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N-NITROSOMELATONIN ENHANCES PHOTIC SYNCHRONIZATION OF MAMMALIAN CIRCADIAN RHYTHMS

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Abstract

Most physiological processes in mammals are synchronized to the daily light:dark cycle by a circadian clock located in the hypothalamic suprachiasmatic nucleus. Signal transduction of light-induced phase advances of the clock is mediated through a neuronal nitric oxide synthase-guanilyl cyclase pathway. We have employed a novel nitric oxide-donor, *N*-nitrosomelatonin, to enhance the photic synchronization of circadian rhythms in hamsters. The intraperitoneal administration of this drug before a sub-saturating light pulse at circadian time 18 generated a two-fold increase of locomotor rhythm phase-advances, having no effect over saturating light pulses. This potentiation was also obtained even when inhibiting suprachiasmatic nitric oxide synthase activity. However, *N*-nitrosomelatonin had no effect on light-induced phase delays at circadian time 14. The photic-enhancing effects were correlated with an increased suprachiasmatic immunoreactivity of cFOS and PER1. Moreover, *in vivo* nitric oxide release by *N*-nitrosomelatonin was verified by measuring nitrate and nitrite levels in suprachiasmatic nuclei homogenates. The compound also accelerated resynchronization to an abrupt 6-h advance in the light:dark cycle (but not resynchronization to a 6-hour delay). Here we demonstrate the chronobiotic properties of *N*-nitrosomelatonin, emphasizing the importance of nitric oxide-mediated transduction for circadian phase advances.

Keywords

circadian; *N*-nitrosomelatonin; suprachiasmatic nucleus; nitric oxide; jet lag

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FMB performed the experiments, analyzed the data and wrote the paper. SAP performed the behavioral experiments of NOMel effects on photic entrainment. FD and SAS synthesized the *N*-nitrosomelatonin and provided advice on its use. DAG and JJC designed the experiments, contributed to data interpretation and analysis, and revised and wrote the final version of the manuscript.

INTRODUCTION

Most mammalian circadian rhythms are orchestrated by a biological clock located in the hypothalamic suprachiasmatic nuclei [(SCN) Moore, 2013 (Stetson & Watson-Whitmyre 1976, Menaker *et al.* 2013)]. Circadian rhythms must be kept in phase (i.e., synchronized) with the 24-h light:dark (LD) cycle to maintain a stable temporal relationship with the environment (Golombek & Rosenstein 2010). Photic information from the LD cycle is the main synchronizing stimulus for the SCN clock, and consequently of its circadian outputs. In behavioral protocols, the circadian phase is modified when a single light pulse is delivered concomitant with nocturnal locomotor activity [i.e., the "subjective night" under constant darkness (DD)]. Specifically, delaying or advancing the clock will occur if light is delivered at the beginning or at the end of the subjective night, respectively (Pittendrigh 1981, Gillette & Mitchell 2002).

The ventrolateral "core" SCN sub-region receives the major retinal afferences from a subset of ganglion cells, responding to light stimulation by glutamatergic transmission through metabotropic glutamate-N-methyl-D-aspartate receptor (NMDAr) (Vileikyte *et al.* 2005). Calcium (Ca2+) influx through NMDAr is followed by activation of both intracellular Ca2+/calmodulin-dependent kinase II and neuronal nitric oxide synthase (nNOS), leading to an increase of nitric oxide (NO) synthesis (Golombek *et al.* 2000, Agostino *et al.* 2004). Pharmacological inhibition of nNOS blocks both light-induced phase-advancing and delaying mechanism (Watanabe *et al.* 1995, Melo *et al.* 1997, Golombek *et al.* 2004). However, it is suggested that downstream of nNOS there is a bifurcation of the pathway. Activation of the guanylyl cyclase-cGMP (GC) and cGMP-dependent protein kinase is involved in phase advances but not in delays (Golombek et al. 2004). However, the activation of the ryanodine receptor (RyR) at the endoplasmatic reticule is involved in light-induced phase delays (Ding *et al.* 1998, Pfeffer *et al.* 2009). Regardless of this difference, both signaling pathways (through GC or RyR) modify the expression of clock genes at the core of the molecular circadian oscillator (Akiyama *et al.* 1999, Albrecht *et al.* 2001).

Besides intracellular signal transduction, the SCN neuronal network coupling is also modulated in circadian synchronization (Quintero *et al.* 2003, Shirakawa *et al.* 2001). Light-induced-glutamatergic transmission sets the phase of the ventrolateral-retinorecipient neurons of the SCN (Antle & Silver 2005). In turn, these neurons release different neurochemical signals [e.g., vasoactive intestinal polypeptide (Ibata *et al.* 1989, Reed *et al.* 2001, Watanabe *et al.* 2000, Aida *et al.* 2002, Vasalou & Henson 2011), gastrin-releasing peptide (Aida et al. 2002, Dardente *et al.* 2002, Vasalou & Henson 2011)] towards the dorsomedial region of the SCN. We have previously demonstrated that NO can also participate as an extracellular messenger, by coordinating the ventrolateral-dorsomedial SCN communication necessary for photic synchronization (Plano *et al.* 2007, Plano *et al.* 2010).

In this work, we have employed a novel NO-donor, *N*-nitrosomelatonin (Kirsch & de Groot 2009, Turjanski *et al.* 2000b) to increase NO levels within the SCN tissue, in order to potentiate photic synchronization of locomotor activity rhythms. Previous studies have demonstrated that this drug can efficiently release NO *in vitro* (Berchner-Pfannschmidt *et al.*

2008, Peyrot *et al.* 2005, Blanchard-Fillion *et al.* 2001, De Biase *et al.* 2005), but there are still no *in vivo* studies assessing its neurochemical effects on circadian synchronization. In particular, we have studied the chronobiotic properties of NOMel using both light-pulse and jet-lag behavioral protocols. These experiments led us to propose some mechanistic insights about NO signal transduction in the photic synchronization pathway.

MATERIALS AND METHODS

Materials and drug treatments

N-nitrosomelatonin (NOMel) was synthesized as previously described (De Biase et al. 2005). Melatonin (Mel) was purchased from Sigma Chem Co. (St. Louis, MO, USA). *N*-nitro-*L*-arginine methyl ester (L-NAME) was obtained from Cayman chemicals ® (Ann Arbor, MI, USA).

Although the half life of NOMel in acqueous solution is in the range of seconds (De Biase et al. 2005), a previous pharmacokinetic experiment in mice with radiolabelled NOMel showed that the drug reaches the brain about 10 minutes after an i.p. injection of 5 mg/kg (Peyrot et al. 2005). We tested a similar dose of 2 mg/kg (see below) and a higher one (20 mg/kg, *see* supplementary materials) and decided to deliver the drug 15 min before the light pulse. Vehicle was composed by 0.5% ethanol in sterile saline (Veh). L-NAME was used at a final concentration of 1mM in 0.9% saline (Weber *et al.* 1995).

