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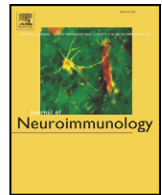

Paying the circadian toll: The circadian response to LPS injection is dependent on the Toll-like receptor 4

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ABSTRACT

Systemic low doses of the endotoxin lipopolysaccharide (LPS) administered at CT15 (circadian time 12 corresponds to locomotor activity onset) induce phase delays of locomotor activity rhythms in mice. To evaluate if this effect was mediated by the Toll-like receptor 4 (TLR4), our present aim was to characterize the circadian behavior and LPS-induced circadian response of TLR4 (LPS receptor)-deficient mice (in C57bl/10 and C3H backgrounds). In mutants, we observed a free-running period and a light-induced phase delay similar to the one observed in their corresponding wild-type (WT) littermates. The LPS-induced phase delay, wheel running inhibition and c-Fos/Per-1 immunoreactivity in the paraventricular nuclei observed in WT mice was absent or significantly decreased in the TLR4-deficient mice. In conclusion, we show that LPS-induced circadian responses are mediated by TLR4.

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1. Introduction

Daily environmental changes have imposed a selective pressure for life on Earth, driving the development of a circadian clock mechanism for the generation and entrainment of rhythms in physiological and behavioral variables (e.g. body temperature, hormonal secretion, sleep, locomotor activity, etc.). In mammals, the master clock resides in the hypothalamic suprachiasmatic nuclei (SCN), and the principal signal that adjusts its activity is the light–dark (LD) cycle (Klein et al., 1991; Reppert and Weaver, 2002).

Although there is substantial information regarding the circadian modulation of many immunological variables (reviewed in Leone et al., 2007 and Coogan and Wyse, 2008), little is known about the possible effect of immune factors on the circadian system itself. In addition, key immune mechanisms might be intrinsically rhythmic, as recently suggested for spleen, lymph nodes and peritoneal macrophages (Davidson et al., 2009; Keller et al., 2009). Moreover, several reports suggest a possible immune feedback regulation of the circadian clock. The sleep–wake cycle (one of the most evident circadian rhythms) is modified by pro- and anti-inflammatory cytokines (Krueger et al., 2001). Introduction of Gram-negative bacteria into the body causes the liberation of toxic, soluble products of the bacterial cell wall, such as lipopolysaccharide (LPS). The administration of LPS has been used to evaluate its somnogenic activity (Mullington et al., 2000), as well as its effect on memory consolidation and on behavior (Gahtan and Overmier, 2001; Pugh et al., 1998). Peripheral administration of LPS exerts profound effects on the sleep–wake cycle and sleep architecture and may produce, at higher doses, fever and a characteristic sickness behavior observed during inflammatory diseases, including sleep pattern changes and fever oscillations along the day (Kluger, 1991; Krueger et al., 1998). In addition, it is now well established that LPS induces autonomic, endocrine and behavioral responses that are controlled by the brain (Linthorst and Reul, 1998; Matsunaga et al., 2000).

In order to investigate the modulatory mechanisms operated by the immune components on the circadian clock, we focused our experiments on the circadian effect of LPS inoculation. Systemic low doses of LPS administered at Circadian Time (CT) 15 (where CT12 corresponds to locomotor activity onset in constant darkness) produce a photic-like phase delay of locomotor rhythm in mice, but it has no significant effect when injected at other CT (Marpegan et al., 2005). Moreover, intracerebroventricular (i.c.v.) injections of interleukin (IL)-1β and tumor necrosis factor (TNF)-α, which are strongly stimulated by LPS, also induce phase delays at CT15, suggesting that LPS may act on the clock by inducing the release of cytokines which finally act on the SCN (Leone et al., unpublished results). The activation of c-Fos in the SCN and paraventricular hypothalamic nuclei (PVN, a relay station of the output circadian pathway originating in the SCN) was previously reported after LPS or cytokines administration (Sadki et al., 2007; Marpegan et al., 2005). Clock gene *per-1* induction in the PVN, but not in the SCN, has been observed after the LPS injection and other circadian phase shifting stimuli (Takahashi et al., 2001; Meza et al., 2008). While the site of action or receptor involved in the circadian LPS responses is unknown, there is data suggesting that the immune-related transcription factor NF-κB is implicated, and its presence and activity in the SCN have been

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demonstrated for both light and LPS-induced phase shifts (Marpegan et al., 2004, 2005).

