Suprachiasmatic Astrocytes Modulate the Circadian Clock in Response to TNF-α

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The immune and the circadian systems interact in a bidirectional fashion. The master circadian oscillator, located in the suprachiasmatic nuclei (SCN) of the hypothalamus, responds to peripheral and local immune stimuli, such as proinflammatory cytokines and bacterial endotoxin. Astrocytes exert several immune functions in the CNS, and there is growing evidence that points toward a role of these cells in the regulation of circadian rhythms. The aim of this work was to assess the response of SCN astrocytes to immune stimuli, particularly to the proinflammatory cytokine TNF-α. TNF-α applied to cultures of SCN astrocytes from Per2[luc] knockin mice altered both the phase and amplitude of PER2 expression rhythms, in a phase-dependent manner. Furthermore, conditioned media from SCN astrocyte cultures transiently challenged with TNF-α induced an increase in Per1 expression in NIH 3T3 cells, which was blocked by TNF-α antagonism. In addition, these conditioned media could induce phase shifts in SCN PER2 rhythms and, when administered intracerebroventricularly, induced phase delays in behavioral circadian rhythms and SCN activation in control mice, but not in TNFR-1 mutants. In summary, our results show that TNF-α modulates the molecular clock of SCN astrocytes in vitro, and also that, in response to this molecule, SCN astrocytes can modulate clock gene expression in other cells and tissues, and induce phase shifts in a circadian behavioral output in vivo. These findings suggest a role for astroglial cells in the alteration of circadian timing by immune activation.   *The Journal of Immunology*, 2013, 191: 000–000.

I n mammals, circadian rhythms are orchestrated by a central oscillator, located in the suprachiasmatic nuclei (SCN) of the hypothalamus that drives daily oscillations in several physiological and behavioral processes (1). Circadian timing is ultimately achieved by a molecular mechanism, consisting in a transcriptional/translational feedback loop. Briefly, in mammals, the heterodimerized transcription factor BMAL1:CLOCK activates *Period* (*Per1*, *Per2*, and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) gene transcription by binding to E-box motifs in their promoters. PER and CRY proteins inhibit BMAL1:CLOCK transcriptional activity, thereby inhibiting their own gene expression. This feedback-loop mechanism generates circadian oscillations of *Per* and *Cry* expression with a period close to 24 h (2). Functional molecular clocks have been found in several tissues and cell types, which have been referred as peripheral oscillators, and are coordinately synchronized by the SCN (3). Indeed, circadian oscillations

are found even in established fibroblast cell lines, such as Rat-1 cells or NIH 3T3 cells, and have been widely used for the study of the effects of different treatments, including immune effectors, on clock gene expression because they are easily grown and maintained (4, 5).

The immune and the circadian systems interplay in a bidirectional fashion. Several immune functions are under circadian control (6, 7); indeed, circadian disruption leads to an altered inflammatory response (8). In contrast, immune factors are able to modulate circadian physiology by acting on central and peripheral oscillators (6). Chronic inflammatory situations lead to alteration both in the responsiveness of the master circadian oscillator to light and in clock gene expression (5, 9). Also, the sleep-wake cycle (partially controlled by the circadian clock) is modulated by immune factors, including TNF-α and IL-1β (10), and SCN physiology can be altered by infections that alter sleep architecture (11, 12). The presence of receptors for the proinflammatory cytokines TNF-α and IL-1β has been reported in the SCN (13, 14), and immune stimuli have been shown to modulate the phase of the master circadian clock (15–17). Interestingly, the circadian response to a peripheral immune challenge is mediated by the action of TNF-α at the SCN level (15), suggesting an important role for this cytokine in immune-circadian communication.

Astrocytes can modulate neuronal activity by controlling ionic environment and the supply of neurotransmitters to synapses, and are also able to directly activate neuronal receptors through the secretion of gliotransmitters (reviewed in Ref. 18). Although astrocytes have a pivotal role in most of the functions of the CNS, their role in circadian physiology is not fully characterized yet. There is a rhythmic expression of glial fibrillary acidic protein (GFAP; a specific astrocyte marker in the adult brain) in the SCN of mice and hamsters (19, 20). Also, primary cultures of cortical astrocytes from mPeriod2::luciferase (Per2[Luc]) knockin mice are rhythmic and can be entrained by temperature (21). In addition, astrocytes also participate in innate immune reactions in the CNS,

secreting proinflammatory cytokines such as IL-1β, TNF-α, IL-6, and several chemokines (CCL2, CXCL1, CXCL10, and CXCL12) upon activation (22–24). Indeed, treatment of SCN astrocytes with LPS or proinflammatory cytokines (TNF-α and IL-1β) leads to the activation of the NF-κB signaling pathway (20), which has also been shown to interact with the molecular circadian clock pathway (25, 26). Considering the capacity of astrocytes to alter neuronal physiology, their responsiveness to immune stimuli, and their not yet fully characterized role in the circadian pacemaker, these cells represent good candidates to act as cellular mediators of the immune-circadian interaction at the SCN level, recognizing proinflammatory signals, and releasing factors that modify SCN physiology. In the present work, we characterized the response of SCN astrocytes to the proinflammatory cytokine TNF-α. We found that treatment with TNF-α produces changes in the molecular clock of SCN astrocytes, as well as inducing the release of CCL2, TNF-α, and IL-6. Furthermore, conditioned media from TNF-α–challenged SCN astrocytes altered clock gene expression in NIH 3T3 cells in vitro, and induced phase shifts in both PER2 expression rhythms in SCN tissue explants and circadian behavioral rhythms in vivo.

Materials and Methods

Animals

C57BL/6J male wild-type (Wt) and TNFR1⁻ knockout (B6.129-Tnfrsf1a^{tm1Mak}/J; The Jackson Laboratory, provided by Dra. Silvia Di Genaro, San Luis National University, Argentina) mice (*Mus musculus*) were raised in the National University of Quilmes colony, and Per2^{Luc} knockin mice (also from C57BL/6J background) (27) were raised at the Morehouse School of Medicine animal facilities. All animals were housed under a 12:12-h light: dark photoperiod with food and water access ad libitum. Animal manipulations and experimental protocols performed in this work were supervised and approved by the University of Quilmes and the Morehouse School of Medicine Institutional Animal Care and Use Committees, in accordance with the National Institutes of Health guide for the care and use of laboratory animals.

Cell cultures and conditioned media preparation

Astrocyte-enriched primary cultures from the SCN were prepared as described in Leone et al. (20). Briefly, brains from mice pups of 0–4 postnatal days were dissected under sterile conditions, and the SCN was obtained with the help of a surgery magnifying glass. Tissue was dissociated and mechanically disrupted, obtaining a cell suspension. Cells were seeded in culture flasks and maintained in DMEM (Life Technologies), supplemented with 10% FBS (PAA, Pasching, Austria) and antibiotic-antimicotic (Life Technologies) until reaching confluence (10–14 d). Flasks were banged and washed with PBS, during every medium change, to detach less adherent cell types. The purity of the cultures was routinely confirmed by GFAP immunoreactivity. NIH 3T3 cells were maintained in DMEM supplemented with 10% bovine calf serum and antibiotic-antimicotic, and passed there times per week until use.

