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CCL2 mediates the circadian response to low dose endotoxin

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ABSTRACT

The mammalian circadian system is mainly originated in a master oscillator located in the suprachiasmatic nuclei (SCN) in the hypothalamus. Previous reports from our and other groups have shown that the SCN are sensitive to systemic immune activation during the early night, through a mechanism that relies on the action of proinflammatory factors within this structure. Chemokine (C-C motif) ligand 2 (CCL2) is induced in the brain upon peripheral immune activation, and it has been shown to modulate neuronal physiology. In the present work we tested whether CCL2 might be involved in the response of the circadian clock to peripheral endotoxin administration. The CCL2 receptor, C-C chemokine receptor type 2 (CCR2), was detected in the SCN of mice, with higher levels of expression during the early night, when the clock is sensitive to immune activation. Ccl2 was induced in the SCN upon intraperitoneal lipopolysaccharide (LPS) administration. Furthermore, mice receiving an intracerebroventricular (Icv) administration of a CCL2 synthesis inhibitor (Bindarit), showed a reduction LPS-induced circadian phase changes and Icv delivery of CCL2 led to phase delays in the circadian clock. In addition, we tested the possibility that CCL2 might also be involved in the photic regulation of the clock. Icv administration of Bindarit did not modify the effects of light pulses on the circadian clock. In summary, we found that CCL2, acting at the SCN level is important for the circadian effects of immune activation.

1. Introduction

Daily environmental changes have imposed a selective pressure for life on Earth, driving the development of a circadian clock mechanism. This temporal system generates and entrains 24-h rhythms in physiological and behavioral variables (e.g. body temperature, hormonal secretion, sleep, locomotor activity, etc.). In mammals, the master circadian clock resides in the hypothalamic suprachiasmatic nuclei (SCN), and the main signal that adjusts its activity is the light-dark (LD) cycle (Golombek and Rosenstein, 2010). Moreover, circadian oscillations ultimately rely in a molecular mechanism, with two interlocked loops of positive and negative elements that regulate their transcription through conserved regulatory regions, including E/E′-boxes and Rev-erb responsive elements (ROREs) (Lowrey and Takahashi, 2011).

Although there is substantial information regarding the circadian modulation of many immunological processes (reviewed in: Cermakian et al., 2013), little is known about the possible effect of immune factors on the circadian system itself. Previous work form our lab has shown that peripheral immune activation, achieved by intraperitoneal low doses of bacterial lipopolysaccharide (LPS), induces changes in the phase of the circadian clock, only if delivered in the early night (Marpegan et al., 2005). The presence of cytokine receptors in the SCN has been described for IL-1 receptor (IL-1R) and IFN-γ receptor (Beynon and Coogan, 2010; Lundkvist et al., 1998), and we have previously shown that the proinflammatory cytokine tumor necrosis factor (TNF)-α, acting in the SCN, has an important role in the circadian response to LPS (Leone et al., 2012). While analyzing several proinflammatory factors that were induced in the SCN by peripheral LPS, we found a strong induction of chemokine (C-C motif) ligand 2 (CCL2), a molecule also known as MCP-1, which leads to recruitment of immune cells and

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promotes proinflammatory signaling (Paladino et al., 2014; Yadav et al., 2010). This finding, together with previous works showing a modulation of neuronal excitability, synaptic transmission and behavior by this chemokine (Guyon et al., 2009; Zhou et al., 2011), suggested that CCL2 could be involved in the effects of immune activation on the behavioral outputs of the SCN.

There are reports showing a strong circadian control on CCL2 expression in tissues and circulating immune cells (Hayashi et al., 2007; Nguyen et al., 2013; Rahman et al., 2015; Scheiermann et al., 2012), however, the possibility that this cytokine could also modulate the circadian clock remains to be explored. In this study we characterized the presence of CCL2 receptor, C-C chemokine receptor type 2 (CCR2), in the SCN of mice, and analyzed the effects on the circadian clock of both administration of CCL2 within the SCN region, and inhibition of CCL2 production upon peripheral LPS administration. Our findings demonstrate an important participation of CCL2 in the pathway that leads from immune-related signaling to changes in the circadian clock.

2. Methods

2.1. Animals

Animal manipulations and experimental protocols performed in this work were supervised and approved by the National University of Quilmes Institutional Animal Care and Use Committee, in accordance with the National Institutes of Health guide for the care and use of laboratory animals.

2.1.1. Housing conditions

Adult (2–5 months, 25–30 gr) male C57-BL/6J mice were kept in our colony at the National University of Quilmes with *ad libitum* access to food and water. For general maintenance, animals were kept under 12 h:12 h light:dark (LD) conditions, and for this lighting condition reference time was defined by the time of lights-off as ZT 12. Manipulations during the lights-off period were performed under dim red light (<1 lux).

For the analysis of phase shifts in locomotor activity rhythms, mice were kept in individual cages equipped with a running wheel, and revolutions were recorded in 5-min bins. After recovery from surgery (see *2.2.2*) animals were transferred to constant darkness (DD). Treatments were applied to animals kept for at least 10 days in DD, and reference time was defined by the onset of locomotor activity as CT (circadian time) 12. Subjective night and day are defined as the period between CT 12 and 0, and CT 0 and 12, respectively.