LPS exerts many of its cellular effects by interacting with a Toll-like receptor 4 (TLR4, Gay et al., 2006). Natural tolerance to the immunostimulatory properties of LPS has been observed in TLR4-deficient (−/−) C3H/HeJ and C57bl/10ScCr strains of mice. The C3H/HeJ mice have a point mutation within the coding region of the *Tlr4* gene, resulting in a nonconservative substitution of a highly conserved proline by histidine at codon 712, whereas C57bl/10ScCr mice exhibit a deletion of the *Tlr4* gene (Qureshi et al., 1999). Althought TLR4 is known to initiate the LPS cascade, other receptors may be implicated in the response to this endotoxin. The CD11b integrin (MAC-1) receptor was identified as a receptor/correceptor for LPS and it can mediate TLR4-independent LPS effects (Perera et al., 1997; Qin et al., 2005; Pei et al., 2007).

To evaluate whether the LPS effect on the locomotor activity rhythm is mediated by this receptor, we characterized the circadian behavior and LPS-circadian response in the previously described TLR4$^{-/-}$ mice (C57bl/10ScCr and C3H/HeJ) and their corresponding wild-type (WT) littermates (C57bl/10ScCn and C3H–HeN). Since we have previously described that LPS produces a light-like effect that might share a common step in the circadian photic entrainment pathway, we also recorded the responses of TLR4$^{-/-}$ mice to light pulses, in addition to the LPS-circadian response. We measured the phase shift of the locomotor activity rhythms and the inhibition of the locomotor activity in response to LPS, as well as its effect on c-Fos and Per-1 expression in the PVN, an area previously shown to respond to the endotoxin (Marpegan et al., 2005). We found a strain dependence of the circadian LPS response, and we show that the LPS-induced phase delay, locomotor activity inhibition and brain c-Fos and Per-1 induction are mediated by TLR4.

2. Materials and methods

2.1. Animals

Adult (2 months old) C57bl/6J male mice (*Mus musculus*) were raised in our colony ($n=9$); wild-type C57bl/10ScCn (C57bl10 WT, $n=7$) and TLR4-deficient C57bl/10ScCr (C57/bl10 TLR4$^{-/-}$, $n=10$) mice were kindly provided by Dr. F. P. Polack, (INFANT Foundation); wild-type C3H–HeN (C3H WT, $n=12$) and TLR4-deficient C3H/HeJ (C3H TLR4$^{-/-}$, $n=8$) were provided by Dr. S. Gonzalez-Cappa, (University of Buenos Aires) and Dr. O. Campetella (National Institute of Industrial Technology, Argentina). All mice were housed under a 12:12-h LD photoperiod (with lights on at 8 AM and light off at 8 PM) or constant darkness (DD) conditions, with food and water *ad libitum*. Mice were transferred to DD conditions in single cages 20 days prior to the treatments. All animal experiments were carried out in accordance with the NIH Guide for the care and use of laboratory animals.

2.2. Behavioral analysis

Mice were housed in individual cages equipped with running wheels, and their locomotor activity circadian rhythm was recorded with a system designed in our laboratory. Wheel revolutions were monitored by magnetic microswitches activated by the wheel and collected every 5 min. Time is expressed as *Zeitgeber time* (ZT), with ZT12 defined as the time of lights off in LD conditions, or CT, with CT12 defined as the moment of locomotor activity onset in DD conditions. Injections in DD were performed under dim red light (<1 lux).

Mice maintained in DD were exposed to a 10-min white light pulse of 100 lx, or injected with a 50 μg/kg of LPS (E. coli serotype 0111:B4 Sigma, St. Louis, MO) or an equal volume of saline solution (vehicle) by intraperitoneal route at CT15. The onset of activity at CT12 was used as a phase reference point to calculate phase shifts. The number of wheel-turns/circadian night in free-running (DD) condition was calculated (CT12–CT24 or CT15–CT24). The relative activity immediately after LPS injection (CT15–CT24 of inoculation day) was obtained as activity at injection day/activity of 5–7 previous days.

