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Potential Protective Role of IL15R α During Inflammation

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Abstract

We have shown that TNF α specifically activates the interleukin-15 (IL15) system in cerebral endothelial cells composing the blood–brain barrier. To determine the functions of cerebral IL15 signaling in inflammation, we first treated mice with lipopolysaccharide (LPS) and determined the expression of the three receptor subtypes of IL15. Robust time-dependent upregulation occurred in all subunits. We then tested whether IL15R α knockout (KO) affected the maintenance of body temperature and activity level after a single dose of LPS. Circadian telemetry data were analyzed by the cosinor method. Both wild-type and KO mice had clear 24-h rhythms of basal temperature and activity. KO mice had a significantly higher midline estimating statistic of rhythm (MESOR;

approximating 24 h mean) of temperature and delayed 24-h acrophase (peak) of activity than the wild-type mice. LPS disrupted the circadian rhythm of activity more severely in the KO group. Besides a decrease in MESOR and 24-h amplitude of activity after LPS, the KO mice showed a significant reduction of MESOR, amplitude, and changed acrophase for temperature on the second of 2 days. The disrupted circadian rhythm of temperature and activity in the KO mice after LPS suggests that upregulated IL15 receptors may serve a beneficial role to counteract the consequences of neuroinflammation.

Keywords

IL15; LPS; Blood–brain barrier; Neuroinflammation; Temperature; Locomotor activity; Telemetry; Cosinor analysis

Introduction

This study examines how the cerebral interleukin (IL)-15 receptor regulates the circadian rhythm of thermoregulation and activity in response to challenge with the endotoxin lipopolysaccharide (LPS). IL15 is a ubiquitously expressed cytokine that activates JAK/STAT signaling through a trimeric receptor complex comprised of IL15R α , IL2R β , and IL2R γ (Hanisch et al. 1997). In fetal human brain, both IL15 and IL15R α mRNAs are higher in the hippocampus and cerebellum than in the cortex and thalamus (Kurowska et al. 2002). Within the CNS, IL15 affects nitric oxide production and growth of microglia (Waldmann and Tagaya 1999). However, IL15R α knockout (KO) mice show astrogliosis and microgliosis at least in the hippocampus, indicating an anti-inflammatory role of IL15 signaling (Wu et al. 2010b). Beside the lack of usual anxiety behavior, the IL15R α KO mice show depressive-like symptoms (Wu et al. 2010a), reduction of memory and defective GABA transmission (He et al. 2010a), and altered locomotor activity and thermoregulation (He et al. 2010b). After facial nerve injury, motor neuron death is 5-fold greater than in the wild-type mice (Huang et al. 2007).

In contrast with IL15R α deletion, IL2R β KO mice exhibit deficits in prepulse inhibition and reduction of fearfulness in response to novelty (Petitto et al. 2002). IL2R γ KO mice also show depressive-like behavior and reduction of contextual memory (Wu et al. 2010c). IL15 ligand KO mice are more susceptible to experimental autoimmune encephalomyelitis (EAE; Wu et al. 2010d), show greater demyelination in the spinal cord, increased immune cell infiltration, and overexpression of fractalkine receptor CX3CR1 in CD8 cells (Gomez-Nicola et al. 2010). Consistently, IL15 treatment improves locomotor behavior in the EAE mice (Wu et al. 2010d). Altogether, the findings indicate that IL15 and its receptors can exert anti-inflammatory roles. We return to the widely used neuro-inflammation model of LPS challenge to test the regulatory changes of IL15 receptors in the CNS and to use telemetry and cosinor analysis to determine the circadian rhythms of body temperature and activity. The results not only show a robust, time-dependent upregulation of IL15 receptors, but also indicate that IL15R α may have essential protective roles to maintain homeostasis in response to LPS.