Animals and surgery

Male adult (3–4 months old) Syrian hamsters (*Mesocricetus auratus* from our own colony derived from the stock at Laboratorios Azul Diagnóstico, Buenos Aires) were raised under a 14-h light:10-h dark cycle [(14:10 LD), lights off at 20.00 hours], averaging 200 lux at bedding level, with food and water *ad libitum* and room temperature set at 20 ± 2°C. For those experiments where intracerebroventricular (i.c.v.) administration was requeried, a 22-gauge steel guide cannulae (Plastic Products, Roanoke, VA) was implanted under ketamine:xylazine [(150:10 mg/kg, intraperitoneal (i.p.)] anesthesia, aimed at 1.0 mm above the target site between the bilateral SCN (coordinates: anterior/posterior = +0.6, medial/lateral = 0.0, dorsal/ventral = −8.0 mm from bregma). After surgery, hamsters were housed in 14:10 LD cycle to recover for 7 to 10 days before being transferred into constant darkness (DD) for the duration of the experiment. All animal procedures were performed in strict accordance with the National Institutes of Health guidelines; in addition, our experiments comply with the ARRIVE guidelines for animal research.

Behavioral experiments

For the light-pulse experiments, hamsters were transferred to individual cages equipped with a running wheel (17-cm diameter) under constant dark (DD) conditions for at least 15 days, with food and water *ad libitum*. Wheel running activity was continuously recorded and data stored at 5-min intervals for further analysis. Hamsters received an i.p. injection of either NOMel, Mel or Veh 15 min, before stimulation with a 10-min pulse of “cool” white light [TL-D Standar, with a moderate infra-red coating (50–75), Philips®] of 50-lux intensity [light-pulse (LP)]. LPs were delivered at circadian time (CT) 14 (i.e., 2 hours after

locomotor activity onset in DD, defined as CT 12) to induce phase delays, and at CT18 to induce phase advances. The CTs were chosen according to the hamster phase-response curve [(PRC) (Dunlap *et al.* 2004, Rosenberg *et al.* 1991, Smith *et al.* 1992)] in order to evaluate drug effects when major phase shifts are obtained. The photic stimulus parameters (10 min, 50 lux) were chosen to produce submaximal phase shifts; however, a saturating light pulse was also evaluated (10 min, 300 lux). To evaluate the mechanism involved in phase-shift potentiation, a co-administration of a NOS inhibitor (L-NAME) and NOMel was performed. L-NAME 1mM was prepared fresh on the day of injection, and a 1-μl volume of drug or vehicle was injected i.c.v. during 1 min. Hamsters received an i.p. injection of either NOMel, Mel or Veh, together with an i.c.v L-NAME or Veh injection 15 min before a LP at CT18. Experimental manipulations in the dark were performed under dim red light (<5 lux). Locomotor activity onset was calculated from actogram-like graphs as a typical marker of the circadian phase. It was defined as the 10-min bin that contained at least 80 wheel revolutions, followed by another bin of at least another 80 wheel revolutions within 15 min (Edelstein *et al.* 2003). Phase shifts were calculated by fitting a line through activity onsets 5 days prior to, and between 5 and 15 days after, light exposure; the time difference between the extrapolation of these two lines (i.e., a phase-shift) was taken on the day of the LP (the mean of the calculations made by three observers, masked to the experimental procedure, was reported). To determine changes in the free-running period of locomotor rhythm, Chi-square periodograms were calculated on data sets corresponding to 10 days before and 10 days after the treatments.

Another group of animals transferred to individual cages equipped with a running wheel were initially maintained under a 14:10 LD cycle [lights off (L-Off) time at 14.00 h and lights on (L-On) at 24.00 h] for at least 10 days. Hamsters were subjected to an abrupt 6-h change in the phase of the LD cycle (i.e., an experimental jet-lag protocol). For the 6 h-phase advance protocol (see Supp Fig. 1 A), animals had a “short-night switch”: on the day of the cycle change, L-Off was maintained at 14.00 h, while L-On was advanced from 24.00 h to 18.00 h. On the following day L-Off was advanced from 14.00 to 8.00 h, to set the new 14:10 LD cycle (L-Off 8.00 h; L-On 18.00 h). For the phase delay protocol (Supp. Fig. 1 B), animals had a “long-day switch”: on the day of the cycle change, L-Off was delayed from 14.00 h to 20.00 h and L-On the following day from 24.00 to 6.00 h, setting the new 14:10 LD (cycle: L-Off 20.00 h; L-On 6:00 h). Drug treatment was done on the same day of the cycle change. Groups of animals were divided to receive an i.p. injection of NOMel, Mel or Veh, at zeitgeber time (ZT) 18 [L-Off of the LD cycle previous to the cycle change was defined as ZT12)] or at ZT14 for the advancing or delaying protocol, respectively. The time for re-entrainment of the locomotor activity rhythm to the new LD cycle was defined as the number of days that it took for each animal to adjust its locomotor rhythm with the new LD cycle (onset at the new time of lights off ± 15 min), reporting the average calculations of three observers masked to the experimental procedure.

Immunohistochemistry

Animals maintained under a 14:10 LD cycle were transferred to DD conditions for an entire day. On the second day under DD, the ZT was extrapolated to CT [i.e., a type-II Aschoff protocol (Aschoff 1965)]. For the cFOS experiments, one group of animal recived and i.p

injections of NOMel, Mel and Veh, 15 min before a 10 min-50 lux LP (sub-saturating), delivered both at CT14 and CT18 (controls only received drug treatment). Another group of animals received the same drug treatment at CT18, but with a saturating light pulse (300 lux, 10min). For the PER1 immunohistochemistry, a third group of animals received the NOMel and Veh i.p. treatment 15 min before a sub-saturating LP at CT18 (controls only received drug treatment). Animals were deeply anesthetized with a ketamine:xylazine (150:10 mg/kg, i.p.) cocktail one or three hours (for cFOS or PER1, respectively) after the LP and then intracardially perfused with 0.01M phosphate buffer saline (PBS) followed by fixative solution (4% w/v paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Brains were then dissected, post-fixed overnight at 4° C in the same solution, cryoprotected in a 30% sucrose-PBS solution for 24 h, and frozen at −80° C for one day. Coronal sections (30 μm thick) were obtained with a cryostat and collected in 0.01 M PBS. Sections were washed with 0.4% Triton X-100 in 0.01 M PBS (PBST). Nonspecific binding sites were blocked with 2% normal horse serum and 5% nonfat dry milk in PBST for 1.5 h at room temperature. Sections were incubated with cFOS (diluted 1:2000, Santa Cruz Biotechnology Inc.) or PER1 (diluted 1:500, Thermo Scientific, Pierce Antibody) primary antibody in PBST for 72h at 4° C. Biotinylated anti-mouse/rabbit IgG (H+L) made in horse (Vector Laboratories) diluted 1:200 in PBST was used as a secondary antibody for 2 h at room temperature. The reaction was amplified with the avidin-biotin complex and visualized with the VIP chromogen substrate (Vectastatin Elite ABC kit, Vector Laboratories). In order to include most of the SCN neurons, quantification of the positive cells was done over four consecutives slices for each brain and the mean value was used for statistical analysis. For the PER1 experiment, a regional ventrolateral and dorsomedial SCN quantification was done (Hamada *et al.* 2001, Abrahamson & Moore 2001). Cell counting was performed with the Image J. 1.29 software (NIH) in hypotalmic sections as previously described (Marpegan *et al.* 2005).

