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Role of G-Substrate in the NO/cGMP/PKG Signal Transduction Pathway for Photic Entrainment of the Hamster Circadian Clock

Santiago Andrés Plano[1,2,#], María Soledad Alessandro[2,#], Laura Lucía Trebucq[2], Shogo Endo[3], Diego Andrés Golombek[2] and Juan José Chiesa[2]

Abstract

The mammalian circadian clock at the hypothalamic suprachiasmatic nuclei (SCN) entrains biological rhythms to the 24-h cyclic environment, by encoding light-dark transitions in SCN neurons. Light pulses induce phase shifts in the clock and in circadian rhythms; photic signaling for circadian phase advances involves a nitric oxide (NO)/cyclic guanosine monophosphate (cGMP)/cGMP-dependent protein kinase (PKG) pathway, increasing the expression of Period (*Per*) genes. Effectors downstream of PKG remain unknown. Here we investigate the role of G-substrate (GS), a PKG substrate, in the hamster SCN. GS and phosphorylated G-substrate (p-GS) were present in a subset of SCN cells. Moreover, GS phosphorylation (p-GS/GS ratio) increased in SCN homogenates after light pulses delivered at circadian time (CT) 18 and intraperitoneal treatment with sildenafil, an inhibitor of phosphodiesterase 5 (a cGMP-specific phosphodiesterase). On the other hand, intracerebroventricular treatment with the PKG inhibitor KT5823, reduced photic phosphorylation of GS to basal levels. Since p-GS could act as a protein phosphatase 2 A (PP2A) inhibitor, we demonstrated physical interaction between p-GS and PP2A in SCN homogenates, and also a light-pulse dependent decrease of PP2A activity. Intracerebroventricular treatment with okadaic acid, a PP2A inhibitor, increased the magnitude of light-induced phase advances of locomotor rhythms. We provide evidence on the physiological phosphorylation of GS as a new downstream effector in the NO/cGMP/PKG photic pathway in the hamster SCN, including its role as a PP2A inhibitor.

Keywords

G-substrate, phase advance, protein phosphatase 2A, suprachiasmatic nuclei

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Daily rhythms in behavior and physiology are regulated in mammals by a hierarchical array of peripheral circadian oscillators controlled by a master clock located at the hypothalamic suprachiasmatic nuclei (SCN), which is entrained by the light-dark (LD) cycle. The SCN is comprised by ∼20.000 gamma-aminobutyric (GABA)-ergic neurons and subdivided into a ventral "core" region with neurons expressing both gastrin-releasing peptide (GRP) and vasoactive intestinal peptide (VIP), which receive the major photic input from the retina, and a dorsal "shell" region which contains mainly neurons expressing arginine

[1]Institute for Biomedical Research (BIOMED), Catholic University of Argentina (UCA) and National Scientific and Technical Research Council (CONICET), Buenos Aires, Argentina
[2]Laboratorio de Cronobiología, Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina
[3]Aging Neuroscience Research Team, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan

[#]These authors contributed equally to this work.

Corresponding Author:
Juan José Chiesa, Laboratorio de Cronobiología, Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes. Roque Sáenz Peña 352, B1876BXD, Bernal, Buenos Aires, Argentina.
Email: juan.chiesa@unq.edu.ar

vasopressin (AVP), and drives the clock rhythmicity (reviewed in Hegazi et al., 2019). SCN neuronal clocks are based on transcription-translation feedback loops (TTFLs) in which circadian locomotor output cycles kaput (CLOCK) and brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1) heterodimers bind to E-box elements of clock genes Period (*Per*) and Cryptochrome (*Cry*), increasing their transcriptional activity (Hegazi et al., 2019). In addition, *Per1-2* are light-inducible genes regulated by cyclic adenosine monophosphate (cAMP) response elements (CRE) (Travnickova-Bendova et al., 2002). Light pulses delivered during the early subjective night (typically circadian time 14, CT14, in hamsters) induce phase delays, while late night (CT18) pulses induce phase advances of the SCN clock, depicting conserved photic response curves in the species. Changing *Per1-2* activity underlying phase delays, or advances, will need separate, phase-dependent transduction pathways that, ultimately, converge at CRE regulation.

Ventral SCN neurons receive dense innervation from intrinsically photosensitive ganglion cells that send efferent projections through the retinohypothalamic tract (RHT). Glutamate released from the RHT transmits photic information for either phase delays or advances to entrain the SCN clock (Purrier et al., 2014), through activation of N-methyl-D-aspartate (NMDA) (and non-NMDA) ionotropic receptors increasing intracellular calcium (Ca^{2+}) levels during the subjective night (Colwell, 2001). Light stimulation at both CT14 and CT18 increases Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) phosphorylation of nitric oxide (NO) synthase (NOS) (Agostino et al., 2004), increasing its activity and NO concentration. Light-induced phase delays at CT14 increase intracellular Ca^{2+} both through T-type voltage-gated channels (Kim et al., 2005) and through the opening of ryanodine receptors at endoplasmic reticulum stores (Ding et al., 1998). In turn, GRP-ergic and VIP-ergic cells activate G-coupled receptors, triggering the adenylate cyclase/cyclic adenosine monophosphate (cAMP)/cAMP-activated protein kinase (PKA) pathway (reviewed in Hegazi et al., 2019, leading to CRE binding protein (CREB) phosphorylation (Gau et al., 2002) and *Per1* induction (Tischkau et al., 2000).

NO is a canonical activator of soluble guanylyl cyclase (sGC), which increases cyclic guanosine monophosphate (cGMP) concentration (Ferreyra & Golombek, 2001) as a further step in the signaling for light-induced phase advances. Increasing cGMP concentration with sildenafil, a phosphodiesterase type 5 (PDE5) inhibitor, potentiates both light-induced phase advances and re-entrainment to advancing LD cycles in hamsters (Agostino et al., 2007). Indeed, cGMP concentration sets a light intensity threshold for phase advances (Plano et al., 2012). Light-increased cGMP concentration induces PKG activity, and further phosphorylation and nuclear translocation of CREB changing *Per1-2* promoter activity (Ding et al., 1997), which is convergent with cAMP/PKA and MAPKs pathways (reviewed in Hegazi et al., 2019). From the two mammalian PKG genes, the type II (PKGII) was consistently found in the SCN (Ferreyra & Golombek, 2001; Tischkau et al., 2004) to modulate *Per1-2* activity and clock resetting (Oster et al., 2003). On the other hand, PKGI did not participate in this process, but seems to be important for the regulation of sleep/wake cycles (Langmesser et al., 2009).