Materials and Methods

Tissue Collection, RNA Extraction, and Quantitative RT-PCR

All experiments were conducted following protocols approved by the Institutional Animal Care and Use Committee. Three-month-old male C57BL/6J mice (B6, Jackson Laboratory, Bar Harbor, ME) received either sterile phosphate buffered saline (PBS) or LPS at 5 mg/kg in 100 μ l of PBS intraperitoneally ($n=4-5$ mice/group). At 4 or 48 h after treatment, the

mice were anesthetized by intraperitoneal injection of ketamine/xylazine and decapitated. The hypothalamus, hippocampus, striatum, cerebellum, cerebral cortex, and spinal cord were dissected. Because of the concern that circumventricular organs contain leakier blood vessels where the blood–brain barrier (BBB) is not tight, only cerebral cortex was used in obtaining crude microvessels by the capillary depletion method previously described (Pan et al. 2006). This method provides enrichment of capillaries with more than 40-fold increase of γ -glutamyl transpeptidase (Yu et al. 2007). The enriched microvessels and the rest of the dissected brain regions were processed on ice in an RNase-free environment, snap frozen in liquid nitrogen, and stored at -80°C until further use.

Total RNA extraction, purification, quantification and reverse-transcription were conducted as previously described (Wu et al. 2010d). The primers and fluorescent probes for real-time PCR are listed in Table 1. The qPCR was conducted on an ABI 7900 HT system (Applied Biosystems, Foster City, CA). The expression level of the target genes was normalized to that of glyceraldehydes-3-phosphate dehydrogenase (GAPDH), and the relative amount of IL15 receptor mRNA was calculated by the $\Delta\Delta\text{CT}$ method (Livak and Schmittgen 2001; Cikos et al. 2007; Schmittgen and Livak 2008).

Western Blotting

The brain tissues were homogenized in CellLytic™ MT Mammalian Tissue lysis buffer (Sigma) supplied with protease inhibitor cocktail at 4°C . The supernatant after centrifugation was collected for protein concentration measurement by the bicinchoninic acid assay. Thirty micrograms of protein from each sample was electrophoresed and transferred to nitrocellulose membranes. After blocking, the membranes were probed with primary antibodies against IL15R α (sc-1524, Santa Cruz Biotechnologies, Santa Cruz, CA) or β -actin (A2228, Sigma) by overnight incubation at 4°C . After thorough wash, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The signals were developed with enhanced chemiluminescence-plus western blotting detection reagents (AmershamBiosciences, Piscataway, NJ). The signal intensity was quantified by use of NIH Image J software and normalized to that of β -actin in the respective samples. All mRNA and protein data are presented with mean $\pm$ standard errors. Student's *t* test was performed to determine the difference between the control and experimental groups.

LPS Treatment, Telemetry, and Cosinor Analysis

The IL15R α KO mice (B6;129X1-IL15ra^{tm1Ama/J}) and their strain background controls (B6; 129 SF2) were purchased from the Jackson Laboratory. Mice were group-housed under a regimen of 12 h of light alternating with 12 h of darkness for at least 2 weeks in the animal care facility before the initiation of experiments. The dark phase was from 7 p.m. to 7 a.m. The room temperature was 24°C . To monitor body temperature and locomotor activity of 3-month-old male KO and wild-type mice ($n=6/\text{group}$), the Mini Mitter (Bend, OR) telemetry system was used as reported previously (He et al. 2010b). The gas-sterilized E-mitters were implanted intraperitoneally into mice anesthetized by isoflurane inhalation. After 7-day recovery from surgery, the mice were single-caged for 3 days before initiation of monitoring. The sampling interval was 5 min. After 3 days of control recording, the mice received 0.8 mg/kg of LPS from *Salmonella enterica* serotype typhimurium (cat L6511, Sigma, St. Louis, MO) intraperitoneally at 4:30 p.m. The mice were monitored for another 3 days. Since the recording did not cover three complete circadian cycles, only the data during 2 days of pre- and post-LPS treatment were analyzed.