2.1.2. Surgery and intracerebroventricular administrations

For cannulae implantation, animals were anesthetized with a ketamine/xylazine cocktail (70/10 mg/kg, respectively), and situated in a stereotactic frame (Stoelting Co. Wood Dale, IL, USA). 26-ga cannulae (Stoelting Co.) were implanted at the bottom of the third ventricle, just above the SCN (coordinates from bregma: −0.5 mm antero-posterior, −5.0 mm dorso-ventral and 0.0 mm from midline). Cannulae were fixed to the skull with two screws (Stoelting Co.) and dental cement. Mice were allowed to recover from surgery for two days in LD conditions before being transferred to experimental conditions.

For intracerebroventricular (Icv) administration, mice implanted with guided cannula, kept in DD conditions, received a total volume of 1 μl of the correspondent solution at 0.2 μl/min by means of a 33-gallon injector (Stoelting Co.) and a Hamilton microsyringe. When the same animal received more than one Icv treatment, each of them were administered at least 10 days apart from the other.

2.2. Bindarit

The CCL2 inhibitor Bindarit (2-methyl-2-[(1-[phenylmethyl]-1*H*-indazol-3yl)methoxy]propanoic acid) was synthesized by and obtained from Angelini (Angelini Research Center-ACRAF, Italy). The drug was dissolved to a 100 mM stock in 1 M NaOH, filtered through 0.22 μm-pore membrane, diluted to working concentrations (100 μM) in sterile PBS and kept at −20 °C until use. As a vehicle, a 1:1000 dilution of 1 M NaOH in sterile PBS was used.

2.3. Analysis of Ccr2 and Ccl2 expression in the SCN

Animals were kept in LD conditions throughout this set of experiments. For immunofluorescence analysis of CCR2 expression in the SCN, mice were sacrificed at ZT 16, and processed as described in 2.6. For the analysis of daily variations in Ccr2 and Ccl2 mRNA levels, animals (N = 4 for each time point) were sacrificed by cervical dislocation at ZT 3, 7, 11, 15 or 19 and processed according to *2.5*. To analyze time-of-day differences in CCR2 expression and induction in the SCN, animals (N = 4 for each condition) received either saline solution (vehicle) or 100 μg/kg LPS (*Escherichia coli*, serotype 0111:B4, Sigma-Aldrich, St Louis, MO, USA) intraperitoneally (Ip) at ZT 2 or 14 and 2 h later (ZT 4 and 16, accordingly) animals were sacrificed and processed as described in *2.6*. Finally, to analyze time-of-day differences in Ccl2 induction, animals (N = 4 for each condition) received either saline or 100 μg/kg LPS Ip at ZT 2 or ZT 14, and were sacrificed by cervical dislocation 60 min later (ZT 3 and 15, accordingly) and processed as described in *2.5*.

2.4. Effects of Bindarit on LPS − and light-induced phase shifts

For concomitant LPS and Bindarit treatments, animals equipped with a guided cannula were kept in DD conditions and treatments were done at least 15 days after surgery. Each mouse received 1 μl of a 100 μM Bindarit solution or Vehicle at CT 14.5, 30 min before Ip LPS or vehicle (Saline solution) administration.

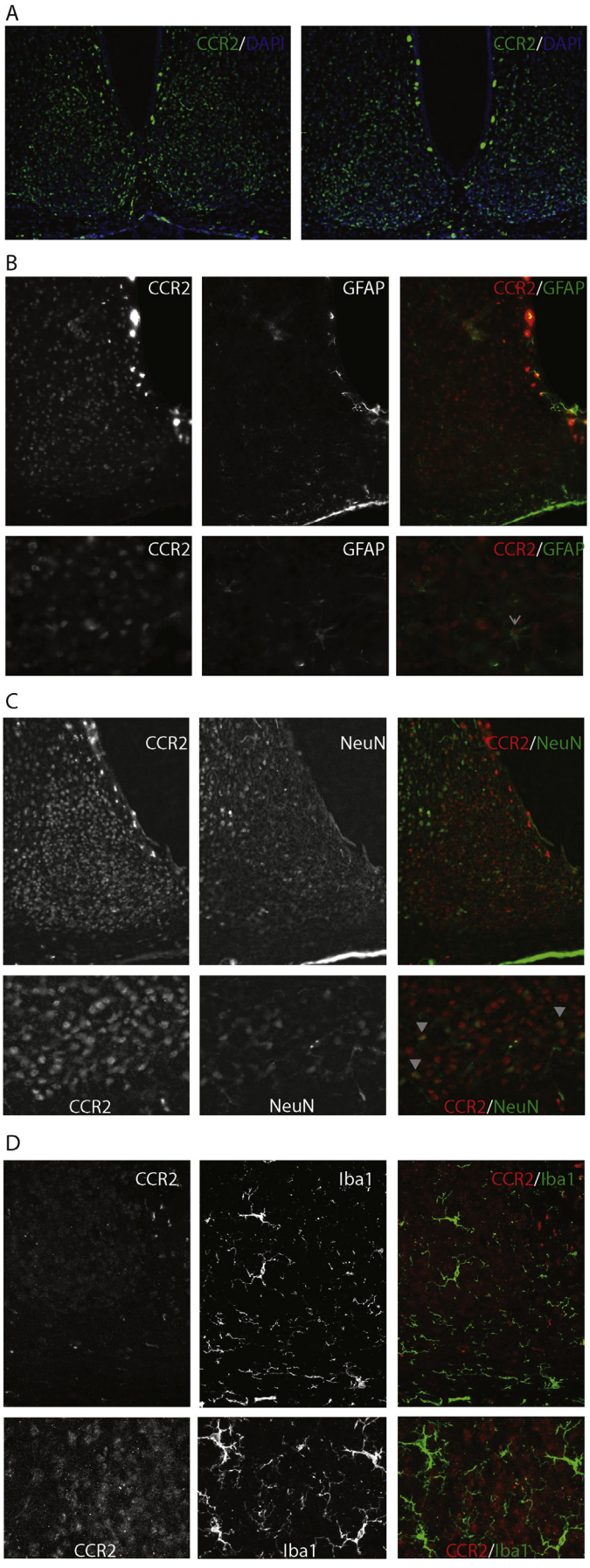
For Bindarit and light-pulse experiments, animals were kept in DD and received 1 μl of a 100 μM Bindarit solution or Vehicle at CT 14.5, 30 min before a 10-min white light pulse (200 lux). As a control of light pulses, animals were manipulated in the same way, but were not exposed to light.