Although there is substantial knowledge on the role of NO/sGC/cGMP/PKG signaling pathway for transducing circadian light advances (reviewed in (Golombek et al., 2004)), putative effectors that may act as substrates downstream of PKGII, are currently unknown. A PKG substrate (i.e., G substrate, GS) peptide with a ~23 kDa molecular weight with preferential phosphorylation by PKG was found in homogenates from the rabbit cerebellum (Schlichter et al., 1978). This specific, high-affinity substrate was also found in cerebellar Purkinje cells regulating motor learning in mice (Endo et al., 2009), in subsets of amacrine cells regulating excitotoxicity (Nakazawa et al., 2009), as well as other brain regions (Endo, 2012). The PKG-phosphorylated GS (p-GS) acts as an inhibitor of serine/threonine phosphoprotein phosphatases 1 (PP1) and 2 A (PP2A), in both mouse (Hall et al., 1999) and rat Purkinje cells (Endo et al., 2003). Hypothalamic areas as the paraventricular nuclei and the SCN show restricted GS expression in mice (Endo, 2012). Therefore, here we examined the role of GS in the hamster SCN as a putative effector of the NO/sGC/cGMP/PKG photic pathway.

Materials and Methods

Animals

Male, 3–4 month-old Syrian hamsters (*Mesocricetus auratus*) (purchased from Laboratorio Azul Diagnostico S. A.) were housed in group cages of 5 individuals and maintained in stock rooms for at least 21 days for acclimation under 14:10 h LD cycles (with zeitgeber time (ZT) 12 defined at lights-OFF), temperature set at $22 \pm 2°C$, with access to balanced rodent chow and water *ad libitum*. All experimental procedures with animals were approved by the Institutional Animal Care and Use Committee of the Universidad Nacional de Quilmes, in accordance with the Guide for the Care and Use of Laboratory Animals (NIH, USA).

Lighting Conditions and Locomotor Data Recordings

For the LD cycles, fluorescent lamps supplying white cool light with 100-200 lux intensity at home cages were used. For light-pulse experiments, hamsters were

transferred from the stock rooms to individual cages into a box designed to supply 50-100 lux for sub-saturating, or 300 lux for saturating light pulses. For locomotor activity rhythm recordings, subjects were provided with running wheels and revolutions were registered and stored into 5-min bins with a data acquisition system designed in our lab. Real time monitoring of locomotor activity time series was used to estimate the typical circadian times (CTs, see below) of the free running rhythms of the individuals under constant darkness (DD), for further manipulations.

Drugs

KT-5823 200 mM (Cayman Chemical, USA) and Sildenafil© 3.5 mg/kg (extracted from commercial preparation, Pfizer, USA) were diluted in 50% DMSO/saline solution. Okadaic acid (Sigma, USA) was diluted from stock solution in 100 μM (high), and 100 nM (low) doses.

Cannula Implantation and Microinjections

Intracerebroventricular (icv.) microinjection of drugs was performed using a guide cannula (PlasticsOne, USA) aimed at the third ventricle just above the SCN (stereotaxic coordinates: +0.6 mm. anterior to bregma, -8 mm. below dura). For cranial surgery, hamsters were anesthetized with isoflurane (5% in oxygen for induction, 2% for maintenance, 200 ml/min) and placed in a stereotaxic device. An incision was made in the skin with a scalpel to access the skull, a trepan was performed with a microdrill, the cannula was placed and attached with screws and acrylic cement to the skull, and the skin was sutured. Hamsters were kept under observation for at least 1 week for post-surgical recovery before the experimental treatments, receiving topic gentamicin/betamethasone. Microinjections were performed under darkness with a Hamilton syringe delivering 1 μl/min flux with gentle restrain of animal mobility during 2 min.

GS and p-GS Immunohistochemistry

Animals were deeply anesthetized with a ketamine:xylazine (150:10 mg/kg ip.) cocktail and then intracardially perfused at CT18 with 0.01 M 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. 30 μm thick coronal sections, containing SCN or cerebellum regions were obtained with a cryostat (Leica, USA) 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 5% nonfat dry milk in PBST for 1.5 h at room temperature. Sections were incubated for 48 h at 4° C with mouse anti-p-GS monoclonal antibody (diluted 1:100 in PBST), and two rabbit polyclonal antibodies that recognize different regions of the GS: N antibody for N-teminal and C antibody for C-terminal of G-substrate (1:4000 for N antibody, and 1:300 for C antibody (Hall et al., 1999). GS and p-GS antibodies were developed by the laboratory of Shogo Endo at the Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan. Biotinylated anti-mouse/rabbit IgG (H + L) made in horse (Vector Laboratories, USA) diluted 1:200 in PBST was used as a secondary antibody for 1.5 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). Images were obtained with a light field microscope (Leica, USA) using 4× and 10× objectives.