Conditioned media from immune-challenged SCN astrocytes were prepared as follows: cells from SCN astrocyte primary cultures were seeded on 24-well plates, at a density of 3 × 10⁴ cells/well. Twenty-four hours later, cultures were treated with LPS from *Escherichia coli* 0111:B4 (2 μg/ml; Sigma-Aldrich), TNF-α (20 ng/ml; PeproTech), IL-1β (5 ng/ml; PeproTech), or vehicle (Veh) (0.01 M PBS) for 2 h, and then washed twice with PBS and left in serum-free DMEM, if the conditioned media were to be used for in vivo administrations, or in 1% serum DMEM for all other experiments. Twenty-four hours later, the conditioned media were collected and processed according with the following experiments: for cytokine/chemokine measures, conditioned media were also collected 2 h after the immune challenge, and samples were conserved at −20°C until use; for ex vivo (SCN slices; see below) and in vivo administration experiments, they were concentrated by a 10× factor with 3000 MWCO Ultrafiltration tubes (Vivaspin 500; Sartorious Stedim, Goettingen, Germany) and were stored at −20°C until use. Finally, for the in vitro experiments, conditioned media were directly applied to NIH 3T3 cultures, with the addition of TNF-α soluble receptor I (Sigma-Aldrich), IL-1R antagonist (Valeant Pharmaceuticals, Mississauga, Ontario, Canada), or Veh, in the corresponding experiments. Conditioned media from TNF-α–, LPS-, IL-1β–, and Veh-treated SCN astrocyte cultures will be further referred to as

TNF-α–conditioned media (CM), LPS-CM, IL-1β-CM, and Veh-CM, respectively. Endotoxin content of conditioned media was verified by *Limulus* test gel clot method (Pyrolab) and resulted negative for LPS presence (data not shown).

Clock gene expression measurements in vitro

For continuous bioluminescence recording of SCN glial cultures, pups from Per2^{Luc} strain were used in the preparation of SCN astrocyte primary cultures, as described above. Cells were seeded on 35-mm culture dishes at a density of 1 × 10⁵ cells/dish and maintained with serum-free DMEM, supplemented with 10 mM HEPES (pH 7.2), B27 (2%; Life Technologies), antibiotic-antimicotic, and 0.1 mM luciferin (Gold Biotechnology). Bioluminescence from the cultures was measured in a Lumicycle apparatus (Actimetrics, Wilmette, IL) at 37°C. For drug treatments, culture dishes were taken out of the Lumicycle for <2 min and kept over a thermal blanket at 37°C. Twenty microliters of drug dissolved on PBS, or Veh, was applied to the culture medium.

For Per1 promoter activity measures, NIH 3T3 cells seeded at 5 × 10⁴ cells/well in 24-well plates were transfected with hPer1-luc [provided by E. Maronde (28)] and CMV-β-gal plasmids using Lipofectamine (Life Technologies). Twenty-four hours after transfection, cells were treated with PBS, TNF-α (2–200 ng/ml), 50 nM PMA (Sigma-Aldrich), or culture medium was replaced with either 50% FBS in DMEM or conditioned medium from SCN astrocytes. After 2 h, the medium was removed from treated cultures, cells were rinsed with PBS, and fresh DMEM 1% serum was added. Twenty-four hours later, cells were lysed, and luciferase and β-galactosidase activities were quantified. Luminescence values were relativized to β-galactosidase activity (and further referred to as relative luciferase units), and then reported relative to the Veh mean for each experiment.

For Per1 mRNA measures, NIH 3T3 cells were seeded at 1.5 × 10⁵ cells/well in 6-well plates. Twenty-four hours after medium change, cells were treated with PBS, TNF-α (2 and 20 ng/ml), or culture medium was replaced with either 50% FBS in DMEM or conditioned medium from SCN astrocytes. After 2 h, the medium was removed from treated cultures, cells were rinsed with PBS, and fresh DMEM 1% serum was added. Twenty-four hours later, cells were harvested with TRIzol reagent (Life Technologies) to extract RNA. The cDNA was synthesized with Superscript II Reverse Trascriptase (Life Technologies) and amplified in a StepOne real-time system (Applied Biosystems) using SYBR Green PCR master mix from (Applied Biosystems), following manufacturer's instructions. Per1 mRNA relative expression was calculated using $2^{-\Delta\Delta CT}$ method (29), normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA levels. Primers sequences are as follows: Per1 forward, 5′-CATTCCGCCTAACCCCATATG-3′; Per1 reverse, 5′-CGGGGAGCTTCATAACCAGAG-3′; HPRT forward, 5′-TGTTGGATACAGGCCAGAC-3′; and HPRT reverse, 5′-TGGCAA-CATCAACAGGACTC-3′.

SCN slices preparation, bioluminescence recordings, and treatment

Male and female PER2^{Luc} mice (7–9 wk of age) were sacrificed with CO₂ and cervical dislocation 2–4 h before lights off because dissections during late subjective day do not reset the phase of the SCN (30). After decapitation, brains were removed and sectioned in the coronal plane while submerged in cold HBSS supplemented with 10 mM HEPES (pH 7.2), B27 (2%), and antibiotic-antimicotic using a motorized vibratome (Campden Instruments, Lafayette, IN). SCN slices (200 μm) were selected from the rostrocaudal center of the SCN, trimmed near the edges of the SCN, and cultured on a membrane (Millicell-CM, PICM030-50; Millipore) in a sealed dish containing 1.2 ml DMEM (Sigma-Aldrich) supplemented with 10 mM HEPES (pH 7.2), 2% B27, antibiotic-antimicotic, and 0.1 mM luciferin (Molecular Imaging Products). Bioluminescence from slices was measured in a Lumicycle apparatus (Actimetrics, Wilmette, IL) at 37°C. On the third cycle of PER2 expression rhythm, 2 μl concentrated conditioned medium from TNF-α– or Veh-treated SCN astrocytes was applied directly to the SCN slice 1–2 h after either the peak or trough phase.

In vivo administration of conditioned media

Mice (Wt and TNFR1⁻ mutants) were stereotactically implanted with 26-gallon stainless steel guide cannula (PlasticsOne) aimed at the bottom of the third ventricle, in the SCN region (coordinates from bregma: −0.5 mm anteroposterior, −5.0 mm dorsoventral, 0.0 mm from midline). After surgery, animals were allowed to recover in light:dark conditions for 72 h with health status being daily controlled, and were transferred to constant dark (DD) conditions afterward. Animals remained in DD conditions for at

least 15 d before the first treatment, and reference time was set by wheel-running activity onset of each animal (circadian time [CT] 12). Intra-cerebroventricular (icv) microinjections were performed under dim red light with a 33-gallon internal injector (PlasticsOne) connected to a microsyringe (Hamilton, Reno, NV). A total of 1 μl concentrated conditioned medium from TNF-α– or Veh-treated SCN astrocytes (TNF-α-CM or Veh-CM, respectively, see above) was delivered at CT 15 at a 0.2 μl/min flux. For the behavioral effects of conditioned media, each animal received two treatments (the order of the treatments was randomly assigned, in a counterbalanced design), separated by 12–13 d.