2.5. CCL2 Icv administration

Recombinant murine CCL2 (ImmunoTools GmbH, Friesoythe, Germany) was diluted in Saline solution to a 0.1 μg/μl final concentration. Animals equipped with a guided cannula (see 2.1.2) were kept in DD conditions and treatments were performed at least 15 days after surgery. On the day of treatment, each animal received a total of 100 ng of Icv-delivered CCL2, or Saline solution as a control, at CT 15.

2.6. mRNA extraction and real time PCR

SCN tissue was collected in 100 μl of Trizol reagent (Life Technologies, Carlsbad, CA, USA) and total RNA was extracted following manufacturer's instructions. Total cDNA was synthetized from SuperScript™ First-Strand Synthesis System (Life Technologies), using polyT primers. Real Time PCR was performed in a Step One Plus (Life Technologies) device using Power SYBR Green PCR Master Mix (Life Technologies). Protocols were performed following manufacturer's instructions. Primer efficiency was calculated by a curve with different concentrations of cDNA pooled from all samples, and products sizes were checked by agarose gel electrophoresis. The following primers were used: Ccl2 Fw: 5′-



CGGCTGGAGCATCCACGTGTT-3′, Ccl2 Rv: 5′-TGGGGTCAGCACAGACCTCTC-3′, Ccr2 Fw: 5′- AAGGAGCCATACCTGTAAATGCC-3′, Ccr2 Rv: 5′- AGTATGCCGTGGATGAACTGAG -3′, Hprt Fw: 5′- TGTTGGATACAGGCCAGAC-3′, Hprt Rv: 5′-TGGCAACATCAACAGGACTC-3′, Gapdh Fw: 5′- TGCACCACCAACTGCTTAG-3′, Gapdh Rv: 5′-GGATGCAGGGATGATGTTC-3′.

Relative mRNA levels were calculated as described in (Pfaffl, 2001), using the geometric mean between the levels of Gapdh and Hprt to normalize target gene expression (Vandesompele et al., 2002).

2.7. Immunostaining

Mice were deeply anesthetized with a mixture containing ketamine (150 mg/kg) and xylazine (10 mg/kg) and perfused intracardially with 4% paraformaldehyde. Brains were carefully removed, postfixed overnight, and cryoprotected in 30% sucrose in 0.01 M PBS for 24 h and 30-μm–thick coronal sections were cut with a freezing microtome. CCR2 immunostaining was achieved as described in (Foresti et al., 2009). Briefly, sections were washed with PBS, incubated with 0.5% H_2O_2 diluted in PBS for 30 min and with 1% H_2O_2 for 60 min. Then, tissue was washed with PBS and nonspecific binding sites were blocked with 3% horse serum and 0.05% Tween 20 in PBS for 60 min and incubated with primary antibody against CCR2 (raised in rabbit, Abcam, Cambridge, UK) or with anti-CCR2 combined either with anti-GFAP (raised in mouse, Sigma-Aldrich), anti NeuN (raised in mouse, a gift from Alejandro Schinder) or anti Iba1 (raised in goat, Abcam) antibody, diluted in blocking solution (1:200), in continuous agitation at 4 °C for 48 h. Then tissue was then washed with PBS and processed either for immunofluorescence or immunohistochemistry. For immunofluorescence, sections were incubated with anti-rabbit secondary antibody conjugated with FITC (1:200, Vector Labs, Burlingame, CA, USA) alone, or with a combination of Alexa-Fluor 594 conjugated anti-rabbit (1:500, Jackson ImmunoResearch, West Grove, PA, USA) and either Alexa-Fluor 488 conjugated anti-mouse (1:500, Jackson ImmunoResearch) or Alexa – Fluor 488 conjugated anti-goat (1:500, Abcam) antibodies, for 90 min, and then counterstained with DAPI (Vectashield, Vector Labs). For immunohistochemistry, sections were treated using the avidin–biotin method with a Vectastain Elite Universal kit containing a biotinylated universal secondary Ab, avidin, and biotinylated HRP (Vector Labs) and vector-VIP peroxidase substrate (SK-4600). Image analysis and cell counting were performed using ImageJ software.