Western Blots

For all western blot assays, after corresponding treatments (see below) hamsters were euthanized by decapitation and their brains were quickly removed and placed at -80°C. For sample preparation, 1 mm brain slices were sectioned just above the optic chiasm with a chopper, the hypothalamic tissue containing the SCN was micro punched, and pools from 3 individuals were homogenized in 50 mM Tris/HCl buffer (pH 7.4), with 0.32 M sucrose, 1 mM EGTA, 1 mM EDTA, 50 mM NaF, a protease inhibitor cocktail (Sigma, USA) and 2 mM sodium orthovanadate. After centrifugation at 12,000 rpm, the supernatants were collected, and protein concentration was determined by Bradford analysis. 30 μg of protein samples for each treatment to obtain a complete set per experiment were loaded in 9% SDS–PAGE gels and transferred to Hybond nitrocellulose membranes (GE Healthcare, USA). Then the membranes were blocked with 5% nonfat dry milk in TTBS for 1 h at room temperature and incubated for 48 h at 4° C with, for GS and p-GS western blots, a monoclonal mouse anti-p-GS antibody (diluted 1:1000 in TTBS). Immunoreactivity was assessed using a secondary antibody coupled to horseradish peroxidase (1:5000, Chemicon Int., USA) and visualized with the ECL kit (GE Healthcare, USA). Blots were stripped and re-incubated with anti-GS antibody diluted 1:350 in TTBS) and tubulin antibody (GE Healthcare, 1:1000). For PP2A western blots, monoclonal rabbit anti-PP2A antibody (diluted 1:1000 in TTBS; ab137825, Abcam), and tubulin antibody (GE Healthcare, 1:1000). Immunoreactivity was assessed using a secondary antibody coupled to horseradish peroxidase (1:5000, Chemicon Int., USA) and visualized with the ECL kit (GE Healthcare, USA). Optical densities obtained for the samples of each treatment were analyzed within the same gel, as follows: first, they were relativized to the loading control (tubulin), then, considered as p-GS/GS

(for phosphorylation determination). For the PP2A determination, values relative to tubulin were compared between day (i.e., CT6) and night (i.e., CT18),

Light Induced Phosphorylation of GS

Animals received icv. injections of KT-5823 200 mM (1.5 µl/min, Cayman Chemical) 15 min before a saturating light pulse (300 lux) or intraperitoneal (ip.) injections of Sildenafil© (3.5 mg/kg, Pfizer) 45 min before a saturating light pulse at ZT18. A group receiving only a saturating light pulse at ZT18 was added as control. Hamsters were sacrificed 15 min after the light pulse, and brains were then quickly removed and placed at -80°C.

p-GS and PP2A Co-Immunoprecipitation Assay

Animals were sacrificed 15 min after a saturating light pulse (300 lux) at CT 18. Control animals did not receive the light pulse. Brains were quickly removed and placed at -80°C. The SCN was punched out and homogenized in 50 mM HEPES, 250 mM NaCl, 1 mM EDTA, 1% NP-40 and centrifuged at 10.000 rpm for 5′ at 4 °C. The supernatants were collected and samples (500 µg) were loaded with 2 µl of PP2A, p-GS antibody or non-immune serum (control) and incubated overnight at 4 °C. Agarose beads (15 µl, A/G PLUS-Agarose, Santa Cruz Biotechnology, USA) were incubated with the mix of the proteins and the antibodies for 90 min at 4 °C. Samples were centrifuged at 3,000 rpm and cracked with loading buffer. A 9% SDS–PAGE was performed, and the proteins were transferred to Hybond nitrocellulose membranes (GE Healthcare, USA). Then the membranes were blocked with 5% nonfat dry milk in TTBS for 1 h at room temperature and incubated for 48 h at 4° C with anti-p-GS antibody (diluted 1:1000 in TTBS) or anti-PP2A antibody (diluted 1:1000 in TTBS). Immunoreactivity was assessed by chemiluminescence using a horseradish peroxidase-labeled secondary antibody (Chemicon Int., 1:5000) and the ECL kit (GE Healthcare). A whole tissue extract was used as a positive control for the western blot procedure.

PP2A Enzymatic Activity Assay

Hamsters were sacrificed at ZT18 after the administration of a saturating light pulse (300 lux, 15 min), or control subjects without light. The SCN tissue obtained from 3 individuals was pooled for each experimental group. Brains were quickly removed and placed at -80°C. The SCN were micro punched and homogenized in 50 mM Tris/HCl buffer (pH 7.4), with 0.32 M sucrose, 1 mM EGTA, 1 mM EDTA, 50 mM NaF, protease inhibitor cocktail (Sigma, USA) and 2 mM sodium orthovanadate. Cell lysates were centrifuged, and the supernatants were collected for measuring PP2A activity, according to the supplier's protocol (ELISA Development Kit, R&D Systems, USA). Bradford analysis was used to quantitate an equal amount of proteins added to all wells. As a control for specificity, okadaic acid (10 nM) was added in an appropriate step of the protocol.

Inhibition of PP2A Activity in Behavioral Experiments

Hamsters were transferred to individual cages, equipped with a running wheel under DD conditions for 24 days, with food and water *ad libitum*. Wheel running activity was continuously recorded and data stored at 5-min intervals for further analysis. At day 25 in DD (see Figure 6), hamsters received an icv. 100 nM microinjection of okadaic acid (Santa Cruz Biotechnology, USA), or vehicle (50:50 CSF: DMSO) 15 min before stimulation with a 15-min, 100 lux light pulse at CT18 (see the following paragraph for CT determination).

Statistical and Chronobiological Analyses

For all experiments, individuals were randomly assigned into their corresponding groups. For western blot experiments, each experimental sample is composed by a pool of SCN tissue obtained from 3 hamsters. Normal distributions were tested with Shapiro-Wilkinson Normality Test and then parametric analyses were performed (one-way and two-way ANOVA as indicated in the text), and post-hoc tests (Dunnett Test and Bonferroni Test) were applied when indicated. Results are presented as Mean ± SEM of experimental datasets. Statistical analyses were performed with GraphPad Prism 7 (GraphPad Software, La Jolla California, USA). P-values lower than 0.05 were considered to reach statistical significance. Time-series of wheel running activity were analyzed in actograms plotted modulo Tau (i.e., using the free running period of corresponding individual). An eye-fitted line set at the onsets of locomotor activity were obtained in the actograms as a typical marker of the circadian phase, to obtain the times for CT12 and CT18. Phase shifts were calculated by fitting a line through activity onsets 15 days prior to, and between 5 and 15 days after light exposure; the time difference between the extrapolation of these two lines on the day of the light pulse stimulation (i.e., a phase shift) was calculated.