2.3. Immunohistochemistry

Mice were deeply anaesthetized with a cocktail containing ketamine (150 mg/kg) and xylazine (10 mg/kg) by intraperitoneal route, 60 min after LPS or vehicle (Veh) treatment at CT15 and perfused intracardially with 4% paraformaldehyde in 0.01 M phosphate buffer (C57bl/6 J: LPS $n=4$, Veh $n=5$; C57bl/10 WT: LPS $n=6$, Veh $n=4$; C57bl/10 TLR4$^{-/-}$: LPS $n=5$, and Veh $n=4$). Brains were carefully removed, post-fixed overnight, cryoprotected in 30% sucrose in 0.01 M phosphate-buffered saline (PBS) for 24 h and 25 μm thick coronal sections were cut with a freezing microtome and collected in 0.01 M PBS. Free floating SCN and PVN-containing sections (10 sections/mouse) were blocked with 5% non-fatty milk in PBS containing 0.4% Triton X-100 and incubated with primary antisera raised in rabbit against c-Fos (Santa Cruz Biotechnology, 1:4000) or Per-1 (Affinity BioReagens, 1:600) diluted in the same solution, for 48 h at 4 °C. Sections were then treated using the avidin–biotin method with a Vectastain Elite Universal kit containing a biotine-conjugated secondary antibody, avidin and biotine-conjugated horse-radish peroxidase (Vector Laboratories, Burlingame, CA) and Vector-VIP peroxidase substrate (SK-4600).

2.4. Statistical analysis

Phase shifts were calculated using the Clocklab program and the wheel-turns/circadian night using El Temps software. Cell counting was performed with the ImageJ 1.29 program (NIH) in hypothalamic sections as previously described (Marpegan et al., 2005). The difference between groups (mean +/− SEM per group) was analyzed by two-way analysis of variance (ANOVA) or the non-parametric ANOVA Kruskal–Wallis test (for data groups with a non-Gaussian distribution). Post-hoc pairwise comparisons were performed by means of a Duncan test. *P* values of 0.05 or less were considered to be statistically significant.

3. Results

3.1. Characterization of circadian responses in TLR4$^{-/-}$ mice

We first analyzed the circadian responses in TLR4$^{-/-}$ mice (C57bl/10ScCr and C3H–HeJ) and their corresponding WT littermates (C57bl/10ScCn and C3H–HeN), in comparison with C57bl/6J mice. We analyzed circadian period, total activity count and the response to light pulses in the free-running condition (DD).

In order to evaluate the free-running period in the different strains of mice, animals were transferred to DD conditions in single cages provided with wheel running, 20 days prior to the treatments. The free-running period of locomotor activity was similar in the different WT and TLR4$^{-/-}$ strain mice analyzed (Fig. 1A and Table 1). As it was previously reported, C57bl/6J mice had a period of 23.55 ± 0.09 h (Schwartz and Zimmerman, 1990; Shimomura et al., 2001).

We next analyzed total locomotor activity as the number of wheel-turns/circadian night under DD condition. We found lower values of total activity in C57bl/10 WT and C3H TLR4$^{-/-}$ mice in comparison with C57bl/6J, C57bl/10 TLR4$^{-/-}$ and C3H WT mice (Fig. 1B and Table 1).

To evaluate the circadian response to light, the different strains of mice were exposed to 10 min-100 lx white light pulse at CT15. We found a differential response to light pulses. The C3H mice had larger phase delays than C57bl/10 mice. In both strain backgrounds, the