2.8. Data analysis and statistics

The effects of different treatments on locomotor activity rhythms were analyzed with the help of El Temps Software. For phase-shift calculations, activity onset was defined as phase-reference adjusted by 4 different observers (blind to experimental conditions), and differences between the phase before and after

Fig. 1. CCR2 is expressed in the master circadian oscillator. A) Expression of CCR2 (green) in cells of the SCN. Cell nuclei are counterstained in blue. **B)** Double immunostaining for CCR2 and GFAP in brain slices obtained at ZT 16. Magnification: 100× in top line; 400× in bottom line. The arrow in the last column of the bottom line highlights the close proximity of a GFAP and a CCR2 positive cells. **C)** Double immunostaining for CCR2 and NeuN in brain slices obtained at ZT 16. Magnification: 100× in top line; 400× in bottom line. The arrows in the last column of the bottom line highlights cells with colocalization between CCR2 and NeuN. **D)** Double immunostaining for CCR2 and Iba1 in brain slices obtained at ZT 16. Magnification: 100× in top line; 400× in bottom line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

treatment were computed. The average value between all the observers for each phase shift was used for further statistical analysis. The effect of Icv CCL2 delivery on circadian phase was analyzed by a Student's *t*-test.

Effect of treatments on the phase of the circadian rhythm, in the number of CCR2-positive cells and in CCL2 mRNA levels in the SCN upon LPS administration were analyzed by two-way ANOVA followed by Newman-Keuls multiple comparison test.

Differences in the mRNA level of Ccr2 and Ccl2 in the SCN were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test.

3. Results

3.1. CCR2 expression in the SCN

CCR2 and CCL2 expression has been described in several regions of the brain, including supraoptic nuclei, dentate gyrus of the hippocampus and cortex for CCR2 and olfactory bulb and cerebellum for CCL2 (Rostene et al., 2007). If CCL2 is involved in the response of the central clock to immune stimuli, then its receptor should be expressed in the SCN. Immunostaining against CCR2, showed that the receptor is present in the mouse SCN (Fig. 1A).

In our first attempt to characterize the cell type expressing CCR2, we analyzed whether CCR2 signal colocalized with GFAP, a specific astrocytic marker. We found no colocalization between this two molecules in the mouse SCN (Fig. 1**B**), suggesting that CCL2 receptor is not expressed in SCN astrocytes. However, we found, in several slices, astrocytes wrapping CCR2-positive cells (Fig. 2**B**, higher magnification), suggesting that CCL2 coming from SCN astrocytes could directly affect CCR2-positive cells. In addition, we found no evidence of CCR2 expressed in SCN microglia, since there was no co-localization with its specific marker Iba1 (Fig. 1**D**), suggesting that CCR2 might be expressed in neuronal cells. In order to describe whether CCR2 is expressed in neurons in the SCN we performed double immunostaining for CCR2 and NeuN. Although NeuN is sparsely expressed in the SCN (Saaltink et al., 2012), we found co-localization between both markers, suggesting that the SCN has CCR2-positive neurons (Fig. 1**C**).

3.2. Daily variation in Ccl2 and Ccr2 expression in mouse SCN

We also determined whether Ccr2 and Ccl2 expression levels varied throughout the day in the SCN. First, we analyzed the mRNA of both Ccr2 and Ccl2 in this tissue, and found a daily variation in the expression of these molecules (Fig. 2). Both molecules showed an increased level of expression during the night, with maximum values at ZT 15 for Ccr2 and at ZT 19 for Ccl2.

Next, we analyzed if the variation observed for Ccr2 at mRNA level was conserved at the protein level. Samples were obtained at ZT 4 and 16, one hour after the trough and peak found for mRNA expression, respectively. We found significant differences between the number of CCR2-positive cells at ZT 4 and ZT 16 in the SCN region (Fig. 3). Furthermore, immune activation by peripheral LPS increased the number of CCR2-positive cells at ZT 4, but not at ZT 16. In addition, bioinformatics analysis of Ccr2 gene promoter region revealed the presence of regulatory elements associated with circadian gene expression control, such as E-box, non-canonical E-box, D-box and RORE (Supplementary Table 1), which could be responsible for the daily oscillations of Ccr2.

3.3. Role of CCL2 in the circadian response to LPS

Since CCL2 induction in the central nervous system upon peripheral LPS administration has been previously documented (Cazareth et al., 2014; Erickson and Banks, 2011), and CCR2 is present in the master circadian clock, we asked whether CCL2 might be involved in the mechanism that leads to phase delays upon peripheral immune activation. We found that Ccl2 mRNA levels are increased upon peripheral LPS administration, suggesting that this molecule might be involved in the circadian response to endotoxin. Noteworthy, Ccl2 induction in the SCN occurred both during the early night as well as during the early day (Fig. 4**A**).