Results

GS and p-GS Immunolocalization in the Hamster SCN

Distribution of G-substrate (GS) and phospho-G-substrate (p-GS) was studied by immunohistochemistry in the SCN. Brain coronal sections taken at ZT18 were incubated with anti-p-GS antibody, and with two

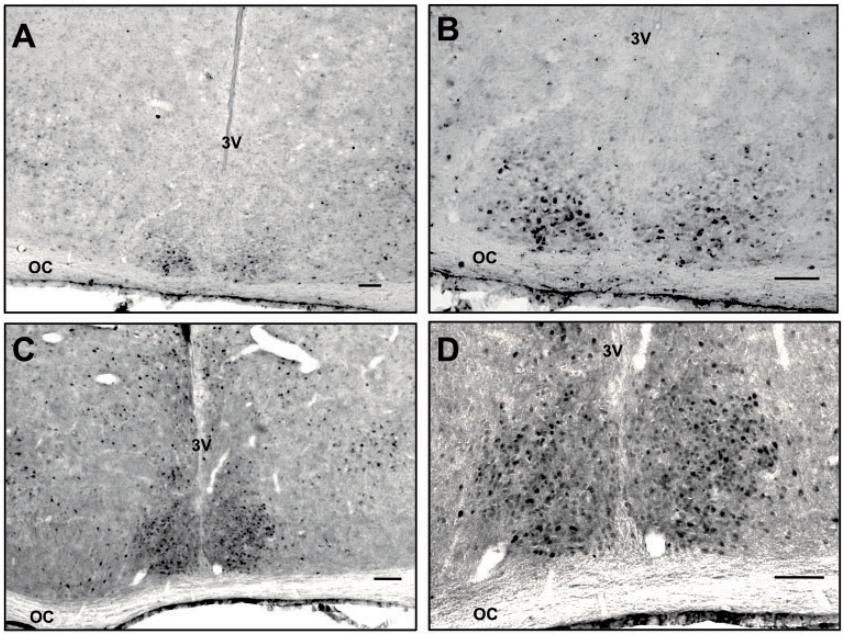

Figure 1. Distribution of GS and p-GS in the SCN After a Light Pulse During the Late Night. Immunostaining in SCN-containing hypothalamic coronal sections from hamsters. A and B: Representative images for GS at low (4×) and medium (10×) magnification, respectively. C and D: Representative images for GS and p-GS at low (4×) and medium (10×) magnification, respectively. (Sale bars: 100 μm in A and C, 50 μm in B and D).

different anti-GS antibodies which recognize the N-terminal, or the C-terminal extreme of GS, respectively. Basal p-GS expression appeared to be restricted to the ventral SCN (Figure 1A and B). GS was clearly detected with the two antibodies showing both dorsal and ventral SCN distribution (Figure 1C and D, only the reaction to N50 is shown).

Light-Induced Phosphorylation of GS in the SCN

The phosphorylation of GS in the hamster SCN was analyzed in western blots by the quantification of the p-GS/GS ratio after the administration of a light pulse at ZT18 (Figure 2A). Compared to dark controls, we observed a higher p-GS/GS ratio after the light pulse (Student's t-test for unpaired samples: $p < 0.0001$, mean $\pm$ SEM in arbitrary units: dark: 0.53 ± 0.03; light pulse: 1.7 ± 0.1; $n = 4$ per group). Then, we tested if photic GS phosphorylation depends on the activation of NO/cGMP/PKG signaling by performing pharmacological treatments affecting the pathway (Figure 2B). As compared to light alone, ip. treatment with the specific PDE5 inhibitor Sildenafil (Agostino et al., 2007) increased, while icv. treatment with the PKG inhibitor KT-5823 decreased the p-GS/GS ratio (one-way ANOVA: $p < 0.0001$, followed by Tukey test for post-hoc comparisons: vehicle + LP vs Sildenafil + LP: $p < 0.01$; vehicle + LP vs KT + LP: $p < 0.0001$; Sildenafil + LP vs KT + LP: $p < 0.0001$; mean $\pm$ SEM: vehicle + LP: 1.4 ± 0.06; Sildenafil + LP: 1.7 ± 0.017; KT + LP: 0.2 ± 0.01; $n = 3$ per group). These results suggest that light activation induces phosphorylation of GS *via* PKG in the SCN.

PP2A Immunodetection in the SCN

The presence of PP2A was studied in hamsters under LD cycles in samples taken at ZT6 (i.e., "Day") and ZT18 ("Night") (Figure 3). When comparing Day *vs*. Night, we found similar expression levels of PP2A in the SCN (Student's t-test for unpaired samples: $p = 0.6$; mean $\pm$ SEM (Arbitrary units): Day: 1.29 ± 0.017; Night: 1.28 ± 0.012, $n = 4$ per group).

Interaction of p-GS and PP2A in the SCN

The interaction of p-GS and PP2A was studied by a co-immunoprecipitation assay in SCN tissue samples from hamsters receiving a light pulse at ZT18 (Figure 4). Homogenates were treated with antibodies anti-p-GS, anti-PP2A, or non-immune serum, and the immunoprecipitated complexes were analyzed by western blots. Samples treated with anti-p-GS were positive in western blots for p-GS, and for its coimmunoprecipitated target PP2A. The same was found for samples immunoprecipitated with anti-PP2A, showing signal for PP2A and for p-GS in respective western blots. These results were independent of the light pulse treatment. In fact, it was quite accurate when p-GS was used for the IP step, but the same signal was generated when PP2A was used.

