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NEUROSYSTEMS

Circadian entrainment to light–dark cycles involves extracellular nitric oxide communication within the suprachiasmatic nuclei

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Abstract

The ability to synchronize to light–dark (LD) cycles is an essential property of the circadian clock, located in mammals within the hypothalamic suprachiasmatic nuclei (SCN). Single light pulses activate nitric oxide (NO) intracellular signaling, leading to circadian phase-shifts required for synchronization. In addition, extracellular NO has a role in the SCN paracrine communication of photic phase advances. In this work, the extracellular nitrergic transmission was assessed in steady-state synchronization to LD cycles of locomotor rhythms in the golden hamster (*Mesocricetus auratus*). Extracellular NO levels were pharmacologically decreased *in vivo* with the specific scavenger, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO). Hamsters were subjected to LD cycles different from normal 24 h (LD 14 : 10) cycles (i.e. T-cycles), with a single 30-min light pulse presented either every 23 h (T23 cycles), or every 25 h (T25 cycles), thus allowing synchronization by advances or delays, respectively. Acute PTIO intracerebroventricular microinjections, delivered 30 min previous to the light pulse, inhibited synchronization by phase advances to T23 cycles, but did not alter phase delays under T25 cycles. In addition, NO scavenging inhibited light-induced expression of PERIOD1 protein at circadian time 18 (i.e. the time for light-induced phase advances). These findings demonstrate the role of extracellular NO communication within the SCN in the steady-state synchronization to LD cycles.

Introduction

Virtually all physiological and behavioral processes in mammals are temporally organized by a master circadian clock located within the hypothalamic suprachiasmatic nuclei (SCN). The 24 h light–dark (LD) cycle is the main signal synchronizing the SCN clock, which sets the phase of its rhythmic outputs. Although the nuclei appear to be composed by single-cell neuronal circadian oscillators (Welsh *et al.*, 1995; Yamaguchi *et al.*, 2003), the SCN are now considered a heterogeneous organ, mainly subdivided into a dorsomedial 'shell' and a ventrolateral 'core' (Silver & Schwartz, 2005). These subregions differ in their cytoarchitecture, connectivity, topography and histochemistry (Leak & Moore, 2001; Moore *et al.*, 2002; Kriegsfeld *et al.*, 2004). The retinorecipient ventrolateral cells respond to a photic stimulus during the subjective night, with an increase in the expression of the clock genes of the Period (Per) family and the immediate-early genes of the Fos family, whereas the dorsomedial cells show a circadian oscillation in the expression of these genes (Antle & Silver, 2005). Although the dorsomedial region acts as a stronger autonomous oscillator, synchronization and phase-resetting of these neurons depends to a great extent on neuropeptide release from the ventrolateral region (reviewed in Aton & Herzog, 2005; Silver & Schwartz, 2005).

A putative messenger that could be involved in such regional interaction is nitric oxide (NO), a highly reactive small gaseous molecule that is able to regulate a set of physiological processes, including vascular tone, blood vessel remodeling and cellular immunity (Marletta, 1989). NO is synthesized in SCN neurons from L-arginine by the enzyme neuronal NO synthase (nNOS; Wang & Morris, 1996), and an endogenous circadian rhythm of NO production in dorsomedial SCN was found in rats (Mitome *et al.*, 2001). NO is a component of intracellular signaling of photic phase-shifts within the SCN (Ding *et al.*, 1994), leading to specific transduction pathways for phase advances or delays (reviewed in Gillette & Mitchell, 2002; Golombek *et al.*, 2004). While NO activation of guanylyl cyclase and cGMP-related mechanisms are necessary for light-induced advances (Ferreyra & Golombek, 2001; Agostino *et al.*, 2007), early night stimulation triggers the NO activation of ryanodine receptors leading to phase delays (Ding *et al.*, 1998; Pfeffer *et al.*, 2009).

Besides its role as an intracellular messenger, the membrane permeability and short half-life of this gas make it an ideal neurotransmitter acting as a fast and specific extracellular signal for the communication of photic information within the SCN (Nathan, 1992; Golombek *et al.*, 2004). Furthermore, *in vivo* evidence for a circadian role of extracellular NO was provided by Plano *et al.* (2007), where inhibiting such communication blocked phase advances in

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response to brief light pulses, together with the ventral-dorsal SCN spreading of the immediate-early gene c-Fos expression.