In addition, Icv administration of CCL2, directed to the SCN region, at CT 15, induced phase delays in locomotor activity rhythms of a similar magnitude of the previously reported phase shifts induced by peripheral LPS (Fig. 5). CT 15 was chosen as the time of administration since the circadian clock has been shown to be sensitive to immune stimuli preferentially at this time point (Marpegan et al., 2005), and because CCR2 expression is higher during the early night (Figs. 3 and 4). To confirm the role of CCL2 on endotoxin-induced phase delays, we analyzed the effects of CCL2 synthesis inhibitor, Bindarit, on the phase shifts produced by 100 μg/kg Ip LPS administration at CT 15, which correspond to the early subjective night in constant darkness. We found that Icv administration of Bindarit 30 min prior to an Ip LPS injection significantly reduced the magnitude of the phase delays caused by the endotoxin (Fig. 4**B** and **C**). Finally, to further characterize the role of CCL2 on the master circadian clock, we tested whether this molecule is involved in the light-induced phase shifts, which leads to photic synchronization of the clock. To test this, we delivered Bindarit icv, 30 min prior to a 200 lux light pulse at CT 15. Light

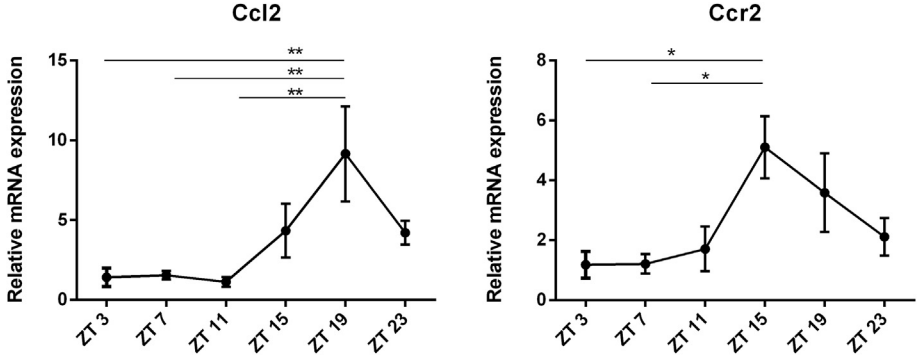


Fig. 2. Daily rhythms in Ccl2 and Ccr2 mRNA expression in the SCN. Relative mRNA levels of Ccl2 (left), and Ccr2 (right) in SCN tissue sampled at different times of the LD cycle. For Ccl2: One-way ANOVA ($F_{5, 16} = 5.118$; $p = 0.0054$), followed by Tukey's test; $p < 0.01$ for ZT 19 vs ZT 3, ZT 7 and ZT 11; $p > 0.05$ for other comparisons. For Ccr2: One-way ANOVA ($F_{5, 15} = 3.702$; $p = 0.0221$), followed by Tukey's test; $p < 0.05$ for ZT 15 vs ZT 3 and ZT 7; $p > 0.05$ for other comparisons. $N = 4$.

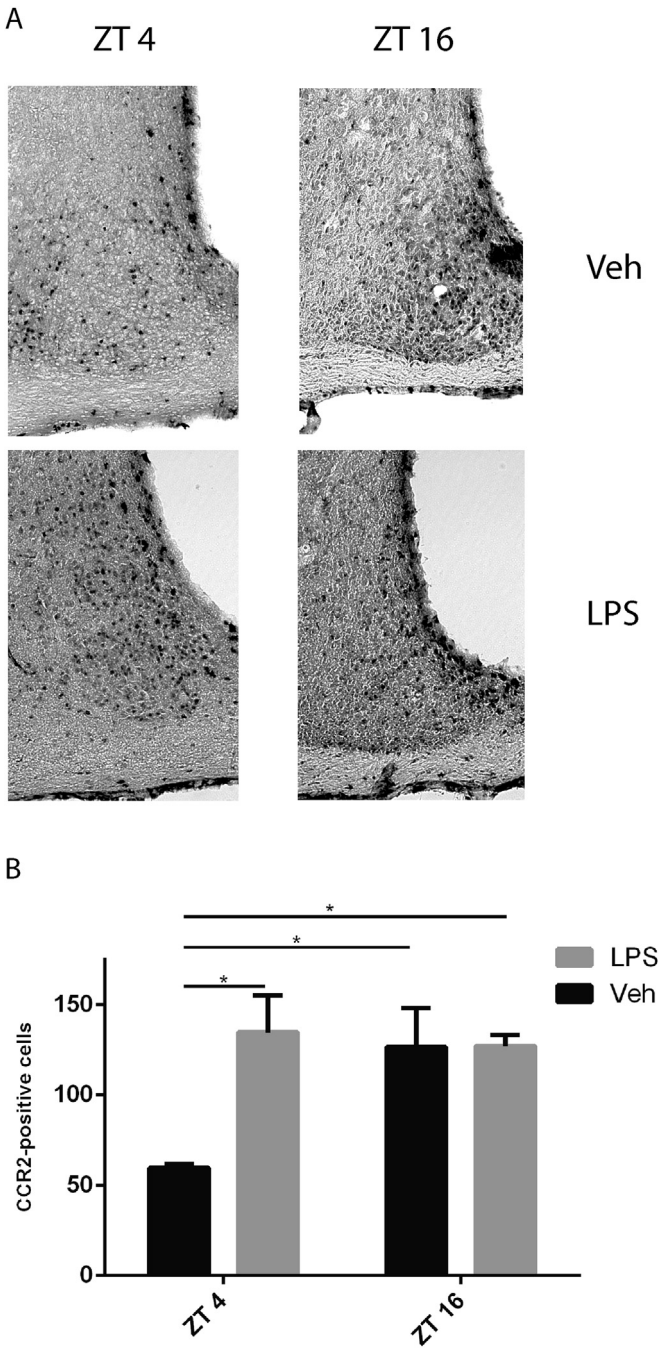


Fig. 3. Day–Night variation in the number of CCR2-positive cells in the SCN. A): Pictures of SCN slices from brains collected at ZT 4 or ZT 16, two hours after an Ip LPS or Vehicle administration, immunostained for CCR2. **B)** Quantification of CCR2-positive cell number on each condition. Two-way ANOVA (Interaction effect: $F_{1, 10} = 5.585$; $p = 0.0397$) followed by Newman-Keuls post hoc test; $p < 0.05$ for ZT 4 Veh vs. ZT 16 Veh, ZT 16 LPS and ZT 4 LPS; $p > 0.05$ for other comparisons. $N = 4$.