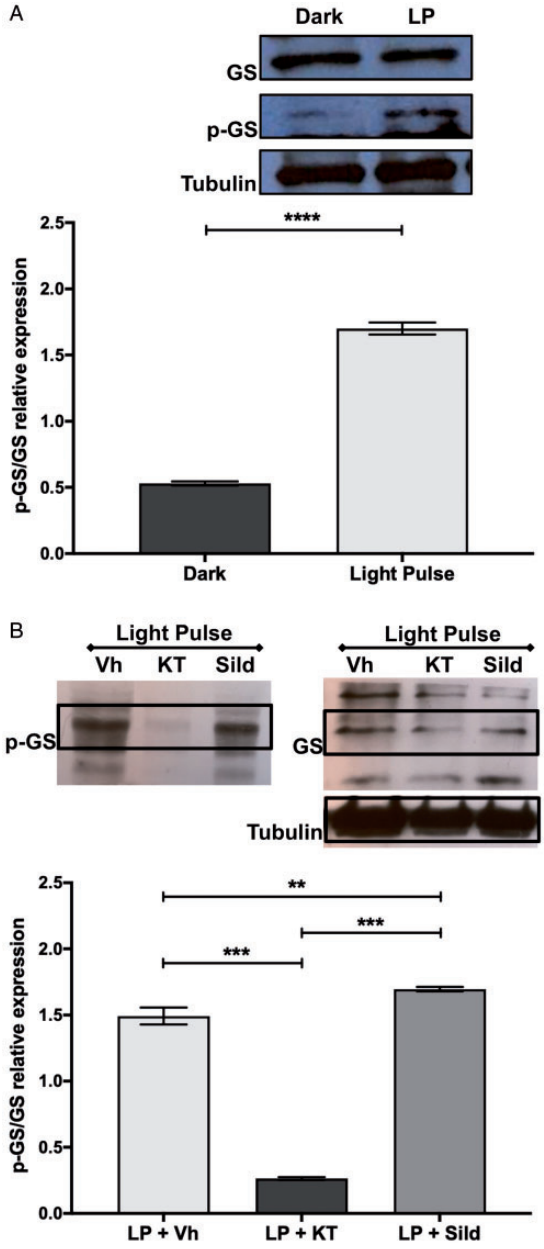

Figure 2. Light Pulse-Induced GS Phosphorylation in the SCN at ZT18 via PKG. A: Western blots and densitometric analysis of GS phosphorylation, at ZT18 in dark conditions or after a saturating light pulse. Densitometric analysis (mean ± SEM of four independent experiments) shows GS phosphorylation, as the p- GS/GS ratio, increases after the administration of a saturating light pulse compared to dark conditions (Dark: 0.53 ± 0.03; LP: 1.7 ± 0.1, n = 4 per group; $p < 0.0001$, Student's unpaired t-test). B: Analysis of the pharmacological modulation of GS phosphorylation after a saturating light pulse at ZT18. Western blots and densitometric analysis (mean ± SEM of three independent experiments) show an increased GS phosphorylation after the administration of Sildenafil compared to vehicle and the inhibition of phosphorylation after KT administration (vehicle + LP: 1.4 ± 0.06; Sildenafil + LP: 1.7 ± 0.017; KT + LP: 0.2 ± 0.01, n = 3 per group; ANOVA: $p < 0.0001$, multiple comparisons: vehicle + LP vs Sildenafil + LP, $p < 0.01$; vehicle + LP vs KT + LP, $p < 0.0001$; Sildenafil + LP vs KT + LP, $p < 0.0001$, Tukey's adjusted p values).

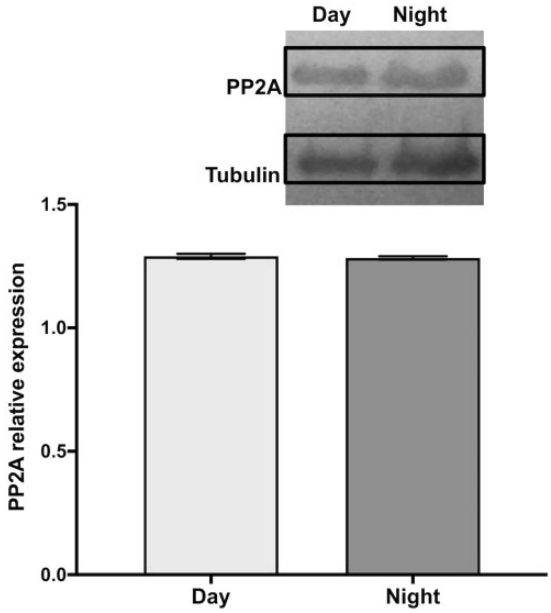

Figure 3. PP2A Expression in the SCN. Western blots and densitometric analysis of PP2A expression during the day (CT6) and night (CT18) in the SCN of hamsters (mean ± SEM of three independent experiments). Densitometric analysis of three independent experiments conducted at night shows no significant differences between day and night expression of PP2A (1.29 ± 0.017 vs 1.28 ± 0.012 arbitrary units, respectively, n = 4 per group; $p = 0.6$, Student's unpaired t-test).

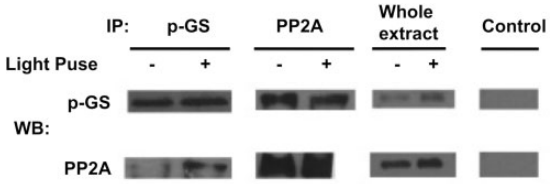

Figure 4. Interaction Between p-GS and PP2A in the SCN With and Without a Light Pulse Stimulus. Western blot (WB) analysis for p-GS and PP2A, of samples immunoprecipitated (IP) with p-GS, PP2A antibodies or with non-immune serum (Control), and whole SCN extract, with or without light pulse at ZT18.

This could be due that the anti PP2A has greater reactivity to form PP2A-p-GS complex, as compared to the anti p-GS. The western blots from samples treated with the non-immune serum showed no signal for both PP2A and p-GS, while the western blot for the whole SCN extract (whole extract) showed the positive signal for the two antibodies as expected.