In this work, a steady-state synchronization protocol was used, based on LD cycles composed by a 30-min light pulse, presented with a period shorter (T = 23 h) or longer (T = 25 h) than 24 h. By means of the NO selective scavenger, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), NO levels were acutely reduced within the SCN *in vivo* in the extracellular matrix, without affecting nNOS activity (Akaike *et al.*, 1993; Maeda *et al.*, 1994; Triguero *et al.*, 2000). In this way, we have studied whether extracellular NO is involved in the synchronization to LD cycles, testing the hypothesis if NO is either participating during steady-state advances (under T = 23 h) or delays (T = 25 h), or both. In addition, we have tested the modulation by extracellular NO of light-induced PER1 expression in the SCN.

Materials and methods

Behavioral recordings

Adult male Syrian hamsters (*Mesocricetus auratus*) were kept under 14-h light, 10-h dark (LD 14 : 10) cycles (room temperature ~20–24°C, humidity ~60–70%). After recovery from surgery (see below), hamsters were placed in light-isolated cabinets with fluorescent lamps (Phillips 300 W) delivering ~150 lux of indirect cool lighting at cage floor level (Mavolux 5032B luxmeter; Camille Bauer, Wohlen, Switzerland). Light intensity was recorded through a photocell within the cabinets. For locomotor activity recording, animals were placed in 40 × 15 × 30 cm aluminum cages with 17-cm-diameter running wheels equipped with magnetic switches for the detection of revolutions. A data acquisition system designed in our laboratory acquired and stored 5-min bin data in a computer. Food pellets and tap water were supplied *ad libitum*. Cage and bedding replacement was done every ~7 days, preferably in darkness.

Surgery and microinjections

Hamsters were surgically implanted with 22-gage stainless steel guide cannula (PlasticsOne®, Roanoke, VA, USA) for intracerebroventricular (i.c.v.) injections. The animals were deeply anesthetized with a 75 mg/kg ketamine and 10 mg/kg xylazine cocktail by intraperitoneal route. Stereotaxic surgery allowed the implantation of a cannula aimed at the bottom of the third ventricle in the SCN region (coordinates from bregma: +0.6 mm anterior, −8.0 mm ventral, 0.0 mm from midline, toothbar set at −2.0 mm; Morin & Wood, 2001). PTIO (Sigma-Aldrich, St Louis, MO, USA) 100 μM was prepared fresh each day by 1 : 10 dilution with saline solution from a 1 mM stock solution in 5% ethanol–saline. Acute i.c.v. microinjections were performed with an internal injector connected to a microsyringe (Hamilton, Reno, NV, USA), delivering a total dose of 1 μL/min. Drug (100 μM) or vehicle (0.5% ethanol–saline) injections were administered 30 min before light onset under dim red light (< 1 lux) in T-cycle protocols, only in those individuals previously showing steady-state synchronization. PTIO 100 μM was also delivered in hamsters under constant darkness (DD protocol), without T-cycle exposure. Animals were free to move during this procedure.

T-cycles protocol

Hamsters (n = 8) were initially exposed to normal LD 14 : 10 cycles for 5 days. Then, a 30-min light pulse (~150 lux) coincident with the beginning of the previous LD photophase, and 22.5 h of darkness, was imposed to allow steady-state synchronization by phase advances. This regime (L: 0.5 h, D: 22.5 h, T23) was kept for 10 cycles (stage T23) Then, animals showing stable synchronization (i.e. period equal to T; n = 5) received an i.c.v. injection of PTIO (T23–PTIO) or vehicle (T23–vehicle), for six successive cycles (n = 4, one animal discarded due to cannula displacement). After this, animals were kept under T-cycles for 10 (T23–post-PTIO) or 6 (T23–post-vehicle) additional days, to observe, respectively, re-synchronization following the drug or vehicle treatments. Finally, hamsters were transferred to DD conditions for 10 days. For synchronization to T25 cycles, hamsters (n = 10) were placed under LD 14 : 10 for 5 days. Then 24.5 h of darkness was followed by 30-min light pulse falling at the end of the previous photophase (L: 0.5 h, D: 24.5 h, T25), for five cycles. After stable synchronization to T25 (n = 4), the animals were injected with either PTIO (T25–PTIO) or vehicle (T25–vehicle) for six cycles. Finally, hamsters were transferred to DD for 10 days. Stable synchronization to T was determined by direct actogram scoring and by periodogram analysis.

Two groups of hamsters were also included to study the effects of PTIO administration on the free-running period (DD protocol). Animals were kept under DD conditions and subjected to the following sequence: 5 days without treatment (DD), 5 days with PTIO administration (DD–PTIO), 5 days without treatment (DD–post PTIO). In one of the groups (n = 4), the DD–PTIO stage involved five PTIO doses administered every 23 h, starting the first at circadian time (CT)18 (thus imposing a T23 schedule of PTIO). The other group (n = 5) received PTIO injections with a T = 25 h schedule, the first starting at CT14.