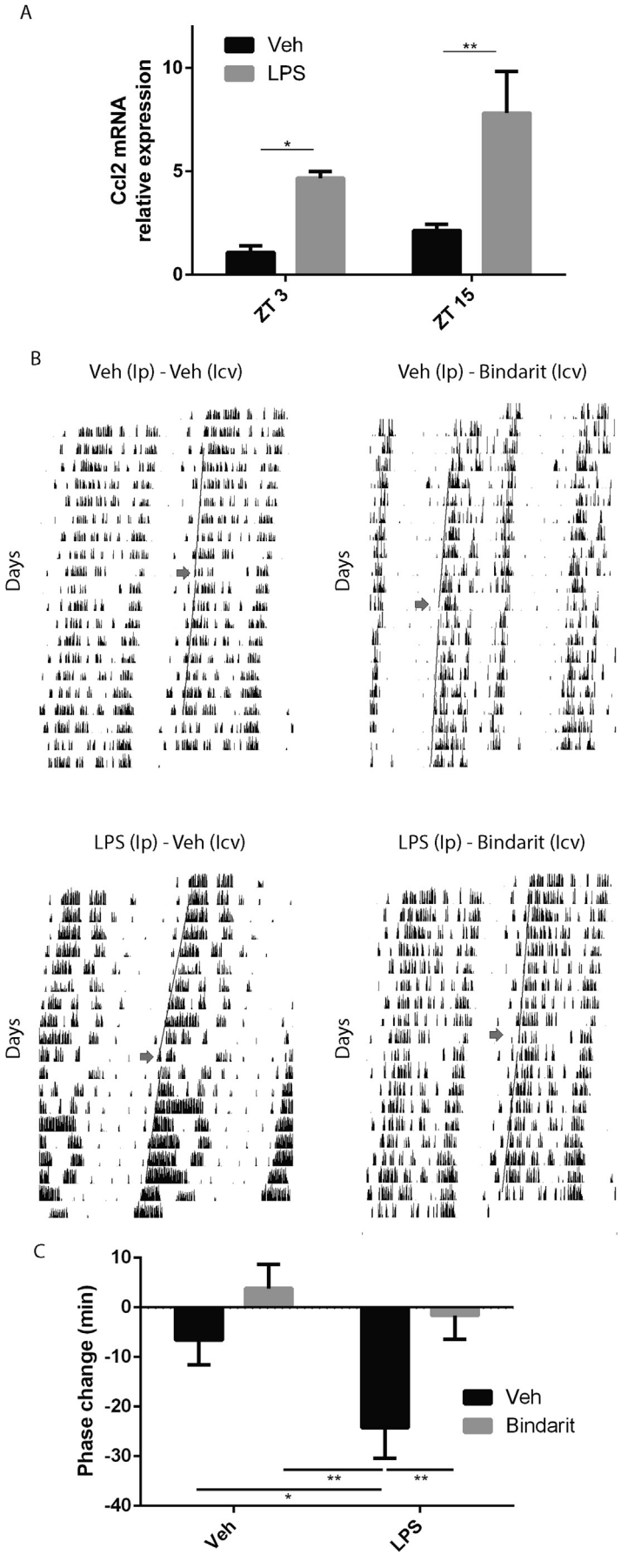
pulses given during the early subjective night, induce phase delay in the circadian clock. Pre-treatment with Bindarit failed to affect the light-induced phase shifts, suggesting that CCL2 is not involved in this phenomenon (Supplementary Fig. 1).

4. Discussion

The role of chemokines in the central nervous system has been under extensive research over the past years, and suggest an important role for these molecules on neurophysiology (reviewed in: Reaux-Le Goazigo et al., 2013). In particular, CCL2 has been shown to modulate neuronal excitability and synaptic transmission, as well as mediating neuroinflammation (Conductier et al., 2010; Guyon et al., 2009; Zhou et al., 2011); however, the possible role of this chemokine in the central circadian pacemaker has not been assessed. The circadian clock is sensitive to LPS administration during the early night (Anderson et al., 2013; Marpegan et al., 2005) and previous studies have shown that TNF-α acting at the SCN has an important role for these interaction between the immune and the circadian systems (Leone et al., 2012; Paladino et al., 2014). Our present results points towards CCL2 acting at the SCN as a necessary factor in the response of the circadian clock to peripheral immune stimuli. We found a diurnal variation in the expression of both CCL2 and CCR2 in the SCN, with higher levels during the night period. The expression of CCR2 peaked, both at the mRNA and protein levels, at the early night (ZT 12–16), matching the time window at which the circadian clock is sensitive to peripheral immune stimuli. Indeed, LPS administration during the early night failed to increase CCR2 levels, in contrast with the effect seen during the day with the endotoxin treatment. This suggests the possibility that maximum expression of this protein occurs in the early night and might contribute to the sensitivity of the clock to immune stimuli in that time period. We found that the promoter region of Ccr2 gene contains regulatory elements associated with circadian gene expression and similar findings have been reported for the Ccl2 gene (Nguyen et al., 2013; Sato et al., 2014), suggesting that these elements might be the responsible for the circadian variation in the expression of Ccr2 and Ccl2.

