Bluthe et al., 1996; Kubota et al., 2001). Any of the previously described pathways may ultimately lead to the release of cytokines in the brain. Once induced, cytokines could initiate a signaling cascade that might produce the phase shift in locomotor activity rhythm. Cytokine antagonist experiments point towards a necessary role for TNF-α acting upon the SCN (or other periventricular hypothalamic nuclei that signal to the SCN) for the circadian outcome of a peripheral LPS challenge. Expression of mRNAs for both TNF receptors I and II was described in the mouse SCN, and treatment with TNF-α altered electrical activity of SCN neurons in a nitric oxide–dependent pathway (Nygard et al., 2009), indicating the master clock is responsive to this cytokine in vitro. Moreover, TNF-α affected clock gene expression in a murine fibroblast cell line (Cavadini et al., 2007; Petrzilka et al., 2009), suggesting that central TNF-α might induce phase shifts through a change in clock gene expression. On the other hand, the action of central IL-1β is less clear. We found that i.c.v. IL-1β delivery can trigger phase shifts, but this cytokine does not seem to be involved in LPS-induced circadian effects. In line with this, a 5 mg/kg LPS i.p. challenge failed to induce IL-1β expression in the mouse SCN, although it correlated with an increase of IL-1R immunoreactivity (Beynon & Coogan, 2010).

Regarding the effects of a peripheral LPS challenge on the circadian clock at the brain level, Takahashi et al. (2001) found Per1 mRNA induction in the PVN, but not in the SCN, after low-dose (50 μg/kg) LPS injection at ZT 22, and Paladino et al. (2010) using the same endotoxin dose found similar induction of PER1 protein in the PVN with challenge at CT 15. Higher doses (1 mg/kg) provoked a transient suppression of Per2 mRNA expression in the rat SCN (Okada et al., 2008), and when applied chronically for 60 d, it altered the photic induction of c-Fos protein in the mouse SCN (Palomba & Bentivoglio, 2008). Variations in the expression of different components of the signaling cascade activated in the brain upon the peripheral immune stimulus could also provide the temporal constraints for the observed effects of LPS. For example, suppressor cytokine signaling (SOCS) 1 and 3, molecules involved in the negative feedback of intracellular cytokine signaling pathways, present daily variations, with a minimum in the early night (Sadki et al., 2007); moreover, a cocktail containing TNF-α and IFN-γ induced higher c-FOS induction during the night (Sadki et al., 2007). The responses to TNF-α in the SCN might represent changes in the induction of proinflammatory cytokines, increased receptor binding, decrease in the levels of negative regulators, or a combination of these or additional factors that could be responsible for the time-dependent effects of peripheral LPS on the circadian system.

Finally, when analyzing the role of central cytokines in endotoxin responses, the possibility of LPS tolerance should not be excluded (because of the within-subject design of these experiments); however, we found no changes in the phase shifting effect of two consecutive administrations of endotoxin, suggesting that there is no tolerance induced under our experimental conditions. In addition, the fact that the effect was specifically related to TNF-α, and not IL-1, suggests that there is no change in the response to LPS in our experimental design.

In summary, our results reveal a necessary role for central TNF-α in the behavioral effects of a peripheral immune challenge, and a direct effect of both IL-1β and TNF-α on circadian phase in vivo. These findings suggest that these cytokines, acting in the central nervous system, could participate in the synchronization of the circadian system by immune molecules, either in physiological or pathological conditions. Additional work should be performed to describe the complete pathways involved in the immune-circadian communication, since this bidirectional crosstalk might be important in situations of both circadian disruption and inflammatory pathologies.

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REFERENCES

Arjona A, Sarkar DK. (2005). Circadian oscillations of clock genes, cytolytic factors, and cytokines in rat NK cells. *J. Immunol.* 174:7618–7624.

Beynon AL, Coogan AN. (2010). Diurnal, age, and immune regulation of interleukin-1beta and interleukin-1 type 1 receptor in the mouse suprachiasmatic nucleus. *Chronobiol. Int.* 27:1546–1563.

Blatteis CM. (1992). Role of the OVLT in the febrile response to circulating pyrogens. *Prog. Brain Res.* 91:409–412.