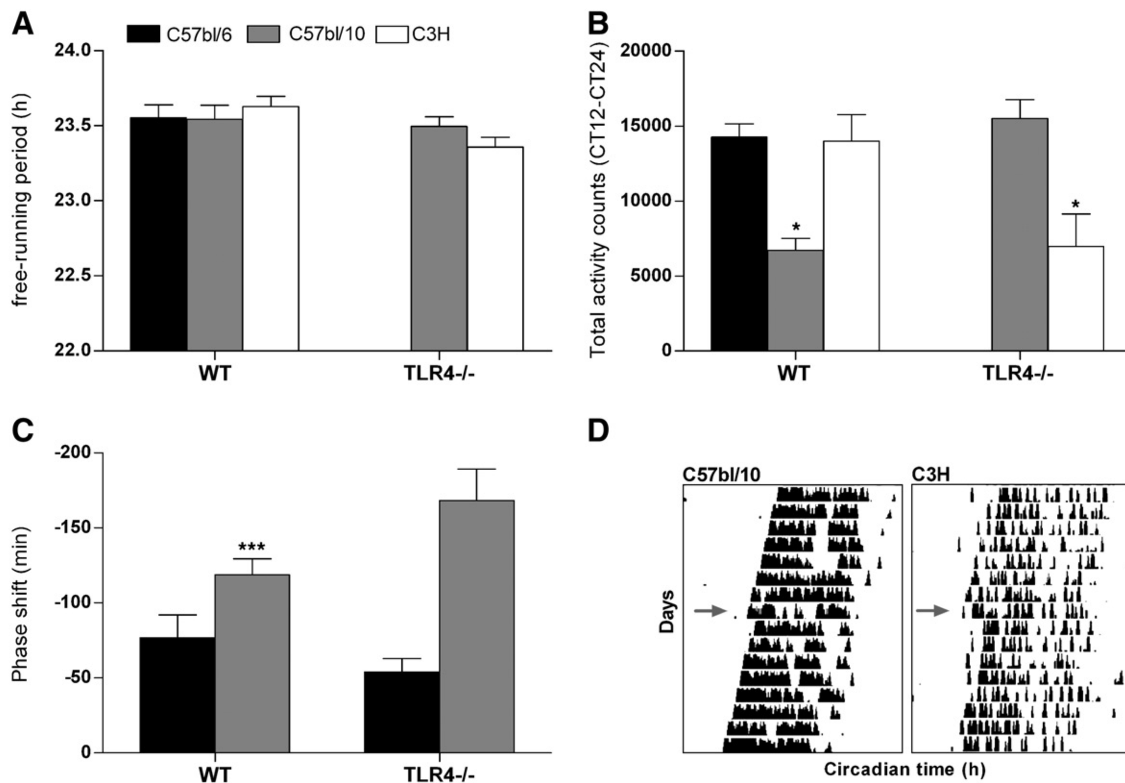

Fig. 1. Characterization of circadian responses in TLR4$^{-/-}$ mice. A) Wheel running circadian period (mean ± SEM) in DD conditions. B) Wheel running total activity in the wake period in DD conditions. Mean ± SEM of wheel-turns/circadian night of 7–10 consecutive days ($p = 0.02$, Kruskal–Wallis test; $^*p<0.05$ C57bl/10 WT and C3H TLR4$^{-/-}$ vs C57bl/6, C3H WT and C57bl/10 TLR4$^{-/-}$. C) Mean ± SEM of light pulse (100 lx, 10 min)-induced phase delay at CT15 in DD condition. Two-way ANOVA indicated a significant difference between mice strains ($^{***}p<0.0001$, C57bl/10 vs C3H) but no differences between WT and TLR4$^{-/-}$ mice. Black bars correspond to C57/Bl10 mice and gray bars to C3H mice. D) Representative actograms of light-induced phase delay in C57bl/10 and C3H mice.

phase delay was similar in WT and TLR4$^{-/-}$ mice (Fig. 1C–D and Table 1).

3.2. Circadian response to LPS in TLR4$^{-/-}$ mice

LPS administration (50 µg/kg, i.p.) at CT15 induced a significant phase delay in all WT mice analyzed (Fig. 2A, C and Table 2). In C57bl/10 WT mice, the LPS inoculation induced a larger phase delay than in C57bl/6J mice, showing a strain-specific LPS response.

The LPS-induced phase delay in both TLR4$^{-/-}$ mice was significantly lower than their specific WT strain mice. Moreover, it was similar to the phase shift observed after Veh inoculation (Fig. 2A, C and Table 2).

In all WT mice a complete inhibition of wheel running was observed immediately after LPS injection (relative activity of the inoculation day between CT15 and CT24, Fig. 2B–C and Table 2). This inhibition was absent in TLR4$^{-/-}$ mice. LPS effects were specific and not directly related to behavioral inhibition, since mechanically stopping the wheel at CT15 did not induce circadian phase shifts (data not shown).

We also analyzed the expression of c-Fos in hypothalamic coronal brain sections that included SCN and PVN regions, 90 min after the LPS or Veh administration at CT15. As we previously reported, LPS induced an increase in the number of c-Fos positive cells in the PVN in WT mice (Fig. 3, ANOVA $p<0.0001$). There were strain-dependent differences in LPS-induced c-Fos induction in the PVN, being higher in C57bl/6J than in the C57bl/10 mice. In TLR4$^{-/-}$ mice, the number of c-Fos positive cells in the PVN was significantly lower than in the WT mice.

As for Per-1 expression, although the average number of cells stained in all mice was high, we found a significant increase in the number of cells in the PVN after LPS inoculation in WT mice (ANOVA $p = 0.0026$. Supp. Fig. 1). TLR4$^{-/-}$ mice exhibited a significantly lower LPS-induced Per-1 expression in the PVN as compared to WT mice. LPS inoculation also induced Per-1 expression in the SCN of C57bl/10 WT mice.