Nitrate and nitrite measurement

Animals following a type II Aschoff protocol (see “immunohistochemistry” subsection) received i.p. injections of NOMel, Mel or Veh 15 min before a LP at CT18 (controls only received drug treatment). Animals were decapitated immediately after the LP and their brains were removed and frozen for one hour at −80° C. A 1 mm thick-single coronal slice of the brain was cut just above the optic chiasm location and a 1 mm diameter-punch containing the SCN was obtained. SCN tissue was disrupted in 500 μl PBS, pH 7.4. Homogenates were filtered using Vivaspin-500 ultrafilters of 3,000 MWCO (Sartorius stedim biotech). Nitrate plus nitrite levels at CT18 were analyzed with the Nitrate/Nitrite Colorimetric Assay Kit, LDH Method (Cayman chemical®) and the Bio-Rad Protein Assay (Bio-Rad) was used to measure the protein concentration of each sample. For statistical analysis, each nitrate and nitrite value was considered first relative to the corresponding protein concentration sample, and then to the mean value of the vehicle group.

Statistical Analysis

A two-way ANOVA was designed for assessing changes in the phase shift, relative nitrate and nitrite level, and the number of cFOS and PER1 positive cells, by taking both the stimulus (LP or darkness), drug treatments (NOMel, L-NAME, L-NAME-NOMel, Mel or

Veh) and dorsomedial and ventrolateral areas (for PER1) as factors, while a one-way ANOVA was designed to study the effect of drug treatment on the number of days to resynchronize in the jet lag protocols. A two-way ANOVA taking repeated measures as factors was done to assess changes in the free-running period. After detecting significant ANOVA factors, post-hoc tests were carried out to study specific contrasts within levels of factors. The level of significance for all statistical tests was set at p=0.05.

RESULTS

Effect of *N*-nitrosomelatonin on phase shifts

All LPs administered at CT18 elicited phase advances of hamster wheel running activity rhythms [Fig. 1a (Two-way ANOVA, for light treatment: $p<0.0001$)]. Furthermore, NOMel-LP animals exhibited a two-fold increase in the magnitude of phase advances as compared to either Veh-LP or Mel-LP groups (Two-way ANOVA, for drug treatment: $p<0.0001$, followed by Bonferroni post-tests: NOMel-LP vs. Veh-LP $p<0.001$, NOMel-LP vs. Mel-LP $p<0.001$; Veh-LP and Mel-LP: n = 8, NOMel-LP: n = 9). No significant differences were found between Mel-LP and Veh-LP groups ($p>0.05$), or between drug treatments without LP (Veh alone vs Mel alone $p>0.05$, Veh alone vs. NOMel alone $p>0.05$, Mel alone vs. NOMel alone $p>0.05$; Mel alone and Veh alone: n = 7, NOMel alone: n=8). The effect of NOMel on saturating light-induced phase advances was also evaluated (Fig. 2a). All LP-treated groups had significant phase shifts as compared to controls (Two-way ANOVA, for light treatment: $p<0.0001$), but no significant differences were found between drug treatments. The phase shifts obtained for the saturating light pulses were similar to those obtained for the NOMel-treated group with sub-saturating LPs ($p>0.05$). Moreover, treatment with NOMel alone or together with LP (both 50 or 300 lux) did not affect the free running period [for NOMel alone: 24,03 ± 0,10 h vs. 24,12 ± 0,33 h ($p>0.05$); and for NOMel-LP_50lux: 23,98 ± 0,15 h vs. 24,16 ± 0,21 h ($p>0.05$); NOMel-LP_300lux: 24,01 ± 0,54 vs. 23,88 ± 0,48; mean ± SEM free-running period before vs. after treatment, respectively].

All LPs administered at CT14 generated a circadian phase delay [Fig. 1b (Two-way ANOVA, for light treatment: $p<0.001$)]. However, no significant differences were found between the LP-treated groups (Veh-LP vs. Mel-LP $p>0.05$, Veh-LP vs. NOMel-LP $p>0.05$, Mel-LP vs. NOMel-LP $p>0.05$; Mel-LP and Veh-LP: n = 6, NOMel-LP: n = 7), or between drug treatments without the LP (Veh alone vs Mel alone $p>0.05$, Veh alone vs. NOMel alone $p>0.05$, Mel alone vs. NOMel alone $p>0.05$; Mel alone and NOMel alone: n = 5; Veh alone: n = 7). A higher dose of NOMel (20mg/kg, see supplementary materials) was also evaluated (Supp. Fig. 2), which also failed to potentiate the light-induced phase delays ($p>0.05$), while no significant differences were found between the higher and the lower dose ($p>0.05$). In addition, no significant changes in the free-running period were obtained at CT14 after the injection of NOMel [for NOMel alone: 23,88 ± 0,10 h vs. 24,03 ± 0,20 h ($p>0.05$); for NOMel-LP: 24.12 ± 0,16 h vs. 24,02 ± 0,35 h ($p>0.05$); mean ± SEM free-running period before vs. after the treatment, respectively].

The effect of NOMel administration on non-photic phase shifts, normally induced during the subjective day (maximum at CT 6), was also evaluated (see supplementary materials).

Regardless the drug treatment, non-photic stimuli generated a small but significant phase advance [(Supp. Fig. 3) Two way ANOVA, for non-photic treatment: $p<0.0001$)]. However, NOMel had no effect on non-photic phase shifts, nor induced any change *per-se* in circadian phase when delivered at CT 6.

Effect of *N*-nitrosomelatonin on cFOS immunohistochemical expression in the suprachiasmatic nucleus

An increased number of cFOS-positive cells was found in the SCN of the hamsters after receiving the light treatment, as compared to those that only received drug treatment, both at CT14 and CT18 [Fig. 3 (Two-way ANOVA, for light treatment: $p<0.0001$, $n = 4$ for all groups)]. Treatment with NOMel before the LP at CT18 induced an increase of cFOS-positive cells with respect to controls ($p<0.001$), which was not observed at CT14 ($p>0.05$). The administration of NOMel before the LP at CT18 generated a ~50% increase of cFOS-positive cells when compared to both Mel-LP or Veh-LP groups (Two-way ANOVA, for drug treatment: $p<0.0001$; Bonferroni post-test: NOMel-LP vs. Veh-LP $p<0.001$, NOMel-LP vs Mel-LP $p<0.001$). No significant differences were found between Mel-LP and Veh-LP groups ($p>0.05$), or between those treated only with the drugs (Veh alone vs. Mel alone $p>0.05$, Veh alone vs. NOMel alone $p>0.05$, Mel alone vs. NOMel alone $p>0.05$) for both CT18 and CT14.

We also evaluated cFOS induction following a saturating LP at CT18 (Fig. 2b). All LP-groups showed an increased number of positive cells as compared to controls (Two-way ANOVA, for light treatment $p<0.0001$). NOMel treatment also increased light-induced cFOS induction as compared to controls (Bonferroni post-test: NOMel-LPsat vs Veh-LPsat $p<0.01$ and NOMel-LPsat vs. Mel-LPsa $p<0.01$; $n=4$ for all groups).