Cytokine/Chemokine measurements

Levels of TNF-α, IFN-γ, CCL2 (also known as MCP-1), IL-6, IL-12p70, and IL-10 were measured in conditioned media from TNF-α– and Veh-treated SCN astrocytes (see above) using the Cytometric Bead Array Mouse Inflammation kit (BD Biosciences), according to manufacturer's instructions.

Immunohistochemistry and immunocytochemistry

Primary cultures of SCN astrocytes were marked for GFAP and TNFR expression. Cells were rinsed with 0.01 M PBS and fixed in 4% paraformaldehyde in 0.01 M PBS for 20 min, washed with PBS, and dehydrated with methanol for 2 min. Cells were blocked with 5% nonfat milk in PBS containing 0.04% Triton X-100 (PBS-T 0.04%) and incubated with a primary Ab against GFAP (1:1000 in 0.04% PBS-T; DakoCytomation, Dlostrup, Denmark) or TNF-R1 (1:50 in 0.04% PBS-T; Santa Cruz Biotechnology) for 24 h at 4°C. After extensive washing with 0.04% PBS-T, cells were incubated with anti-rabbit secondary Ab conjugated with FITC for 2 h at room temperature, washed again, and stained with DAPI.

For cFos immunohistochemistry, mice were deeply anesthetized with a mixture containing ketamine (150 mg/kg) and xylazine (10 mg/kg) 90 min after receiving 1 μl either TNF-α-CM or Veh-CM at CT15. Animals were 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 40-μm–thick coronal sections were cut with a freezing microtome. Free floating slices were blocked with 5% nonfat milk in PBS containing 0.4% Triton X-100 and incubated with primary antisera raised in rabbit against c-Fos (Santa Cruz Biotechnology; 1:2000) 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 biotinylated universal secondary Ab, avidin, and biotinylated HRP (Vector Laboratories, Burlingame, CA) and vector-vasoactive intestinal polypeptide peroxidase substrate (SK-4600). Image analysis and cell counting were performed using ImageJ software, as described previously (31).

Data analysis and statistics

Bioluminescence recordings were made in a Lumicycle apparatus with the corresponding data acquisition program (Actimetrics). CT 12 was defined as the peak in Per2^{Luc} expression. Phase shifts were calculated by adjusting a damped sine wave by the Levenberg–Marquardt algorithm to the cycles previous to the treatment. Using the period given by this fitting, and the time of the last peak before the treatment, we calculated the expected next peak. The difference between the expected peak and the first peak occurring after treatment was considered the phase shift. For relative amplitude calculation, peak to trough amplitude of each cycle after treatment was divided by the amplitude of the last cycle before treatment.

Phase shifts on wheel-running activity were calculated by five observers masked to the experimental procedure with *El Temps* software (Antoni Díez Noguera, University of Barcelona), using activity onset as a phase reference point. The average value between all the observers for each phase shift was used for further statistical analysis.

Phase shifts in bioluminescence rhythms were analyzed by one-way ANOVA, followed by Tukey's test in the astrocyte culture experiments, and by two-way ANOVA, followed by Sidak's post hoc test in the SCN slice experiments. The effect of TNF-α on relative amplitude of Per2^{Luc} rhythms was analyzed by repeated measures two-way ANOVA. Effects on Per1 expression in NIH 3T3 cells were analyzed by one-way ANOVA, followed by Dunnett's test, or by Tukey's test, as appropriate. Cytokine levels in conditioned media were analyzed with two-way ANOVA, followed by Sidak's post hoc test. In vivo phase shifts produced by conditioned media were analyzed by two-way ANOVA, followed by Sidak's post hoc test. Induction of cFos in the SCN region was analyzed by two-way ANOVA, followed by Tukey's multiple comparisons test. Data are presented as mean ± SEM. The *p* values ≤ 0.05 were considered to be statistically significant.

Results

TNF-α alters Per2^{Luc} expression rhythms in SCN astrocytes

TNF-α has been previously described to induce phase shifts in locomotor activity rhythms, and to mediate the effects of a peripheral immune stimulus on the circadian clock (15), as well as altering clock gene expression in murine fibroblasts (5), suggesting an important role for this cytokine in the communication between the immune and the circadian systems. Therefore, we focused in the response of SCN astrocytes to TNF-α. First, we analyzed whether the molecular rhythms of these cells could be modulated by this cytokine. We monitored Per2^{Luc} expression in primary cultures of SCN astrocytes; these cells showed a rhythmic expression in PER2 for >6 d, with an average period of 22.76 ± 0.83 h (Fig. 1A, 1D), in accordance with the rhythm described in cortical cultures (22.4 h) (21). Treatment of SCN astrocyte primary cultures with 20 ng/ml TNF-α at the trough of Per2^{Luc} expression (CT 0) resulted in phase delays when compared with Veh control (−4.3 ± 0.4 and −0.9 ± 0.7 h, respectively) (Fig. 1A, 1B). Moreover, treatment with higher doses of TNF-α (200 ng/ml) at the same phase of Per2^{Luc} rhythms induced phase delays of −9.1 ± 1.1 h, suggesting a dose-dependent response of the molecular clock of SCN astrocytes to TNF-α (ANOVA followed by Tukey's test, *p* < 0.01 for Veh versus 20 ng/ml TNF-α, *p* < 0.001 for Veh versus 200 ng/ml TNF-α, and *p* < 0.001 for 20 ng/ml TNF-α versus 200 ng/ml TNF-α, *n* = 4, Fig. 1B). We found no effect on the amplitude of the rhythms in cultures treated at CT 0, either with 20 (repeated measures two-way ANOVA, *p* > 0.05 for treatment factor, *p* < 0.01 for cycle factor, *p* > 0.05 for interaction, *n* = 4, Fig. 1C) or 200 ng/ml (data not shown) TNF-α. In contrast, treatment with 20 ng/ml TNF-α 2 h after the peak of Per2^{Luc} expression (CT 14) resulted in a small, yet significant, increase in the amplitude of the rhythm (repeated measures two-way ANOVA, *p* < 0.01 treatment factor, *p* < 0.0001 for cycle factor, *p* > 0.05 for the interaction, Fig. 1D, 1F). This effect was also found in cultures treated with 200 ng/ml TNF-α, but did not reach statistical significance when compared with the effect of 20 ng/ml TNF-α (data not shown). Treatment with Veh at CT 14 induced phase delays of −3.7 ± 0.2 h. We found no differential effect for 20 ng/ml TNF-α at this time, compared with the Veh control, although 200 ng/ml TNF-α induced smaller phase delays, compared both with Veh and 20 ng/ml TNF-α (ANOVA followed by Tukey's test, *p* < 0.001 for 200 ng/ml TNF-α versus Veh, *p* < 0.001 for 200 ng/ml TNF-α versus 20 ng/ml TNF-α, *n* = 6, Fig. 1E). Primary cultures showed to be >95% GFAP-positive cells, and also expressed TNF-R1 (Fig. 1G).