The cosinor analysis assessed the circadian parameters. Assuming a period (length of a cycle) of 24 h, the midline estimating statistic of rhythm (MESOR, a rhythm-adjusted 24-h

mean), amplitude (half the extent of predictable change within a day), and acrophase (ϕ , the time of peak within the cycle) were estimated by least squares. The activity data were log-transformed before analysis ($Y' = \log_{10}(Y + 1)$). To respect the requirement of independence of residuals from the fitted model, data were decimated. The individual 20-min data of activity and temperature were recorded over two consecutive days before and after LPS treatment (0.8 mg/kg), and the individual records were analyzed by least squares spectra. Since the data were equidistant, this approach amounts to computing the discrete Fourier transform. Spectra were computed for the entire 2-day record, using a fundamental period of 48 h, up to one cycle per hour. Spectra were also computed separately for the first and second day, using a fundamental period of 24 h, up to one cycle per hour.

Amplitudes at each frequency were averaged for each group (wild-type or KO) separately, before and after LPS treatment. The Student *t* test was used to compare the mean amplitudes at each frequency between wild-type and KO mice before and after LPS treatment. Similarly, the effect of LPS treatment was assessed at each frequency by comparing the mean amplitudes before and after treatment separately for the wild-type and the KO mice. Paired *t* tests were also applied on amplitude differences between groups across all frequencies. Amplitude differences were further linearly regressed as a function of frequency.

The above analyses serve to assess any differences in the time structures of temperature and activity. For a comparison of MESORs, 24-h amplitudes, and/or acrophases, parameter tests (Bingham et al. 1982) were used. In view of the inter-individual differences in the time course of temperature and activity, comparisons of population parameters were performed in preference to comparisons of average profiles.

Results

Regional and Temporal Patterns of Increased IL15R α mRNA in Response to LPS

qPCR showed that basal levels of IL15R α mRNA differed among the tissues examined. LPS increased IL15R α mRNA in cerebral microvessels obtained from the cortex at 4 h, but not at 48 h. At 4 h after LPS treatment, there was a significant increase of IL15R α mRNA in the striatum, hypothalamus, and spinal cord, with a trend ($p=0.058$) toward a significant increase in the cerebellum (Fig. 1a). At 48 h, similar significant elevation of IL15R α mRNA was seen not only in the striatum, hypothalamus, and spinal cord, but also in the hippocampus and cerebellum (Fig. 1b).

Increased IL2R β mRNA After LPS Challenge

Significant increases in IL2R β mRNA were seen in the striatum, cerebellum, and enriched cerebral microvessels at 4 h after LPS challenge (Fig. 2a). At 48 h, most brain regions and microvessels had a significant increase whereas the hypothalamus showed a similar trend ($p=0.06$). The spinal cord did not show a significant change (Fig. 2b).

Increased IL2R γ mRNA in Response to LPS

At both 4 and 48 h after LPS injection, there was a striking increase of IL2R γ mRNA in all CNS regions tested. This included cerebral microvessels, all brain regions, and the spinal cord (Fig. 3a and b).

IL15 Receptor Protein Levels After LPS Challenge

Western blotting was performed on hypothalamic and hippocampal samples in which LPS challenge had induced a significant increase of IL15R α mRNA at 48 h as shown in Fig. 1a. The level of IL15R α protein in the hypothalamus tended to be increased ($p=0.057$ by

densitometric analysis). There were no changes of IL15R α in the hippocampus and housekeeping gene β -actin in both regions (Fig. 4a–d).

Circadian Rhythm of Body Temperature in the KO and Wild-Type Mice and the Changes After LPS Challenge

The average body temperature of all mice within the same group is presented as Fig. 1. Despite a large variation on day 1 and loss of data points because of a drop of temperature below the detection range after LPS challenge, the results clearly show a difference induced by LPS. In the wild-type mice, there was a distinctive circadian pattern of temperature oscillation, with a nadir in the light span and peak in the dark span. LPS disrupted the 24-h rhythm, most apparent on day 1. There were greater changes in KO mice (Fig. 5b) than wild-type mice (Fig. 5a).