PER1 immunohistochemistry

Free-running animals were divided into subgroups to receive i.c.v. microinjection of PTIO (PTIO + LP, n = 6) or vehicle solution (LP, n = 5) 30 min before a light pulse (150 lux, 30 min) at CT18, and PTIO (PTIO, n = 3) or vehicle solution (Dark, n = 4) at CT18, without light exposure. Hamsters were anesthetized 180 min later, perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, and their brains were removed and fixed for 5 additional hours. The tissue was cryoprotected in 30% sucrose in phosphate-buffered saline (PBS) solution, obtaining 30-μm coronal sections with a freezing microtome to perform a standard immunohistochemical procedure. Briefly, sections were incubated for 72 h in PBS with 0.4% Triton (PBST) at 4°C with rabbit anti-PER1 antibody (Affinity BioReagents, Rockford, IL, USA) diluted 1 : 200, washed in PBST, incubated with biotinylated universal secondary antibody (Vector Labs, Burlingame, CA, USA) diluted 1 : 200, and finally incubated with the avidin-biotin complex (Vectastain Elite ABC kit; Vector Labs). The sections were then reacted with Vector Vip reagents (Vector Labs) to visualize the peroxidase reaction, and mounted on microscope slides. The number of PER1-immunoreactive SCN cells (PER1-ir cells) was determined using the IMAGEJ program (NIH).

Locomotor activity rhythm and statistical analyses

Locomotor activity data were binned into 15-min sets by performing a moving average procedure. Period was estimated by means of χ^2 periodogram calculated in data sections corresponding to each stage (highest significant peak for $P < 0.05$, range: 20–28 h), obtaining also the percentage of variance explained by this period (i.e. robustness of rhythm). Also, locomotor data sections with a length equal to T were averaged (i.e. T-module waveforms) at each stage to calculate the phase relationship between the rhythm and the T-cycle (Aschoff,

1981). Activity onset was determined as the time when the waveform crossed the average level for at least 2 h. Then, the phase relationship was calculated as the difference between the time of lights-ON and the activity onset, hence resulting in positive values for T23 and negative for T25 cycles, due to synchronization by phase advances or delays, respectively. In all individuals, data sections corresponding to the last five cycles of each T-cycle stage were used for both period and percentage of variance calculated from periodogram analysis, and for the phase relationship calculated from waveforms. Circadian phase control by the previous T-cycle was evaluated at T-cycle–DD transitions. First, an eye-fitted line was drawn on the actograms by taking the activity onset during the first 7 days in DD, and projected backwards to the last T-cycle. Then, these projected activity onsets were studied with the Rayleigh z-test (Batschelet, 1981), which calculates the significance in the clustering of individual phases along the T-cycle. If clustering is statistically significant, this indicates the homogeneity of T-cycle effects on the individual phases (i.e. phase control).

All variables (rhythm period, percentage of variance, phase relationship and the number of PER1-ir cells) were reported using the mean value, and standard error of the mean (SEM) for the respective stages or groups. Two-way ANOVA considering the T-cycle stage, and the treatment (PTIO or vehicle) as factors was used to compare the behavioral variables in both T23 and T25 protocols. Planned comparisons were used in the case of significance for treatment. One-way ANOVA analysed differences in the number of PER1-ir cells between groups, and in both the period and percentage of variance in the DD protocols (T23 and T25 PTIO schedules) between stages. Time-series analysis was done by means of an integrated package of tools for chronobiology, 'El Temps' (© Antoni Díez Noguera, University of Barcelona).

All animal manipulations and experimental protocols used here were supervised and approved by the University Institutional Animal Care and Use Committee, in agreement with policies and laws of the Office of Laboratory Animal Welfare, National Institutes of Health, USA.

Results

T23 cycles protocol

Exposure of naive animals from LD 14 : 10 to T23 cycles generated a decrease in the circadian period in 50% of the animals, which maintained a stable 23-h period (T23 stage, 23.1 ± 0.05 h), with the onset of locomotor activity occurring about 6–7 h before the time of lights-ON (Fig. 1A). These animals showed a consistent