Effect of *N*-nitrosomelatonin on PER1 immunohistochemical expression in the suprachiasmatic nucleus

An increased number of PER1-positive cells was found in the SCN of the hamsters after receiving the light treatment, as compared to those that only received drug treatment at CT18 [Fig. 4 (Two-way ANOVA, for light treatment: $p<0.0001$, $n = 5$ for all groups)]. Treatment with NOMel before the LP at CT18 induced an increase of PER1-positive cells with respect to control at the dorsomedial (DM) region of the SCN (Two-way ANOVA, for drug treatment: $p<0.001$; Bonferroni post-test: DM-NOMel-LP vs. DM-Veh-LP $p<0.001$), without affecting the PER1 expression in the ventrolateral region (VL-Veh-LP vs. VL-NOMel-LP $p>0.05$). No significant differences were found between those treated only with the drugs both at the ventrolateral and dorsomedial regions (DM-Veh alone vs. DM-NOMel alone $p>0.05$; VL-Veh alone vs. NOMel alone $p>0.05$).

Effect of *N*-nitrosomelatonin on the photic induction of suprachiasmatic nitrate and nitrite levels

The analysis of vehicle-treated groups show that the light treatment *per se* had no significant effect on SCN-nitrate and nitrite levels (Fig. 5). In addition, Mel treatment by itself did not affect SCN nitrate and nitrite levels. However, regardless of the photic stimulation, the treatment with NOMel generated a significant increase in nitrite-nitrate levels when

compared either to Veh or Mel-treated animals (Two-way ANOVA, for drug treatment: $p<0.0001$; Bonferroni post-test: Veh alone vs Veh-LP, $p>0.05$, Mel alone vs Mel-LP, $p>0.05$, NOMel alone vs. Veh alone or Veh-LP, $p<0.05$, NOMel alone vs. Mel alone or Mel-LP, $p<0.05$, NOMel-LP vs. Veh alone or Veh-LP, $p<0.05$, NOMel-LP vs. Mel alone or Mel-LP, $p<0.01$, Veh alone and Mel-LP: n = 9, NOMel alone: n = 10, Mel alone and NOMel-LP: n = 11, Veh-LP: n = 12). To verify if the availability of NOMel in the brain is dependent on CT, SCN-nitrite levels were also measured both at CT14 and CT18 after the administration of NOMel or vehicle (see supplementary materials). NOMel increased SCN nitrite leves similarly at both CTs (Supp. Fig. 4; Two way ANOVA, for drug treatment: $p<0.005$; for CT: $p>0.05$).

Effect of the co-administration of NOMel with a NOS inhibitor

Light pulses induced significant phase advances in vehicle-treated animals [Fig. 6 (Two-way ANOVA, $p<0.0001$ for light, and $p<0.05$ for drug treatment; Bonferroni post-test: Veh+Veh alone vs. Veh+Veh-LP, $p<0.05$)]. These phase advances were blocked by the previous i.c.v. treatment with L-NAME (Veh+Veh-LP vs. L-NAME+Veh-LP, $p<0.05$). However, when NOMel was co-administered with the NOS inhibitor before the light stimulation, significant phase advances were still obtained (L-NAME+Veh-LP vs L-NAME-NOMel-LP, $p<0.05$). In addition, no differences were found between the Veh+Veh-LP and the L-NAME-NOMel-LP groups ($p>0.05$), or between all the groups treated only with the drugs (Veh+Veh alone vs. L-NAME+Veh alone, $p>0.05$, Veh+Veh alone vs. L-NAME-NOMel alone, $p>0.05$, L-NAME+Veh alone vs. L-NAME-NOMel, $p>0.05$; Veh+Veh alone, Veh+Veh-LP: n=3, L-NAME-NOMel alone and L-NAME+Veh-LP: n = 4, L-NAME+Veh alone: n = 5, L-NAME-NOMel-LP: n = 7).

Effect of *N*-nitrosomelatonin on resynchronization to an experimental jet-lag protocol

Animals treated with NOMel at ZT18 on the day of a 6-hour phase advance in the LD cycle significantly accelerated the resynchronization rate to the new cycle, when compared either to the Mel or Veh groups [Fig. 7 (One-Way ANOVA, $p<0.001$, followed by Tukey Test: NOMel vs. Veh $p<0.05$, NOMel vs. Mel $p<0.05$, Veh vs. Mel $p>0.05$; Mel: n = 6, Veh: n = 7, NOMel: n = 8)]. On the other hand, no differences were found between the drug treatments in the 6-hour phase delay protocol (Veh vs. Mel $p>0.05$, Veh vs. NOMel $p>0.05$, Mel vs. NOMel $p>0.05$; Mel: n = 10, NOMel and Veh: n = 12).

DISCUSSION

Our behavioral experiments consistently show that NOMel potentiates light-induced phase advances of locomotor activity rhythms of hamsters, without affecting photic phase delays. This phase-dependent effect was observed in both LP (Fig. 1) and simulated jet-lag protocols (Fig. 7). Even when a 10x higher dose was evaluated for light-induced phase delays, no potentiation was obtained (see Supp. Fig. 2). Taking these results into account, we only evaluated the lowest dose for the rest of the experiments. It should also be stated that all animals showed a normal behavior (e.g., stereotypical grooming, exploration, and gross motor activity) after the injection of the drug, even for the higher dose (data not shown).

Even though not directly correlated with the magnitude of the phase shifts (Trávnícková et al. 1996), light exposure during the night induces the expression of several immediate early genes, including c-fos (Golombek *et al.* 2003, Kornhauser *et al.* 1996, Porterfield & Mintz 2009), which is considered as a marker of neuronal activation in the circadian clock. Moreover, PER1 is a key component of the molecular clock (Buhr & Takahashi 2013), strongly induced by light and strictly correlated with photic phase shifts (Shigeyoshi et al. 1997). We have previously demonstrated that the blockade of extracellular NO communication within the SCN impeded both photic behavioral advances as well as the ventral-dorsal spreading of cFOS expression within the SCN, while it does not affect phase delays (Plano *et al*. 2007). In addition, the same treatment also inhibited both photic advances for the steady-state synchronization to LD cycles, and SCN-PER1 expression at CT18, without affecting the synchronization by phase delays (Plano *et al*. 2010). In agreement with our previous reports, here we show that NOMel-treated animals only exhibited an increased number of photically-induced SCN cFOS-positive cells at CT18, and importantly, an increased number of PER1 immunoreactivity at the dorsomedial region of the SCN, demonstrating the same kind of neuronal activation as when modulating extracellular NO communication. Therefore, our results support the idea that NO, as an extracelullar messenger, is involved only in the signalling of photic phase advances. On the other hand, NOMel did not potentiate phase advances induced by saturating light pulses (Fig. 2a) but did increase cFOS induction in response to such stimulation (Fig. 2b). As stated before, cFOS induction is not necessarily correlated with the magnitud of the phase shift. It is possible that the saturating light-induced phase changes in locomotor activity may be limited by the circadian pacemaking system, reaching a ceiling effect that cannot be further modulated by our pharmacological treatment. In contrast, NOMel increased even further the number of cFOS-expressing cells in response to bright light pulses. We assume that the increased levels of cFOS induction could be due to an enhanced extracellular NO communication; however, this effect does not correlate with behavioral phase shifts because of the ceiling effect mentioned before. In all cases, NOMel potentiates the effects of light on gene expression, but lacks any circadian effects *per se*, suggesting that NO is capable of modulating the signal transduction pathway of photic entrainment, but is not sufficient to drive changes in the circadian pacemaker.