Conditioned media from immune-challenged SCN astrocytes alters Per1 expression through the action of TNF-α

Astrocytes respond to immune stimuli secreting several proinflammatory factors, including a variety of cytokines and chemokines (32). To further characterize the role of SCN glia on immune-circadian communications, we tested whether this response could produce alterations in the circadian clock of another cell type. We analyzed the effects of conditioned media from primary cultures of SCN astrocytes transiently challenged with TNF-α, LPS, and IL-1β, on NIH 3T3 cells carrying a hPer1-luc reporter plasmid. We found that conditioned media from TNF-α– and LPS (but not IL-1β)-stimulated glia produced an increase in Per1 promoter activity, measured 24 h after treatment (ANOVA followed by Dunnett's test with Glia-PBS as control, *p* < 0.05 for TNF-α-CM and LPS-CM, *n* = 8, Fig. 2A). TNF-α–stimulated glia showed an induction of TNF-α secretion 2 and 24 h after treatment (two-way ANOVA, *p* < 0.0001 for drug effect, *p* < 0.001
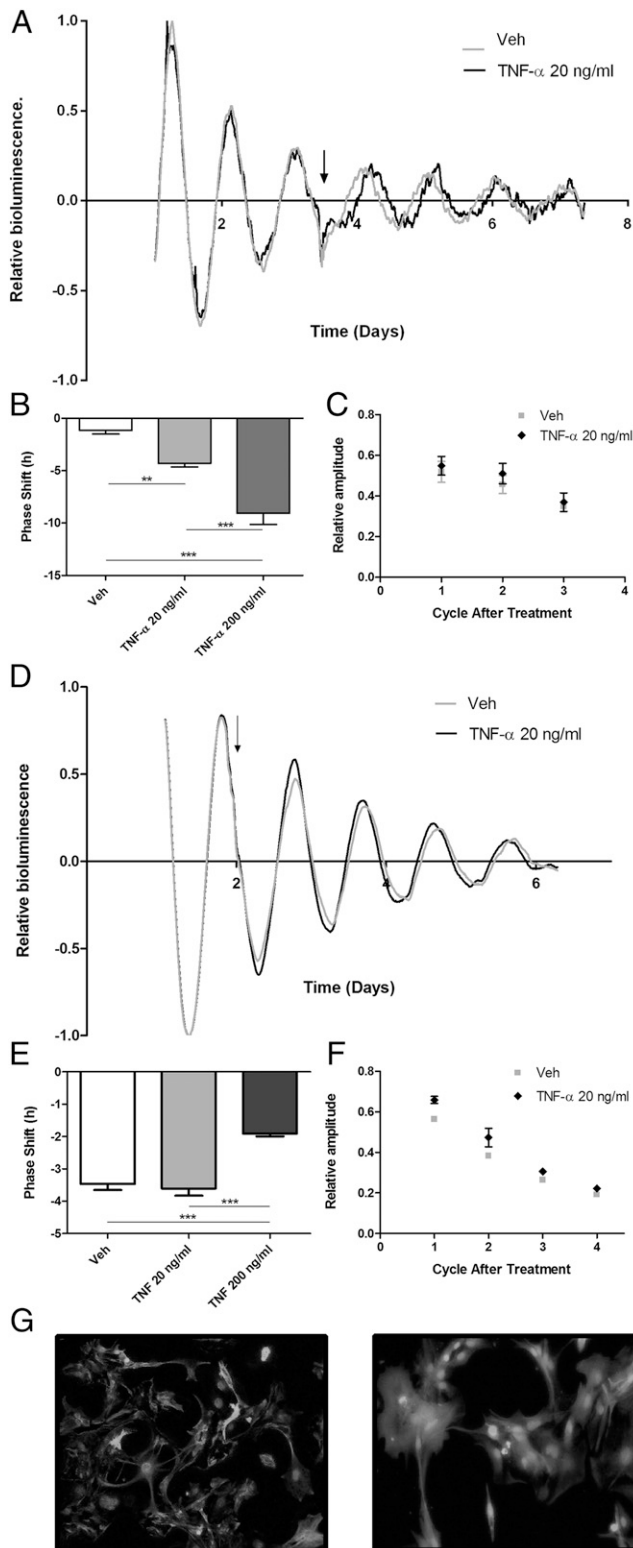
FIGURE 1. TNF-α alters PER2 expression rhythms in SCN astrocyte cultures. Representative bioluminescence records of SCN astrocyte cultures from Per2^{Luc} mice, treated with 20 ng/ml TNF-α either at the trough or 2 h after the peak of PER2 expression [(**A**) and (**D**), respectively]. (**B**) Treatment with TNF-α at CT 0 (trough of the bioluminescence rhythm) produced phase delays in PER2 rhythms, and this effect was dose dependent (ANOVA, followed by Tukey's test, **$p < 0.01$ for Veh versus 20 ng/ml TNF-α, ***$p < 0.001$ for Veh versus 200 ng/ml TNF-α, and ***$p < 0.001$ for 20 ng/ml TNF-α versus 200 ng/ml TNF-α, $n = 4$). (**C**) Treatment with 20 ng/ml TNF-α at CT 0 produced no effect in the amplitude of PER2 rhythms (repeated measures two-way ANOVA, $p > 0.05$ for treatment

factor, $p < 0.01$ for cycle factor, $p > 0.05$ for interaction, $n = 4$). (**E**) Treatment with 20 ng/ml TNF-α at CT 14 (2 h after the peak of the bioluminescence rhythm) produced phase delays that were not different from the Veh control, but treatment with 200 ng/ml TNF-α resulted in phase delays of smaller magnitude (ANOVA followed by Tukey's test, ***$p < 0.001$ for 200 ng/ml TNF-α versus Veh, ***$p < 0.001$ for 200 ng/ml TNF-α versus 20 ng/ml TNF-α, $n = 6$). (**F**) Treatment with 20 ng/ml TNF-α at CT 14 produced a small increase in the amplitude of PER2 rhythms (repeated measures two-way ANOVA, $p < 0.01$ treatment factor, $p < 0.0001$ for cycle factor, $p > 0.05$ for the interaction, $n = 6$). (**G**) SCN astrocyte cultures were >95% GFAP positive (*left panel*) and express TNFRI (*right panel*). Original magnification ×200.