Table 1
Circadian parameters in different strains of mice.

Parameter	C57bl/6	C57bl/10		C3H		p
	WT	WT	TLR4$^{-/-}$	WT	TLR4$^{-/-}$	
Free-running period	23.55 ± 0.09 (n = 5)	23.54 ± 0.09 (n = 7)	23.50 ± 0.06 (n = 10)	23.63 ± 0.07 (n = 12)	23.36 ± 0.07 (n = 8)	ns
Total activity counts	14260 ± 882 (n = 5)	6716 ± 784* (n = 7)	15509 ± 1253 (n = 10)	13987 ± 1767 (n = 12)	6965 ± 2158* (n = 8)	0.02[a]
Light-induced phase shift	nd	-77 ± 34# (n = 5)	-54 ± 25# (n = 8)	-118 ± 10.6 (n = 11)	-168 ± 21 (n = 8)	<0.0001[b]

Wheel running circadian period and wheel running total activity (wheel-turns/circadian night of 7–10 consecutive days) were evaluated in constant darkness. Ten min-100 lx white light-induced phase shifts were assessed at CT15 in DD conditions. nd: not determined. Data correspond to mean ± SEM.

[a] Non-parametric ANOVA Kruskal–Wallis test was used to analyze total activity counts; $^*p<0.05$ in comparison with all other groups.

[b] Two-way ANOVA test was used for light-induced phase shift, p value correspond to strain mice way (C57/bl10 vs. C3H mice).

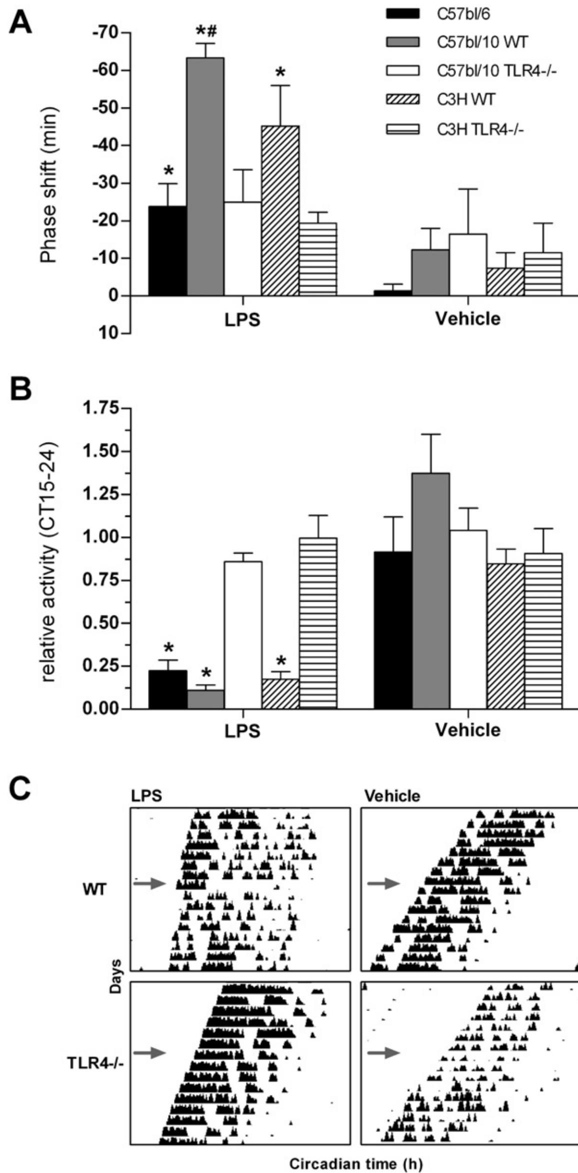

Fig. 2. LPS-induced phase delay at CT15 in DD condition. A) Mean ± SEM of phase delay after LPS (50 mg/kg, i.p.) or vehicle (Veh) injection at CT15 in DD condition. B) Relative activity immediately after LPS injection: CT15-CT24 of inoculation day (activity at injection day/activity of 5–7 previous days). C) Representative actograms of WT and TLR4$^{-/-}$ mice challenged with LPS or Veh. (A) $p<0.0001$, ANOVA, (B) $p<0.0001$, Kruskal–Wallis test. Post-hoc comparisons: $^{\#}p<0.05$ C57bl/10 WT vs C57bl/6; $^{*}p<0.05$ LPS vs Veh in all WT mice; and $^{*}p<0.05$ LPS in WT vs LPS in their respective TLR4$^{-/-}$ strain.