We hypothesized that the NOMel potentiation observed in both behavioral and immunohistochemical results might be due to an increase of NO levels acting both as an intracelullar as well as an extracellular messenger. Therefore, in order to evaluate if this compound is indeed working as a NO-donor, we decided to measure nitrate and nitrite levels (i.e., the main NO metabolites) in the SCN after the drug administration (Fig. 5). SCN nitrate and nitrite levels confirm that NOMel is working as an efficient NO-donor *in vivo*. In addition, taking into account the behavioral, the immunohistochemical and the nitrate-nitrite results, we can confirm that the increase of NO levels *per se*, without light stimulation, is not enough to generate a circadian phase advance. On the other hand, nitrate levels are used as an indirect way to measure NOS activity (Kleinbongard *et al.* 2003), and *ex vivo* measurement of brain tissue nitrate and nitrite levels accurately reflects NOS activity *in vivo* (Salter *et al.* 1996). However, in our experiments light stimulation did not generate a significant increase of SCN nitrate and nitrite levels as compared to controls. In order to

evaluate NO synthesis, it would be necessary to take SCN samples several minutes after light stimulation. However, as this was not one of the objectives of the present work, where SCN tissue was immediately obtained after the LP, future experiments will be necessary to confirm this hypothesis. In addition, since SCN nitrite leves measured both at CT14 and CT18 after NOMel treatment were similar (see Supp. Fig. 4), we can discard any circadian-dependent differences in the bioavailability of the drug, in relation to the timing of NOMel administration.

In order to determine the mechanism by which NOMel is modulating the photic pathway in the SCN, we co-administered L-NAME, a well-known NOS inhibitor (Weber et al. 1995), together with an i.p. injection of NOMel, followed by a light pulse. We hypothesized that the NO levels generated by the NOMel treatment, regardless of the photic NOS activation, would allow the photic signal transduction cascade to continue, by activating the required substrates downstream of NOS, thus generating the phase shift. The present results agreed with our hypothesis (Fig. 6). Therefore, NOMel should be delivering the appropriate NO levels for the continuation of the photic pathway, but it can not activate the pathway by itself. Although not measured in this work, we assumed that NO levels in other tissues would be increased due to the systemic NOMel administration. However, the central inhibiton of the NOS activity performed in the present experiments, together with the increased nitrate and nitrite levels found, allow us to suggest that the main effect of the NOMel is due to its action at the SCN.

We also evaluated the effect of the drug on the collectively named “non-photic” phase shifts, using a cage and bedding change as stimulus [(Supp. Fig. 3) (Mistlberger 1992, Yannielli & Harrington 2004)]. The administration of NOMel at CT6 did not affect non-photic phase advances induced by such stimulus, nor generated a phase advance *per se*. To our knowledge, there is no data about the participation of NOS in the transduction of these circadian phase shifts.

The photic-signal transduction pathway bifurcates downstream of NO: while GC is involved in circadian phase advances, the opening of the RyR is related to the phase-delaying mechanism. It has been shown that another type of NO-donor, S-Nitroso-N-acetylpenciallamine (SNAP), can potentiate both phase advances and delays, at the same CTs as we have studied in this work (Melo et al. 1997). However, our results with NOMel indicate that this drug can only enhance phase advances. Therefore, NOMel could only be potentiating the activation of the GC, but not that of the RyR. Indeed, SNAP can stimulate the opening of RyR in other tissues (Hart & Dulhunty 2000, Wang *et al.* 2010), and this mechanism involves the S-nitrosylation of the thiol group of cysteine residues, generating a S-NO group (Gonzalez *et al.* 2008, Kakizawa *et al.* 2012). S-nitrosilation by NOMel has only been demonstrated for the glyceraldehyde 3-phosphate dehydrogenase under *in vitro* conditions (Kirsch & de Groot 2008). We presume that under *in vivo* conditions, the NO levels released by NOMel had little thiol reactivity able to increase the S-nitrosylation of the RyR, which is likely involved in the aperture of the channel for enhancing the phase-delaying effects of light. Another important difference is that the activation of the GC is much more sensitive to NO activation, within the nanomolar range (Russwurm & Koesling 2004, Rodriguez-Juarez *et al.* 2007, Hall & Garthwaite 2009). Instead, S-nitrosylation is

considered as a short-ranged mechanism in which a high concentration of NO is necessary (Evangelista *et al.* 2013, Martinez-Ruiz *et al.* 2013). This was taken into account for the inclusion of the experiment in which a 10-times higher dose of NOMel was evaluated at CT14. However, NOMel still failed to potentiate the phase-delaying effects of light. In addition, SNAP can actually be considered as a low molecular weight S-nitrosothiol (Oliveira *et al.* 2008), since the NO-group is linked to the rest of the molecule through a sulfur bond. Therefore, a trans-nitrosyaltion (Matsumoto & Gow 2011, Nakamura & Lipton 2013) of the RyR could also be hypothesized, since SNAP trans-nitrosylates other substrates by de-nitrosylating itself [similar to the glutathion or thioredoxin systems (Sengupta & Holmgren 2013)]. The NOMel compound does not have this ability, because the NO-group is linked to the rest of the molecule through a nitrogen-nitrogen bond [*N*-nitrosomelatonin (Turjanski et al. 2000a)]. Therefore, we hypothesize that it is because of the nature of the NO released, and/or of the mechanism by which it is released, that NOMel can only potentiate the light-induced phase advances.

In conclusion, we have demonstrated the chronobiotic properties of NOMel, enhancing photic synchronization of circadian rhythms. Importantly, these are the first *in-vivo* experiments showing the NO-donor property of this drug. We propose that this drug can eventually be tested in other mammalian models, in order to evaluate its ability for the treatment of different types of circadian disorders which affect human health, such as those occuring in shift-work or jet-lag. Melatonin by itself has been reported to be adequate for treating and preventing jet lag in human trials (Caspi 2004, Herxheimer & Petrie 2002). We are introducing here the nitroso version of this compound, which has a promising future for studies aimed to treat circadian alterations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

14:10 LD	14-h light:10-h dark
CREB	Ca2+/cAMP response element binding protein
CT	circadian time
DD	constant darkness
GC	guanylyl cyclase
i.p	intraperitoneal
L-NAME	*N*-nitro-*L*-arginine methyl ester

LD	light:dark
LP	light pulse
L-Off	lights off
L-On	lights on
Mel	melatonin
nNOS	neuronal nitric oxide synthase
NOMel	*N*-nitrosomelatonin
NO	nitric oxide
Per1	period1
PBS	phosphate buffer saline
PBST	phosphate buffer saline-tween
PRC	phase response curve
RHT	retino-hypothalamic tract
RyR	ryanodine receptor
SCN	suprachiasmatic nuclei
SNAP	S-nitroso-N-acetylpenicillamine
Veh	vehicle
ZT	zeitgeber time