for time effect, $p > 0.05$ for interaction, $n = 5$, Fig. 2B). CCL2 and IL-6 secretion by SCN astrocytes was also upregulated by TNF-α treatment, but no effect was found for IFN-γ, IL-10, and IL-12p70 (Supplemental Fig. 1). Because TNF-α secretion was upregulated in conditioned media from immune-challenged SCN astrocytes, and, as stated before, this cytokine has been shown to be involved in the response of the circadian clock to an immune stimulus, we tested whether a transient stimulus with TNF-α could alter clock gene expression in NIH 3T3 cells. PMA and a 50% serum shock were used as positive controls, because they have been described to synchronize Per1 expression in murine fibroblasts (4). We found that Per1-luc activity on NIH 3T3 cells was increased by treatment with 2 ng/ml and 200 ng/ml TNF-α (ANOVA followed by Dunnett's post hoc test with Veh as negative control, $p < 0.01$ for PMA, $p < 0.05$ for 50% FBS shock, TNF-α 2 and 200 ng/ml, $n = 4$, Fig. 2C). We also evaluated the effects of conditioned media and TNF-α itself on the expression of Per1 mRNA of NIH-3T3 cells. We found that both 20 ng/ml TNF-α and TNF-α-CM induced Per1 mRNA expression, in accordance with the results from the hPer1-luc construct (ANOVA followed by Dunnett's post hoc test with Veh as negative control, $p < 0.05$ for FBS shock, $p < 0.01$ for 20 ng/ml TNF-α, $p < 0.05$ for TNF-α-CM, $n = 4$, Fig. 3). Because TNF-α transient treatment induced its own secretion in SCN astrocytes, and this cytokine could induce Per1 expression in NIH 3T3 cells, we hypothesized that TNF-α secreted by SCN astrocytes to the conditioned media could be responsible for the effects on Per1 expression on fibroblast cells. To confirm this, TNF-α activity was blocked by addition of TNF soluble receptor (TNFsR) to the conditioned media. The effect of TNF-α-CM on clock gene expression in NIH 3T3 cells was inhibited by the addition of TNFsR (ANOVA followed by Tukey's post hoc test, $p < 0.05$ for Veh-CM versus TNF-α-CM, $p < 0.05$ for TNF-α-CM versus TNF-α-CM + TNFsR, $p > 0.05$ for all other contrasts, $n = 14$, Fig. 2D). Furthermore, because IL-1β delivered into the SCN region can produce phase shifts in locomotor activity circadian rhythms (15), we tested whether the effects of conditioned media from TNF-α–challenged astrocytes on clock gene expression could also implicate the action of IL-1β. Although IL-1β induced the expression of Per1 in NIH 3T3 cells (Supplemental Fig. 2), IL-1 antagonism did not alter the effects of TNF-α-CM applied to these cells (Fig. 2D).

Conditioned media from immune-challenged SCN astrocytes induces phase shifts in the master circadian oscillator

Per1/2 induction in the SCN is part of the mechanism leading to phase shifts in the circadian clock (33). Because the humoral consequence of SCN astrocyte response to TNF-α was able to induce Per1 in other cell types through the secretion of TNF-α, we tested whether this could be extended to the modulation of circadian rhythms in the SCN both in ex vivo and in vivo approaches.
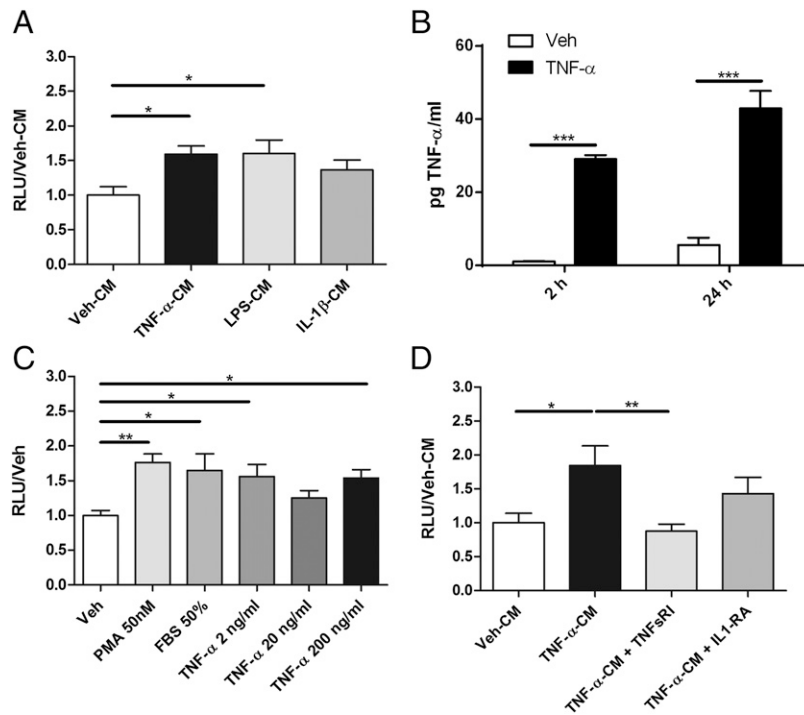
FIGURE 2. Conditioned media from immune-challenged SCN astrocytes induces Per1 promoter activity in NIH 3T3 cells. SCN astrocyte primary cultures were challenged with TNF-α (20 ng/ml), LPS (2 μg/ml), IL-1β (5 ng/ml), or Veh (PBS) for 2 h, and then treatment was washed out and conditioned media were collected 24 h later. Conditioned media from TNF-α, LPS, IL-1β, and Veh are referred as TNF-α-CM, LPS-CM, IL-1β, and Veh-CM, respectively. (**A**) TNF-α-CM and LPS-CM induced Per1 expression in NIH 3T3 cells transfected with hPer1-luc plasmid (ANOVA followed by Dunnett's test with Veh-CM as control, *$p < 0.05$ for TNF-α-CM and LPS-CM, $n = 8$; RLU, relative luciferase units). (**B**) Primary cultures of SCN astrocytes transiently treated with TNF-α (20 ng/ml) showed an induction of TNF-α secretion measured both 2 and 24 h after treatment (two-way ANOVA, $p < 0.0001$ for drug effect, $p < 0.001$ for time effect, $p > 0.05$ for interaction, followed by Sidak's test, ***$p < 0.001$, $n = 5$). (**C**) NIH 3T3 cells transiently treated with 2 and 200 ng/ml TNF-α showed an induction of Per1 expression. Positive controls: 50 nM PMA and 50% FBS (ANOVA followed by Dunnett's test, **$p < 0.01$ for PMA, *$p < 0.05$ for 50% FBS shock, 2 and 200 ng/ml TNF-α, $n = 4$). (**D**) The effects of conditioned media from TNF-α–challenged SCN astrocytes on NIH 3T3 Per1 expression were inhibited by TNFsR, but not by IL-1 antagonism (ANOVA followed by Tukey's test, *$p < 0.05$ for glia-PBS versus glia-TNF-α, **$p < 0.01$ for glia-TNF-α versus glia-TNF-α + TNFsR, $p > 0.05$ for all other contrasts, $n = 14$). RLU, Relative luciferase units.

Rhythms of PER2 expression in SCN slices from PER2^{Luc} mice were shifted by treatment with TNF-α-CM, only when the stimulus was applied at CT 13–14 (-0.62 ± 0.15 h for TNF-α-CM, 0.22 ± 0.26 h for Veh-CM, Fig. 4A, 4C), but not at CT 1–2 ($0.05 \pm$
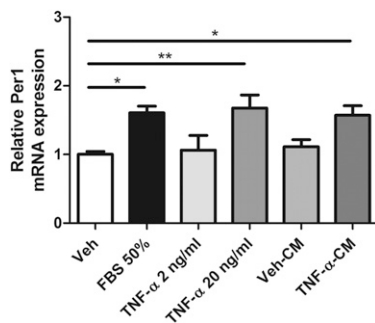

FIGURE 3. TNF-α and conditioned media from TNF-α–challenged SCN astrocytes induce Per1 mRNA expression on NIH 3T3 cells. SCN astrocyte primary cultures were treated as expressed for Fig. 2. NIH 3T3 cell cultures were transiently treated with Veh (PBS), TNF-α (2–20 ng/ml), TNF-α-CM, Veh-CM, or 50% serum shock (FBS 50%) as positive control for 2 h, and then treatment was washed out and mRNA expression was quantified 24 h later. Serum shock, 20 ng/ml TNF-α and TNF-α-CM–induced mPer1 expression on NIH 3T3 cells (ANOVA followed by Dunnett's post hoc test with Veh as negative control, *$p < 0.05$ for FBS shock, **$p < 0.01$ for 20 ng/ml TNF-α, *$p < 0.05$ for TNF-α-CM, $n = 4$).