4. Discussion

4.1. LPS effects on the circadian system

Systemic low doses of LPS delivered at CT15 induce a photic-like phase delay of locomotor rhythm in mice (Marpegan et al., 2005). In previous reports, we suggested that LPS may act on the clock by inducing the release of the cytokines IL-1β and TNF-α which finally act on the SCN (Leone et al., 2006 and unpublished results). As previously reported, peripheral LPS inoculation induces a phase-response curve (PRC) similar to the one observed for the light pulses, inducing phase delays in the early subjective night (CT15; Marpegan et al., 2005). The phase shift observed after LPS inoculation provides evidence of the response of the circadian system to an immune challenge. Indeed, phase shifts can be considered as a reliable read-out of the circadian system, since they reflect changes in clock-controlled output variables, and are necessary for the adequate entrainment to the environment. Since we used relatively low doses of the endotoxin, this might reflect the modulatory effect of an increased activity of immune effectors on the circadian system. By increasing the release of pro-inflammatory cytokines, LPS might activate a signaling mechanism to the circadian system, and therefore adjust the phase of different clock-controlled variables. In this sense, the endotoxin might be considered a zeitgeber that changes humoral mechanisms that feedback into the clock. A related mechanism could be activated in response to infections, with the important difference of the chronic stimulation of the immune system (Marpegan et al., 2009).

In order to further characterize this immune–circadian interaction, we analyzed the participation of the LPS receptor TLR4 in the LPS-induced phase delay model. We studied the circadian behavior and LPS-induced circadian response of TLR4$^{-/-}$ mice. We found a strain dependence of the circadian LPS response, and we showed that the LPS-induced phase delay, locomotor activity inhibition and brain c-Fos and Per-1 induction are mediated by TLR4.

4.2. Circadian responses in different strains of mice

We first analyzed the circadian responses in TLR4$^{-/-}$ mice and their corresponding control strain mice. Under DD conditions, circadian rhythms assume free-running periods that are close to 24 h. While the free-running period in BALB/c mice was close to 23 h (Shimomura et al., 2001), in C57bl/6J and C57bl/10 mice it ranged between 23.3 and 23.9 h (Schwartz and Zimmerman, 1990). In line with this report, we did not find any difference in the free-running period between C57bl/6J and C57bl/10 mice. Moreover, the circadian period was similar between WT and TLR4$^{-/-}$ mice. On the other hand, strain differences in the shape of the circadian pattern for activity were observed between C3H and C57bl/6J mice (Tankersley et al., 2002). Although the mean of activity counts in the early subjective night was similar in both strain of mice, only C57bl/6J mice showed a second activity peak during the late night. We observed lower values of total activity in C3H TLR4$^{-/-}$ but not in the C3H WT mice.

Circadian entrainment is mediated through daily phase shifts induced by light on the pacemaker. Indeed, under DD conditions, light pulses delivered in the early subjective night (CT15) induce phase delays in behavioral rhythms controlled by the clock, while late night pulses (CT18) advance the phase. The PRC to light pulse is relatively similar between C57bl/6J and BALB/c mice (Schwartz and Zimmerman, 1990). Here we show that C3H mice exhibit larger phase delays than the C57bl/10 mice after a light pulse at CT15. We have previously described that LPS produces a photic-like effect that might share a common step in the circadian entrainment pathway (which may be mediated by an NFκB pathway) since the co-administration of LPS and light pulse at CT15 shows no additive phase shift responses (Marpegan et al., 2005). Therefore, we compared the light pulse response in TLR4$^{-/-}$ mice but found no differences with their respective WT littermates.

4.3. LPS-related pathways within the central nervous system

The mechanism by which peripheral administration of LPS affects the central nervous system remains controversial. It has been shown that LPS (100 μg/kg) is not capable to cross the brain–blood barrier in rats but it can bind to specific receptors in cerebral vascular endothelium, inducing pro-inflammatory responses (Singh and Jiang, 2004). Cerebral expression of TLR4 has been demonstrated in rat circumventricular organs and in some parenchymal structures, especially within the regions lining the cerebroventricular systems, including the PVN of the hypothalamus (Laflamme and Rivest, 2001). Moreover, cytokines induced by LPS may also enter the brain through the *organum vasculosum laminae terminalis* where the

Table 2
LPS-induced circadian responses.