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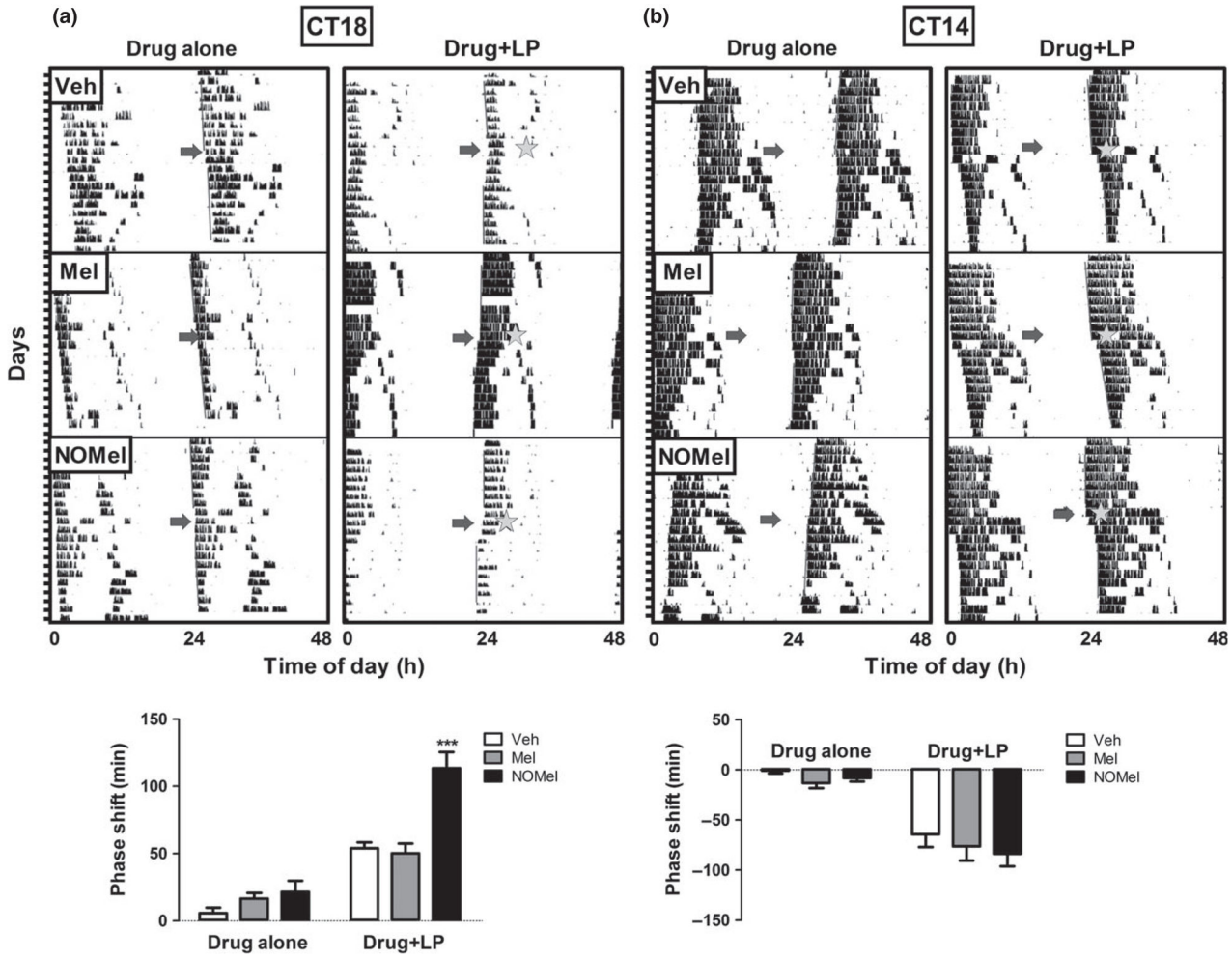

Figure 1. Effect of NOMel on light-induced phase shifts

Representative double-plotted actograms showing wheel-running activity rhythms of hamsters under constant darkness treated with vehicle (Veh), melatonin (Mel) or *N*-nitrosomelatonin (NOMel) i.p. injections 15 min before a light pulse (LP) of 10 min, 50 lux at circadian time (CT) 18 (a) or CT14 (b). Control animals received the drug treatment in the dark. The day of treatment is indicated by an arrow. Light stimulation time is indicated by a star. Activity onsets are indicated by grey straight lines drawn over the actograms, defining the phase of the rhythm. The mean ± SEM of the phase shifts is shown (for the CT18 group: ***p<0.001).

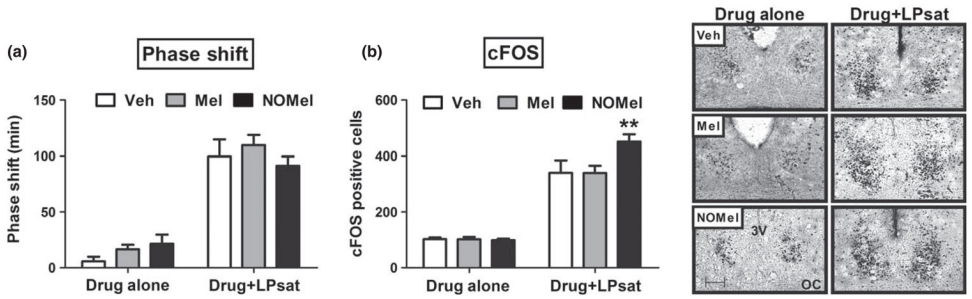

Figure 2. Effect of NOMel on saturating light-pulses

Hamsters under constant darkness received intra-peritoneal injections of vehicle (Veh), melatonin (Mel) or N-nitrosomelatonin (NOMel) 15 min before a saturating light pulse [300 lux, 10 min (LPsat)] at circadian time (CT) 18. **(a)** The mean ± SEM of the phase shifts is shown (Mel-LPsat and NOMel-LPsat: n=6; Veh alone and Veh-LP: n = 7, NOMel alone and Mel alone: n=8 Mel). (**b)** The mean ± SEM of the cFOS-positive cells is shown (Bonferroni post-test **p<0.01; n=4 for all groups). Representative brain coronal sections illustrating cFOS expression at the SCN 60 min after a saturating light pulse at circadian time (CT) 18. 3V, third ventricle; OC, optic chiasm. Scale bar = 200 μm.