Bluthe RM, Michaud B, Kelley KW, Dantzer R. (1996). Vagotomy blocks behavioural effects of interleukin-1 injected via the intraperitoneal route but not via other systemic routes. *Neuroreport* 7:2823–2827.

Born J, Lange T, Hansen K, Molle M, Fehm HL. (1997). Effects of sleep and circadian rhythm on human circulating immune cells. *J. Immunol.* 158:4454–4464.

Castanon-Cervantes O, Wu M, Ehlen JC, Paul K, Gamble KL, Johnson RL, Besing RC, Menaker M, Gewirtz AT, Davidson AJ. (2010). Dysregulation of inflammatory responses by chronic circadian disruption. *J. Immunol.* 185:5796–5805.

Cavadini G, Petrzilka S, Kohler P, Jud C, Tobler I, Birchler T, Fontana A. (2007). TNF-alpha suppresses the expression of clock genes by interfering with E-box-mediated transcription. *Proc. Natl. Acad. Sci. U. S. A.* 104:12843–12848.

Coogan AN, Wyse CA. (2008). Neuroimmunology of the circadian clock. *Brain Res.* 1232:104–112.

Davidson AJ, Castanon-Cervantes O, Leise TL, Molyneux PC, Harrington ME. (2009). Visualizing jet lag in the mouse suprachiasmatic nucleus and peripheral circadian timing system. *Eur. J. Neurosci.* 29:171–180.

Golombek DA, Rosenstein RE. (2010). Physiology of circadian entrainment. *Physiol. Rev.* 90:1063–1102.

Halberg F, Johnson EA, Brown BW, Bittner JJ. (1960). Susceptibility rhythm to *E. coli* endotoxin and bioassay. *Proc. Soc. Exp. Biol. Med.* 103:142–144.

Hayashi M, Shimba S, Tezuka M. (2007). Characterization of the molecular clock in mouse peritoneal macrophages. *Biol. Pharm. Bull.* 30:621–626.

Hrushesky WJ, Langevin T, Kim YJ, Wood PA. (1994). Circadian dynamics of tumor necrosis factor alpha (cachectin) lethality. *J. Exp. Med.* 180:1059–1065.

Keller M, Mazuch J, Abraham U, Eom GD, Herzog ED, Volk HD, Kramer A, Maier B. (2009). A circadian clock in macrophages controls inflammatory immune responses. *Proc. Natl. Acad. Sci. U. S. A.* 106:21407–21412.

Koyanagi S, Ohdo S. (2002). Alteration of intrinsic biological rhythms during interferon treatment and its possible mechanism. *Mol. Pharmacol.* 62:1393–1399.

Kubota T, Fang J, Guan Z, Brown RA, Krueger JM. (2001). Vagotomy attenuates tumor necrosis factor-alpha-induced sleep and EEG delta-activity in rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 280:R1213–R1220.

Kwak Y, Lundkvist GB, Brask J, Davidson A, Menaker M, Kristensson K, Block GD. (2008). Interferon-gamma alters electrical activity and clock gene expression in suprachiasmatic nucleus neurons. *J. Biol. Rhythms* 23:150–159.

Laflamme N, Rivest S. (1999). Effects of systemic immunogenic insults and circulating proinflammatory cytokines on the transcription of the inhibitory factor kappaB alpha within specific cellular populations of the rat brain. *J. Neurochem.* 73:309–321.

Leone MJ, Chiesa JJ, Marpegan L, Golombek DA. (2007). A time to kill, and a time to heal. Pathophysiological interactions

between the circadian and the immune systems. *Physiol. Mini-Rev.* 2:60–69.

Lundkvist GB, Hill RH, Kristensson K. (2002). Disruption of circadian rhythms in synaptic activity of the suprachiasmatic nuclei by African trypanosomes and cytokines. *Neurobiol. Dis.* 11:20–27.

Marpegan L, Bekinschtein TA, Costas MA, Golombek DA. (2005). Circadian responses to endotoxin treatment in mice. *J. Neuroimmunol.* 160:102–109.

Marpegan L, Leone MJ, Katz ME, Sobrero PM, Bekinschtein TA, Golombek DA. (2009). Diurnal variation in endotoxin-induced mortality in mice: correlation with proinflammatory factors. *Chronobiol. Int.* 26:1430–1342.