By cosinor analysis, we determined the least squares spectra of the combined 2-day records (Fig. 6a), as well as days 1 and 2 considered separately (not shown). The 24-h curves were reconstructed (Fig. 6b), and amplitudes across all frequencies were compared (Fig. 6c). With the exception of one wild-type mouse after LPS treatment when the 2-day record was analyzed, all mice showed a statistically significant circadian rhythm for temperature. On a group basis, both wild-type and KO mice showed a strong 24-h temperature oscillation before LPS treatment ($p < 0.005$ from zero-amplitude test). The KO mice had a higher MESOR ($p = 0.008$) and a tendency toward a later acrophase (-16°) by comparison with the wild-type (-355° ; $p = 0.062$). After LPS challenge, the circadian rhythm was no longer detectable in both groups on day 1, and to a lesser extent, on day 2. On day 2, there was no difference in circadian characteristics of the wild-type mice after vs. before LPS treatment. However, KO mice still showed a decreased MESOR ($p = 0.026$), a reduced circadian amplitude ($p = 0.022$), and an advanced circadian acrophase ($p = 0.024$).

LPS treatment of the wild-type mice was associated with a reduction in the harmonic content of the temperature spectra (Fig. 6a). As seen in Fig. 6c (top left), most amplitude differences after vs. before treatment are negative. When the 2-day records were analyzed, amplitudes across all frequencies tended to be reduced (paired $t = 1.987$, $p = 0.053$). This result stems primarily from day 2 (paired $t = 6.198$, $p < 0.001$); on day 1, the circadian amplitude of temperature tended to be larger ($p = 0.083$), probably in view of the large but transient drop in temperature shortly after the treatment was administered. Results from day 2 suggest that restoration of the time structure of temperature after treatment occurred at all frequencies, and that ultradians adjust faster than the circadian component and its first few harmonic terms ($r = 0.635$, $p < 0.001$).

LPS treatment in the KO mice was also associated with an overall reduction in temperature amplitudes across all frequencies on day 2, although less than in the control mice (paired $t = 2.728$, $p = 0.012$). In particular, the 24-h amplitude of temperature on day 2 did not rebound to its value before LPS treatment ($p = 0.009$). On day 1, the 12-h (rather than the 24-h) amplitude tended to be larger ($p = 0.063$), probably related to the large but transient drop in temperature shortly after treatment. As observed for the wild-type mice, the restoration of the time structure of temperature for the KO mice after treatment on day 2 involved all frequencies, ultradian adjusting faster than the circadian component and its first few harmonic terms ($r = 0.475$, $p = 0.019$; Fig. 6c, bottom left). Although there was no major difference in the overall time structure of temperature between the KO and wild-type mice during the control period, KO mice had larger amplitudes than the wild-type after LPS treatment (paired $t = 3.208$, $p = 0.002$ from 2-day analyses).

On a population basis, a circadian rhythm was detected with statistical significance for temperature only before treatment. Before treatment, KO mice had a higher mean

temperature (MESOR) as compared with control mice ($p < 0.02$). They also showed a tendency toward a delayed circadian acrophase. In the wild-type mice, LPS treatment was associated with a change in the amplitude–acrophase pair, the amplitude being decreased and the acrophase delayed (Table 2).

Circadian Rhythm of Activity Changes After LPS Challenge

As seen in plots of average activity levels of the original data (Fig. 7a, b), there were differences in the circadian rhythm between the wild-type and KO mice and a greater suppression of rhythmicity in the KO mice than in the wildtype mice. By cosinor analysis, we determined the least squares spectra of the combined 2-day records (Fig. 8a), as well as separate analyses of days 1 and 2 (not shown). We also reconstructed the 24-h curves (Fig. 8b) and compared amplitudes across all frequencies (Fig. 8c). Overall, LPS treatment was associated with a decrease in 24-h amplitude of activity, statistically significant in the analyses of 2-day records ($p = 0.007$) and on day 2 ($p = 0.001$), but not on day 1. The wild-type control mice maintained a circadian rhythm of activity even after LPS treatment. By contrast, the KO mice showed greater circadian disruption after LPS treatment on days 1 and 2 and the combined 2 days.