Strain	Phase delay (min)		Relative activity	
	LPS	Vehicle	LPS	Vehicle
C57bl/6 WT	$-23.8\pm6^*$ ($n=9$)	-1.27 ± 1.83 ($n=7$)	$0.22\pm0.06^*$ ($n=9$)	0.92 ± 0.2 ($n=7$)
C57bl/10 WT	$-63.3\pm3.9^{\#*}$ ($n=3$)	-12.2 ± 5.74 ($n=3$)	$0.08\pm0.04^*$ ($n=3$)	1.32 ± 0.14 ($n=3$)
C57bl/10 TLR4$^{-/-}$	-24.9 ± 8.7 ($n=5$)	-16.4 ± 12.05 ($n=3$)	0.86 ± 0.05 ($n=5$)	1.04 ± 0.13 ($n=3$)
C3H WT	$-45.11\pm10.8^*$ ($n=11$)	-7.3 ± 4.2 ($n=10$)	$0.17\pm0.04^*$ ($n=11$)	0.85 ± 0.09 ($n=10$)
C3H TLR4$^{-/-}$	-19.3 ± 3 ($n=6$)	-11.5 ± 7.8 ($n=8$)	0.99 ± 0.13 ($n=6$)	0.91 ± 0.14 ($n=8$)
p	<0.0001		<0.0001	

Circadian responses to LPS. Phase shifts were calculated in response to LPS administration at CT15 (see text for details). Relative activity was calculated immediately after LPS or vehicle injection from CT15 to CT24 of inoculation day and corresponds to activity counts at injection day/activity counts of 5–7 previous days in constant darkness. ANOVA was used to compare phase shifts and non-parametric ANOVA Kruskal–Wallis test was used for relative activity comparison. Post-hoc pairwise comparisons: $^{\#}p<0.05$ in comparison with C57bl6; $^*p<0.05$ LPS vs vehicle inoculation in all WT mice, and LPS inoculation in WT mice in comparison with their respective TLR4$^{-/-}$ strain mice. Data correspond to mean ± SEM in all cases.

blood–brain barrier is leaky due to the presence of a fenestrated endothelium (Romanovsky et al., 2003) or may act through neural terminals of peripheral tissue, e.g., those of the vagus nerve (Maier et al., 1998; Simons et al., 1998). A role for cytokines in the regulation of the molecular circadian clock has been suggested. Cavadini et al. (2007) have shown that treatment of fibroblasts with TNF-α downregulates expression of E-box–driven clock genes in cultured fibroblasts. Furthermore, the interaction of TNF-α with TNFR (Receptor) 1, but not TNFR2, leads to fast downregulation of gene expression of *Dbp* and upregulation of negative regulators of the molecular clock, Period (*Per*)-1 and *Per-2*, Cryptochrome-1 (*Cry-1*), and differentiated embryo chondrocytes-1 (*Dec-1*) (Petrzilka et al., 2009). Sadki et al. (2007) examined the effect of TNF-α/IFN-γ i.c.v. microinjection on SCN expressed c-Fos and reported an interaction between cytokine treatment and c-Fos expression in core and shell regions of the SCN.

As for the effects of endotoxin on the brain, it has been reported that LPS injection (50 μg/kg) at ZT22 caused a rapid induction of Per-1, but not mPer-2 mRNA levels in the mouse PVN without any change of SCN and liver mRNA levels (Takahashi et al., 2001). Changes in Per-2 and Dbp rhythms were also found in rats after inoculation with high doses of LPS (Okada et al., 2008). In line with these reports, we observed an increase in number of cells that express Per-1 and c-Fos in the PVN in LPS-injected WT mice. These nuclei have been described