Mathias S, Schiffelholz T, Linthorst AC, Pollmacher T, Lancel M. (2000). Diurnal variations in lipopolysaccharide-induced sleep, sickness behavior and changes in corticosterone levels in the rat. *Neuroendocrinology* 71:375–385.

Motzkus D, Albrecht U, Maronde E. (2002). The human PER1 gene is inducible by interleukin-6. *J. Mol. Neurosci* 18:105–109.

Mullington J, Korth C, Hermann DM, Orth A, Galanos C, Holsboer F, Pollmacher T. (2000). Dose-dependent effects of endotoxin on human sleep. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 278:R947–R955.

Nava F, Carta G, Haynes LW. (2000). Lipopolysaccharide increases arginine-vasopressin release from rat suprachiasmatic nucleus slice cultures. *Neurosci. Lett.* 288:228–230.

Nygard M, Lundkvist GB, Hill RH, Kristensson K. (2009). Rapid nitric oxide-dependent effects of tumor necrosis factor-alpha on suprachiasmatic nuclei neuronal activity. *Neuroreport* 20:213–217.

Ohdo S, Koyanagi S, Suyama H, Higuchi S, Aramaki H. (2001). Changing the dosing schedule minimizes the disruptive effects of interferon on clock function. *Nat. Med.* 7:356–360.

Okada K, Yano M, Doki Y, Azama T, Iwanaga H, Miki H, Nakayama M, Miyata H, Takiguchi S, Fujiwara Y, Yasuda T, Ishida N, Monden M. (2008). Injection of LPS causes transient suppression of biological clock genes in rats. *J. Surg. Res.* 145:5–12.

Okamura H, Doi M, Fustin JM, Yamaguchi Y, Matsuo M. (2010). Mammalian circadian clock system: molecular mechanisms for pharmaceutical and medical sciences. *Adv. Drug Deliv. Rev.* 62:876–884.

Paladino N, Leone MJ, Plano SA, Golombek DA. (2010). Paying the circadian toll: the circadian response to LPS injection is dependent on the Toll-like receptor 4. *J. Neuroimmunol.* 225:62–67.

Palomba M, Bentivoglio M. (2008). Chronic inflammation affects the photic response of the suprachiasmatic nucleus. *J. Neuroimmunol.* 193:24–27.

Pan W, Zadina JE, Harlan RE, Weber JT, Banks WA, Kastin AJ. (1997). Tumor necrosis factor-alpha: a neuromodulator in the CNS. *Neurosci. Biobehav. Rev.* 21:603–613.

Petrzilka S, Taraborrelli C, Cavadini G, Fontana A, Birchler T. (2009). Clock gene modulation by TNF-{alpha} depends on calcium andp38 MAP kinase signaling. *J. Biol. Rhythms* 24:283–294.

Portaluppi F, Smolensky MH, Touitou Y. (2010). Ethics and methods for biological rhythm research on animals and human beings. *Chronobiol. Int.* 27:1911–1929.

Romanovsky AA, Sugimoto N, Simons CT, Hunter WS. (2003). The organum vasculosum laminae terminalis in immune-to-brain febrigenic signaling: a reappraisal of lesion experiments. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 285: R420–R428.

Sadki A, Bentivoglio M, Kristensson K, Nygard M. (2007). Suppressors, receptors and effects of cytokines on the aging mouse biological clock. *Neurobiol. Aging* 28:296–305.

Singh AK, Jiang Y. (2004). How does peripheral lipopolysaccharide induce gene expression in the brain of rats? *Toxicology* 201:197–207.

Thomson LM, Sutherland RJ. (2005). Systemic administration of lipopolysaccharide and interleukin-1beta have different effects on memory consolidation. *Brain Res. Bull.* 67:24–29.

Toedter G, Hayden K, Wagner C, Brodmerkel C. (2008). Simultaneous detection of eight analytes in human serum by two commercially available platforms for multiplex cytokine analysis. *Clin. Vaccine Immunol.* 15:42–48.

Wang X, Reece SP, Van Scott MR, Brown JM. (2011). A circadian clock in murine bone marrow-derived mast cells modulates IgE-dependent activation in vitro. *Brain Behav. Immun.* 25:127–134.