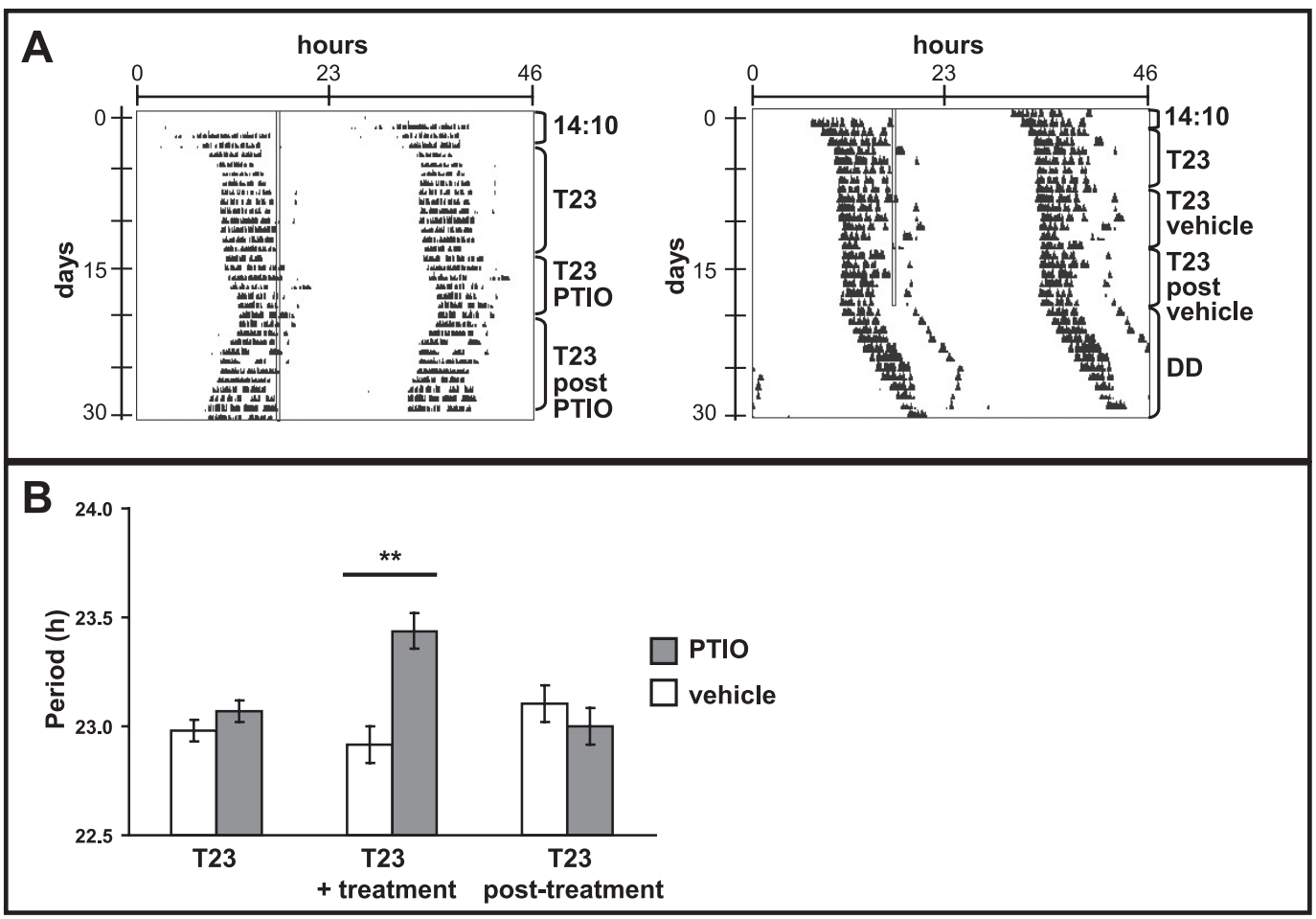

FIG. 1. Locomotor activity rhythms of hamsters under T23 cycles. (A) Panels exhibit representative actograms double-plotted at 23 h format of 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO; left) and vehicle-treated (right) groups. The white bar depicts the T23 cycle photoperiod (30 min of light), experimental stages ranges are indicated with brackets. Note the period increment at the T23–PTIO stage, the following transients and the re-acquisition of the 23-h period after treatment withdrawal (T23–post PTIO). Vehicle-treated hamsters kept a 23-h period at all T23 stages. (B) Period calculated with periodogram (mean ± SEM) of each corresponding T23 stage, for both PTIO (gray bars) and vehicle (white) groups (**$P < 0.01$, planned comparison, two-way ANOVA).