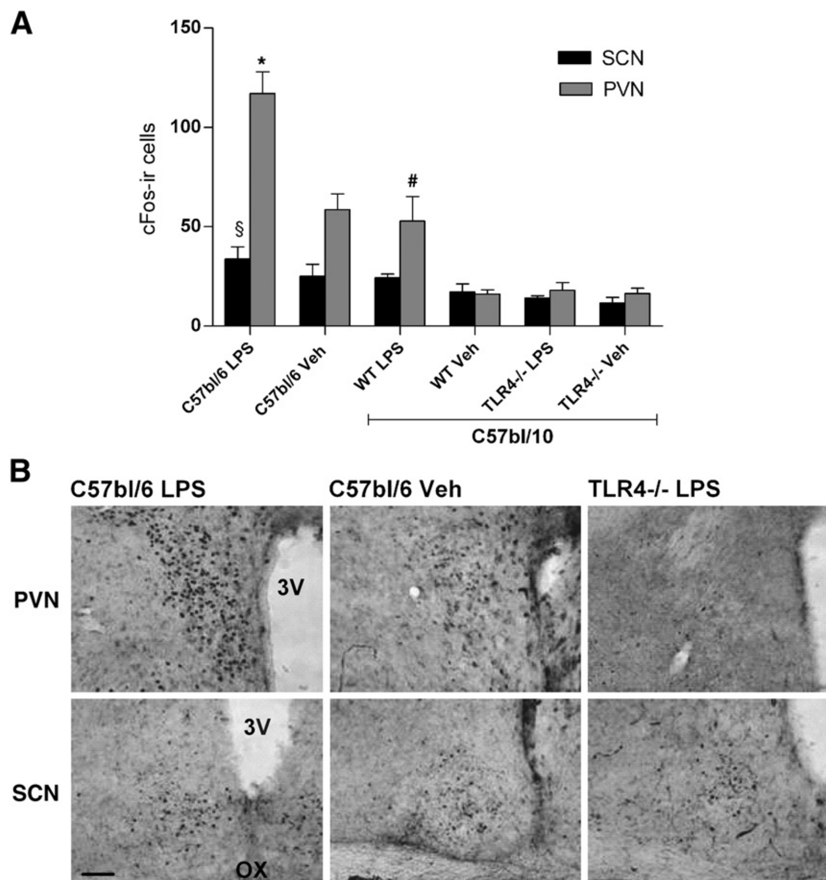

Fig. 3. LPS-induced c-Fos brain expression. Immunohistochemistry of c-Fos in SCN and PVN in mice challenged with LPS (50 μg/kg, i.p.) or vehicle (Veh) at CT15 respectively. (A) Mean + SEM of positive cells number of c-Fos and (B) representative pictures. SCN: $p=0.03$, Kruskall–Wallis test; post-hoc comparisons: $^{\S}p<0.05$ C57bl/6 LPS vs C57bl/10 TLR4$^{-/-}$ LPS. PVN: $p<0.0001$, ANOVA; post-hoc comparisons: $^*p<0.05$ C57bl/6 LPS vs all groups; $^{\#}p<0.05$ C57bl/10 WT LPS vs C57bl/10 WT Veh, TLR4$^{-/-}$ LPS and TLR4$^{-/-}$ Veh. C57bl/6: LPS $n=4$, Veh $n=5$; C57bl/10 WT: LPS $n=4$, Veh $n=4$; TLR4$^{-/-}$: LPS $n=5$, and Veh $n=4$. Scale bar = 100 μm.

as an output relay station for the SCN (Kalsbeek et al., in press), and its stimulation affects clock-controlled rhythms such as pineal melatonin secretion (Isobe and Nishino, 2004). In addition, certain circadian phase shifting stimuli have also been found to induce Per-1 expression in the PVN but not in the SCN (Meza et al., 2008). The role of LPS-induced clock genes in the PVN for circadian entrainment remains to be examined in more detail.

4.4. TLR4-dependent and independent LPS effects

Although TLR4 is known to initiate the LPS cascade, other receptors may be implicated in the response to this endotoxin. The CD11b integrin (MAC-1) receptor was previously identified as receptor/correceptor for LPS in phagocytes (Perera et al., 1997). It has been recently reported that the LPS-induced activation of NADPH oxidase in microglial cell cultures is TLR4-independent (Qin et al., 2005). Furthermore, this report showed that LPS-induced TNF-α production by microglial cells is TLR4-dependent at low endotoxin dosis, but it is TLR4-independent at higher LPS concentrations. MAC-1 receptor expression was reported in microglia (Akiyama and McGeer, 1990), and may be responsible for microglia production of TNF-α, nitric oxide and superoxide in response to LPS (Pei et al., 2007). Indeed, we have previously reported that astrocytes may mediate input signals to mouse SCN coming from the immune sistem (Leone et al., 2006). In this context, MAC-1 (expressed in glial cells) could be a possible LPS receptor implied in this immune–circadian interaction.

In summary, our present results show that TLR4 is the main receptor involved in the pathway through which LPS modifies the setting of the circadian clock. Additional work should be performed in order to determine the complete pathways in this circadian–immune cross talk.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jneuroim.2010.04.015.

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