0.16 h for TNF-α-CM, 0.56 ± 0.27 h for Veh-CM, Fig. 4B, 4C), showing that the master circadian oscillator is sensitive to the factors released by SCN astroglia upon immune stimulation in a time-dependent manner. Furthermore, we assessed the effects of the conditioned media on behavioral circadian outputs. TNF-α-CM or Veh-CM was injected icv into the SCN region at CT 15 in Wt or Tnfrsf1a^{tm1Mak}/J mice to test the effect on circadian locomotor activity. The time of administration was chosen based on previous findings that showed that the circadian clock is susceptible to both peripheral and central immune stimuli during the early subjective night (15, 16). We found that in Wt mice the administration of TNF-α-CM resulted in a small, albeit significant phase delay in locomotor activity circadian rhythms, whereas no phase shifts were induced by icv administration of Veh-CM (-25.3 ± 6.8 versus -6.7 ± 7.4 min, respectively, Fig. 5A, 5B). Moreover, when injected into mice carrying a mutation for the TNF-α receptor 1, there was no statistically significant difference between the TNF-α-CM and Veh-CM (-6.8 ± 7.4 min for TNF-α-CM versus 11.4 ± 8.2 Veh-CM, Fig. 5A, 5B). Besides the effects on circadian outputs, the activation of SCN cells by peripheral or central immune stimuli can be evidenced by cFos expression (13, 16). We found that icv delivery of TNF-α-CM to Wt mice increased the number of cFos-expressing cells in the SCN, both in the core (ventrolateral) region and shell (dorsomedial) region, but produced no effect when injected into TNFR$^-$ mice (Fig. 5C, 5D). No statistically significant difference was found
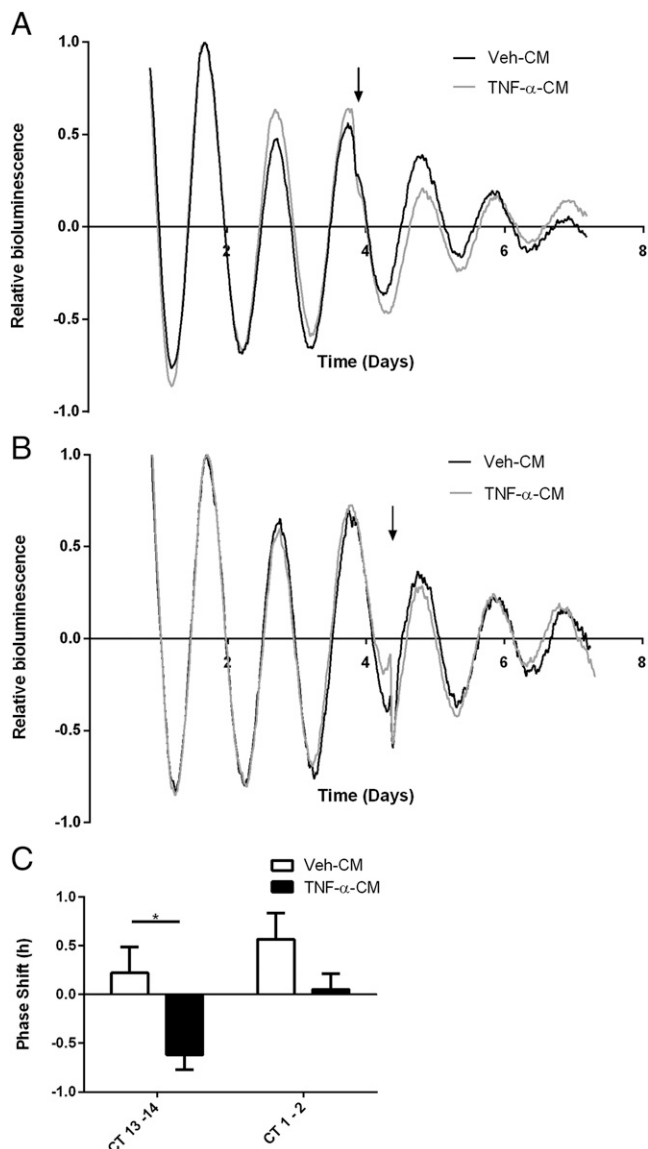
FIGURE 4. Conditioned media from TNF-α–challenged SCN astrocyte phase shift PER2 expression rhythms in SCN slices. SCN astrocyte primary cultures were treated as expressed for Fig. 2. Representative bioluminescence records of SCN slices from Per2^{luc} mice, treated with conditioned media from TNF-α– or Veh-treated SCN astrocytes 1–2 h after the peak (**A**) or 1–2 h after the trough of PER2 expression (**B**). (**C**) TNF-α-CM induced significant phase delays of PER2 expression rhythms in mouse SCN slices when applied 1–2 h after the peak, but not 1–2 h after the trough. The −0.62 ± 0.15 h for slices receiving TNF-α-CM at CT 13–14, 0.22 ± 0.26 h for slices receiving Veh-CM at CT 13–14; 0.05 ± 0.16 h for slices receiving TNF-α-CM at CT 1–2, 0.56 ± 0.27 h for slices receiving Veh-CM at CT 1–2 (two-way ANOVA, followed by Bonferroni's multiple comparisons post hoc test; *$p < 0.05$ for TNF-α-CM versus Veh-CM at CT 13–14, $p > 0.05$ for TNF-α versus Veh-CM at CT 1–2); $n = 6–8$.

between cFos levels in core and shell regions, on each of the treatments performed (data not shown). A model summarizing our results appears in Fig. 6.

Discussion

The master circadian oscillator has been shown to be sensitive to a variety of immune stimuli. Particularly, the proinflammatory cytokine TNF-α (alone or in combination with other proinflammatory factors) has been shown to modify SCN functioning in vivo (13, 15)

or to alter SCN electrical properties in vitro (34, 35). Astrocytes mediate innate immunity within the CNS, sensing and expressing several proinflammatory factors (reviewed in 36), and there is an increasing amount of evidence supporting an important role of these cells in circadian physiology. Indeed, SCN astrocytes are activated upon immune stimulation, both in vitro and in vivo (20, 37). In this study, we show that SCN astrocytes exhibit rhythmic Per2^{luc} expression, which can be modulated by the proinflammatory cytokine TNF-α. The effects of TNF-α on the molecular clock of SCN astrocytes were phase dependent. We found an increase in the amplitude of Per2^{Luc} rhythm only when applied 2 h after the peak, but not when applied at the trough. Conversely, the effect on the phase was enhanced when the cells were treated at the trough, when compared with that found 2 h after the peak. This inverse relationship between the magnitude of the phase shift and the amplitude of the Per2^{Luc} rhythms has been previously described in other system (i.e., photic stimulus on light-sensitive transgenic fibroblasts) (38). Differential circadian control over the expression of the components of the signaling pathway activated by TNF-α, which ultimately affect clock gene expression, could account for the phase dependency of the effects of TNF-α. Indeed, members of both NF-κB and p38 pathways (which are activated by TNF-α) present circadian rhythmicity on murine macrophages (39), and the TNFR-α type 1 presents diurnal variations on the mouse SCN (13). To which extent the effects of alterations in the astrocyte circadian clock affect SCN outputs is still unknown, and should be subject of further investigation.