We have previously reported that, at the protein level, CCL2 is induced in the SCN upon Ip LPS injection, with no differences in the levels of induction between the early night and the early day (Paladino et al., 2014), and the same was found for Ccl2 mRNA in this work. In addition, we show that CCL2 administration to the SCN region can induce phase delays in locomotor activity rhythms demonstrating that the master circadian clock is sensitive to this chemokine, and that CCL2 synthesis inhibition blocks the phase shifts produced by endotoxin administration in the early night, suggesting an important role for CCL2 in the response of the SCN to peripheral LPS. Thus, CCL2 induction in the SCN might be necessary (since its inhibition reduces the phase shifts), but not sufficient (since CCL2 is induced at time points when LPS does not affect the clock) for the effects of peripheral immune activation on the circadian system. This could be due to the variation in the levels of its CCR2 receptor, which would provide a specific time window in which the SCN is sensitive to the chemokine, or to a circadian variation in the ability to activate downstream signaling pathways upon binding to CCR2, or a combination of these (and other) factors.

Bindarit is an indazolic derivative that has been shown to inhibit proinflammatory processes trough the inhibition of the induction of monocyte chemoattractant chemokines (Mirolo et al., 2008). Although not fully characterized, its mechanisms relies in the down-regulation of NF-kB pathway, producing a reduced binding of p65 and p65/p50 to the proximal regulatory region of the murine Ccl2 promoter (Mora et al., 2012). Bindarit has been shown effective to ameliorate symptoms related to inflammation and monocyte infiltration in different diseases, including animal models of chikungunya infection (Chen et al., 2015; Rulli et al., 2011), pancreatitis (Bhatia et al., 2005; Zhou et al., 2010), arthritis (Guglielmotti et al., 2002), lupus (Guglielmotti et al., 1998), experimental autoimmune encephalomyelitis (Ge et al., 2012), prostate, breast and skin cancer (Gazzaniga et al., 2007; Zollo et al., 2012) and in a clinical study of lupus nephritis (Ble et al., 2011). Although Bindarit has been described to be a selective inhibitor for MCPs, mainly CCL2, but also including CCL7 and CCL8, (Mirolo et al., 2008), we are confident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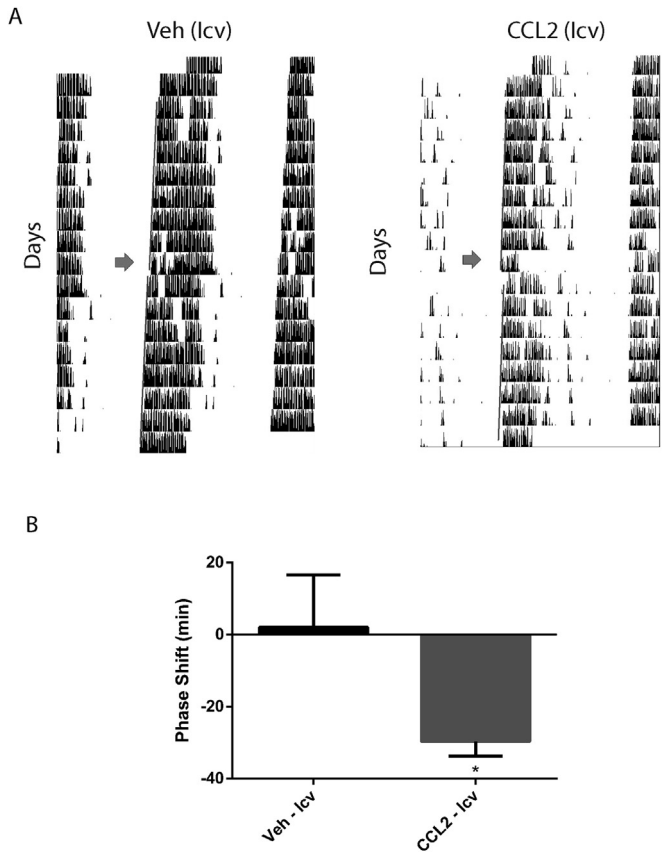

Fig. 5. Intracerebroventricular administration of CCL2 can shift the circadian clock. A) Representative actograms of animals receiving either 100 ng of CCL2 or Vehicle Icv, at CT 15. Arrows indicate the day of treatment. **B)** Icv CCL2 induced significant phase delays in wheel running activity rhythms, compared with the Vehicle control. Student's t-test; $p < 0.05$. $N = 6$.

that the blockade of LPS-induced phase shifts by Bindarit is through interfering with CCL2 synthesis. Reports of CCL7 or CCL8 induction in the brain upon peripheral immune activation are scarce, with only one work showing upregulation of CCL7 in the brain upon endotoxin treatment, using either a high dose of LPS, or repeated low doses (Homji et al., 2012), without analyzing regional differential induction. Moreover, no behavioral effect has been attributed to either of the two chemokines in a context of inflammatory response. On the other hand, we have demonstrated that CCL2 is upregulated in the SCN upon peripheral immune activation (Fig. 4**A** and Paladino et al., 2014), and that CCL2 administrated in the SCN region leads to phase shifts in circadian locomotor activity rhythms (Fig. 5). This, combined with the blockade of the phase shifts in response to Ip LPS, by a pharmacological tool that inhibits Ccl2 synthesis, makes strong evidence in favor of a necessary role for CCL2 in the mechanism of endotoxin-induced phase delays. To our knowledge, this is the first report for the use of this pharmacological tool in the amelioration of the circadian outcomes of proinflammatory activation.