Photic Regulation of PP2A Activity

PP2A enzymatic activity was assayed in SCN tissue homogenates obtained from hamsters during the late night (Figure 5). Brains were dissected 15 min after the

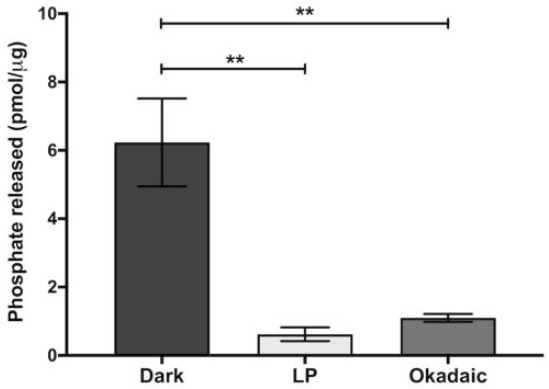

Figure 5. A Light Pulse During the Late Night Inhibits Enzymatic Activity of PP2A in the SCN. The PP2A activity was measured in SCN lysates after the administration of a sub-saturating light pulse to hamsters at ZT18, or controls kept in the dark (mean ± SEM of three independent experiments). Okadaic acid was administered in samples taken from dark-exposed hamsters, as an internal control. Okadaic acid has a similar effect on PP2A activity than light. A significant decrease of the PP2A activity for both the light pulse (LP) and the Okadaic acid administration was found compared to the Dark group, and there were no differences between LP and Okadaic (Dark: 6.23 ± 2.23 pmol/μg; LP: 0.62 ± 0.33 pmol/μg; Okadaic: 1.01 ± 0.20 pmol/μg; n = 3 per group; ANOVA: $p < 0.01$; multiple comparisons: Dark vs LP $p < 0.01$; Dark vs Okadaic, $p < 0.01$; LP vs Okadaic $p = 0.89$, Tukey's adjusted p values).

administration of light pulse (or dark controls) at ZT18, and the enzymatic activity was measured in lysates of SCN cells. Samples from hamsters exposed to dark were also treated with okadaic acid, a specific inhibitor of PP2A, as a control for specificity. Compared to hamsters that did not receive the light stimulation, we observed a significant decrease of PP2A activity after the light pulse. This level of activity was similar to that obtained after administration of the inhibitor okadaic acid (one-way ANOVA: $p < 0.01$; Tukey test for post-hoc comparisons: Dark vs. light pulse: $p < 0.01$; Dark vs. okadaic acid: $p < 0.01$; light pulse vs. okadaic acid: $p = 0.89$; mean ± SEM: Dark: 6.23 ± 2.23 pmol/μg; light pulse: 0.62 ± 0.33 pmol/μg; okadaic acid: 1.01 ± 0.20 pmol/μg; n = 3 per group).

Inhibition of PP2A Activity In Vivo in Phase-Advance Experiments

The effect of PP2A activity inhibition in light resetting of the circadian clock was tested. For that aim, groups of hamsters received an icv. microinjection of 100 nM okadaic acid (Santa Cruz Biotechnology) or vehicle (50:50 CSF: DMSO) 15 min before stimulation with a sub-saturating light pulse (100 lux, 15 min) at CT18 (Figure 6). In vehicle-treated subjects, the light pulse generated typical phase advances found in hamsters (Figure 6, Panel A). Inhibition of PP2A activity in the SCN greatly enhanced the photic response, increasing phase advances 3-fold as compared to vehicle (Mean ± SEM: LP + vehicle: 158.5 ± 38.73 minutes; LP + okadaic acid: 467.5 ± 124.4 minutes, n = 4 per group; Student's t-test for unpaired samples, $p < 0.03$). Phase advances in hamsters treated with okadaic acid reached up to 12 h in two subjects (Figure 6, Panel B), and one individual showed a similar response to that of the controls (panel C). This dose was effective to modulate the photic response, without producing toxic effects, at least at the level of locomotor activity rhythms, while a greater dose (100 μM) generated arrhythmicity (Supplementary Figure 1).

Discussion

In this work we studied the participation of GS as a putative downstream component in the NO/cGMP/PKG signaling pathway for photic entrainment in the hamster SCN. GS, and phosphorylated GS, were detected in both the SCN and cerebellar Purkinje cells (not shown) by immunohistochemistry. Photic stimulation of the SCN clock led to a significant increase in p-GS/GS ratio in western blots. This increased ratio was also observed when the light pulse was paired with Sildenafil, a specific inhibitor of PDE5 that increases cGMP concentration in the SCN and in turn stimulates PKG activation (Agostino et al., 2007). In addition, after treatment with the PKG inhibitor KT5823, GS phosphorylation was decreased to basal levels (i.e., non-stimulated condition). These evidences indicate that GS phosphorylation is induced by light-induced activation of the cGMP/PKG entrainment pathway at the SCN. Furthermore, the results demonstrate the participation of GS as a newly downstream substrate of PKG in the mammalian SCN clock.

Phosphorylated GS was reported as a strong inhibitor of the PP2A holoenzyme in Purkinje cells, among other protein phosphatases (Endo et al., 1999; Hall et al., 1999; Endo et al., 2003). Thus, we hypothesized a similar role of GS in the SCN photic pathway. First, we analyzed by western blots the presence of PP2A in the SCN and found that there is a similar day/night expression of the phosphatase. Then, we performed a co-immunoprecipitation assay which evidenced that the phosphorylated GS interacts with PP2A in the SCN at ZT18; however, the resolution of this assay is not enough to demonstrate if this colocalization depends on photic activation. We also assessed if PP2A enzymatic activity in the SCN was modulated by photic stimulation, and we found that a light pulse at ZT18 induced a substantial drop from basal levels (without light stimulation), similar to those generated after pharmacologic inhibition with okadaic acid. Furthermore, we tested the physiological