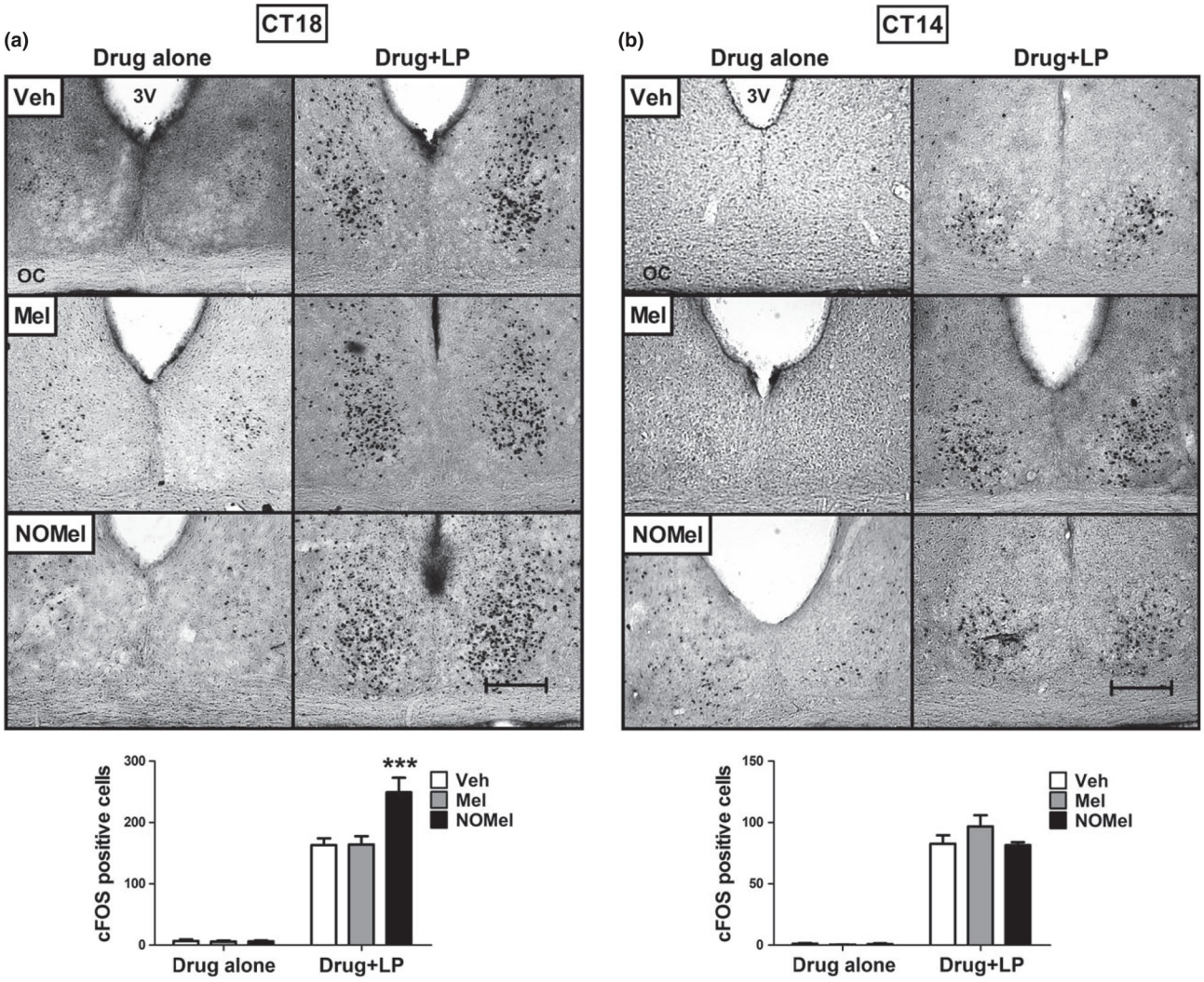

Figure 3. Effect of NOMel on photic induction of cFOS expression in the SCN

Representative brain coronal sections illustrating cFOS expression at the SCN 60 min after a 10 min-light pulse (LP) of 50 lux at **a.** circadian time (CT) 18 and **b.** CT14. Animals received the drug treatment 15 min before the LP. Control animals only received the drug treatment in the dark. The mean ± SEM of the number of cFOS-positive cells is shown (for the CT18: ***p<0.001). 3V, third ventricle; OC, optic chiasm. Scale bar = 200 μm.

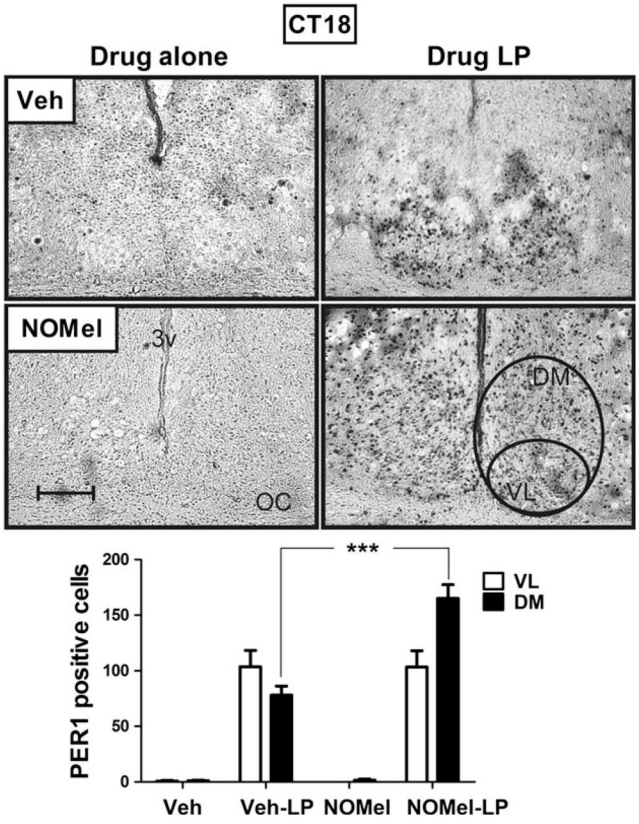

Figure 4. Effect of NOMel on photic induction of PER1 expression in the SCN

Representative brain coronal sections illustrating PER1 expression at the SCN 180 min after a 10 min-light pulse (LP) of 50 lux at circadian time (CT) 18. Animals received the drug treatment 15 min before the LP. Control animals only received the drug treatment in the dark. Continuous line delimits the ventrolateral (VL) and dorsomedial (DM) region of the SCN. The mean ± SEM of the number of PER1-positive cells is shown (for drug treatment: ***$p<0.001$). 3V, third ventricle; OC, optic chiasm. Scale bar = 200 μm.

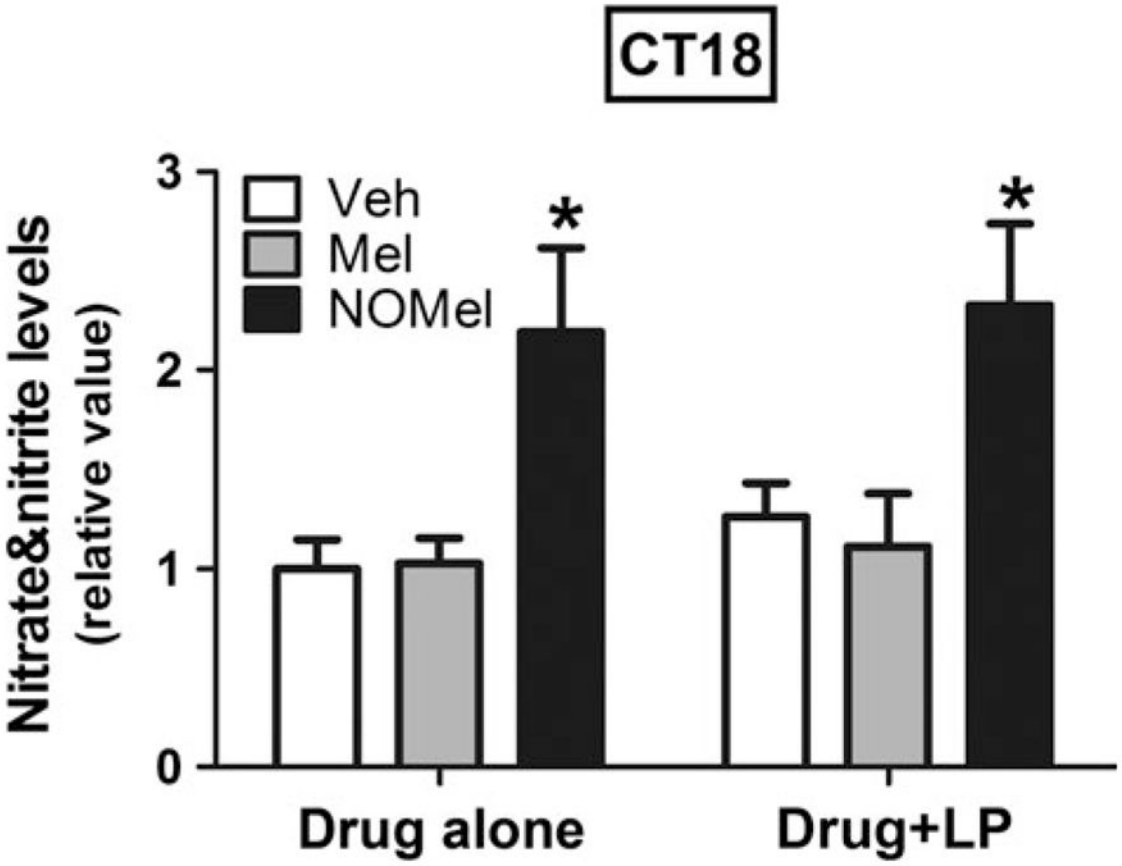

Figure 5. Effect of NOMel on photic induction of nitrate and nitrite levels in the SCN