response to the NO scavenger, which elicited a period lengthening (T23–PTIO, 23.44 ± 0.08 h) after drug microinjections for six successive cycles (Fig. 1A). Furthermore, transient advances could be observed in the actograms immediately after removing drug treatment, followed by stable synchronization (T23–post-PTIO, 23.00 ± 0.08 h), indicating the effect of the recovery of T-cycle on the circadian phase. Vehicle injections did not modify the synchronized period during T23 cycles (Table 1) (Fig. 1A). Two-way ANOVA demonstrated significance in treatment ($P < 0.05$) as well as in treatment–stage interaction ($P < 0.01$), but not in stage ($P > 0.05$). A significant period increment due to PTIO was demonstrated when comparing between T23–PTIO and T23–vehicle ($P < 0.01$; Fig. 1B). The percentage of variance explained by the T23 rhythm was unaffected by either treatment or stage ($P > 0.01$, two-way ANOVA). In addition, a similar phase relationship was calculated under all T23 stages (N/A for T23–PTIO; $P > 0.05$, two-way ANOVA). Significant phase grouping at the T23–DD transition verified the phase control of locomotor rhythms by previous T23 cycle [Rayleigh z-test: $r = 0.97$, $P < 0.05$, r(alpha) = 0.84]. All subjects showing a T23 period free-run in DD from the expected phase relationship with the T-cycle (Fig. 1A, actogram on the right).

T25 cycles protocol

In the animals subjected to T25 cycles, circadian periods lengthened from 24 h at LD 14 : 10 to close to 25 h, matching the T-cycle in about 50% of the animals tested. These periods synchronized to T25 were unaffected by either PTIO or vehicle (Fig. 2). Actograms also exhibited locomotor activity bouts anticipating light onset, followed by a transient inhibition of locomotion (i.e. negative masking) after lights-ON. T25 periods were not significantly dependent on either treatment or stage ($P > 0.01$, two-way ANOVA; Fig. 2B). Either the percentage of variance of the rhythm or the phase relationship were independent of both treatment and stage ($P > 0.1$, two-way ANOVA). After being released under DD, both groups exhibited significant phase grouping in the Rayleigh z-test [for T25–PTIO: $r = 0.8$, $P < 0.05$, r(alpha) = 0.76; for T25–vehicle: $r = 0.95$, $P < 0.05$, r(alpha) = 0.76]. Subjects also showed the expected phase relationship in the actograms when they were allowed to free-run in DD (Table 1) (see Fig. 2A).

DD protocol

Animals receiving daily i.c.v. PTIO treatment under DD conditions did not exhibit a significant change in their free-running periods