Astrocytes have been described to be important for the regulation of complex brain mechanisms, such as sleep homeostasis (40), memory (41–44), and circadian rhythms (45–47). Considering the sensitivity of the SCN to immune stimuli and the capacity of astrocytes to respond to proinflammatory factors and to modulate neuronal physiology, we hypothesized that SCN astrocytes could act as mediators in the communication between the immune and the circadian systems. Using conditioned media from SCN astrocytes, we showed that, in response to a transient TNF-α stimulus, SCN astrocytes can modulate the expression of Per1 on mouse fibroblast cell cultures, through the release of TNF-α. The effects of TNF-α on the molecular clock of NIH-3T3 cells have been previously described (5, 48), producing a transient increase in Per1 mRNA expression within the first hour after treatment, followed by a suppression lasting for 36 h. Our results show an increase in Per1 promoter activity and Per1 mRNA expression, measured 24 h after the end of the treatment with TNF-α or TNF-α-CM. The main difference between the two experimental designs is the duration of the treatment. In the work of Cavadini et al. (5), cells were synchronized with a serum shock and left with the cytokine afterward. In contrast, our approach was to perform a transient treatment with TNF-α or TNF-α-CM, followed by a medium change. Medium change has been shown to synchronize fibroblast cells (49), and TNF-α has been described to induce Per1 expression at short times after treatment in NIH-3T3 cells (48). This last effect could enhance the synchronization due to the medium change and therefore produce the higher Per1 levels upon TNF-α treatment found under our experimental design.

Factors secreted by SCN astrocytes, in response to a transient immune stimulus, also produced important changes in the master circadian oscillator. TNF-α-CM treatment of SCN slices resulted in phase shifts of PER2 expression rhythms, only when the treatment was applied 1–2 h after the peak, but not close to the trough of PER2 expression. PER2 expression in the SCN peaks at about CT 12 in vivo (50), so treatments within the first hours after the PER2 peak in SCN slices would mimic early subjective night in vivo treatments, in terms of PER2 phase. Indeed, the moment of
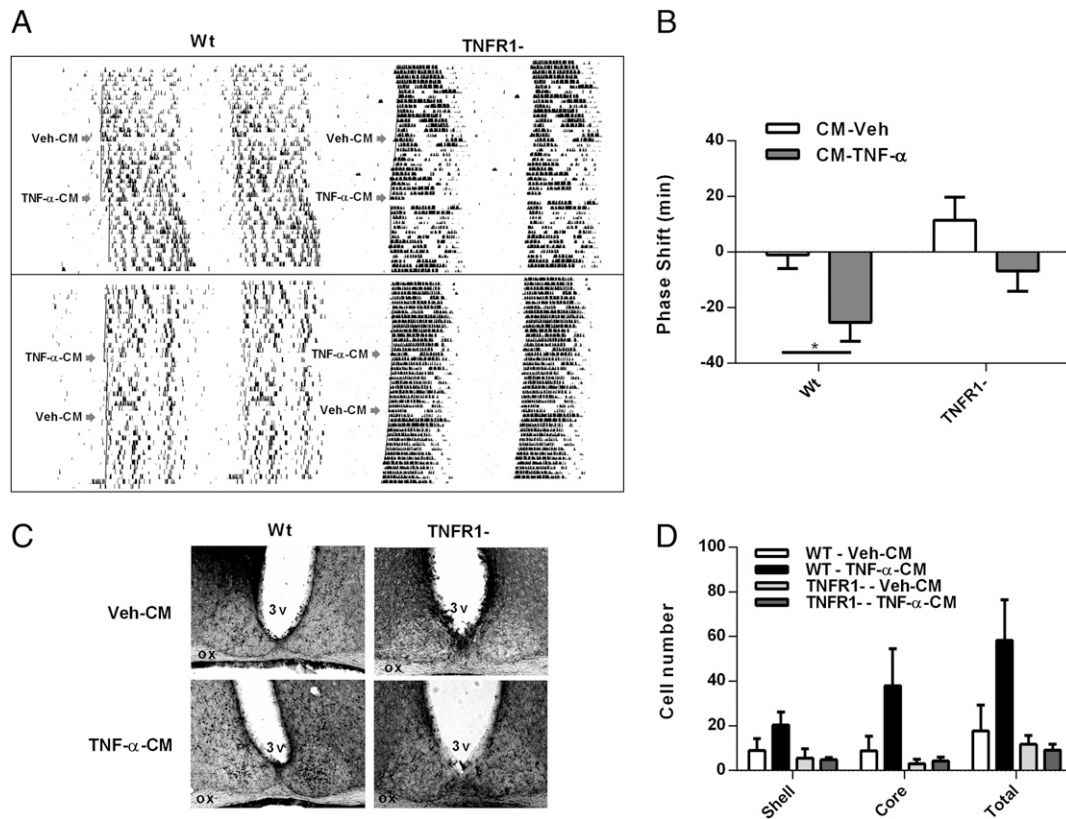
FIGURE 5. Conditioned media from TNF-α–challenged SCN astrocytes induce phase shifts in locomotor activity rhythms. SCN astrocyte primary cultures were treated as expressed for Fig. 2. (**A**) Representative actograms of Wt (*left panel*) or TNFR⁻ (*right panel*) animals receiving icv administration of conditioned media from TNF-α– or Veh-treated SCN astrocytes, at CT 15. Arrows indicate the day of treatment. Animals were in DD conditions throughout the days represented in the actograms. (**B**) TNF-α-CM delivered icv at CT 15 produced significant phase delays on locomotor activity rhythms in Wt mice, but not in TNFR- mutants. The -25.3 ± 6.8 min for Wt mice receiving TNF-α-CM, -1.1 ± 4.9 min for Wt mice receiving Veh-CM, -6.8 ± 7.4 min for TNFR⁻ mice receiving TNF-α-CM and versus 11.4 ± 8.2 min for TNFR- mice receiving Veh-CM (two-way ANOVA analysis followed by Sidak's multiple comparisons post hoc test; *$p < 0.01$ for Wt-TNF-α-CM versus Wt-Veh-CM; $p > 0.05$ for TNFR⁻, TNF-α-CM versus TNFR⁻, Veh-CM; $n = 8$–9). (**C**) Representative SCN coronal sections illustrating cFos expression 90 min after icv administration of TNF-α-CM or Veh-CM in Wt and TNFR⁻ mice. (**D**) TNF-α-CM increased the number of cFos-expressing cells in the SCN core (two-way ANOVA, followed by Tukey's multiple comparisons post hoc test; $p < 0.001$ for Wt-TNF-α-CM versus Wt-Veh, $p < 0.0001$ for Wt-TNF-α-CM versus TNFR⁻, TNF-α-CM, $p < 0.001$ for Wt-TNF-α-CM versus TNFR⁻, Veh-CM, $p > 0.05$ for all other pairwise comparisons) and shell (two-way ANOVA, followed by Tukey's multiple comparisons post hoc test; $p < 0.05$ for Wt-TNF-α-CM versus Wt-Veh, $p < 0.01$ for Wt-TNF-α-CM versus TNFR⁻, TNF-α-CM, $p < 0.01$ for Wt-TNF-α-CM versus TNFR⁻, Veh-CM, $p > 0.05$ for all other pairwise comparisons) regions and in the whole SCN area (two-way ANOVA, followed by Tukey's multiple comparisons post hoc test; $p < 0.01$ for Wt-TNF-α-CM versus Wt-Veh, $p < 0.001$ for Wt-TNF-α-CM versus TNFR⁻, TNF-α-CM, $p < 0.01$ for Wt-TNF-α-CM versus TNFR⁻, Veh-CM, $p > 0.05$ for all other pairwise comparisons); $n = 3$–4.