The mean ± SEM of the nitrate and nitrite levels detected in SCN homogenates immediately after a 10 min-light pulse (LP) of 50 lux at circadian time (CT) 18 is shown. Data are relativized first to the protein concentration of each sample, and second to the mean value of the Veh-Drug Alone group (*$p<0.05$).

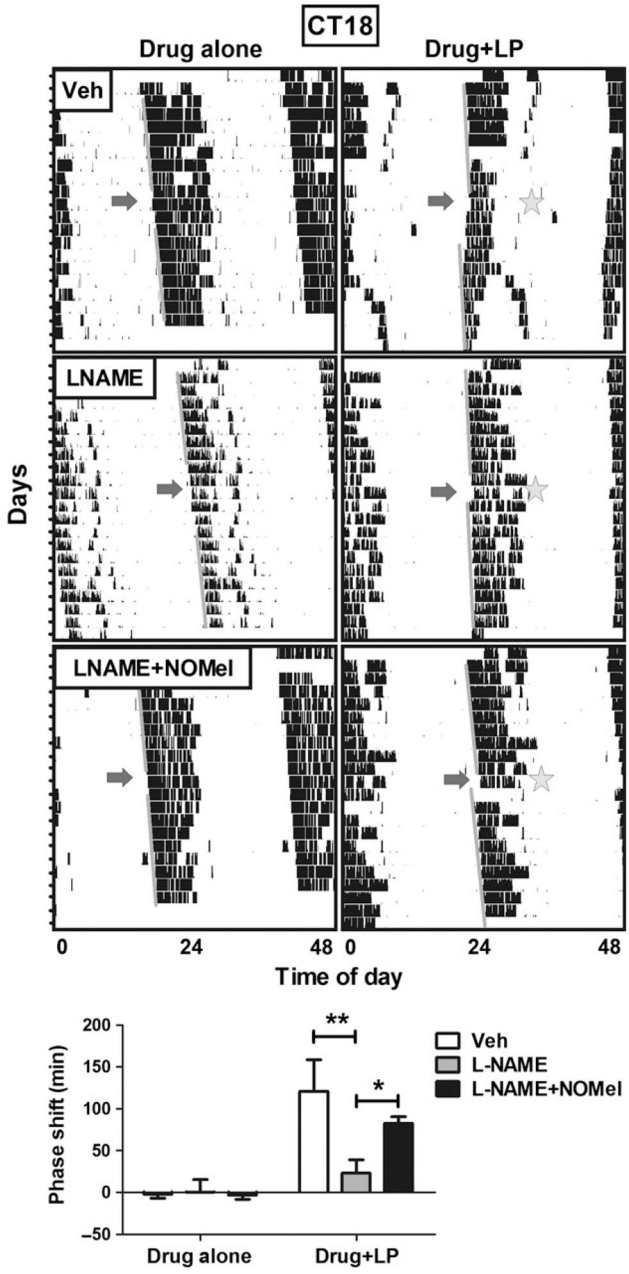

Figure 6. Effect of co-administration of NOMel and L-NAME on photic phase shifts

Representative double-plotted actograms showing wheel-running activity rhythms of hamsters under constant darkness treated with i.c.v. injection of vehicle (Veh), *N*-nitro-*L*-arginine methyl ester (L-NAME), and i.p. injections of *N*-nitrosomelatonin (NOMel) or vehicle 15 min before a light pulse (LP) of 10 min, 50 lux at circadian time (CT) 18. Control animals received the drug treatment in the dark. The day of treatment is indicated by an arrow. Light stimulation time is indicated by a star. Activity onsets are indicated by grey straight lines drawn over the actograms, defining the phase of the rhythm. The mean ± SEM of the phase shifts is shown (*p<0.05).

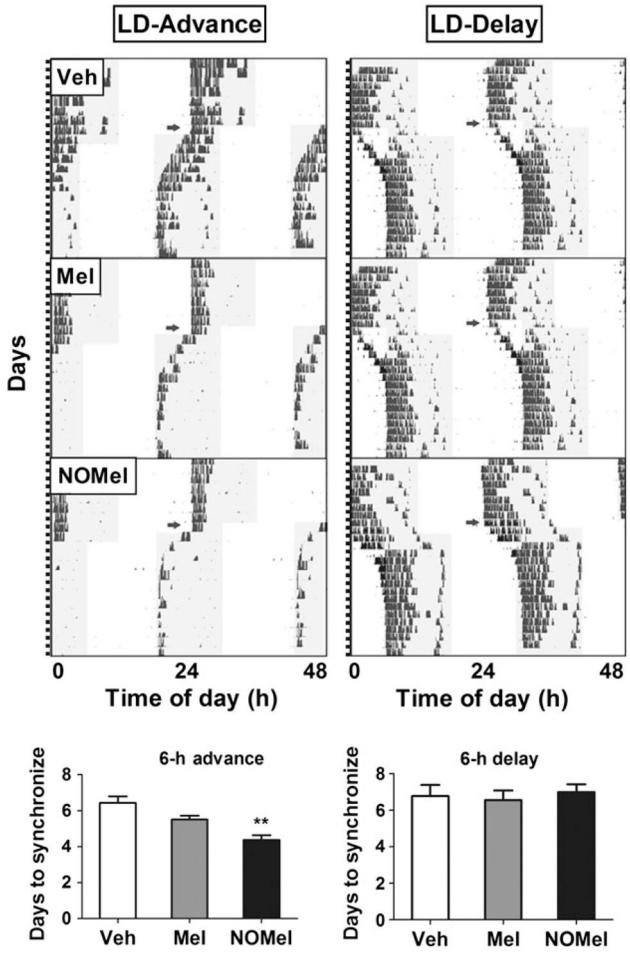

Figure 7. Effect of NOMel on resynchronization in an experimental jet-lag protocol

Representative double-plotted actograms showing wheel-running activity rhythms of hamsters showing resynchronization to a 6-h LD-phase **a.** advance (left actograms) or **b.** delay (right actograms) after the injection of vehicle (Veh), melatonin (Mel) or *N*-nitrosomelatonin (NOMel). The shaded area indicates darkness. Injections were given at zeitgeber time (ZT) 18 for the advancing and ZT14 for the delaying protocol on the day of the cycle change (indicated by an arrow). The mean ± SEM days to resynchronize to the shifted cycle for both protocols is shown (for LD-advance: *$p<0.05$).