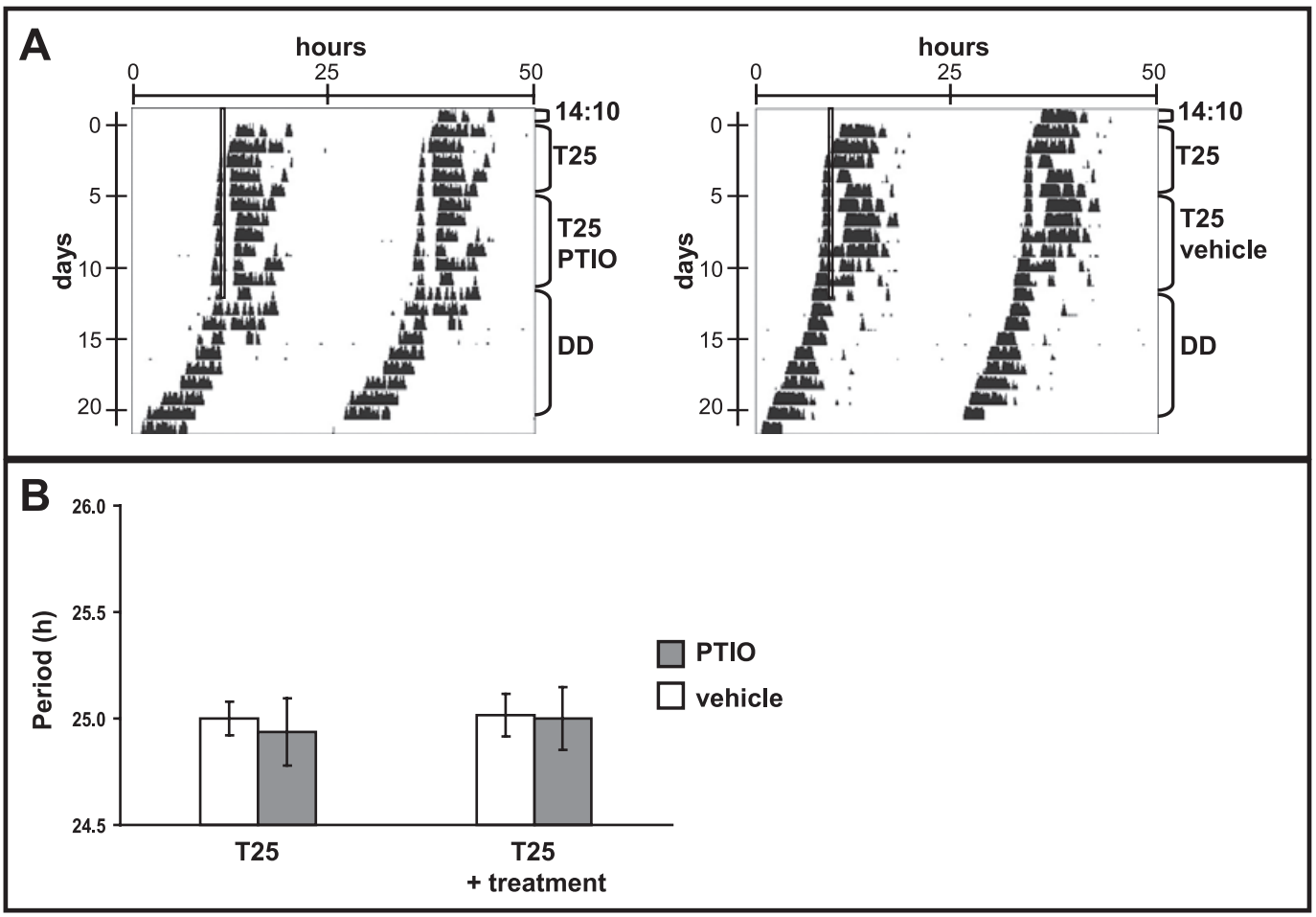

FIG. 2. Locomotor activity rhythms of hamsters under T25 cycles double-plotted in actograms in 25-h format (same description as for Fig. 1). (A) Hamsters receiving drug (left panel) at T25–2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) stage, and vehicle (right panel) at T25–vehicle stage. Treatment did not modify the period synchronized to T25 cycles. (B) Mean ± SEM of period values obtained from periodogram of both PTIO (gray bars) and vehicle (white) groups for the experimental stages. T25-h period was independent of both treatment and stage.

(Fig. S1), neither when they were subjected to the T23 schedule of PTIO injections ($P > 0.1$, one-way ANOVA), nor under the T25 schedule ($P > 0.1$, one-way ANOVA).

PER1 immunohistochemistry

Immunohistochemical detection of PER1 protein within the SCN revealed a marked inhibition of the light-induced expression due to the treatment with the NO scavenger PTIO (Fig. 3). An approximately 10-fold increment was induced after light pulses at CT18 (LP). Administration of PTIO 30 min before the light pulse (PTIO + LP) inhibited this photic induction of PER1. Moreover, PTIO alone did not affect PER1. Statistical comparison between groups demonstrated higher significant expression for the light pulse group ($P < 0.01$, ANOVA).

Discussion

In this work, a design of T23 and T25 cycles composed by a 30-min light pulse was performed to impose steady-state phase advances or delays, respectively, obtaining synchronized locomotor rhythms to both T-cycles. The phase relationships, as well as the coincidence between the rhythm phase under the T-cycle and that after being released in DD, suggest that the endogenous clock activity was driven by the T-cycle, discarding any masking effects (Johnson *et al.*, 2003). A 23-h T-cycle was established for this work based on previous experiments in the laboratory using a T23.5 cycle (data not shown), closer to τ, which is more effective to synchronize, but does not allow to reveal changes due to pharmacological treatment. Selection of a 30-min light pulse was also based on previous work (Plano *et al.*, 2007). With a similar criterion, a T25 cycle was imposed to induce synchronization by delays.

TABLE 1. Variables obtained from locomotor activity rhythm analyses under the different treatments and stages of T23 and T25 protocols

Protocols, treatments and stages	Period (h)	Variance (%)	Phase relationship (h)
T23 protocol			
PTIO treatment			
T23 ($n = 4$)	23.10 ± 0.05	65.82 ± 2.15	6.53 ± 0.53
T23-PTIO ($n = 4$)	23.44 ± 0.08**	52.97 ± 5.89	n.a.
T23-post PTIO ($n = 4$)	23.00 ± 0.09	60.84 ± 5.17	6.33 ± 1.39
Vehicle			
T23 ($n = 4$)	22.98 ± 0.04	58.69 ± 1.65	6.41 ± 0.34
T23-vehicle ($n = 4$)	22.92 ± 0.06	56.96 ± 3.77	6.33 ± 0.43
T23-post vehicle ($n = 4$)	23.10 ± 0.09	49.09 ± 5.15	6.31 ± 0.51
T25 protocol			
PTIO treatment			
T25 ($n = 4$)	24.94 ± 0.16	53.4 ± 2.70	−1.32 ± 0.37
T25-PTIO ($n = 4$)	25.00 ± 0.14	62.6 ± 8.00	−1.92 ± 0.71
Vehicle			
T25 ($n = 4$)	25.00 ± 0.08	65.10 ± 6.61	1.62 ± 0.14
T25-vehicle ($n = 4$)	25.02 ± 0.10	57.10 ± 7.20	−1.40 ± 0.34