All mice in both groups showed a significant circadian rhythm for activity before LPS treatment. Before LPS treatment, the KO mice showed a smaller 12-h amplitude than the wild-type. The difference was statistically significant in analyses of 2-day records ($p = 0.001$) and in separate analyses of data on day 1 ($p = 0.008$) and on day 2 ($p = 0.005$; Fig. 8c, top right). After LPS treatment, only three wild-type mice (out of six) and one KO mouse (out of six) maintained a statistically significant circadian rhythm when the 2-day records were analyzed. Then, the KO mice no longer differed in their time structure from wild-type mice. Decreased amplitudes across all frequencies were detected on day 2 after treatment for wild-type mice (paired $t = 2.297$, $p = 0.031$). In these mice, a faster adjustment was observed for the higher than for the lower frequencies (day 1— $r = 0.575$, $p = 0.003$; day 2— $r = 0.518$, $p = 0.009$; Fig. 8c, upper left).

On a population basis, a statistically significant circadian rhythm in activity was present in both wild-type and KO mice before treatment. The KO mice had a later circadian acrophase than the control mice before treatment ($p = 0.05$). After LPS treatment, a circadian rhythm was found for the control mice when the 2-day records were considered, but not when data from days 1 and 2 were analyzed separately. A small decrease in activity (MESOR) was only observed on day 1 for wildtype mice ($p = 0.049$). It was more pronounced in KO mice, seen on both days (day 1— $p = 0.001$; day 2— $p = 0.004$) and overall ($p = 0.002$). In these mice, the decrease in MESOR was accompanied by a sharp decrease in circadian amplitude, also observed on both days (day 1— $p = 0.012$; day 2— $p < 0.001$) and overall ($p = 0.002$), and by a delayed acrophase detected on day 1 ($p = 0.024$) but not on day 2 (Table 2).

Discussion

We have shown regulatory changes of the IL15 system at the BBB level (Pan et al. 2009; Wu et al. 2010e), and that IL15 permeation across the BBB can be upregulated by LPS (Pan et al. 2009; Wu et al. 2010e). In this study, qPCR showed that the level of expression of IL15 receptors was highest in enriched cortical microvessels, the main component of the BBB. The basal differences of mRNA in the 4- and 48-h studies differ from each other, probably caused by different tissue preparations and slight variations in amplification efficiency of the genes in different assays. Since different CNS regions also showed upregulation of IL15R α , activation of IL15 signaling in CNS parenchyma may be the major mediator of altered thermoregulation and activity. Indeed, IL15-induced c-Fos activation in the medial preoptic area and increased the mRNA for the transient receptor potential

vanilloid-4 cation channel in the hypothalamus of the KO mice (He et al. 2010b). Although this indicates that IL15 signaling participates in thermoregulation, the slight reduction of locomotor activity appears to be different from that found in the open field test (Wu et al. 2010b) and metabolic chamber (He et al. 2010b). It is possible that the stress from anesthesia and telemetry probe placement 7 days before the recording had a persistent effect on the KO mice. The circadian response to LPS was greater in the KO mice than in the wild-type mice. Thus, the deteriorating control of biopotentials and disrupted circadian rhythm in the IL15R α KO mice supports a protective role of IL15R α against the deleterious results of neuroinflammation.

The upregulation of IL15 receptor mRNA appears to occur in all CNS regions tested. While the spinal cord is the pathway for sensorimotor integration, the selected brain areas represent different behavioral outcomes: the hippocampus for memory retention and consolidation, the striatum for extrapyramidal motor function, the hypothalamus for neuroendocrine control, and the cerebellum for coordination of movement. The generalized increase is consistent with the involvement of the BBB in increasing IL15 permeation after LPS challenge (Pan et al. 2009). By sampling the CNS regions and cerebral vasculature at 4 and 48 h after LPS challenge, we were able to determine the relative contributions from cerebral endothelial cells that interface the blood and CNS parenchyma. The higher upregulation of the co-receptor IL2R β and even greater increase of IL2R γ than IL15R α may reflect the summation of the signals conferred by many IL2R γ -sharing cytokines that are activated by LPS.