Regarding the cell types involved in the process, we have previously reported that SCN astrocytes secrete CCL2 in response to immune activation *in vitro* (Duhart et al., 2013a). Double immunostaining for NeuN and CCR2 (Fig. 1**C**) shows that, albeit NeuN is sparsely expressed in the SCN, some of the NeuN-positive cells also express CCR2, suggesting that there is at least a population of SCN neurons that might be sensitive to CCL2. Under our immunohistochemical protocol, we could not find co-localization of CCR2 with specific astroglial (GFAP, Fig. 1**B**) or microglial (Iba1, Fig. 1**D**) markers, which suggests that CCL2, likely secreted by glial cells, will be mainly acting directly on neurons, inducing changes in clock properties. However, we cannot discard that some CCR2 positive astroglial or microglial cells could have been missed in our protocol, due to the mild permeabilization used in order to protect the membrane-receptor integrity. In addition, given the strong CCR2 signal found in the periventricular region (Figs. 1 and 3**A**), we cannot discard ependymal cell expression of CCL2 receptor, which could be important for the functional consequences of CCL2 acting at the SCN.

Finally, we have shown that inhibition of CCL2 synthesis in the SCN does not alter the phase delays induced by light pulses during the early night (Supplementary Fig. 1). This suggests a specific participation of this chemokine in SCN activation upon immune stimuli, and not as part of a general synchronization mechanism. The signaling pathways activated by CCL2 upon LPS peripheral administration might include MAPK, PKC or Ca-CAM pathways (Bonsall and Lall, 2013; Bose and Cho, 2013). These pathways have been described in the photic synchronization of the circadian clock (Golombek and Rosenstein, 2010), which suggests that CCL2 induction (which would occur upon immune activation) could lead to activation of signals that lead to phase shifts, similar to the activation of the nuclei by light. This is supported by our previous results showing that the effects of light and immune stimuli on the clock are not additive (Marpegan et al., 2005), suggesting an interaction or overlapping of both pathways.

5. Conclusions

The present work shows that the interaction between the immune and the circadian systems relies on the induction of CCL2 at the SCN level, although the clock response to this chemokine might be modulated by the level of its receptor, CCR2. Our results point towards a possible pharmacological modulation of the effects of immune activation on the circadian clock. Several pathologies which involve inflammatory activation, including sepsis and cancer, as well as different inflammation models, have been shown to alter the circadian clock (reviewed in: Duhart et al., 2013b). A role for CCL2 acting in the CNS has been proposed for these pathologies (Carrillo-de Sauvage et al., 2012; Dominguez-Punaro et al., 2007), and it has been reported that peripheral administration of Bindarit is capable of inhibiting Ccl2 synthesis in the CNS (Ge et al., 2012), which suggests that this pharmacological tool could be used to mitigate the circadian (and CNS-related) consequences of inflammatory situations.

Fig. 4. CCL2 in the circadian response to LPS. A) Ccl2 mRNA is induced in the SCN upon intraperitoneal LPS administration. Relative mRNA levels of Ccl2 in SCN tissue sampled at ZT 3 or ZT 15, one hour after an ip LPS or Vehicle administration. Two-way ANOVA (No effect for Interaction or Time of day: $F_{1, 12} = 1.007$, $p = 0.3355$ and $F_{1, 12} = 4.084$, $p = 0.0662$, respectively; Drug effect: $F_{1, 12} = 19.84$, $p = 0.0008$) followed by Newman-Keuls post hoc test; $p < 0.05$ for ZT 3 Veh vs ZT 3 LPS; $p < 0.01$ for ZT 15 Veh vs ZT 15 LPS. $N = 4$. **B) and C) CCL2 synthesis inhibition blocks LPS-induced phase shifts. B)** Representative actograms of animals receiving different combinations of icv (at CT 14.5) and ip (at CT 15) treatments. Arrows indicate the day of treatment. **C)** Icv delivery of Bindarit inhibits Ip LPS-induced phase delays. Two-way ANOVA (No effect for Interaction: $F_{1, 30} = 1.370$, $p = 0.2509$; Ip Drug effect: $F_{1, 30} = 4.839$, $p = 0.0357$; Icv Drug effect: $F_{1, 30} = 9.895$, $p = 0.0037$) followed by Newman-Keuls post hoc test; $p < 0.05$ for Veh Icv + LPS Ip vs Veh Icv + Veh Ip; $p < 0.01$ for Veh Icv + LPS Ip vs Bindarit Icv + Veh Ip; $p < 0.05$ for Veh Icv + LPS Ip vs Bindarit Icv + LPS Ip; $p > 0.05$ for other comparisons). $N = 9$.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2016.05.005.

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