Values are means ± SEM. For period analysis, two-way ANOVA demonstrated significance for treatment as factor ($P > 0.05$, $^{**}P < 0.01$, planned comparison for T23–PTIO vs. T23–vehicle). T23, a 23-h cycle with a single 30-min light pulse every 23 h; T25, a 25-h cycle with a single 30-min light pulse every 25 h; PTIO, the specific NO scavenger, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; T23-post PTIO, animals were kept under T23-cycles for 10 additional days; T23-post vehicle, animals were kept under T23-cycles for 6 additional days.

Under T23 cycles, phase advances underlying steady-state synchronization were systematically blocked due to NO scavenger treatment. Because the percentage of variance did not differ under T-cycle stages, our results suggest a brief, specific drug effect on phase-advancing mechanisms, without disruptions in circadian rhyth-

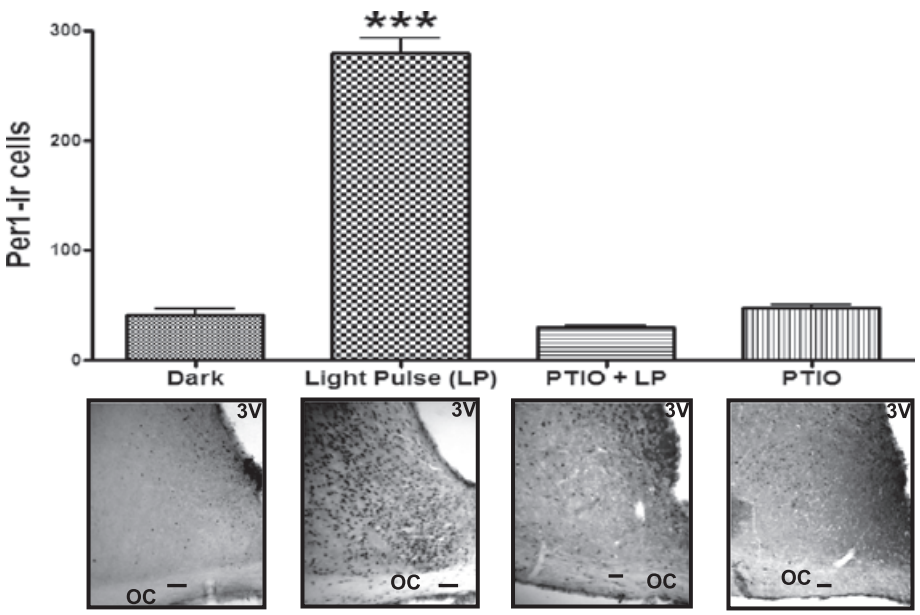

FIG. 3. Immunohistochemical detection of light-induced PER1 protein within the SCN under NO scavenger treatment. The number of SCN PER1-ir cells (mean ± SEM) are shown for the following groups: vehicle (Dark, $n = 4$), light pulse (LP, $n = 6$) at CT18, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) 30 min prior to light pulse at CT18 (PTIO + LP, $n = 5$) and PTIO at CT17.5 (PTIO, $n = 3$). About a 10-fold increment from basal (Dark) PER1 expression was induced by light (LP). This photic induction was strongly inhibited to basal levels by previous NO scavenging (PTIO + LP). $^{***}P < 0.001$, Tukey test for unequal N, light pulse vs. dark, PTIO + LP or PTIO alone. Representative SCN-sections are shown in the photographs in the lower panels. 3V, third ventricle; OC, optic chiasm.

micity. On the other hand, under T25 cycles NO scavenger treatment did not modify the synchronized period. Although it is possible that our results respond to unspecific effects of NO scavenging, such as a change in vascular tone, or even pharmacokinetics changes, the phase-specificity of its effects suggests that PTIO is directly affecting the circadian phase-resetting. Moreover, the aforementioned results are in agreement with previous work using single light pulses, in which PTIO blocked both light-induced phase advances and the cFos protein expression as a marker of the ventral-dorsal spread of neuronal activity in the SCN after photic stimulation (Plano *et al.*, 2007). The present work studied the immunohistochemical light-induced expression of PER1 at late subjective night, finding a reliable increase after a 30-min pulse, in agreement with previous studies (Hamada *et al.*, 2001). PER1 induction was shown to be strongly inhibited by NO scavenging treatment. The *per1* gene is a key participant in the molecular feedback loop that generates circadian rhythms, and is critically involved in resetting the endogenous clock to light signals (Shigeyoshi *et al.*, 1997; Albrecht *et al.*, 2001). Thus, these results suggest that reduced NO communication within the SCN at late subjective night modify the molecular phase-resetting mechanism driving steady-state advances of locomotor rhythm.