Despite the robust increase of IL15 receptor mRNAs, the increase in IL15R α protein expression did not reach statistical significance in the hypothalamus ($p=0.057$) and clearly not in the hippocampus at 48 h. This is probably related to the sensitivity of detection and discrepancy of onset of translational and transcriptional regulation.

Biological rhythms are essential for survival of an organism. Body temperature has a distinct 24-h rhythm, with a nadir corresponding to sleep. The circadian rhythm of body temperature is affected by locomotor activity as well as many other factors. IL15R α KO mice not only had different circadian characteristics of body temperature and locomotor activity from the wild-type mice under control conditions, but also showed an exaggerated response to the endotoxin LPS with greater disruption of 24-h rhythms. These results from circadian analyses suggest that IL15R α signaling may be beneficial in response to neuroinflammation by helping to maintain homeostasis of thermoregulation and activity.

In summary, LPS induced a robust and time-dependent upregulation of IL15R α , IL2R β , and IL2R γ in BBB microvessels and to a lesser extent in CNS parenchyma. IL15R α KO mice showed an exaggerated response to even a small dose of LPS, with greater reduction of body temperature and locomotor activity and disruption of the circadian rhythm.

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Fig. 1. Increased mRNA expression of the cognate IL15R α receptor for IL15 in most CNS regions and enriched cerebral microvessels in response to LPS treatment at **a** 4 and **b** 48 h. $n=5$ /group. * $p<0.05$; ** $p<0.01$; *** $p<0.005$; + $p<0.1$

Fig. 2.

Increased mRNA expression of the co-receptor IL2R β for IL15 in most CNS regions and enriched cerebral microvessels in response to LPS treatment at **a** 4 and **b** 48 h. $n=5$ /group.

* $p<0.05$; *** $p<0.005$; + $p<0.1$

Fig. 3. Increased mRNA expression of the co-receptor IL2R γ for IL15 in different CNS regions and enriched cerebral microvessels in response to LPS treatment at **a** 4 and **b** 48 h. $n=5$ /group. ** $p<0.01$; *** $p<0.005$

Fig. 4. IL15Rα protein expression in mice 48 h after LPS or PBS treatment did not reflect the increased mRNA. **a, c** Hypothalamus with densitometric analysis ($+p=0.057$). **b, d** Hippocampus with densitometric analysis. $n=5$ /group (one control hypothalamic sample was lost)

Fig. 5.

Plots of 2-day average temperature of (a) wild-type and (b) IL15R α KO mice *before* and *after* LPS challenge (0.8 mg/kg, $n=4$ /group). Wildtype mice showed a distinctive circadian pattern of temperature oscillation, with a nadir in the light span and peak in the dark span. LPS treatment disrupted the 24-h rhythm, most apparent on day 1. There were greater changes in KO mice (b) than in wild-type mice (a)

Fig. 6.

Cosinor analysis of circadian rhythm of temperature in wildtype and IL15Ra KO mice *before* and *after* LPS treatment. **a** Least squares spectra of 2-day records. **b** Average curves shown with fit of 24-h cosine curve. **c** Comparison of amplitudes across all frequencies. The strong circadian rhythm of temperature before treatment was disrupted by LPS on day 2 in the KO mice. Because of large individual variations, changes on day 1 were not statistically significant.

Fig. 7.

Plots of 2-day average activity in (a) wild-type and (b) IL15R α KO mice *before* and *after* LPS treatment (0.8 mg/kg, $n=6$ /group). **a** In the wild-type mice, LPS reduced activity level mainly on day 1. The changes were more apparent in the active dark span than the light span. **b** In the KO mice, the circadian rhythm was blunted and persisted until the end of day 2. The inactivity in the KO mice indicates a greater effect of LPS on these mice than on the wild-type controls

Fig. 8.