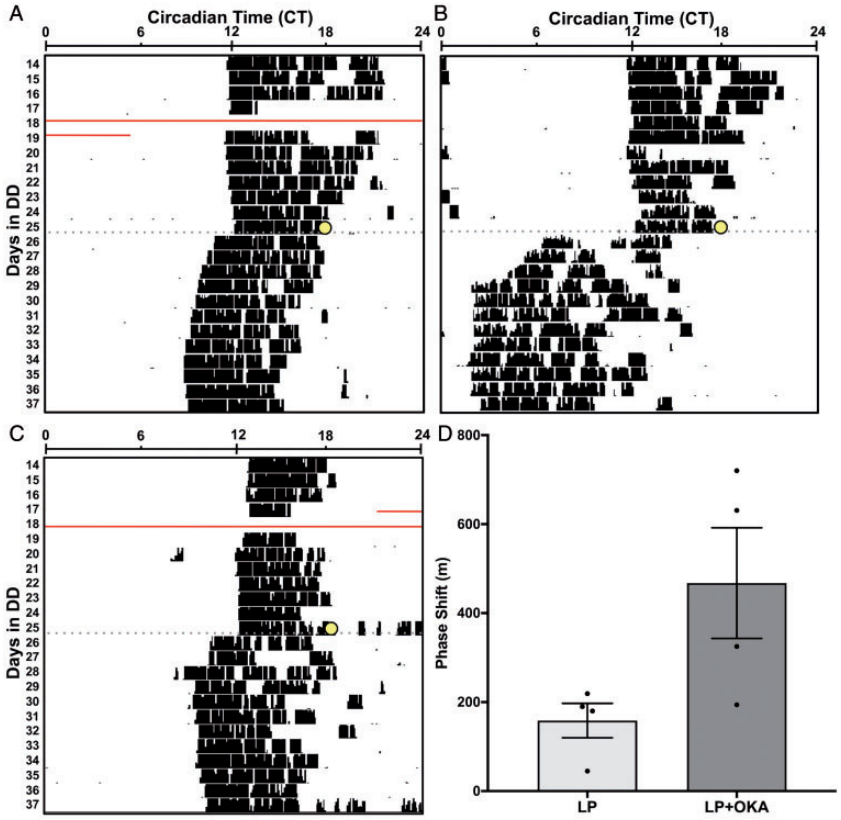

Figure 6. PP2A Inhibition Increased the Light-Induced Phase Advance of the Circadian Rhythms. A–C: Actograms, plotted modulo tau, showing wheel-running activity rhythms of hamsters under constant darkness treated with an icv. administration of vehicle (A) or 100 nM Okadaic acid (B, one animal with the maximum response, and C, individual with a response no different to vehicle) 15 min before a subsaturating LP (100 lux, 15 min) at circadian time CT18, yellow circles mark the moment of the icv. administration. The top bar with ticks marks the circadian time (CT) for the pre-treatment period, numbers at the y-axis dentate the number of days at DD. D: The panel shows a scatter dot plot with the mean ± SEM for the phase advances. The treatment with Okadaic acid significantly increased the phase advance compared to the vehicle (LP: 158.5 ± 38.73 min; LP + Oka: 467.5 ± 124.4 min, $n = 4$ per group; $p < 0.05$, Student's unpaired t-test).

role of PP2A *in vivo* on locomotor rhythm entrainment. Phase advances induced by light were enhanced when the light pulse was delivered with okadaic acid, a pharmacological PP2A inhibitor. While there is substantial evidence on the role of protein kinases in the circadian clockwork (Alessandro et al., 2019), the importance of phosphoprotein phosphatase regulation in the mammalian circadian clock is still elusive. Schmutz et al. demonstrated a similar enhancing effect of light resetting in transgenic mice with inducible inhibition of PP1 (Schmutz et al., 2011), an effect related to dephosphorylation-dependent stabilization (Gallego et al., 2006) and subcellular localization (Schmutz et al., 2011) of PER2 found *in vitro*. The balance of casein kinase I delta/epsilon (CKIδ/ε) and PP1 activity regulates speed and rhythmicity of PER1-2 phosphorylation and clock period oscillation *in vitro* (Lee et al., 2011). Also, PP1 was found to be involved in BMAL1 destabilization, by its interaction with CLOCK/BMAL1 heterodimer in high-throughput yeast two-hybrid interaction experiments (Wallach et al., 2013). In addition, PP5 demonstrated a key role regulating core molecular clock activity at the SCN by its interaction with cryptochromes, *via* its regulation by dephosphorylation of CKIε-mediated PER phosphorylation (Partch et al., 2006). Another PP tested in the circadian clock is the calcium-dependent protein phosphatase calcineurin (PP2B), whose SCN pharmacological inhibition by peripheral immunosuppressants reduced the size of light-induced delays at CT14 in mice (Katz et al., 2008). Regarding PP2A, to date there is no evidence of its role in the circadian clock. Here we demonstrated that light-induced phosphorylation of GS by PKG interacts with PP2A in the SCN, decreasing its activity, and our results put forward the physiological role of this interaction as a positive loop-enhancing photic signaling pathway.

We hypothesized that GS, a bona fide PKG substrate, is a downstream component of NO/cGMP/PKG pathway in the SCN. Other PKG substrates as the