The intracellular transduction mechanism by which NO is able to modify the circadian phase was the subject of extensive investigation. Photic increment of NO levels activates GC-cGMP-dependent kinase (PKG), specifically during the late subjective night for light-induced phase advances (reviewed in Golombek *et al.*, 2004). Modulation of this pathway reaches the molecular clock by modifying behavioral, neuronal activity, cGMP and Per1 rhythms (Tischkau *et al.*, 2003b; Agostino *et al.*, 2007). Moreover, NO and glutamatergic transmission induced phosphorylation of Ca^{2+}/cAMP response element-binding protein (CREB; Ding *et al.*, 1997), which activates Per1 transcription for photic advances (Tischkau *et al.*, 2003a). In addition to NO regulation of Per1, it was recently shown that transcription levels of brain, muscle Arnt-like protein-1 (Bmal1), which heterodimerizes with CLOCK to inhibit Per1, can be regulated by NO through reversion of orphan nuclear hormone receptors (REV-ERBs)-mediated repression (Pardee *et al.*, 2009). In the present work, we show that extracellular NO is necessary for steady-state synchronization only by phase advances, which correlates with photic Per1 activation. Although this mechanism is unclear, it is interesting that NO scavenging exerts a different effect on synchronization that inhibits intracellular NO-generating mechanisms by NO synthase inhibitors (Ding *et al.*, 1994; Weber *et al.*, 1995). This suggests a different role for intracellular NO as a second messenger or for extracellular NO as a paracrine neurotransmitter. Although pharmacokinetic explanations cannot be ruled out at present, an alternative hypothesis could be that extracellular NO is not equally available for intracellular signaling at different times of day, thus allowing for a temporal gating of NO modulation of light-induced phase changes.

Based on our current results, extracellular NO could be proposed as an anterograde diffusible signal in the SCN tissue, acting through the GC-cGMP-PKG pathway by which light synchronizes circadian rhythms. However, NO could also act independently of the light signal, as a messenger for the coupling between autonomous clock cells setting the circadian period (Liu *et al.*, 1997; Chiesa *et al.*, 2006; Vasalou *et al.*, 2009) for synchronization. Our experimental results suggest that this latter hypothesis should be discarded, as the circadian period under DD did not change with PTIO treatment (Fig. S1). Intercellular communication mechanisms are necessary for the SCN to act *in vivo* as a tissue clock with coherent circadian outputs. Several mechanisms, including voltage-gated Na^+ channels-dependent action potentials (Yamaguchi *et al.*, 2003), electrical synapses (Long *et al.*, 2005) or vasoactive intestinal peptide (Maywood *et al.*, 2006), have been implicated in such communication. NO transmission was also proposed for cellular/regional oscillator coupling (Michel & Colwell, 2001; Golombek *et al.*, 2004). Indeed, SCN phase-resetting should be taken as a tissue property, involving photic modulation of intercellular coupling (Quintero *et al.*, 2003; Silver & Schwartz, 2005). This could be achieved by decreasing gap-junctional currents (Long *et al.*, 2005) through extracellular NO-dependent alterations of connexins, as it occurs in the retina (Bloomfield & Völgyi, 2009).

Finally, further experiments using NO scavengers or donors (e.g. on Per1-luc transgenic tissue) would certainly be useful to study extracellular NO communication by either synchronizing (coupling) individual cells, or by gating ventral-dorsal photic information. Also, chronic treatment with these drugs might be useful to study the putative role of NO communication in the regulation of circadian period, or in parametric protocols (i.e. synchronization to complete LD cycles).

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Locomotor activity rhythms under DD protocols.

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Abbreviations

CT, circadian time; DD, constant darkness; i.c.v., intracerebroventricular; ir, immunoreactive; LD, light–dark; nNOS, neuronal NO synthase; NO, nitric oxide; PBS, phosphate-buffered saline; PBST, PBS with 0.4% Triton; Per, Period; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; SCN, suprachiasmatic nuclei; T23, a 23-h cycle with a single 30-min light pulse every 23 h; T25, a 25-h cycle with a single 30-min light pulse every 25 h.

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