Cosinor analysis of circadian rhythm of activity comparison in IL15R α KO and wild-type control mice *before* and *after* LPS treatment. **a** Least squares spectra of 2-day records. **b** Average curves shown with fit of 24-h cosine curve. **c** Comparison of amplitudes across all frequencies. The strong circadian rhythm of activity before treatment was disrupted by LPS on both days in the KO mice

Table 1

Taqman primers and fluorescent probes for real-time PCR

Gene	Primer and probe sequences
IL15R α	Forward: 5'-CCAGGCCATTCCTGTGTTG Reverse: 5'-GATGTCAGCATGCTCAATAGATACG Probe: 5'-6-FAM-CCGGGCACCACGTGTCCACC-TAMRA
IL2R β	Forward: 5'-CGACTTCCATCCCTTTGACAA Reverse: 5'-TGGGTATCAATGTGCAGAACTTG Probe: 5'-6-FAM-CTTCGCCTGGTGGCCCTCATT-TAMRA
IL2R γ	Forward: 5'-GAAGCTGGACGGAACTAATAGTGAA Reverse: 5'-CTCCGAACCCGAAATGTGTAC Probe: 5'-6-FAM-GAACCTAGATTCTCCCTGCCTAGTGTGGA-TAMRA
GAPDH	Forward: 5'-TGTGTCCGTCGTGGATCTGA Reverse: 5'-CCTGCTTACCACCTTCTTGA Probe: 5'-6-FAM-CCGCCTGGAGAAACCTGCCAAGTATG-TAMRA

Table 2

Comparison of circadian rhythm parameters of temperature and activity of wild-type (WT) and KO mice before and after LPS treatment^a

		Temperature			Activity		
		Rx	M	A	Φ	$P(H_0:A=0)$	$P(H_0:A=0)$
2-day record							
WT							
1	Before	36.64	0.674	-355	0.005	1.485	0.315
2	After	36.52	0.317	-11	0.446	1.199	0.169
KO							
3	Before	36.95	0.804	-16	0.002	1.570	0.312
4	After	36.48	0.175	-242	0.178	1.005	0.085
Comparison							
2 vs. 1						0.081	
4 vs. 3			0.060			0.006	0.002
3 vs. 1		0.008		0.062		0.076	0.005
4 vs. 2						0.063	0.075
Day 1							
WT							
1	Before	36.63	0.556	-356	0.004	1.504	0.260
2	After	36.37	0.210	-102	0.217	1.065	0.139
KO							
3	Before	36.88	0.832	-14	0.024	1.573	0.313
4	After	36.30	0.544	-195	0.229	0.904	0.154
Comparison							
2 vs. 1						0.049	
4 vs. 3			0.064			0.005	0.001
3 vs. 1		0.018				0.092	0.002
4 vs. 2						0.034	0.077
Day 2							
WT							
1	Before	36.64	0.790	-354	0.012	1.467	0.369
							0.010

\$watermark-text

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Temperature				Activity						
Rx	M	A	ϕ	$P(H_0:A=0)$	(A, ϕ)	M	A	ϕ	$P(H_0:A=0)$	(A, ϕ)
2	After	36.68	0.673	-353	0.108	1.334	0.198	-353	0.150	
KO										
3	Before	37.01	0.778	-18	0.008	1.566	0.318	-8	<0.001	
4	After	36.66	0.403	-334	0.116	1.106	0.051	-89	0.466	
Comparison										
2 vs. 1										
4 vs. 3		0.026	0.022	0.024	0.004	0.004	0.001			0.002
3 vs. 1		0.008		0.051	0.099			0.018		0.073
4 vs. 2										

M midline estimating statistic of rhythm (MESOR, a rhythm-adjusted mean), A 24-h amplitude, half the extent of predictable change within 24 h, ϕ 24-h acrophase, a measure of the timing of overall high values recurring each day

^a P values for test of equality of parameters considered singly (M, A, ϕ) and jointly ((A, ϕ) pairs). P values from test of $H_0:A=0$ correspond to detection of circadian rhythm on a population basis (similarity of 24-h (A, ϕ) pairs across animals in WT or KO group before or after LPS treatment)