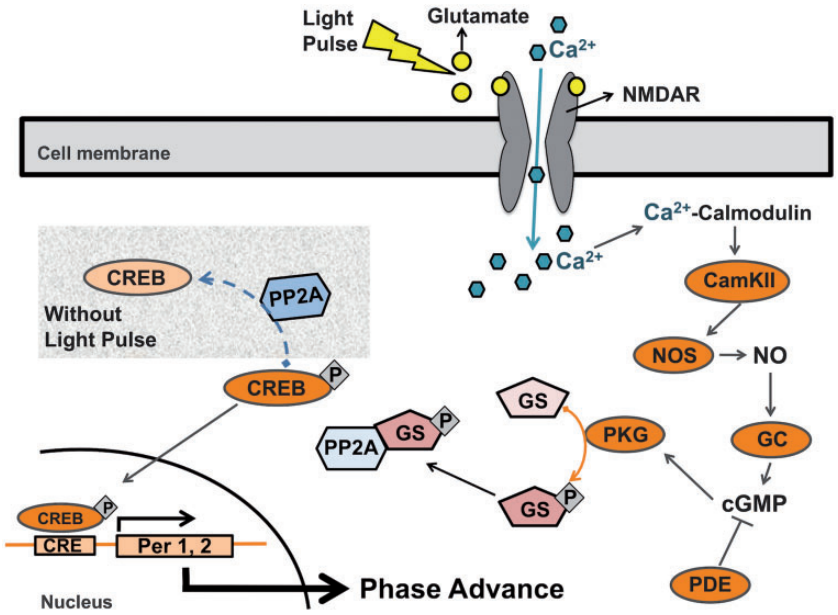

Figure 7. Model of the NO/cGMP/PKG/GS/PP2A Signal Transduction Pathway Responsible for Photic Entrainment by Advances of the Circadian Clock. Light stimulation at late night reaches the SCN retinorecipient neurons by glutamatergic neurotransmission, opening NMDAR postsynaptic receptor increasing intracellular calcium (Ca2+) and activates cascade of kinases and second messengers [Ca2+-calmodulin dependent kinase II (CamKII), nitric oxide synthase (NOS), NO, guanylate cyclase (GC), cyclic guanosine monophosphate (cGMP)], which increases cGMP dependent protein kinase (PKG) activity to phosphorylate GS. The phosphorylated GS peptide (p-GS) interacts with PP2A, inhibiting its activity. This will stabilize putative substrates of PP2A, such as phosphorylated cyclic adenosine monophosphate responding element binding protein (p-CREB), otherwise dephosphorylated by basal PP2A (shaded area without light pulse). p-CREB interacts with E-boxes increasing per1-2 genes transcription, a key endpoint needed for resetting the circadian clock. In the figure, the signal transduction pathway players already confirmed are shown in orange, darker colors mark the activated form of the proteins, while lighter colors indicate the inactive forms. The dotted arrow indicates the hypothetical p-CREB-PP2A interaction.

vasodilator-stimulated phosphoprotein (VASP) (Wang et al., 2005) were found in brain areas regulating synaptic plasticity, such as the hippocampus, as well as in the amygdala and cerebellum (reviewed in Kleppisch & Feil, 2009). Nigrostriatal neurons receiving dopaminergic input are enriched in dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa (DARPP-32). DARPP-32 is an excellent substrate for PKA/PKG and phosphorylated DARPP-32 acts as a potent PP1 inhibitor (Nishi et al., 2005). Even though both phosphorylated DARPP-32 and GS have similar characteristics as PP inhibitors (Endo et al., 2003), the SCN lacks DARPP-32 mRNA, and normal entrainment was observed in DARPP-32 knock-out (KO) mice (Yan et al., 2006). As mentioned, the NO/cGMP/PKG pathway is essential for advancing the SCN clock with late night light stimulation. A circadian peak in cGMP concentration at the late night is regulated through enzymatic hydrolysis of cGMP by PDEs and affects the enzymatic activity of PKGII (Tischkau et al., 2003). This rise in PKG activity seems to be of importance for the state of the clock, as inhibition of PKGII by KT5823 generates phase delays in locomotor activity, SCN neuronal firing, cGMP concentration, and *Per1* mRNA expression (Tischkau et al., 2003). In this sense, entrainment could be favored by these cycling pathway components that predict (i.e., creates a favorable environment for) photic input at late night.

A model summarizing the main findings of this work is depicted in Figure 7. First, we have supplied evidence on GS phosphorylation as a new NO/cGMP/PKG pathway effector. Co-immunoprecipitation of p-GS with PP2A, light-induced inhibition of PP2A enzymatic activity, and enhanced locomotor rhythm advances by pharmacological inhibition of PP2A, indicate that p-GS acts as a PP2A inhibitor modulating photic entrainment in the SCN. This could sustain phosphorylation levels of key effectors such as CREB, increasing its stability and nuclear entry. Indeed, the nuclear entry of p-CREB and its interaction with E-boxes increase the transcription of per1-2 genes, a key feature for resetting the circadian clock (Ding et al., 1997). It was found that p-CREB dephosphorylation by either the activity of PP2A (Wadzinski et al., 1993) or PP1 (Alberts et al., 1994), decreases CREB activity and gene transcription *in vitro*. Evidence *in vivo* is limited to PP1 regulating CREB

activity in learning and memory (Genoux et al., 2002), and okadaic acid inhibition of PP1/2A, which increases p-CREB levels in the striatum (Choe et al., 2004).

To conclude, the discovery of this new role for GS, a well-known substrate for PKG, as a PP2A inhibitor downstream in the circadian light entrainment pathway, opens new questions to investigate additional interactions. Finding a new player of the circadian entrainment pathway could help in the screening for novel drug targets. New strategies are based on the modulation of core circadian genes, as the drugs Nobiletin, which enhances molecular clock amplitude (He et al., 2016), and SR9009, a REV-ERBα agonist that alters the expression of many genes involved in metabolism (Solt et al., 2012). However, the modulation of these targets in the core molecular clock may have pleiotropic effects in the outputs. Drugs that modulate the photic response itself could be more efficient for alleviating entrainment disorders such as jetlag, as is the case of PDEs inhibitors (Plano et al., 2012), or potential downstream targets of the pathway such as phosphorylated GS-PP2A. It is worth to notice that the interplay of loops formed by serine/threonine kinases and phosphatases regulating phosphorylation kinetics, and thus stability and subcellular localization of key effectors, should be taken as essential nodes in transduction networks setting the timing and amplification of physiological responses of the circadian clock. Screening of genetic alterations, and genomic analysis of posttranslational regulation of these components may shed light on mechanisms underlying pathophysiology of circadian entrainment (as for instance in Familial Advanced Sleep Phase Disorders, in which the phosphorylation state of PER2 is altered, shortening the period of the circadian clock; Vanselow et al., 2006). The evidence we present on the role of novel regulatory circadian pathways as PKG-GS-PP2A might find its way in the discovery of novel treatments for such disorders.

Declaration of Conflicting Interests

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ORCID iDs

Santiago Andrés Plano https://orcid.org/0000-0002-7984-8826
Shogo Endo https://orcid.org/0000-0002-3948-8723

Supplemental material

Supplemental material for this article is available online.

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