sensitivity for immune stimuli in the ex vivo study correlates with the time window at which the circadian system can be modulated by immune stimuli in vivo (16). Furthermore, in vivo administration of TNF-α-CM into the SCN region during the early subjective night (CT 15) induced activation of SCN cells and elicited phase shifts on wheel-running activity rhythms. It is important to state that the SCN can be divided into two general areas, based on the expression of different neuropeptides: 1) the ventrolateral, retinorecipient area (also named core), rich in vasoactive intestinal

polypeptide and gastrin-releasing peptide-expressing neurons; and 2) the dorsomedial (also named shell) region, which presents arginine-vasopressin–expressing neurons (51, 52). Increased cFos immunoreactivity in the SCN has been described upon both peripheral (i.p. LPS) (14, 16) and central [cytokine cocktail, icv (13)] immune stimulation during the early night, with predominant expression in the shell region in the first case and in the core region in the later. Our results also showed that a central stimulus (icv TNF-α-CM delivery) produced an increase in the number of

FIGURE 6. Astrocytes modulate circadian rhythms through TNF-α. In response to an inflammatory condition, induction of TNF-α in the CNS may induce changes in the molecular clock of SCN astroglia and induce the release of TNF-α from these cells, amplificating the initial signal. This cytokine, released by SCN astrocytes, may induce clock gene expression, activation of SCN cells, and shifts in behavioral output rhythms.
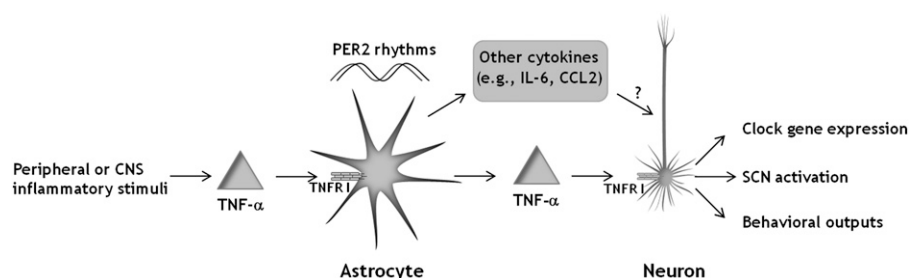
cFos-expressing cells in both SCN regions, suggesting that there are different mechanisms of SCN activation, depending on the nature and administration route of immune modulators.

The analysis of the conditioned media showed that TNF-α treatment induced its own secretion in SCN astroglia, and blockade of this cytokine with the addition of TNFsR attenuated the effects of conditioned media on clock gene expression in fibroblasts. Furthermore, the in vivo responses to TNF-α-CM delivered icv disappeared in mice lacking functional TNF-α receptors. These results suggest that TNF-α is at least partially responsible for the circadian effects of the conditioned media from immune-challenged astrocytes (although we cannot discard the possibility that TNF-α secretion could be induced in NIH 3T3 cells or cells within the CNS by other molecules present in the TNF-α-CM). Besides its role in pathological inflammation, a physiologic role for TNF-α in normal brain function has also been proposed (53); indeed, TNF-α from unchallenged astrocytes influenced synaptic strength (54), supporting the idea that glial cells can modulate neuronal functioning through the action of this cytokine. Both SCN PER2^{Luc} rhythms and behavioral circadian output are mediated by neuronal activity (55, 56), which implies that the effects of the TNF-α-CM on both outputs involve changes in SCN neuronal functioning. Our present findings imply that SCN astrocytes could be mediators of circadian modulation possibly functioning as amplifiers of the TNF-α signal, which would ultimately induce changes in clock gene expression in SCN cells and in the phase of the master circadian clock.

Besides the secretion of TNF-α, other mechanisms exerted by astrocytes could be responsible for the effects of conditioned media on clock gene expression and circadian behavioral outputs. The expression of IL-1β signaling receptor, IL-1R1, presents diurnal variation in the mouse SCN, and icv delivery of this cytokine into the SCN resulted in phase shifts of locomotor activity rhythms (14, 15). We found that Per1 expression in fibroblast cells could be induced by 5 and 50 ng/ml IL-1β (Supplemental Fig. 2); however, IL-1 antagonism failed to attenuate the effects of TNF-α-CM on NIH 3T3 clock gene expression. These results suggest that, although the molecular clock of murine fibroblasts is sensitive to IL-1β, this cytokine is not primarily involved in the alteration of clock gene expression by SCN astrocyte-conditioned media. In addition, CCL2 and IL-6 secretion was upregulated after TNF-α treatment of SCN astrocytes (Supplemental Fig. 1). These molecules have been shown to play a role on different brain functions (57, 58) and in circadian physiology (28, 59). The precise role of CCL2 and IL-6 in the interaction between the immune and the circadian system remains to be investigated.

Glial regulation of circadian physiology has been a subject of investigation over the last years. There is a differential day/night glial coverage of SCN neurons dendrites that has been suggested to be important to the pacemaker-resetting mechanism (60). Moreover, SCN astrocytes respond to different neurotransmitters (glutamate, serotonin) increasing intracellular calcium (61), and inhibition of glial metabolism transiently disrupts circadian rhythms in neuronal and locomotor activity (45), further supporting the idea that glial cells might be important for adequate circadian pacekeeping. Recent works have demonstrated circadian rhythms in extracellular ATP from astrocyte cultures, which depends on clock gene machinery (62, 63), and this gliotransmitter could act as a circadian output from astroglial cells, with effects on both glia itself and neurons. Our results show, for the first time to our knowledge, that factors secreted by SCN astrocytes (particularly TNF-α) are able to modulate circadian physiology, stressing the importance of these cells in the functioning of the master biological clock (Fig. 6). In addition, several pathologies implicating proinflammatory

situations can lead to dysregulation of circadian rhythms, and also involve activation of glial cells (11, 64–68). Our results suggest that, upon activation by a proinflammatory signal produced by a pathological state, SCN astrocytes could be an important cellular substrate mediating the circadian outcome of the disease. These findings are important for contributing both to the understanding of the role of glial cells in the circadian clock and to the characterization of the mechanisms by which the immune system modulates biological rhythms.

Disclosures

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